UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

Characterization of Insect Bite Hypersensitivity in a Population of Lusitano Horses: contribution for future implementation of Skin Prick Tests (SPT) in IBH diagnosis

Vera Purificação Carvalho Pessoa

Orientador: Professora Doutora Paula Alexandra Botelho Garcia de Andrade Pimenta Tilley

Co-orientador: Professor Doutor Manuel Branco Ferreira

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na especialidade Clínica

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To my beloved mother, to my sister and father,

To all my closest friends and family,

To my Dear Camila, a dog that was never just a dog,

Who I miss so much everyday…

To my professors and mentors.

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Hipersensibilidade à picada de insetos (HPI) em equinos Puro-Sangue Lusitano: contribuição para o desenvolvimento de testes cutâneos por picada (TCP) no diagnóstico da HPI

Resumo:

A Hipersensibilidade à Picada de Insetos (HPI) é uma dermatite pruriginosa sazonal, que afeta um elevado número de cavalos a nível mundial. É uma reação de hipersensibilidade tipo I e IV a alergénios presentes na saliva *Culicoides spp.* É uma doença multifatorial, que afeta diferentes raças com uma prevalência variável. O tratamento é limitado, mas sendo uma doença alérgica imunomediada por IgEs, há a possibilidade de se desenvolver imunoterapia específica. O trabalho apresentado visou a caracterização da HPI em cavalos Lusitanos em Portugal continental, através de um estudo de caso controlo envolvendo 30 animais com HPI e 30 animais saudáveis. A população de cavalos estudados foi caracterizada pela avaliação de questionários individuais. Testes intradérmicos (TID) e cutâneos por picada (TCP) foram efetuados, sendo testados 14 alergénios específicos, 13 proteínas recombinantes de *Culicoides nubeculosus* (Cul n) *e Culicoides obsoletus* (Cul o) designadas de Cul n 1 a Cul n 11, Cul o 1P and Cul o 2P, e extrato de Cul n (WBE). Adicionalmente 12 destes 30 cavalos, 6 teste e 6 controlo, foram testados com alergénios produzidos em células de insetos (Cul n 3Bac, Cul n 4Bac) e cevada (Cul n 3 Bar, Cul n 4Bar) bem como Cul o 3 e Cul o WBE. Foram ainda realizados testes *in vitro,* nomeadamente determinação sorológica de IgEs específicas por ELISA, para os alergénios Cul n 3, 4, 9, 10, Cul o 2, 3, Cul o1P e Cul o2P, e determinação de sulfitoleucotrienos (sLT) produzidos por leucócitos do sangue periférico (CAST), quando estimulados por Cul n WBE e Cul o WBE. O nosso estudo indicou que os TCP apresentam uma matriz de discriminação superior aos TID, sendo por isso recomendados, podendo ser um potencial avanço no estabelecimento de testes de diagnóstico mais adequados. Relativamente aos testes *in vitro*, embora a determinação sorológica de IgEs específicas demonstrasse que os cavalos afetados tinham níveis séricos de IgEs mais elevados para os alergénios significativos, o teste de libertação de sulfidoleucotrienos (sLT) teve melhor desempenho. Além disso foi possível estudar e identificar as espécies de culicóides encontradas em coudelarias, que podem estar relacionadas com a HPI nos equinos estudados, sendo as espécies do grupo *Obsoletus*, *C. nubeculosus* e *C. imicola* as mais prevalentes.

Este estudo visa contribuir para a melhoria de métodos de diagnóstico da HPI, representando um avanço para a futura implementação de painéis de diagnóstico de alergénios específicos e, eventualmente, para a futura implementação de imunoterapia específica.

Palavras-chave: HPI, *Culicoides,* Testes "*in vitro*", Testes cutâneos (TCP e IDT).

Characterization of Insect Bite Hypersensitivity (IBH) in a population of Lusitano horses: contribution for future implementation of SPT in IBH diagnosis

Abstract:

Insect Bite Hypersensitivity (IBH) is a recurrent seasonal pruritic dermatitis affecting many horses worldwide. It is mainly a type I hypersensitivity, but type IV may also occur, to allergens present in the saliva of *Culicoides spp.* It is a multifactorial disease affecting different breeds, with a variable prevalence. Treatment options are very limited, but being an allergic disease IgE-mediated, there is the possibility of developing specific immunotherapy. This study aimed to characterize IBH in Lusitano horses living in Portugal mainland, through a case control study involving 30 IBH-affected animals and 30 healthy controls. Questionnaires were performed for all the horses involved in the study in order to characterize the studied population. *In vivo* allergen testing was evaluated by performing skin tests, both skin prick tests (SPT) and intradermal tests (IDT), using 14 specific allergens, including 13 different recombinant (r-) proteins from C*ulicoides nubeculosus* (Cul n) or *Culicoides obsoletus* (Cul o) salivary glands (termed Cul n 1 to Cul n 11, Cul o 1P and Cul o 2P) as well as Cul n whole body extract (WBE). Moreover, a cluster of 12 of these horses, 6 IBH-affected and 6 from the control group, were also tested with allergens produced in insect cells (Cul n 3Bac, Cul n 4Bac) and barley (Cul n 3 Bar, Cul n 4Bar) as well as Cul o 3 and Cul o whole body extract (WBE). Furthermore, *in vitro* diagnostic tests have been performed, namely, serum IgEs that were measured by ELISA for the allergens Cul n 3, 4, 9, 10 and Cul o 2, 3, as well as Cul o1P and Cul o2P and *in vitro* sulfidoleukotriene (sLT) release assay which were carried out with Cul n WBE and Cul o WBE. In our study SPTs presented a higher discriminatory diagnostic potential than IDTs, and should be preferred, being a potential breakthrough in the establishment of more suitable allergen avoidance measures in IBH diagnosis. Regarding the *in vitro* tests, even though serology measurement of specific IgEs showed that IBH-affected horses had higher serum IgE levels for the significant allergens, sLT release assay performed best. It was also possible to study and identify *Culicoides spp* found in the stud farms, which may be related to IBH in the studied horses. The most frequently found were *Obsoletus* group species, *C. nubeculosus* and *C. imicola*.

This study contributed to improve IBH diagnosis, representing a step forward for the future implementation of locally relevant diagnostic allergen panels and eventually for the establishment of patient tailored, component resolved specific immunotherapy.

Key words: IBH, *Culicoides*, *In vitro* tests, Skin tests (SPT, IDT).

Hipersensibilidade à picada de insetos (HPI) em equinos Puro-Sangue Lusitano: contribuição para o desenvolvimento de testes cutâneos por picada no diagnóstico da HPI

Resumo Alargado:

Introdução:

A Hipersensibilidade à Picada de Insetos (HPI) é uma dermatite, alérgica, pruriginosa, recorrente e sazonal causada principalmente por reações de hipersensibilidade do tipo I mediadas por IgEs aos alergénios presentes na saliva de *Culicoides spp*. Reações de hipersensibilidade retardada do tipo IV podem também ocorrer como resultado da ativação de linfócitos T, após sensibilizados a um antígeno específico.

Esta doença afeta cavalos em todo o mundo, com prevalências muito variadas, não apresentando especificidade por raça, cor ou espécies. Embora a prevalência de HPI em Portugal e no cavalo Puro-sangue Lusitano (PSL) ainda não seja conhecida, as características ambientais são favoráveis à atividade dos culicóides, sendo cada vez mais do conhecimento geral um aumento do número de cavalos afetados em coudelarias nacionais. Até à realização desta investigação não existiam estudos sobre o impacto da HPI nos cavalos Lusitanos, nem em Portugal Continental, nem noutros países onde existem cavalos Lusitanos. A criação do cavalo Lusitano desempenha um papel importante na economia portuguesa e os cavaleiros estão a fazer uso crescente dos Lusitanos em todo o mundo para diferentes disciplinas de desporto equestre. A HPI pode resultar em redução do valor comercial dos cavalos afetados, bem como aumentar os custos de tratamento e maneio ambiental para controlar os sinais clínicos da doença, causando também desconforto e alterações de comportamento. Quando os sinais clínicos são muito graves e sem forma de controlar o impacto negativo da doença no bem-estar animal, a eutanásia pode ser recomendada.

Objetivos:

O presente estudo teve como objetivos:

• Caracterizar os cavalos estudados, as coudelarias e alguns aspetos do maneio dos equinos, principalmente o alojamento, através da avaliação de questionários.

• Caracterizar a população de *Culicoides* presente nas coudelarias de cavalos Lusitanos com história de HPI.

• Identificar fatores imunoalérgicos relevantes, caracterizando e comparando os resultados de testes de alergia *in vivo*, testes intradérmicos (TID) e cutâneos por picada (TCP), e testes *in vitro* pela determinação sorológica das IgEs séricas específicas para

os alergénios testados, e determinação de sulfidoleucotrienos (sLT) por testes de libertação em plasma rico em leucócitos, em cavalos Lusitanos de coudelarias em Portugal continental com diagnóstico clínico de HPI (grupo teste – T), em comparação com cavalos coabitantes saudáveis (grupo controlo – C).

Material e métodos:

Os cavalos foram incluídos neste estudo com base na história pregressa e no exame físico. Foram realizados questionários para a caracterização da população estudada, com base em estatísticas descritivas, nomeadamente frequências absolutas e relativas, média, mediana e desvio padrão sempre que aplicável.

Para caracterizar a população de *Culicoides*, foram selecionadas treze coudelarias de PSL em várias regiões de Portugal Continental, que foram incluídas por apresentarem antecedentes de cavalos Lusitanos com HPI. Os *Culicoides* foram coletados entre maio e junho de 2016 usando armadilhas específicas: OviTrap "Onderstepoort Veterinary". As armadilhas foram colocadas em coudelarias com um mínimo de 5 cavalos Lusitanos. Os espécimes de *Culicoides* foram inicialmente identificados pelo padrão de asa, usando para tal microscopia estereoscópica e chaves de identificação apropriadas. Quando os espécimes de *Culicoides* não puderam ser identificados desta forma, cada um foi separado em diferentes regiões corporais e posteriormente identificados.

Relativamente à identificação de fatores imunoalérgicos relevantes, 30 cavalos C e 30 cavalos T foram submetidos a testes cutâneos, intradérmicos e por picada, TID e TCP respetivamente, no pescoço testando-se 14 alergénios salivares específicos, incluindo 13 diferentes proteínas recombinantes (r-) de *Culicoides nubeculosus* (Cul n) ou *Culicoides obsoletus* (Cul o) (denominados Cul n 1 a Cul n 11, Cul o 1P e Cul o 2P), bem como extrato de Cul n ("Whole Body Extract" - WBE). Adicionalmente, um grupo de 12 destes cavalos, 6 T e 6 C, foram testados com alergénios produzidos em células de insetos (Cul n 3Bac, Cul n 4Bac) e cevada (Cul n 3 Bar, Cul n 4Bar), bem como Cul o 3 e Cul o WBE. As concentrações de alergénios utilizadas foram 10µg/ml para os TID e 100µg/ml para os TCP. Os diâmetros das pápulas foram avaliados aos 20 min, 6 e 48 horas (TID). Os TCP foram considerados positivos quando o diâmetro da pápula era ≥0,9cm e os TID quando o diâmetro da pápula era pelo menos 50% do diâmetro da pápula de histamina (controlo positivo). Além disso, as IgEs séricas foram medidas por ELISA para os alergénios Cul n 3, 4, 9, 10 e Cul o 2, 3, bem como Cul o1P e Cul o2P, e os sLT foram determinados por testes de estimulação em plasma enriquecido por leucócitos com o Cul n WBE e Cul o WBE.

Resultados:

Relativamente à avaliação de questionários os cavalos T incluídos neste estudo estavam em conformidade com os critérios de inclusão, apresentando evidências de dermatite pruriginosa sazonal e lesões no momento da avaliação, neste caso um "score" clínico de HPI >1.

Quanto à identificação de *Culicoides spp* junto de coudelarias com história de HPI em cavalos Lusitanos, foram capturadas um total de 7 espécies de *Culicoides* durante este estudo, juntamente com aquelas pertencentes ao grupo *Obsoletus*. Algumas destas espécies de *Culicoides*, no entanto, foram encontradas apenas em coudelarias nacionais, nomeadamente *C. corsicus*, *C. fascipennis*, *C. harati, C. odiatus, C. santonicus e C. tbiliscus*. Além disso, a maioria destas espécies já haviam sido identificadas em estudos anteriores realizados em Portugal.

Relativamente à identificação de fatores imunoalérgicos relevantes foi possível determinar:

- Testes *in vivo*: em relação aos TCP para o primeiro painel de alergénios, as reações mais fortes foram observadas no grupo T em comparação com o grupo C para os alergénios: Cul n WBE, Cul n 7, 8, 9, Cul o1P e Cul o 2P ($p \leq 0,0001$). No segundo painel de alergénios, foram observadas diferenças estatisticamente significativas para o Cul o WBE, Cul n 3 Bar e Cul n 4 Bac ($p \leq 0,05$). Setenta por cento (70%) dos cavalos afetados por HPI (T) e apenas 11% dos controles foram positivos para pelo menos 4 alergénios no caso dos TCP. O Cul n WBE foi o alérgeno para o qual todos os cavalos T foram positivos, mas apenas 20% do grupo de controle foram positivos para este alérgeno. Sessenta por cento (60%) dos cavalos T e apenas 13% dos cavalos C foram positivos para o Cul n 9.
- Testes *in vitro*: Na serologia determinou-se que os cavalos T apresentaram níveis séricos de IgEs mais elevados do que os cavalos C para os seguintes alergénios: Cul n 3, Cul n 4, Cul n 10, Cul o 1P, Cul o 2P, Cul o 2 e Cul o 3. A análise ROC mostrou que Cul n 3 e Cul o 1P foram os alergénios que apresentaram melhor desempenho na determinação de IgEs específicas. A determinação da concentração de sulfidoleucotrienos (sLT) com Cul o WBE teve o melhor desempenho dos ensaios *in vitro*, com uma área sob a curva (análise de ROC, "area under curve" - AUC) de 0,90 (95% CL 0,74-0,96).

Discussão:

Foi possível caracterizar os cavalos estudados através da avaliação dos questionários de acordo com vários parâmetros. Os cavalos incluídos no grupo teste

apresentavam na sua maioria apresentavam sinais clínicos de HPI discretos a moderados, provavelmente devido à altura da realização dos testes, início da primavera, em que os culicóides ainda tinham atividade reduzida.

Durante este estudo foram identificadas várias espécies de *Culicoides*. As espécies do grupo *Obsoletus, C. nubeculosus e C. imicola* foram as mais frequentemente implicadas na HPI em cavalos Lusitanos.

Em relação aos testes de alergia *in vitro* e *in vivo*, os TCP apresentaram maior potencial de diagnóstico com uma maior matriz de discriminação do que os TID, e por isso devem ser privilegiados, sendo um potencial e promissor avanço no estabelecimento de medidas de prevenção mais adequadas ao diagnóstico da HPI. Com base nestes resultados os alergénios determinados como estatisticamente significativos, nomeadamente o Cul n WBE, Cul n 7, Cul n 8, Cul n 9, Cul o 1P e Cul o 2P, parecem ser fortes candidatos para inclusão num painel de TCP para o diagnóstico da HPI.

Em relação aos testes *in vitro*, embora a medição sorológica das IgEs específicas tenha mostrado que os cavalos T apresentavam níveis séricos de IgEs mais elevados para os alergénios significativos, ou seja, Cul n 3, Cul n 4, Cul n 10, Cul o 1P, Cul o 2P, Cul o 3 e Cul o 2, o Teste de libertação de sLT teve o melhor desempenho com o Cul o WBE. Estes alergénios também devem ser considerados como candidatos a serem incluídos num painel de diagnóstico.

Os resultados relevantes deste estudo representam um avanço não só na determinação dos alergénios a serem incluídos num painel de diagnóstico específico para a HPI, mas também para a implementação de imunoterapia específica com uma seleção de alergénios individualizados para cada caso específico. Ainda assim, estudos adicionais usando alergénios recombinantes de *Culicoides* recentemente identificados são necessários, e podem até melhorar o desempenho dos testes alérgicos, nomeadamente os TCP para o diagnóstico da HPI.

Conclusão:

Em suma, este estudo pode ter uma contribuição relevante para a implementação futura de painéis de alergénios localmente relevantes para o diagnóstico da HPI e, eventualmente, para a implementação de imunoterapia específica. Além disso, estes resultados também podem ter aplicações práticas na medicina humana, reconhecendo a importância do estudo de doenças alérgicas em animais considerando o cavalo como um modelo animal translacional de relevo. Isto foi recentemente reconhecido pela constituição de um grupo de interesse em Alergologia Comparada e

Veterinária na Academia Europeia de Alergia e Imunologia Clínica (EAACI) e na Sociedade Portuguesa de Alergologia e Imunologia Clínica (SPAIC).

Palavras-chave: HPI, Questionários, *Culicoides,* alergénios, Testes alérgicos de diagnóstico *in vitro* e *in vivo*, TCP, TID.

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APC - Antigen Presenting Cell
APSL - Portuguese Lusitano Breed Association
ASIT - Allergen specific immunotherapy
AUC – Area Under Curve
BPA - Best Performing Allergens
C – Healthy/Control group
CAST – Cellular Antigen Stimulation Test
CD4+ - Helper Tcells
CD8+ - Cytotoxic T lymphocytes
Cul n - *Culicoides nubeculosus* (extract/recombinant)
Cul n Bar/Bac - Barley or insect cell expressed allergens.
Cul o – *Culicoides obsoletus* (extract/recombinant)
cTh2 - Conventional Th2 Cells
E. coli - Escherichia coli
EAS - Equine Asthma Syndrome
ELISA – Enzyme-Linked Immunosorbent Assay
FcƐRI - High-affinity IgE receptors
FoxP3: Forkhead box P3
IBH - Insect Bite Hypersensitivity
IDT - Intradermal Test
IFN-g – Interferon Gamma
IgE – Immunoglobulin E
IgG4 – Immunoglobulin G4
IL - Interleukins
ILC2 - Innate Lymphoid Cells
IgA, IgM, IgG and IgE – Immunoglobulins A, M, G and E
MA - Major Allergens
MCP-1/CCL2 - Monocyte Chemoattractant Protein 1
MHC – Major Histocompatibility Complex
n - Sample Size
OD values – Optical density values
PAF – Platelet-Activating Factor
PBS – Phosphate-buffered saline
PRE - Pure Spanish Breed
PSL – Pure Lusitano Breed
r- Allergens - Recombinant Allergens
ROC - Receiver Operating Curve
RT - Room Temperature
SD - standard deviation
s-IgE - Specific Immunoglobulin E
sLT - Sulfidoleukotrienes
SPT - Skin Prick Test
T - IBH-affected/ Test group
TGF-β – Transformation Growth Factor beta
TH2 - T helper cells 2
TNF– Tumor Necrosis Factor
TSLP – Thymic Stromal Lymphopoietin
peTh2 - Effector Th2 Cells
WBE – Whole Body Extract
WBFSH - World Breeding Federation for Sport Horses
WHO - World Health Organization

"Animal herbivurum, rarissime carnivorum, generosum, superbum, fortissimum in currendo, punctando, truhendo; optissimum equitando, cursu furens; sylvis delectatur, hinnita sociam vocat; calcitrando pugnat." (Ervideira 2000)

Vera Purificação Carvalho Pessoa

2024

Introduction

This dissertation focuses on the study of Insect Bite Hypersensitivity (IBH) in a population of Lusitano horses in Portugal mainland. IBH is a highly prevalent and recurrent allergic disease, that induces significant economic losses in the equine sector.

The main goals of the experimental work were the characterization of the studied IBH-affected Lusitano horses by the evaluation of questionnaires, the identification of the most frequent *Culicoides spp.* close to Lusitano stud farms that may have an impact in IBH, the development of a diagnostic allergen panel establishing more suitable allergen avoidance measures in IBH, and contribute to the establishment of patient tailored, component resolved specific immunotherapy.

For this purpose, this dissertation will be divided in 5 chapters:

Chapter I is mainly a comprehensive review of IBH and includes:

a) a brief history of IBH
b) pathophysiology, etiology, immune mechanisms, and genetics
c) current diagnostic approaches, and therapy
d) the relevance of IBH in Lusitano horses, among other subjects.

Additionally, the role and relevance of the Lusitano horse is also approached, as well as the fundaments of hypersensitivity reactions and most common allergies in horses.

This chapter provides the reader the necessary information to understand the experimental clinical work here presented and is divided in a general introduction and objectives.

Chapter II: "Characterization of a population of Lusitano horses living in Portugal mainland, taking part in a case control study involving 30 IBH affected animals (test group) and 30 healthy ones (control group): questionnaire evaluation."

It aims to evaluate the questionnaires and characterize the studied population of IBH-affected Lusitano horses, in Portugal mainland.

The objectives of the experimental work and the respective results are reported in the third and fourth chapters, and presented in the form of published or submitted scientific publications:

- **"Chapter III**: "*Culicoides* species found in Portugal that can be related to IBH.**"**

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- **Chapter IV**: "Comparison of Skin Prick tests (SPT), Intradermal tests (IDT) and *in vitro* tests in the characterization of Insect Bite Hypersensitivity (IBH) in a population of Lusitano horses: contribution for future implementation of SPT in IBH diagnosis".

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Chapter V: includes a general discussion regarding the results of the experimental work, as well as future areas that must be addressed to further improve our knowledge of IBH. Lastly there is a conclusion, future perspectives and other considerations, that can empower the study, in the light of the presented results.

Chapter I:
General introduction and objectives

"(...)In summary, our study contributes to understanding the heterogeneity of immunity in nature and adds important information on the equine immune system, which is clearly distinct from men(...)" (Manuel et al 2006)

1. General Introduction:

1.1. Brief history, demographic characterization, and importance of the Lusitano (PSL) horse:

The horse always had an extreme relevance in Portugal, and a great importance for our history and consolidation of the territory. Portugal is one of the oldest European countries, and part of the success in establishing our territory is due to the Lusitano horse (Cordeiro 1997). The Lusitano had a common origin with the Andalusian horse, developing analogous morpho-functional characteristics (Monteiro 1983). They were considered a unique breed commonly designated by Iberian horse. In 1942 Spain decided to create the denomination of Pure Spanish Breed (PRE) hence dividing the animals born in these two countries (Soares 1998). After the creation of the PRE-Studbook, Portugal officially adopted the name Lusitano horses to those born in Portugal, with their own morph-functional characteristics and genealogy (Monteiro 1983). From then onwards, these horses began to be recognized as two different breeds: PRE in Spain and the Lusitano (PSL) in Portugal (Soares 1998). Although these two Iberian breeds have the same base and origin, the differences between them are notorious due to different selection goals based on certain characteristics of interest (Bowling and Ruvinsky 2000). Even though designation of Lusitano was established in 1942, the Stud Book was only officially established in 1967, with a small number of animals, aiming the preservation and improvement of the Lusitano breed. In 1989, the studbook was “closed” allowing only the registration of descendants of previously registered stallions and mares, and since 1992 it is necessary to carry out filiations tests before an animal can be registered (Vicente et al. 2012).

The national and international organism responsible for the management of the Lusitano’s studbook and for the defense, promotion and dissemination of the breed is the Portuguese Lusitano Breed Association (APSL) and was founded in 1989. APSL is responsible for the approval of breeders for the Portuguese Lusitano studbook, by performing morphological functional tests evaluated by accredited judges, in which they assign scores to different body regions, according to a scoring grid (Vicente 2015). The visual evaluation of the qualitative morphological characteristics includes the general profile and different morphological regions, namely the head profile, type of neck, coat and shape of the eyes, orientation and implantation of the ear, line of the back, limbs, gait quality, hoof quality and body symmetry (Saastamoinen and Barrey 2000). Hence, the importance of well morphologically conformed animals according to the breed standards, to the genealogic book of approved breeders.

Figure 1: Lusitano thoroughbred standard individual (APSL, accessed on the 8th of July 2023)

Nowadays thoroughbred Lusitano (PSL) is the most important Portuguese equine breed (figure 1). According to Vicente et al. (2015) the estimated population, until 2009, was composed of ≤5000 breeding females, half of which approximately were found in Portugal and the rest distributed in other countries. The PSL population has been increasing in Portugal and throughout the world. In mainland Portugal, the highest concentration of PSL occurs mainly in the central and southern regions of the country, with the district of Santarém registering the highest number of births until 2009, followed by the district of Portalegre due to the Alter Real stud farm (figure 2). In addition, there has been an increase in the breeding of PSL in the north of Portugal, as well as in the Azores islands (Vicente et al. 2015).

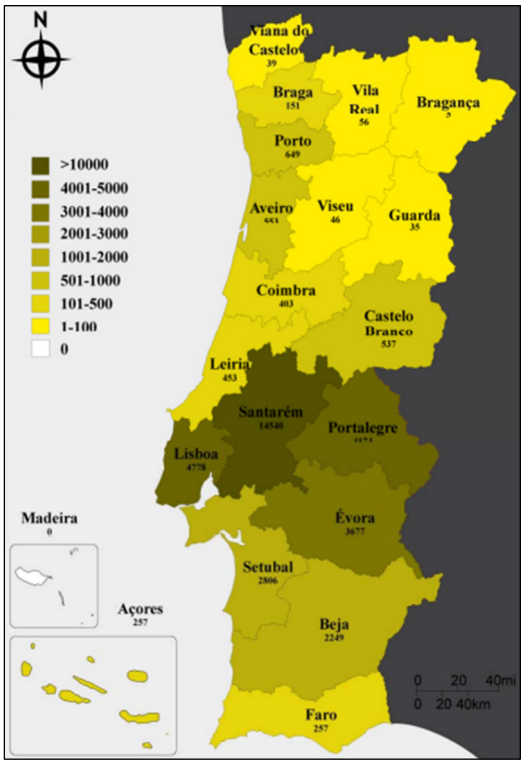

Figure 2: Total births in Portugal, overtime (n=35206, until 2009) (Adapted from: Vicente et al. 2011).

According to Vicente et al. (2020), there are 33 different countries with birth records of PSL horses registered in the Portuguese studbook, and there are records of Lusitano horses spread around the world in 63 countries as seen bellow in figure 3.

Figure 3: World's distribution of PSL (Adapted from: Vicente et al. 2011).

In 2015 the Lusitano equine breed was considered as endangered, grade B – intermediate risk, according to the Regulation of Rural Development adopted by the European Commission. Portugal would then establish the necessary conditions in order to reach the status of risk of abandonment, within the scope of regulatory requirements and objectives of the Rural Development Program 2014-2020. This was based on the number of horses that existed in 2013, namely 2000 females exploited in Pure line, 1500 males, 300 breeders, with an effective population size of 28.1 and with an "insufficient" amount of cryopreserved genetic material at the Portuguese Animal Germplasm bank (Carolino et al. 2013).

Since then, Lusitano breed has been expanding, with a growing number of breeding females per year. The number of breeding females currently exceeds 5000 animals. This is quite important as Lusitano breed presently is in not considered as endangered, grade B – intermediate risk (Vicente et al. 2022).

The Lusitano is a very functional and versatile horse with a brave, vigorous, docile, and noble temperament. These characteristics made this breed ideal for leisure and sport (Vicente 2015) in various equestrian disciplines, namely show jumping, work, driving, dressage, and high school (Cordeiro 1997). According to the World Breeding Federation for Sport Horses (WBFSH) (2021), the Lusitano is in 8th place in the world ranking of breeds used in dressage and the first as a breed with a closed herd book.

These characteristics and its quite solid and consecrated standard, lead to a high worldwide dispersion of the Lusitano (Vicente et al. 2020), as seen above.

The horse sector has assumed an increasingly relevance in the Portuguese economy, enhancing its importance in the promotion of ecological and economic sustainability. Horse breeding and management can be a sustainable agricultural activity and contribute to biodiversity. On the other hand, horse riding has a significant impact on the Portuguese economy, being one of the most important sectors of rural tourism and playing a major and increasingly role in equestrian competition sports. In this way, the horse sector in Portugal has a high potential in promoting economic and environmental sustainability, by promoting employment and income, equestrian tourism, and sports, and a more efficiency and transparent trading market.

1.2. Allergic reactions – the enhanced immune system response

The immune system is most of the times associated with the protection of the organisms against pathological agents. Sometimes overactive responses of the immune system can cause undesirable reactions like tissue injuries and the establishment of disease. These are called hypersensitivity reactions and is the base of allergic diseases. According to the WHO (2023) allergy is a hypersensitivity reaction initiated by immune mechanisms. Allergic diseases can be divided into four types of hypersensitivity reactions, Type I, II, III and Type IV (IV a-d) (Dispenza 2019), as seen bellow in figure 4.

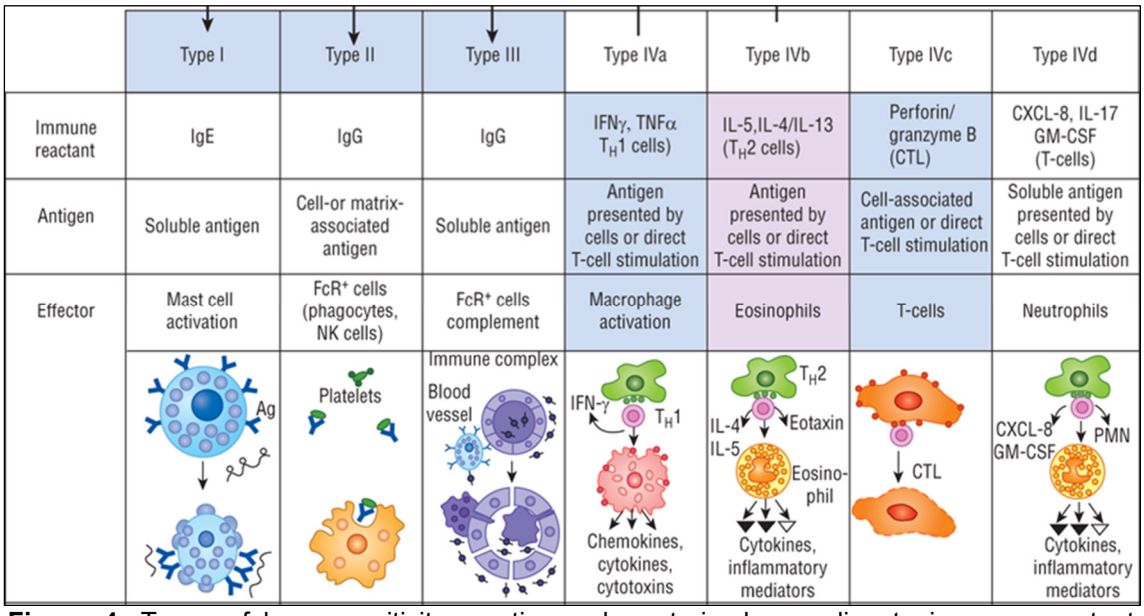

Figure 4: Types of hypersensitivity reactions, characterized according to immune reactant, antigen, and effector mechanism. (Adapted from: DiPiro et al. 2020).

Type I hypersensitivity is an immediate reaction IgE mediated, caused by the release of inflammatory mediators from mast cells and basophils (Dispenza et al. 2019). It is also known as anaphylactic hypersensitivity. It is mediated by allergen specific IgE antibodies that bound to high affinity receptors present on the surface of mast cells and basophils. The first exposure to the allergen results in sensitization. Re-exposure to the same allergen cross links with the cell's high affinity receptors, causing degranulation and release of inflammatory mediators (Ley et al. 2019).

Type II hypersensitivity is an antibody-dependent process in which specific antibodies bind to antigens, resulting in tissue damage or destruction. It is divided in:

2a (cytolytic/cytotoxic reactions): occur when pathological antibodies bind to the antigens located on the surface of cells (leucocytes and RBCs), causing their lysis through the *in-situ* fixation of complement. It is mediated, primarily, by IgM (multimeric)

or IgGs (monomeric). IgM are often more effective in fixing complement (Celik et al. 2014; Dispenza 2019).

2b: the binding of immunoglobulins to cellular receptors causes cell function alteration/loss (Dispenza 2019).

Type III hypersensitivity is an immune-mediated reaction by soluble immune complexes. Mostly IgG, but IgM may also be involved. These immune complexes are formed in blood and deposited in various tissues and organs. This will activate the classical complement pathway causing inflammatory damage (Celik et al. 2014; Dispenza 2019).

Type IV hypersensitivity is also known as delayed hypersensitivity reaction. Usually takes more than 12 hours to develop. Typically, the maximal reaction time occurs between 48 to 72 hours. It is an immune response cellular mediated (Abbas et al. 2004) causing an inflammatory reaction to either exogenous or endogenous antigens. The major cells involved are T lymphocytes and monocytes/macrophages. Reaction to exogenous antigens involves T cells and antigen-presenting cells (APC). These cells produce cytokines that stimulate a local inflammatory response in a sensitized individual. Type IV hypersensitivity can be divided in to 4 subtypes (Pichler et al. 2003):

Type IV a – TH1 cell producing interferon gamma with macrophage predominance.

Type IV b –TH2 cell producing IL 4 and 5, with eosinophil predominance.

Type IV c – CD8 T cell predominance (cytotoxicity).

Type IV d - IL8-producing lymphocytes, with neutrophil predominance.

Hypersensitivity reactions like Insect Byte Hypersensitivity (IBH) are mainly type I and IV (subtype b) (Marsella et al. 2023).

According to the World's Health Organization (WHO) definition, "an allergen is a protein that elicits a specific IgE antibody response in at least five individuals". Allergens are foreign and apparently harmless molecules (antigens) that induce an abnormal immunological response. This immune response is usually IgE mediated and may result in clinical allergy. It is currently assumed that molecules with high IgE antibody binding frequencies (>50% of allergic individuals) are major allergens (MA), whereas those with low frequencies (<50% of allergic individuals) are considered minor allergens (Deifl and Bohle 2011; Caraballo et al. 2020).

The ability of an allergen to induce allergic symptoms is called allergenicity. Allergens' clinical importance is determined by the frequency and intensity of their IgE antibody binding (Caraballo et al. 2020). The cross-link of IgE requires at least 2 IgE epitopes on an allergen molecule to activate the effector cells. These effector cells are

mainly mast cells and basophils and their activation cause degranulation and release of inflammatory mediators (Gieras et al. 2016).

Antigenic epitopes can be classified as either B or T cell reactive epitopes. T cell epitopes are linear amino acid sequences, distributed throughout the primary structure of the allergen. They are recognized by T cell receptors after the whole allergen is processed by APCs (antigen presenting cells) and presented as small linear peptides (8 to 18 amino acids) in complex with MHC I or II molecules. B cell epitopes are recognized by IgE antibodies and can be either linear or conformational. The amino acids are not in a linear sequence. They can be brought together by folding the amino acid chain, which is dependent on the 3-dimensional structure of the protein. Changes in folding of the protein may lead to loss of B-cell epitopes (Meno 2011; Pomés 2010)

Immunoglobulin E (IgE) plays a major role in allergic diseases. Horse's IgE, just as in humans, is critically involved in allergic diseases (Wagner et al. 2006). In human serum, IgE is the least abundant antibody class with a concentration of only around 50 ng/ml, whereas in horses the total IgE concentration is much higher, reaching the µg/ml range, and variations between individuals are high (Van der Meide 2013). These higher IgE levels in horses are most likely caused by generally higher parasitic loads in horses than in humans (Wagner 2009).

Basophils and mast cells are important effector cells in response to allergic inflammation (Nakanishi et al. 2010). Both mast cells and basophils have numerous granules that contain histamine, interleukins (IL) amongst other inflammatory mediators. These cells present high-affinity IgE receptors (FcεRI), which can bind to the IgE. The bidding of an antigen to IgEs bound to FcεRI receptor on the surface of a mast cell or a basophil leads to cell's degranulation. Histamine and other inflammatory mediators are released. This is associated to immediate hypersensitivity reactions (Falcone et al. 2011).

Basophils produce inflammatory mediators such as interleukins, namely IL4, 13 among others, contributing to allergic inflammation process (Stone et al. 2010). These cells can also function as antigen-presenting cells (APC) with their MHC class II, mediating Th2 cell differentiation (Karasuyama et al. 2011; Perrigoue 2009; Yoshimoto et al. 2009).

Allergies can be diagnosed *in vivo* by skin tests (intradermal – IDT, and skin prick tests - SPT) and *in vitro* by histamine or sLT release tests and serological tests.

Allergen extracts are heterogeneous mixtures composed of major allergens (MA), cross-reactive allergens and non-allergenic substances. These extracts allow to estimate the reactivity of a patient's serum towards all potentially allergenic components. Sometimes identification of the primary allergy source may be difficult (Treudler and

Simon 2013). This is due to the presence of both allergic and other non-allergic components, which may also present reactivity, and may affect testing for allergenicity (Valenta et al. 2018). Standardization of extracts can be challenging, because when produced by different manufacturers, allergen extracts present considerably differences in composition and activity (Casset et al. 2013; Larenas-Linnemann et al. 2011;).

According to Smoldovskaya et al. (2016) *in vitro* allergy diagnostics with standardized recombinant allergens (r-allergens) enables to get more reproducible results. Nevertheless, it should be noted that r-allergens do not cover the entire spectrum of potentially allergenic proteins that are present in the extracts. For that reason, the use of only individual r-allergens is not recommended to identify the allergy source, as it may give false-negative results (Smoldovskaya et al. 2016).

According to Smoldovskaya et al. (2016) allergy diagnostics should be carried out considering clinical history, results of skin tests and/or measurement of allergen specific IgEs, to allergen extracts. The use of individual r-allergens should be applied to identify the main allergens, for subsequent allergen specific immunotherapy (ASIT) (Math et al. 2014; Valenta 2012) and for prediction and monitoring of treatment efficiency (Marcucci et al. 2012).

According to Focke et al. (2010) immunotherapy is the only causative treatment for allergies, based on the administration of increasing doses of allergens until a desensitization is reached. It targets the mechanisms underlying the allergy, by increasing the frequency of regulatory T cells and suppressing the allergen-specific Th2-cells, and hence the allergen-specific IgEs. Allergen specific immunotherapy (ASIT) has been reported to be successful in inducing tolerance for different type I allergies (Chen et al. 2012; Senti et al. 2012).

Horses can suffer from several types of allergies with respiratory and skin allergies the most common. Insect Bite Hypersensitivity (IBH) is the most common skin allergy in horses (Littlewood 2014) and mainly involves a Type I (IgE mediated) hypersensitivity reaction (figure 5).

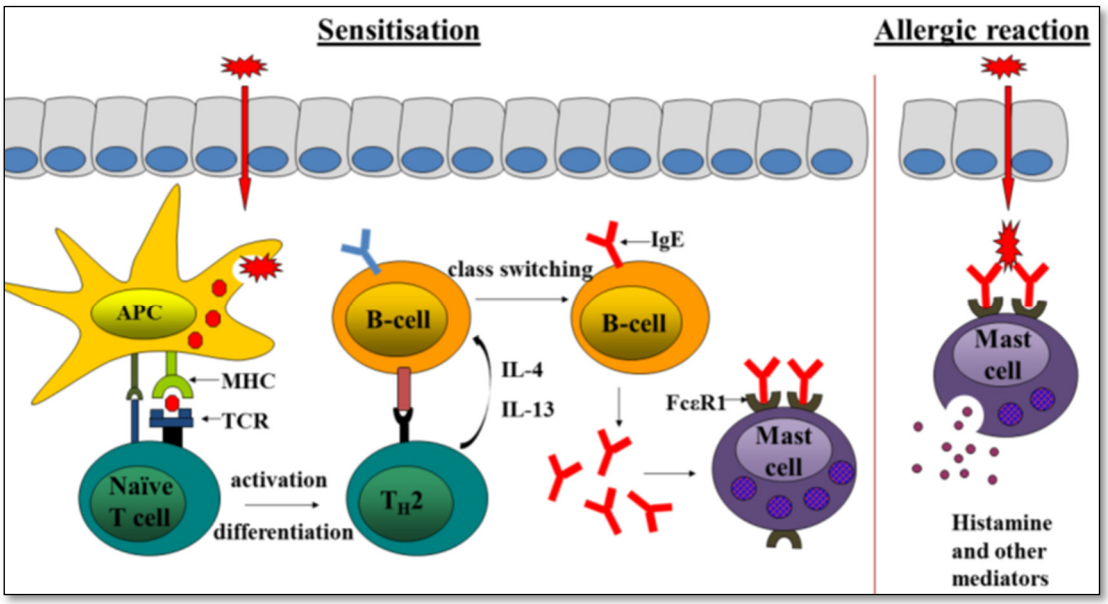

Figure 5: Schematic representation of a type I allergic reaction (Adapted from: van der Meide, 2013). An allergen is deposited on the skin, taken by antigen presenting cells (APC), that will process the allergen into smaller peptides. These peptides will bound to the major histocompatibility complex (MHC) class II on the surface of APC cells and then presented to Naïve T cell (CD4+), leading to their activation. CD4+ cells will traduce interleukins (IL) 2, 4 and 13, and induce the production of IgEs by B-cells. IgEs will bind to high affinity receptors (FcƐR1) on the surface of mast cells and basophils, finalizing the sensitization phase. When a subsequent exposition to the allergen occurs, it will bind to the mast cells coated with the specific IgEs, leading to degranulation and release of histamine cytokines, leukotrienes, and other inflammatory mediators (Van der Meide, 2013).

A delayed hypersensitive reaction type IV may also occur most likely due to cell infiltration (Cox et al 2023).

1.3. Equine Allergies

Allergic diseases are frequently observed in veterinary practice. With increasing standards in veterinary care, intradermal testing and allergen immunotherapy were introduced to small animal practice in the mid-nineteen hundreds. Later, serum testing for allergen specific IgE was developed for dogs, cats, and horses (Mueller et al. 2016).

Horses develop skin and respiratory disorders that have been attributed to allergy. The most common allergic diseases are equine asthma syndrome (EAS) and insect bite hypersensitivity (IBH). IBH is the best characterized allergic skin disease, but an effective treatment for this disease still remains elusive (Muller et al. 2016).

The concept of an individual animal having an allergic threshold, above which clinical signs are seen, has been long established. However, there is now a greater understanding that allergies often occur combined. This cumulative effect may push them above the threshold. This is why when dealing with the allergic horse, even if IBH is suspected to be the primary trigger, it's important to also consider the presence of other insect hypersensitivities namely, atopic dermatitis (AD), equine asthma (EA), and food hypersensitivity (Marsella et al. 2013).

Lanz et al. (2017) suggested that different manifestations of hypersensitivity may occur simultaneously in horses. IBH-affected horses have shown to have a higher risk of being concurrently affected by EA, and vice versa. Atopic dermatitis (AD) in horses is also becoming more commonly recognized and many IBH-affected horses also present AD.

Allergies can affect any breed, age, or sex of horses. However, IBH is particularly common in Icelandic and other native ponies and cobs (White 2017).

The age of the onset of clinical signs may differ. Dermatological and gastrointestinal signs usually occur in horses with 2 to 5 years old, whereas severe respiratory signs tend to occur at a later stage, usually around 7 years old or even in later ages (Couetil et al. 2016).

Changes in the environment that induce a heavier allergen load should be considered when taking the animal's history such as moving to pastures with a higher incidence of midges, stabling with high dust levels or seasonally higher pollen counts.

Clinical signs differ according to the type of allergy, and organic system affected.

- Dermatological signs:

The main clinical sign is pruritus, which may lead to alopecia, scaling, and subcutaneous edema. Most cases of pruritus in the allergic horse are due to atopic dermatitis, biting midges' salivary protein allergies, especially *Culicoides spp*. (Fadok and Greiner 1990), contact allergy or food allergy, but less common. Other lesions can

also occur, namely, papules, scales/pustules suggestive of an infectious cause secondary to an underlying primary issue.

- Respiratory signs:

Cough, increased respiratory rate and effort at rest, and exercise intolerance are the most frequent respiratory signs (Couetil et al. 2016). Equine asthma is mostly associated with airway inflammation and narrowing of the airways along with increased mucus production. It can be caused by mold/fungal spores, dust mites and pollens, both outdoors and in the stable (Couetil et al. 2020).

- Gastrointestinal signs:

Diarrhea, colic, and long-term weight loss are the most common signs associated with food allergy in horses. This condition is much less understood than in humans or dogs (Dupont et al. 2016; Lloyd et al. 2003). The prevalence is not currently known in the equine population. As previously noted, dermatological signs can also be associated with food allergy.

An allergic disease in a horse is a lifelong condition. Treatment's main goal is to achieve control of the clinical signs. Hence, a multimodal therapeutic approach is probably the best way to achieve long-term management. Nevertheless, decreasing allergen exposure is probably the best treatment option (Marsella et al. 2023), and should always be incorporated in any long-term multimodal treatment plan. This normally involves changing the horse's overall environment to reduce exposure to allergens, which in real-life situations proves to be challenging (Marsella et al. 2023).

1.4. Insect Bite Hypersensitivity (IBH): a brief approach to the disease

Insect bite hypersensitivity (IBH) is a recurrent seasonal pruritic dermatitis, and the most common cutaneous disease in horses (Scott & Miller 2003). Many names have been given to IBH, including summer eczema, sweet itch, summer seasonal recurrent dermatitis and Queensland itch (Wagner et al. 2015).

IBH is an allergic reaction, caused mainly by insects of the genus *Culicoides*. Hypersensitivity reactions to other insects are much less well studied in horses, but they seem to play a role in equine recurrent urticaria and atopic dermatitis (AD) (Mueller et al. 2016).

1.4.1. Brief history of IBH:

Insect bite hypersensitivity descriptions are cited in the veterinary literature for over 160 years (Marsella et al. 2023). The first description of the disease was presented at the proceedings of the British Veterinary Medical Association in 1841. VMA Felix Delany presented a paper entitled “On the skin of the horse, its functions and some of its diseases” in which he describes a disease ‘Surfeit’ that manifests as small “papules on the skin with inflammation, deposition under the cuticle and peeling with loss of hair, which in some cases may progress to resemble mange” (Marsella et al. 2023). During the meeting several other participants reported that in their experience, ‘Surfeit’ was more common in the warmer months with a peak incidence in spring and autumn. (Marsella et al. 2023).

The disease was first associated with insect bites in 1891, by Bancroft which demonstrated that horses in Queensland were protected from ‘Queensland Itch’ if they were stabled from before dusk until after dawn.

In the early 20th century papers describe a condition of horses resembling IBH referred to as “sweet itch” in the UK (Annon 1918) in which lesions began as papules associated with severe itching leading to further traumatic injury to the skin, affecting the mane, base of the tail, and ventral midline of the affected horses.

In 1928, Allen and Kingstone introduced the term *lichen tropicus* to describe the condition seen commonly in India, stating to be more common in imported artillery horses.

Several other authors had similar descriptions, and some even associated this condition with filarial parasites. By this time, *Culicoides nubeculosus* was known to be the intermediate host of *Onchocerca cervicalis* (Mellor 1975). Nevertheless, the importance of filarial parasites was questioned, and no exact explanation was given to justify why filaria would only occur in some exposed horses.

Building on the observations of Bancroft (1891) that Queensland itch was associated with biting flies, Riek (1953) demonstrated positive allergic reactions to intradermal skin tests with *Culicoides robertsi* antigens and went on to show that sensitivity could be transferred passively to the skin of unaffected horses by a heat labile serum antibody.

From then on, some authors referred to a condition resembling IBH in which intensely pruritic lesions are distributed along the mane, withers, back and tailhead caused by an allergic reaction to insect bites (Insect Bite Hypersensitivity), while others used the term summer sores for a more localized granulomatous lesion frequently seen on the face and lips of horses associated with the larvae of *Habronema* spp. (Marsella et al. 2023).

1.4.2. IBH distribution:

IBH has a worldwide distribution, with an incidence of 2.8% in the United Kingdom, 3% in Switzerland, 4.4% in Japan, 21.8% in Israel, 26% in Canada, 29% in Germany and 32% in Australia (Scott & Miller 2003). IBH has been described worldwide, except in Iceland, and affects approximately 10% of horses of all breeds (Jonsdottir et al. 2019). It is thought that the variability of incidence is related to the influence of environmental factors on the activity of the insect species involved (Schurink et al. 2010; van der Rijt et al. 2008). Despite the great variability, the most reported values for prevalence are 5 to 10% (Schaffartzik et al. 2009; Schurink et al. 2011).

Currently there are no values for prevalence in Portugal. However, the environmental characteristics are favourable to the activity of the hematophagous insects most frequently involved in this dermatitis, as they are present throughout the national territory (Ramilo et al. 2012).

1.4.3. Etiology:

Insects of the genus *Culicoides* are considered the main causal agents of hypersensitivity to insect bites. Females are hematophagous insects that mechanically damage the horse's skin. They inject saliva that contains important pharmacological active components that help the females "feed on horse's blood". These components are digestive enzymes that include hyaluronidase, trypsin, and chymotrypsin (Wilson et al. 2008; Russel et al. 2009) and are likely to have a dual role: assisting the disruption of the skin barrier and connective tissue during biting, and subsequently in digestion of the blood meal (Marsela et al. 2023).

The genus *Culicoides*, Order *Diptera*, Family *Ceratopogonidae,* consists in 800-1000 different species of *Culicoides spp.* that can be found worldwide, except in Iceland (Craig, 2011).

These small sized insects, 1-3 mm, also known as “biting midges,” live preferably in moisty and muddy grounds. They are most active during early morning and early evening, and prefer a hot, humid, and windless environment as they are poor flyers (figure 6) (Craig 2011).

Figure 6: Representation of an optimal environment to *Culicoides spp.*

When *Culicoides* are active they can feed for long periods, and the horses may be bitten by many hundreds if not thousands of *Culicoides***.** Nevertheless, severe systemic illness or anaphylactic shock caused by *Culicoides* bites has not yet been reported (Marsella et al. 2023).

IBH can affect any horse’s breed and it does not seem to have any preference by gender (Hallamaa 2009; Halldórdsóttir & Larsen 1991; Steinman et al. 2003;) or colour of the coat (Halldórdsóttir & Larsen 1991). Also, *Culicoides spp.* shown no preferences for hosts, and besides horses, several other species can be affected, namely donkeys, ruminants, and others (Yeruham, Perl & Braverman 2004). There are some breeds that seem to have a higher risk of developing IBH, namely Icelandic horses born in Iceland after being exported to countries where *Culicoides* are endemic. This is probably due to the absence of *Culicoides* in Iceland (Wagner, 2015; Ziegler et al. 2018).

The Culicoides that are mainly associated with IBH are *C. nubeculosus, C. obsoletus* and with less expression *C. sonorensis*. Each species has distinct environmental and food preferences, which may explain, in part, the differences in the

incidence of the disease in different countries (Anderson et al. 2011; Schaffartzik et al. 2010; van der Rijt et al. 2008).

In Portugal, several species of *Culicoides* circulate, being the most common *C. imicola* and those belonging to the *Obsoletus* group (figure 7) (Ramilo et al. 2012).

Figure 7: *Culicoides obsoletus* (Adapted from: van der Meide et al. 2012)

It is also possible that some horses exhibit hypersensitivity to multiple insects, other than *Culicoides spp.* (Helberg et al. 2006; Schaffartzik et al. 2010; Scott and Miller 2003; Wagner, 2009) such as *Simulium spp, Stomoxys calcitrans* and *Haematobia irritans* (Hellberg et al. 2006; Schaffartzik et al. 2010; Scott & Miller 2003). Schaffartzik et al. (2010) concluded that a homologous allergen identified in the salivary glands of *Culicoides nubeculosus* and *Similium spp.* showed extensive cross-reactivity mediated by IgE *in vitro* and *in vivo*.

1.4.4. Pathology of IBH

a) Mechanisms involved in IBH sensitization:

IBH is mainly a type I hypersensitivity, Th2 immune response IgE-mediated that involves mast cell and basophil degranulation. Clinical signs may vary depending on the exposure to the allergen (Janeway et al. 2005) and the IgE bind to high affinity receptors on effector cells (FcεRI). These cells are mainly basophils and mast cells that degranulate releasing inflammatory mediators like histamine and interleukins among others (Deifl &

Bohle 2011). This event is accompanied by eosinophil skin infiltration (Benarafa et al. 2002).

According to (Jonsdottir et al. 2019) the mechanisms involved in IBH are:

I. Sensitization:

Culicoides spp. while feeding inject saliva that contains a variety of allergens. Upon inflammation or damage, the epithelium produces cytokines, TSLP, IL-33, and IL-25, which act as alarmins and activate group 2 innate lymphoid cells (ILC2). These lymphoid cells secrete interleukins, IL-5, and IL-13. Antigen presenting cells (APC) are modulated by IL2 as they take up salivary gland allergens. APCs migrate to the draining regional lymph node, while presenting the allergen peptides on MHC class II to naïve CD4+ T helper cells. Naïve CD4+ T helper cells differentiate into T helper type 2 cells (Th2), that produce IL-4 and IL-13. IL-4 and IL-13 will instruct the B cells to undergo class switching. Upon class switching, B cells produce allergen specific IgEs, binding to the high affinity IgE receptor (FcεRI) on the surface of effector cells, sensitizing the horse to *Culicoides* allergens.

II. Re-exposure:

During re-exposure, an immediate and/or late-phase reaction can occur. Upon re-exposure to *Culicoides spp.* allergens, they bind to the mast cell–bound allergen-specific IgEs, causing cross-linking between the receptors. This will induce mast cells to degranulate and release inflammatory mediators, such as histamine, leukotrienes, prostaglandins, and Platelet-activating factor (PAF). The release of inflammatory mediators will lead to the development of oedema, erythema, and pruritus, within minutes. Furthermore, activated mast cells also release chemokines and cytokines responsible for the recruitment of effector cells to the allergy site, mainly eosinophils, Th2 cells, and basophils, causing the late phase reaction, which starts 2–4 h after exposure and reaches its peak at 24 h. Th2 cells produce cytokines, in particular IL-5 that further recruits' eosinophils to the site of allergic inflammation.

These mechanisms are displayed in figures 5 and 8.

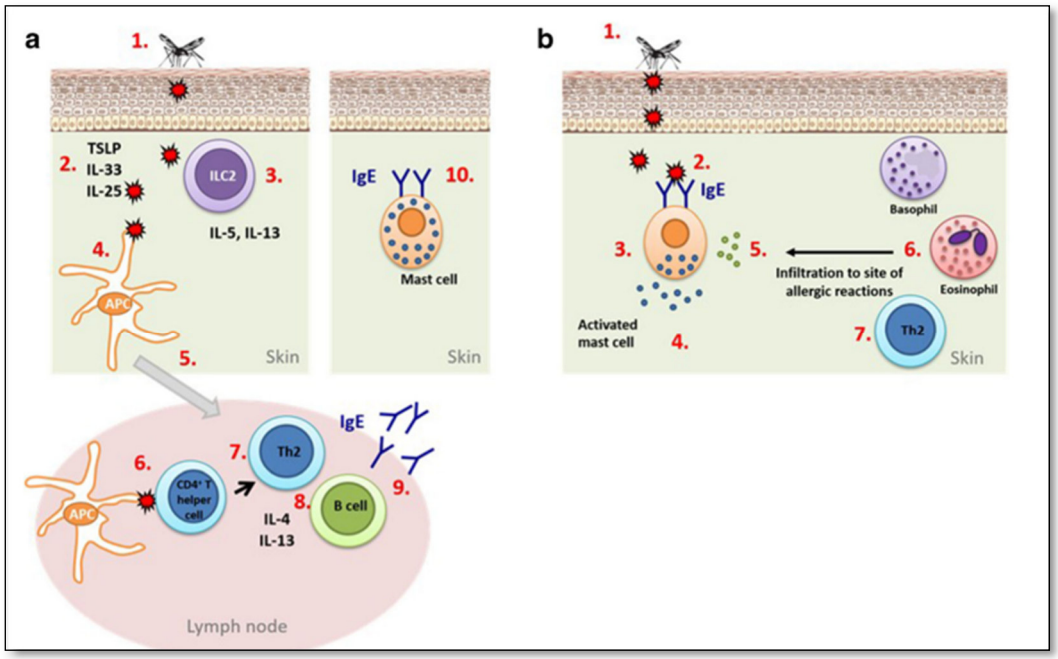

Figure 8: Simplified scheme of Type I hypersensitivity mechanisms involved in IBH (Adapted from: Jordonsttir et al. 2019). Abbreviations: TSLP – tymic stromal lymphopietin; IL 4, 5, 13, 25 and 33 – interleukines; APC – antigen presenting cells; TH2 – T helper cells; IgE – immunoglobuline E.

Also, a type IV delayed hypersensitivity reaction may occur, as result of the activation of sensitized T lymphocytes to a specific antigen. It involves a sensitization and an effector phase (Snyder 2017). During the sensitization phase, antigen-specific memory T lymphocytes (CD4+) are formed (Snyder 2017), and CD8+ lymphocytes (often called cytotoxic T lymphocytes, or CTLs) may also be activated, but in lower quantities. Once CD4+ T lymphocytes are activated in response to antigen presentation, they can develop into Th1 lymphocytes initiating the effector phase. The Th1 response is also enhanced by the production of inflammatory mediators, namely, IL-2, IL-3 and γ-IFN by CD4+ lymphocytes. γ-IFN activates macrophages enhancing phagocytic mechanisms. Macrophages are also effective as antigen-presenting cells (APC), as they present class II MHC and cell adhesion molecules.

According to Benarafa et al. (2002) and Klumplerova et al. (2013) thymic stromal lymphopoietin (TSLP) and monocyte chemoattractant protein 1 (MCP-1 or CCL2) are also associated with IBH's pathology.

Eosinophils contribute to IBH's pathogenic mechanism playing a role in late-phase type I hypersensitivity and in cell-mediated hypersensitivity (Type IV b) (Marsella et al. 2023). In the late phase of IgE-mediated type I hypersensitivity they form dense perivascular clusters in the deeper dermis, resulting in acute skin inflammation (Benarafa et al. 2002). During delayed type IV b hypersensitivity, a phenomenon called T-cell

plasticity occurs. This leads to a shift from conventional Th2 to pathogenic effector Th2 cells (peTh2), with the production of high levels of IL-5, which promotes eosinophil differentiation, migration, activation, and survival (Marsella et al. 2023).

Receptors for several cytokines produced by lymphocytes and other inflammatory cells have been identified on nociceptor nerves in skin. Some of the cytokines produced by lymphocytes and other inflammatory cells have been reported to directly stimulate itching or to play a role in chronic itch. IL-31 is known to play a key role in pruritus development in IBH (Marsella et al. 2023; Olomski et al. 2019).

b) Effects of chronic exposure in IBH:

Lam et al. (2020) suggested that allergic mechanisms of IBH are influenced by the duration of allergen exposure, and somehow, it is likely that allergic horses when chronically exposed to the allergens may also experience a shift in their immune response.

The early onset of IBH is classified as an IgE-mediated or type I allergy, which is primary implied by the dominant role of conventional Th2 (cTh2) cells. As such, cTh2 expresses IL-4 and IL-13, promoting B cell class switch towards IgE antibody producing plasma cells and causing type I allergic reactions.

As the allergy develops, chronic allergen exposure during the natural course of IBH might shift the differentiation of cTh2 into pathogenic effector (peTh2) cells, highly positive for IL-5 and a major source for eosinophil production, recruitment, and activation (figure 9). Nevertheless, pruritus remains constant throughout the course of disease, however, its source may differ, from histaminic or non-histaminic origin (figure 9).

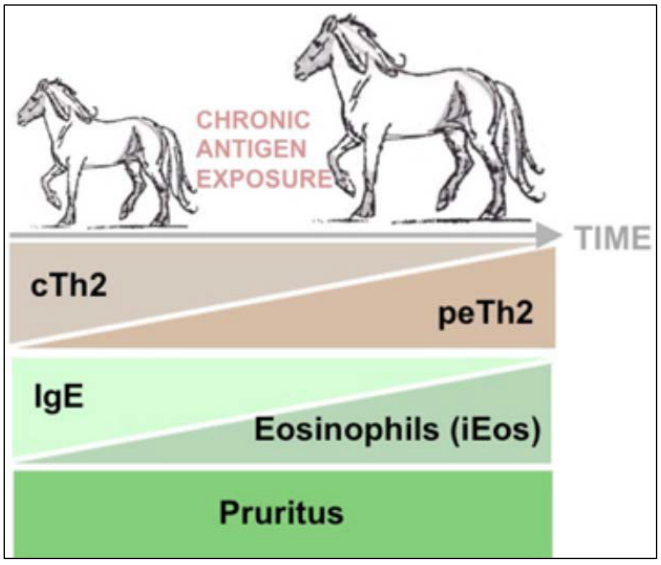

Figure 9: Model of switching allergy mechanisms through chronic allergen exposure during the natural course of IBH. (Adapted from: Lam et al. 2020)

c) The epithelial barrier

Little is known about the potential disturbance of the epithelial barrier in IBH (Marsella et al. 2023). It is a fact that IBH-affected horses present skin barrier's alterations at different levels namely, epithelial cell differentiation, epidermal lipid metabolism, and a decreased tight junction formation. Two mechanisms are involved in the skin's immune response in IBH:

a. Cellular response, with increased number of dendritic cells (Langerhans), IgE positive cells (mast cells) and CD4+ T cells.
b. Production of inflammatory mediators, with an increased expression of IL13 and IL 31 (very important in pruritus) and a decreased expression of FoxP3 mRNA (marker of regulatory immune response).

Cvitas et al. (2020) recently published a study, where thymic stromal lymphopoietin (TSLP) was hypothesized to play a role in the disturbance of the epithelial barrier. It was demonstrated an increased mRNA expression in skin biopsies of lesions of IBH-affected horses when compared with healthy controls. Though, it was not possible to determine whether IBH horses were sensitized to environmental allergens, or it could be due to overlapping atopic dermatitis (AD). IBH-affected horses frequently present simultaneously AD (Marsella et al. 2023) and it is known that the epithelial barrier plays an important role in atopic dermatitis (AD) in horses.

1.4.5. Clinical signs:

Clinical signs usually manifest in horses from two to four years of age and are distinctly seasonal (from spring to autumn in temperate climates), according to the presence of insects, accentuating at dawn and dusk. In addition, a marked exacerbation of clinical signs with age was observed (Scott & Miller 2003). Horses exposed to *Culicoides* allergens are often sensitized without, however, showing clinical signs of allergy. On the other hand, the manifestation of the disease at a later age may occur even with the presence of an early sensitization to the allergen (Wagner et al. 2009).

IBH is mainly characterized by the presence of alopecia and broken hairs, and cutaneous lesions with or without crusty papules, due to pruritus. Primary lesions are pruritic papules and/ or wheals. Some horses may develop secondary bacterial infections, enhancing pruritus and the other clinical signs (Marsella 2019).

Affected areas depend on the feeding habits of the *Culicoides* spp. (Marteles et al. 2019). However, lesions have a typical dorsal distribution, generally beginning at the mane and base of the tail, and can then extend to the face, neck, shoulders, and dorsal thorax. Less often we may observe a more ventral location of the lesions, mainly in the

thorax and abdomen, axillary regions, and inguinal area. Lesions can also extend to the limbs, face, intermandibular space, chest, and ventral midline (Fadok 2013; Scott & Miller 2003). The presence of atypical areas of pruritic lesions may be associated with specificities related to feeding patterns of different species of *Culicoides* (van der Rijt et al. 2008). Chronic and recurrent lesions are characterized by extensive alopecia, crusting and lichenification (hyperpigmentation, thickening of the skin and exaggerated skin lines). In the most severe cases, horses may lose all the hair from the mane and proximal third of the tail due to self-trauma. IBH horses chronically affected may present rugal folds, leukoderma (vitiligo-like depigmentation) and leukotrichia (white hairs in the affected regions) (Marsella et al. 2023).

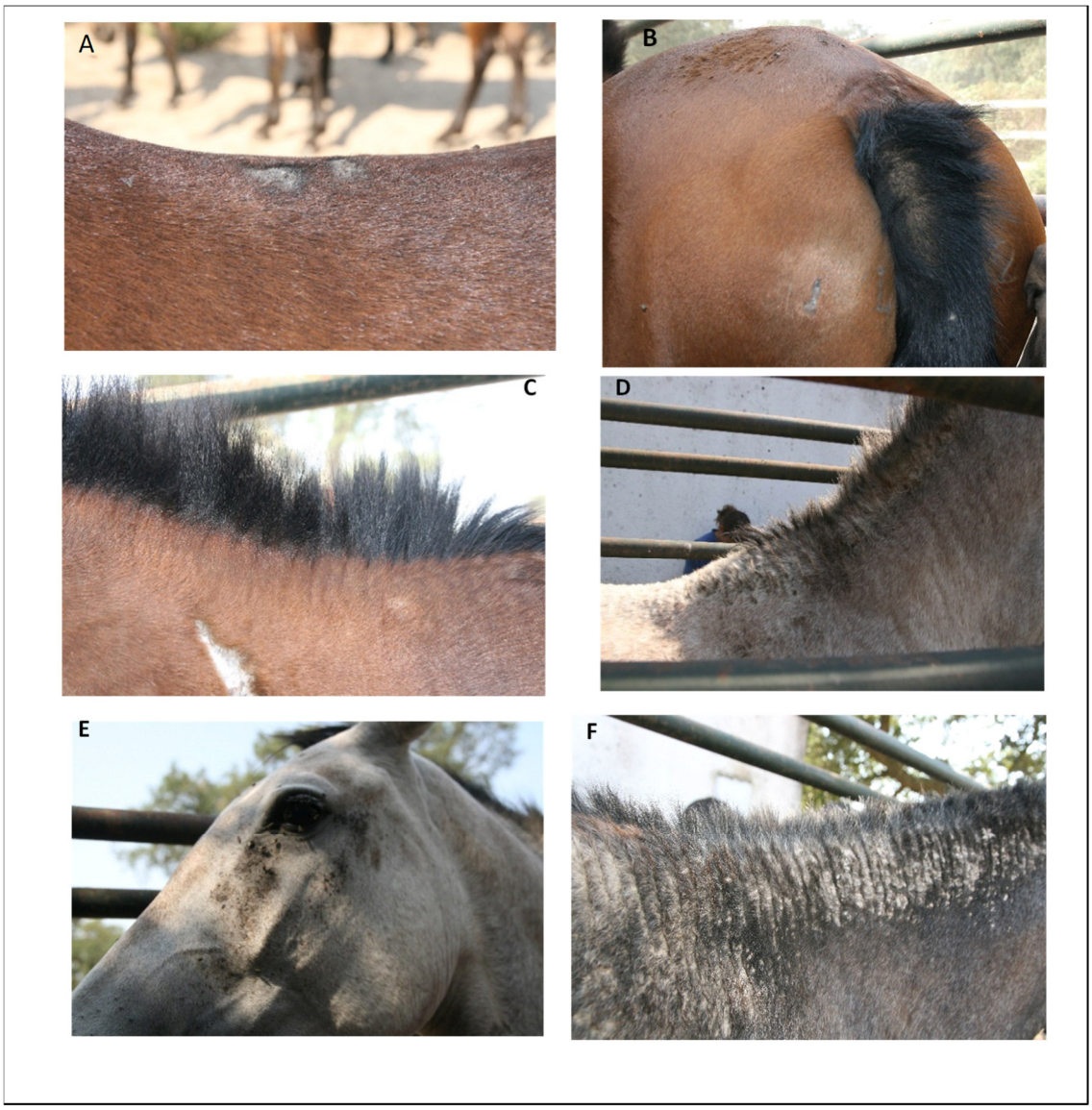

Figure 10: Lesions, type, distribution, and severity in IBH-affected horses, namely alopecia (A), broken hairs on the mane and tail (B, D and D), scales and hyperkeratosis (F).

IBH is one of the most pruritic diseases in horses. Pruritus can sometimes be extreme leading to self-trauma and hyperesthesia. Affected animals scratch and bite frequently, rubbing on objects present in the environment such as fences, poles, trees, pit walls, doors, mangers and drinking fountains. According to Scott & Miller (2003) "if pruritus involves the mane and tail, the horse will rub the areas until the hairs are broken or barbed, leaving a "buzzed mane" and "rat tail" appearance, respectively". This behavior perpetuates and aggravates skin lesions by enhancing the development of secondary infections (impetiginisation). Intense itching can even lead to behavioral changes such as anxiety, nervousness, restlessness or even aggression, making horses unfit to ride. They often lose weight due to constant irritability (Scott & Miller 2003). Thus, this dermatitis has a negative impact in animal welfare and also in the commercial value of affected animals (Schurink et al. 2009). IBH is of great importance for horses' well-being as the clinical signs can be very painful. Sometimes the horse cannot be trained or ridden, and in the most severe cases euthanasia may be considered (Anderson et al. 1988).

1.4.6. Diagnostic:

1.4.6.1 Histopathology:

Insect bite hypersensitivity (IBH) is mainly characterized by a skin infiltration with mast cells and eosinophils (van der Haegen et al. 2001). Chronic allergen exposure leads to an increasing role for eosinophils (Fettelschoss-Gabriel et al. 2018). Blood eosinophil counts correlate with IBH severity. Increased numbers of tryptase-positive and IgE protein-positive mast cells as well as IgE mRNA-expressing cells, have been demonstrated also in skin lesions of IBH supporting the role of IgE-mediated reactions in IBH (Fettelschoss-Gabriel et al. 2018).

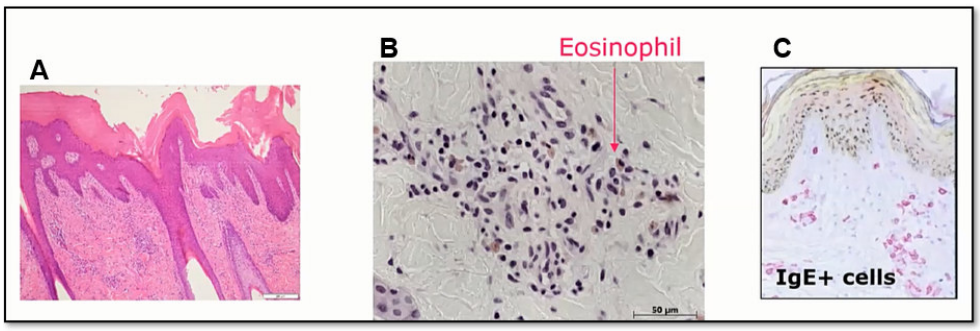

Figure 11: Acanthosis and hyperkeratosis (A); diffused infiltration with eosinophils (typical in IBH's skin biopsy) (B). HE staining, 40X magnification. Skin biopsy fragments stained with an IgE developed with fast red, also show presence of subdermal IgE positive cells, mainly mast cells (C). (Adapted from: Schaffartzik et al. 2012).

The histopathological findings consist mainly of epidermal orthokeratotic hyperkeratosis associated with acanthosis and spongiosis as well as dermal edema, vascular congestion, and eosinophilic inflammation (Oliveira Filho et al. 2012; Schild et al. 2003) as seen in figure bellow.

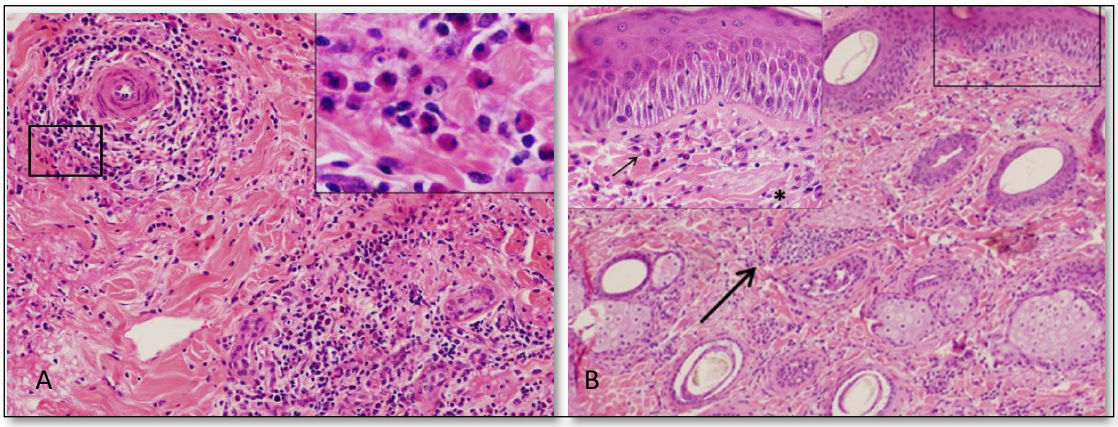

Figure 12: Histological skin section, HE stained, of a horse with insect bite hypersensitivity. A: Lympho-histiocytic inflammation with an elevated number of eosinophils (magnification 10X and 400X in detail); B: A site of intercellular edema (spongiosis) associated with inflammatory infiltrate foci, which are perivascular within the superficial dermis – arrows (magnification 5X and 15X in detail) (Adapted from: Oliveira Filho et al. 2012).

Confirming the clinical and epidemiological findings by performing histopathological examinations of the lesions may also contribute to the diagnosis of IBH (Scott & Miller 2003).

1.4.6.2. Allergy tests:

IBH diagnosis is still based on expert assessment comparing the presence of the representative lesions with the history of seasonal pruritus, mainly in the summer, and improvement of clinical signs in response to environmental management (Miller et al. 2019; Scott & Miller 2003; van Damme et al. 2012). However, misdiagnosis can occur and because clinical symptoms are absent in colder months, there is a great need for an accurate diagnostic. Various techniques are available to detect allergic sensitization, commonly designated as allergy tests, *in vivo* and *in vitro* tests. Until a few years ago allergy diagnostic tests included Intradermal tests (IDT) and serological assays to determine allergen specific IgE (Wagner et al. 2009), the later using mostly ELISA techniques, and others *in vitro* functional tests. These tests are mostly performed with non-standardized crude allergen extracts from *Culicoides* and other hematophagous insects, leading to currently unreliable and/or poorly repeatable diagnostic tests.

I. Functional in vivo tests:

a) Intradermal Tests (IDT):

IDT using multiple allergens have been used as a diagnostic tool in IBH-affected horses, for over 20 years (Wagner et al. 2009). A few studies have shown that IBH-affected horses more frequently have positive IDT results with *Culicoides* extract, and sometimes also with other insect extracts, than healthy control horses (Mueller et al. 2016; Schaffartzik et al. 2012).

Intradermal skin tests with r-allergens and Cul WBE have been shown to induce mast cell reactivity in horses with and without IBH (Sloet-van-Oldruitenborgh-Oosterbaan et al. 2009; Wagner et al. 2008).

IDTs are useful in IBH diagnostic, as they assess cell involvement in delayed hypersensitivity reactions (figure 13).

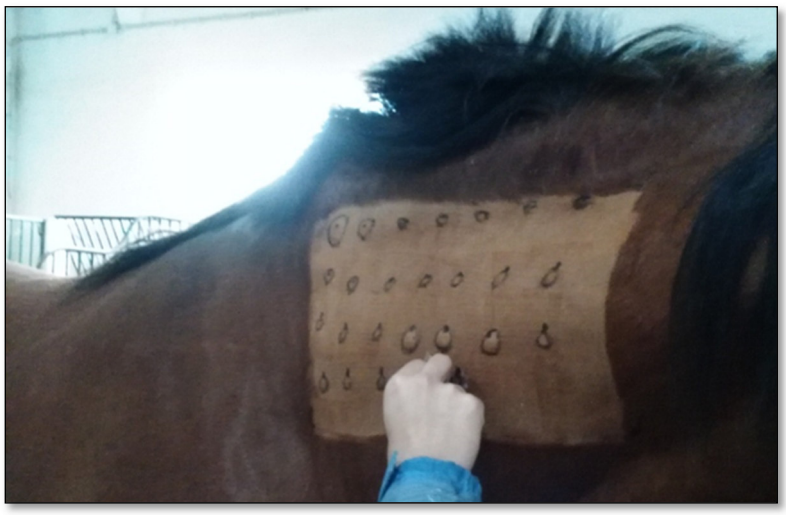

Figure 13: Intradermal test in a horse, with papules induced by allergens after 20 min.

b) Skin Prick tests (SPT):

Skin prick tests (SPT) are the indicated diagnostic test for type I immediate allergy, based on the medical history and clinical symptoms. SPTs can confirm sensitization in IgE-mediated allergic disease (Heinzerling et al. 2013).

SPTs are minimally invasive, inexpensive, and results are immediately available and reproducible when carried out by trained professionals. The recommended method to perform SPT includes the use of specific allergens, positive and negative controls, and interpretation of the tests after 15 to 20 minutes of inoculation, with a positive result defined as a determined cut-off value. When relevant allergens are inoculated in the skin, specific IgE bound to the surface receptors on mast cells, leading to their degranulation, and histamine and other inflammatory mediators are released. This induces the formation of a wheal/papule in the site of inoculation which can be quantified. Many different allergens can be tested simultaneously because the resultant

reaction to a specific allergen is localized to the immediate area of the SPT (Heinzerling et al. 2013).

SPTs provide an objective confirmation of sensitivity but should always be carefully interpreted based on the previous history and clinical signs. SPTs are also very useful in providing information about the correct allergen(s) prescribed for allergen specific immunotherapy (ASIT) (Heinzerling et al. 2013).

SPTs are more specific than Intradermal tests (Wood et al. 1999). IDTs are more labour-intensive and require more precise techniques when compared with SPTs. Also, IDTs have occasionally been associated with serious systemic allergic reactions and even death from anaphylaxis (Riezzo et al. 2010).

In vitro tests may be less sensitive and/or less specific than SPTs depending on the method and the allergens tested (Chung et al. 2010).

Even though SPTs are the gold standard in the diagnostic of type I hypersensitivity reactions in human medicine, still no studies about the use of SPTs in IBH diagnosis were published, until the present experimental study (figure 14). The use of SPTs were first described by Tilley et al. (2012) in the study of allergens concerning equine asthma.

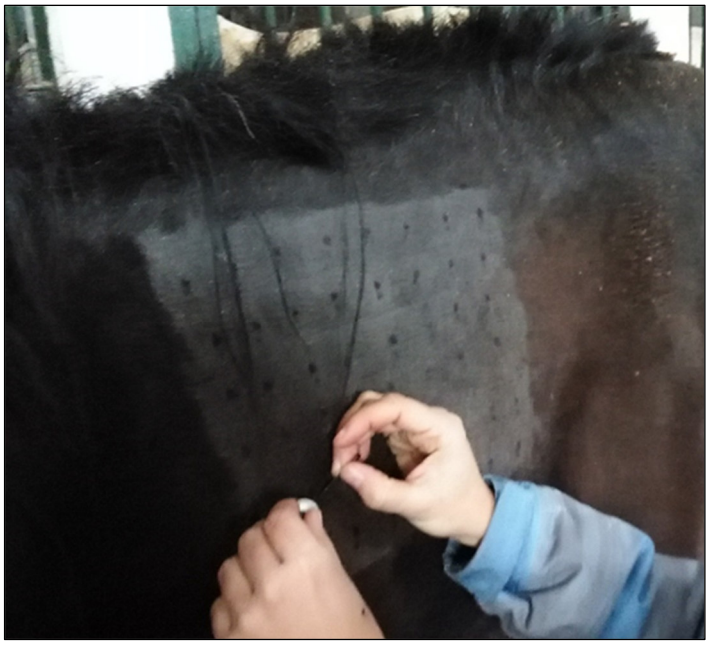

Figure 14: Skin Prick test (SPT) performed in an IBH-affected horse.

II. Functional *in vitro* tests:

Functional *in vitro* diagnostic tests that are available to test the allergenicity of the allergens in allergic patients are:

a) Cellular assays (histamine or sLT release):

The Cellular Antigen Stimulation Test (CAST) is performed on blood samples and based on inflammatory mediators' release from peripheral blood leukocytes, namely sulfidoleukotriene (sLT) (Baselgia et al. 2006), using *Culicoides* allergens (*C. nubeculosus, C. sonorensis* or *C. obsoletus* extracts) (Langner et al. 2008; van der Meide et al. 2012). sLT are more frequently released in IBH-affected horses than in healthy controls (Mueller et al. 2016). A previous study had shown a high correlation between *in vitro* sLT and histamine release in peripheral blood leukocytes from horses after a 40-minute stimulation with *Culicoides* allergens (Marti et al. 1999) indicating that the sLT release can be used to detect immediate type I reaction *in vitro* to certain allergens. These tests use different allergens such as whole-body extract (WBE) and recombinant allergens (r-allergens) (Ferroglio et al. 2006; Marti et al. 2008).

Histamine release assays revealed basophil degranulation in response to *Culicoides* WBE stimulation of cells from the blood of both IBH and healthy horses (Baselgia et al. 2006; Wagner et al. 2008). Basophils comprise less than 1% of white blood cells in peripheral blood (Chirumbolo et al. 2018). Recent studies report higher percentage of basophils in allergic horses when compared to healthy controls (Raza 2020). Equine basophils rapidly release interleukin 4 (IL-4) in response to *Culicoides* specific stimulation of peripheral blood mononuclear cells (PBMC) (Raza 2020).

b) Specific serological IgE determination:

Allergy serological tests are based on the knowledge that allergic individuals have higher allergen specific-IgE levels in their serum than healthy individuals. Total serum IgE detection is used for humans, but in animals the detection of allergen specific IgE is the one used. In case of IBH, serological assays have shown higher serum IgE concentrations against *Culicoides* salivary allergens in IBH-affected horses. Nevertheless, serological allergen specific IgE detection show sensitization not allergy, as some animals may have allergen specific IgEs but may not present clinical signs.

The detection of allergen specific IgE antibodies in serum is often performed by Enzyme Linked Immuno Sorbent Assay (ELISA). Wilson et al. (2008), point out the fact that greater IgE detection occurs when allergens from native "wild" species of *Culicoides* are used.

1.4.7. Treatment and control:

IBH management involves insect control and the use of topical and systemic antipruritic and anti-inflammatory agents (glucocorticoids, antihistamines, omega-3 and fatty acids).

Systemic long-term treatment with corticosteroids may have potentially side-effects, and antihistamines are not licensed and are not very effective. On the other hand, essential fatty acids may be considered with prolonged use duration.

Topical treatments can also alleviate clinical signs of IBH, for example use of creams containing omega-3-fatty acids, humectants, and emollients has been shown to improve clinical lesions of IBH but did not influence the pruritic score (Huhmann et al. 2019). Topical treatments may also include repellents (topical insecticides), antibacterial and/or antifungal shampoos and topical steroids for small areas of inflammation. Shower with cool water may be beneficial, as it rehydrates the skin, improves the integrity of the epidermal skin barrier, causes vasoconstriction, which minimizes the number of inflammatory mediators reaching the skin. Also, cool water showers may wash off allergens, reducing percutaneous absorption, and any bacteria or yet, helping to control secondary infections. Cutaneous application of paraffin or mineral oil prevents *Culicoides* from contacting and biting the epidermis (Scott & Miller 2003).

Affected horses must remain in pastures with copings and head and neck protectors and must be collected from pasture before sunset, as seen in figure bellow.

Figure 15: Net blankets to prevent insect bites.

It is also possible to use fans above the horses' body level to disperse the *Culicoides*, whose flight power is reduced. Mostly, treatment relies on avoidance or decreased insect exposure of the horses and insect management in the environment (Marsella et al. 2023). Different approaches are described, namely:

- Fly rugs covering the horse’s head, neck, and tail base.
- Repellents with permethrin, and simultaneously vitamin E (when applied simultaneously prevents permethrin-induced paresthesia).

- Tags and collars repellents (can be tied into the mane and tail).
- Topical oil-based products may help by providing a barrier on the skin.
- Installation of large fans to help air circulation in the stables and insects' dispersion as midges are poor fliers.
- Good ventilation of the stables.
- Insecticide sprays released by a timer can be used within enclosed areas.
- Use midge trapping machines in the proximity of the stables or shelters.

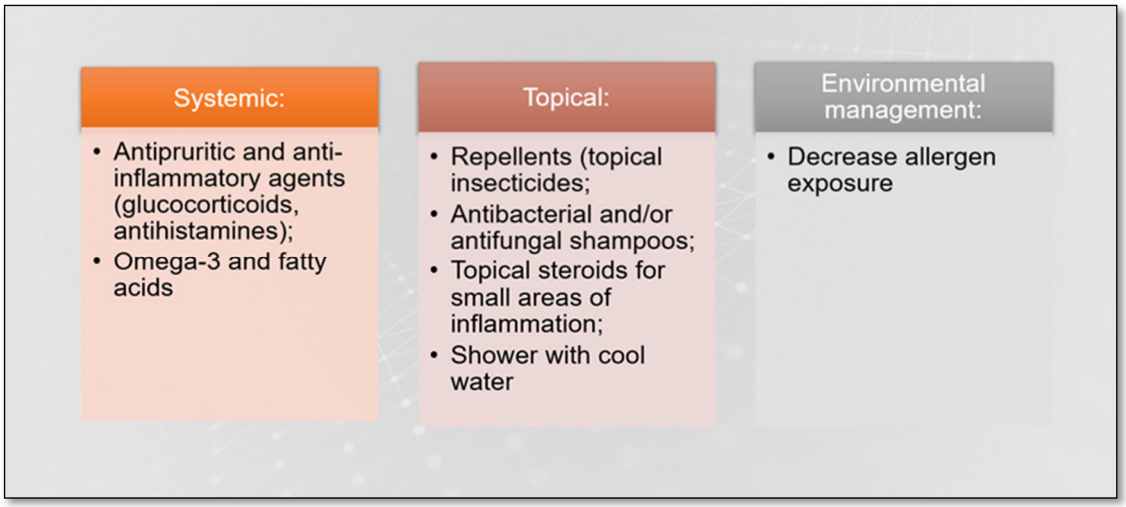

Figure 16: Schematic representation of IBH treatment and environmental management.

Overall, the main disadvantage of current treatment options is that they fail to target the mechanisms underlying the allergy. Also, treatments often only present a limited and short activity and may have several side-effects. Management and treatment of IBH is challenging, and the available approaches to control IBH have limitations (Marsella et al. 2023). Control plans as well as therapeutic approaches must be tailored to the individual situation and may involve some adaptation.

To date an effective treatment for this disease remains elusive (Mueller et al. 2016). However, as IBH is an allergic disease mostly IgE mediated, there is the possibility of developing specific immunotherapy (Scott & Miller 2003; Wagner et al. 2006) and it is very important to develop research in this area.

1.4.8. Immunotherapy:

Allergen-specific immunotherapy (ASIT) is the only causative treatment of allergy to the moment. It is based on the repeated administration of the disease-eliciting allergens until the sensitivity to the administered allergens is reduced through various immunological mechanisms (Focke et al. 2010). According to Marsella (2013) immunotherapy is a long-term treatment option.

In case of type I hypersensitivities allergen-specific immunotherapy (ASIT) acts by reducing the progression of the disease and decreasing the risk of development of new allergic conditions (Devillier et al. 2019; Fujita et al. 2012).

The mechanism underlying ASIT is a shift of the immune response from Th2 towards a regulatory and/or Th1 response immune response. In response to ASIT, IgG antibodies are produced, specially IgG4, which blocks the binding of the allergen with the specific IgEs preventing mast cell degranulation (Marsella et al. 2023). Additionally, ASIT is associated with decreased production of IL-4 and IL-5, cytokines produced by Th2 CD4+ T cells (Akdis et al. 2006). Naïve CD4+ T helper cells differentiate into T helper type 1 (Th1) cells and/or T regulatory cells (Tregs). The Th1 cells produce IFN-γ, and instruct the B cells to undergo class switching, and start producing IgG and IgA. Additionally, Treg cells produce regulatory cytokines IL-10 and TGFβ (figure 17) (Akdis et al. 2014; Jonsdottir et al. 2019).

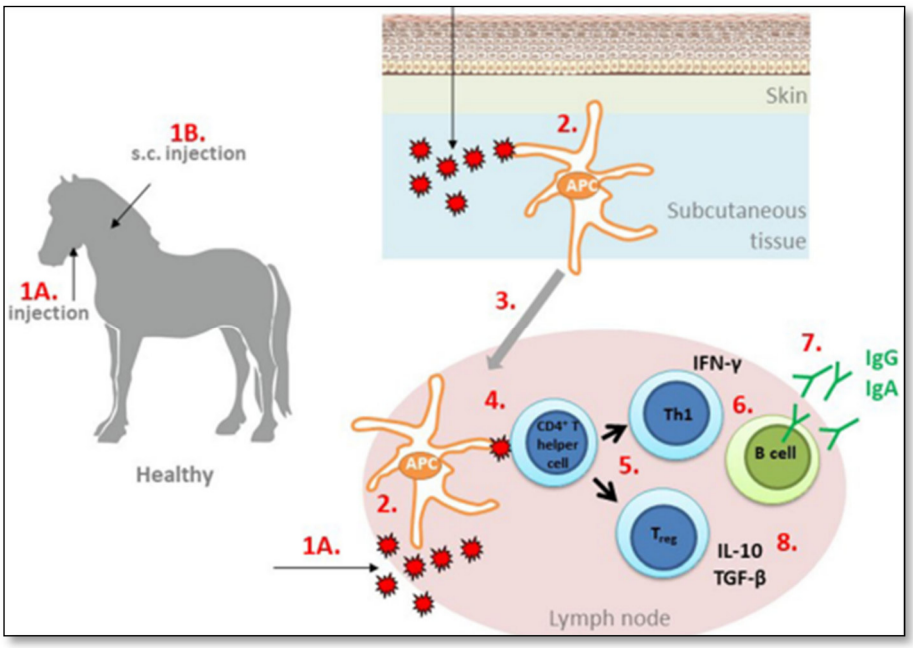

Figure 17: Simplified scheme of preventive allergen specific immunotherapy (Adapted from: Jonsdottir et al. 2019). The vaccine, consisting of r- Cul allergens, is injected subcutaneously, intradermal or into the submandibular lymph node. Antigen-presenting cells (APCs) take up the allergens and bring them to the draining lymph node. In the lymph node, APCs present allergen peptides on MHC class II to naïve CD4+ T helper cellswhich differentiate into T helper type 1 (Th1) cells and/or T regulatory cells (Tregs). Th1 cells produce IFN-γ, and instruct the B cells to undergo class switching, and start producing IgG and IgA antibodies, whereas Treg cells produce regulatory cytokines IL-10 and TGFβ (Jonsdottir et al. 2019).

ASIT has proven to be relatively inexpensive, highly effective, and long-lasting when high-quality antigens are used (Zhernov et al. 2019). Allergen extracts have been used with a good outcome. However, the success of the immunotherapy depends on the quality of the extracts used. Standardization of allergen extracts can be difficult, and may present variations in the amount, potency, and immunogenicity. Also, major allergens may be lacking (Curin et al. 2017). The use of natural allergen extracts, for example *Culicoides* whole body extract (WBE), can result in local and severe systemic side effects. These extracts are composed of a mixture of different proteins and other substances (Focke et al. 2010), that may decrease their immunogenicity (Focke et al. 2008). The use of recombinant allergens (r-allergens) can overcome some problems related with natural extracts and improve the success of allergen specific immunotherapy. Hellberg et al. (2006) stated that each horse has a unique pattern of antibody binding, so specific immunotherapy with recombinant salivary gland allergens will vary from horse to horse, and a tailored immunotherapy to each individual case may be needed.

The ability to prevent the development of IBH clinical signs by vaccinating horses with recombinant allergens before natural exposure is unknown. Intradermal, intralymphatic and oral exposure to recombinant allergens leads to an IgG response that appears to partly block binding of *Culicoides*-specific IgE in IBH horses (Eliane Marti, personal communication).

Active immunization against interleukins (IL5 and IL31) is being developed, with good outcomes, leading to an improve of IBH clinical signs. Some authors report absence of side effects; no eosinophilia is observed as well as no evidence of auto-reactive T-cells (Jonsdottir et al. 2019). Also, no evidence of problems caused by immune complexes, and no changes in hepatic and renal parameters were reported.

1.4.9. Allergens in IBH:

Allergens' present on *Culicoides spp.* saliva are the main cause of IBH, and this importance has enhanced research to identify the salivary proteins with major implication in IBH (Russell et al. 2009).

In vitro tests show that there is some variation in the different *Culicoides* specie's ability to cause an IgE mediated response. Nevertheless, there are also evidences that some of these allergens are common to different *Culicoides* species, as IBH-affected horses may present clinical signs in the presence of both native and exotic *Culicoides* species.

Allergens tests show that IBH-affected horses can either react to native/local and exotic species of *Culicoides*, but local species induce a stronger IgE-mediated immune response, and better sensitivity assays, as seen in figure bellow (van der Meide et al. 2012).

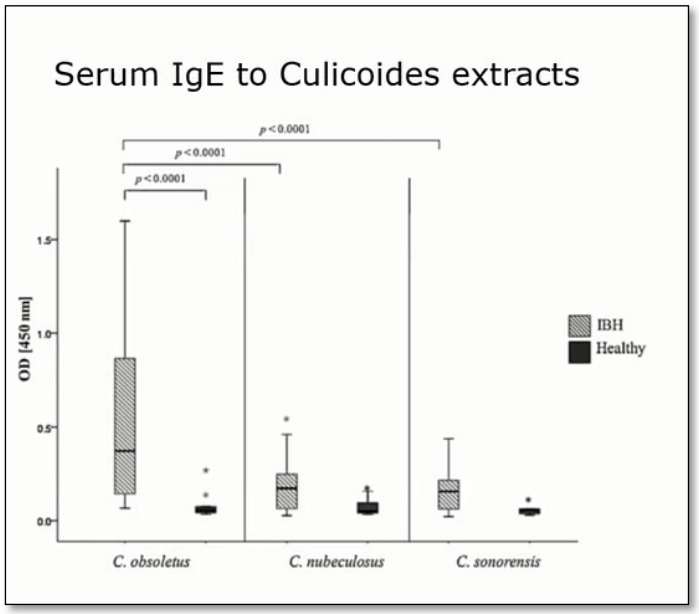

Figure 18: Determination of serum IgE in IBH-affected and healthy horses to different *Culicoides* species extracts in the Netherlands. IgE levels were higher in IBH-affected horses for C. *obsoletus*, the most prevalent native *Culicoides*, when compared to *C. nubeculosus* and *C. sonorensis.* (Adapted from: Van der Meide et al. 2012).

There have been identified 54 new protein sequences from 19 *Culicoides nubeculosus* salivary glands, and many of these are implicated in IBH (Campbell et al. 2005; Russell et al. 2009).

The first *Culicoides* allergen to be isolated and published was Cul s 1, maltase from *C. sonorensis*, expressed in insect cells using the Baculovirus system (Langner et al. 2009). Ten allergens were then isolated from 1400 dissected salivary glands of laboratory bred female of *C. nubeculosus* using a phage display cDNA library. These were termed Cul n 2-Cul n 11. Cul n 1 had been isolated before (Schaffartzik et al. 2010). The sensitivity of the allergens ranged from 13-57%. Major allergens identified were Cul n 1-Cul n 4 (Schaffartzik et al. 2011). Van der Meide et al. (2012) isolated seven genes from *C. obsoletus* using alignment to *C. sonorensis* and *C. nubeculosus* sequences, and the allergens were expressed as recombinant proteins in *E. coli*. The seven allergens were named Cul o 1-Cul o 7.

Eleven recombinant proteins from *C. nubeculosus* have been expressed and recognized as allergens for IBH, with a frequency of IgE sensitization against the single allergens ranging from 13-57% (Schaffartzik et al. 2011), and seven recombinant

proteins from *C. obsoletus*, with a percentage of positive tested IBH affected horses ranging from 38-67 % (van der Meide et al. 2013).

According to Jordonttir et al. (2019) a lot of work has been done into identifying the major allergens in IBH and producing them as pure recombinant proteins. Allergens from the three *Culicoides* species *C. nubeculosus* (Schaffartzik et al. 2010; Schaffartzik et al. 2011), *C. sonorensis* (Langner et al. 2009), and *C. obsoletus* (van der Meide et al. 2013; Peeters et al. 2013) have been identified and produced. All of them have been expressed in *E. coli*, some in insect cells and barley (van der Meide et al. 2018) With a first available panel of allergens, Marti et al. (2015) have set up a protein microarray for detection of the IgE antibodies of IBH-affected horses, which has now been improved with the addition of further *Culicoides* allergens, resulting in a total of 27 different *Culicoides* salivary proteins, as seen in figure bellow.

Allergens	*C. nubeculosus*	kDa	Allergens	*C. obsoletus*	kDa	Allergens	*C. sonorensis*	kDa
Cul n 1	Antigen 5–like protein	25	Cul o 1p	Kunitz protease inhibitor		Cul s 1	Maltase	69
Cul n 2	Hyaluronidase	47	Cul o 2p	D7-related salivary protein				
Cul n 3	Putative cystein endopeptidase	45	Cul o 1	Maltase				
Cul n 4	Secreted salivary protein	18	Cul o 2	Hyaluronidase				
Cul n 5	Secreted salivary protein	46	Cul o 3	Antigen 5–like protein				
Cul n 6	Secreted salivary protein	17	Cul o 4	Trypsin				
Cul n 7	Unknown salivary protein	21	Cul o 5	Unknown salivary protein				
Cul n 8	Maltase	69	Cul o 6	D7-related salivary protein				
Cul n 9	D7-related salivary protein	16	Cul o 7	Secreted salivary protein				
Cul n 10	Secreted salivary protein	48						
Cul n 11	Trypsin	30						

Figure 19: Table of published r-*C. nubeculosus* allergens (Adapted from: Jordonstir et al. 2019).

Allergens are named by the first three letters of the genus, the first letter of the species name and a number according to the nomenclature system allergens (http://www.allergen.org).

1.4.10. Genetics background and other risk factors

IBH is a multifactorial disease, where both environmental and genetic factors play an important role in the onset and establishment of the disease (Marsella et al. 2023).

IBH has a polygenetic nature, and even though the genes that are linked to IBH have not been identified (Marsella et al. 2023), some studies have found that certain breeds are more susceptible than others. Eriksson et al. (2008) studied Swedish-born Icelandic horses and found a prevalence of IBH of 8% in the breed, and Schurink et al. (2012) identified several genomic regions associated with IBH in both Shetland pony mares and Icelandic horses.

IBH has a genetic background, and it has been shown that the offspring has higher risk to develop the condition if the mare is IBH-affected (Eriksson et al. 2008).

Few studies have been published on how to reduce the risk of IBH through selective breeding. So far, there is no evidence of a simple dominant or recessive mode of inheritance, which makes selective breeding difficult to achieve, as there is uncertainty as to what genes to selectively breed for.

Nevertheless, Schurink et al. (2012) suggested that the knowledge of the genes associated with IBH may contribute to understanding IBH's biology, enabling more efficient therapy, prevention, and selection in order to decrease its prevalence.

Steinman et al. (2003) and Van Grevenhof et al. (2007) stated that coat colour had no significant effect on IBH incidence. Even though it seems to affect all breeds of horses, there are some breeds that seem to have a higher risk of developing IBH, namely Icelandic horses born in Iceland after being exported to countries where *Culicoides* are endemic, probably due to the absence of *Culicoides* in Iceland (Wagner 2015). It remains to be determined whether these apparent breed differences are due to genetic factors, environmental factors, or a combination of the two, and whether there are specific genes that contribute to disease susceptibility.

Vychodilova et al. (2013) found that the presence and expression of IBH can be influenced by many non-genetic factors, including the degree of exposure to insect bites, seasonal and climatic variations between years. Concomitant health conditions may also have an effect and Kehrli et al. (2015) demonstrated that occurrences of IBH were increased in horses that suffered from Equine Asthma when compared to healthy individuals.

IBH has a genetic basis, but it is a very complex disease, with multiple genes involved and even though various genetic studies have been performed further investigation needs to be done in order to identify the genes responsible for the disease. Also, being a multifactorial disease the development of genetic tests to diagnose IBH is not possible (Eliane Marti, personal communication).

1.4.11. Economic impact and importance of IBH in horses' welfare

IBH is one of the equine diseases that most commonly affects the quality of life of horses and is therefore a welfare issue with the potential to cause significant economic losses for horse owners with IBH-affected animals (Hallamaa 2009).

Lusitano horse breeding plays an important role in the Portuguese economy and riders are making increasing use of the Lusitanos around the world (Cordeiro Raposo 2002). Since IBH is a recurrent disease with no effective treatment so far, it will inevitably lead to economic losses, due to reduced commercial value of the affected horses, as well as extra costs to control the disease. Moreover, euthanasia may be considered when the symptoms are too severe.

IBH is a serious condition that can cause a significant level of pain and discomfort in affected horses and is therefore a welfare concern. Control of IBH is difficult due to the complex interplay of both hereditary and environmental factors in its pathogenesis (Peeterson 2009). Its tendency is to get progressively worse in succeeding years requiring continued research to establish effective prevention and treatment for the disease.

To summarize IBH:

In accordance with Clinical consensus guidelines of the World Association for Veterinary Dermatology (Marsella et al. 2023) for IBH it is possible to summarize and highlight some aspects of the disease:

- IBH is multifactorial disease resulting from a combination of environmental and genetic factors. Warm humid climates with heavy exposure to *Culicoides* and proximity to water increase the risk for development of IBH in predisposed horses. Lack of exposure to *Culicoides* in the early stages of life significantly increases the risk for development of clinical disease.
- Heritability varies among breeds, yet it is widely accepted that predisposition for IBH is genetically inherited as a polygenetic disease.
- IBH is an extremely pruritic disease, and papular eruptions, hives, eosinophilic granulomas and hyperreactive airways can be seen in horses with IBH. The distribution of clinical signs often reflects the feeding sites of the *Culicoides spp.* present in the geographical region and can be dorsal, ventral or a combination of both. The most common affected areas are face, ears, mane, tail, chest, ventral abdomen, and legs. Secondary infections are common and can contribute significantly to the level of pruritus.
- The role of IgE, the conventional Th2 response, and the effector Th2 response promoting eosinophilia is documented and accepted in the pathogenesis of IBH.
- IBH is characterized by the existence of a lymphocytic response with an increase of Th2 cells and a decrease of Tregs cells. Th1 response is considered protective against IBH.
- IL-4, IL-5, IL-13, and IL-31 are cytokines of major importance in IBH and as target for therapeutic intervention (IL-5, 31).
- Insufficient information is available to draw conclusions on the role of skin barrier dysfunction in the pathogenesis of IBH.

- Currently, nine *Culicoides* antigens have been identified as Major Allergens (MA) in IBH, through studies focusing on IgE binding from sera of affected horses. To standardize the nomenclature of the *Culicoides* allergens (recombinant and/or extracts) is essential to avoid confusion.
- IBH is a clinical diagnosis based on compatible history, clinical signs, exclusion of other pruritic skin diseases and favorable response to insect control measures.
- Use of insect repellents and other means of insect avoidance largely remains the most effective long-term approach for treatment of IBH in clinical practice. Current evidence does not support the use of antihistamines as a monotherapy in any clinical phases of IBH.
- Evidence is still lacking in recommending immunotherapy as a treatment for IBH and further studies are needed to explore benefits of ASIT for the treatment of IBH.

2. Objectives and Theme Relevance:

2.1 Theme Relevance:

The Lusitano horse, which is a considerable and important part of the national economy, is mainly raised in an extensive outdoor regime with high insect exposure. The team's clinical experience in the field revealed a considerable number of cases involving Lusitano horse stud farms, sometimes affecting many animals from the same holding. The severity of the clinical signs presented by the affected horses is often reflected in economic losses due to permanent skin lesions. Additionally, there are obvious negative effects on animal welfare. Over time, horses' owners have expressed their concern and interest in solving this problem.

In the past, members of this project developed studies with relevance in IBH, namely:

- Production and characterization of the allergens involved in IBH (Schaffartzik et al. 2010 and 2011).
- *In vitro* identification and quantification of sulfidoleukotrienes (sLT) released from leukocytes present in the blood of IBH-affected horses.
- Use of skin prick tests (SPT) as a diagnostic method for use in horses with allergic disease (Tilley et al. 2010; Tilley 2011).

So far there have been no studies on IBH in Portugal, although the environmental characteristics in the country have been proven to be favorable to *Culicoides* activity (Ramilo et al. 2012).

We intended to develop a research project that could contribute to a more effective diagnostic protocol for IBH in Lusitano horses and were fortunate to earn the collaboration of a Swiss team for this purpose.

Therefore, this project represents the first step towards an effective resolution of the drawbacks associated with IBH and is the first reported study of IBH in Lusitano horses comparing *in vitro* (serological determination of allergen-specific IgEs and sLT release essay - CAST) and *in vivo* (skin allergy tests, both Skin Prick Tests – SPTs, and Intradermal Tests - IDTs) diagnostic tests, in their ability to distinguish between healthy (control, n=30) and IBH-affected (Test, n=30) horses.

In this way, this study contributes to the improvement of IBH's diagnosis by using skin tests, namely SPTs, and may also be a step forward in the development of specific immunotherapy for IBH.

2.2 Objectives of the study:

The main objectives of this study were:

- To characterize IBH affected Lusitano horses in Portugal mainland, through a control case study involving 30 animals IBH affected (test group), and 30 healthy animals (control group). For this it was done systematically:
  I. Completion and evaluation of standardized questionnaires, which includes data on clinical history, the environment, and associated clinical signs. The standardized questionnaire was based on a previous protocols described by other authors (Babel et al. 2014; Lanz et al. 2017).
  II. Clinical examination of the horses.
  III. Photographic record of lesions.
- To identify *Culicoides spp.* found in the studied stud farms in Portugal mainland, which may be related to IBH in the studied horses.
- To evaluate *in vivo* relevance of allergens by performing skin prick tests (SPT) and intradermal tests (IDT).
- To perform and evaluate two *in-vitro* diagnostic tests:
  (i) quantification of sulfidoleukotrienes (sLT) produced by peripheral blood leukocytes using CAST-ELISA.
  (ii) serum specific IgE assay using a specific ELISA technique, described by Eliane Marti (personal communication), for *Culicoides nubeculosus* and *Culicoides obsoletus* allergens.

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Chapter II:

Characterization of a population of Lusitano horses living in Portugal mainland, taking part in a case control study involving 30 IBH affected animals (test group) and 30 healthy ones (control group): questionnaire evaluation.

Characterization of a population of Lusitano horses living in Portugal mainland, taking part in a case control study involving 30 IBH affected animals (test group) and 30 healthy ones (control group): questionnaire evaluation.

1. Abstract

Insect bite hypersensitivity (IBH) is an IgE-mediated allergic skin disorder in horses and ponies caused mainly by *Culicoides spp* midges. IBH occurs in most breeds worldwide and prevalence can vary between 3 and 60% depending on different factors, namely environment and genetics. The prevalence of IBH in Portugal, particularly in Lusitano horses, is presently unknown. The Lusitano horse has an important role in the Portuguese economy and our teams' experience in the field revealed a considerable number of IBH cases involving Lusitano horse stud farms, resulting in serious owner's concern and economic losses.

In this study we established inclusion/exclusion criteria in order to assess and characterized 30 IBH-horses and 30 cohabitant controls, lodged in 11 Lusitano Stud farms, distributed throughout Portugal mainland. We carried out standardized owner questionnaires, clinical exams, and photographic registers in order to evaluate the severity of the clinical signs and skin lesions of the IBH-affected Lusitano horses. Furthermore, the horse's environment was characterized.

It was possible to confirm that all horses included in the present study complied with the set inclusion/exclusion criteria and the characterization of these horses and their environment should contribute to a better knowledge of IBH in Lusitano horses that were living in Portugal mainland.

Keywords: Lusitano horses, IBH, epidemiological characterization, Portugal.

2. Introduction

Insect bite hypersensitivity (IBH) is an IgE-mediated allergic skin disorder in horses and ponies caused mainly by *Culicoides spp* midges. It is also known as summer eczema, sweet itch and Queensland itch (Braverman et al. 2008; Broström et al. 1987; Eriksson et al. 2008; Riek et al. 1954). The onset of the disease occurs usually between 2 and 4 years of age (Eriksson et al. 2008; van den Boom et al. 2008) and the most common symptoms are severe pruritus with edema, papules, crust formation, and alopecia (Schaffartzik et al. 2012). Severely affected horses cannot be shown, are not suitable to ride due to the great discomfort they endure and are sometimes euthanized (Fadok et al. 1994; Gorkel et al. 1998).

IBH is a serious problem worldwide except for Antarctica, New Zealand, and Iceland (Mellor et al. 2000). Although IBH occurs in most breeds, it is more prevalent in

Shetland ponies, Friesian, and Icelandic horses (Schurink et al. 2009; Schurink et al. 2011). IBH prevalence varies, in average, between 3 and 60% depending on the environment and on the genetic background of the horse (Schaffartzik et al. 2012; Muller et al. 2016). For example, in the UK (Schurink et al. 1973) the prevalence of the disease can vary from 3-11%, being 37% in Germany (Littlewood et al. 1998), 10-60% in Australia (Riek, 1954) and up to 71% in the Netherlands (Van Grevenhof et al. 2007).

On a breed level, horses imported from Iceland may have a prevalence of more than 50%. This increased prevalence in imported Icelandic horses compared to other breeds or EU-born Icelandic horses is not yet fully understood, but it is suggested that the lack of *Culicoides spp.* exposure before export and the increased environmental pressure after export, may play an important role (Björnsdóttir et al. 2009; Broström et al. 1987).

The Lusitano horse is known for its great functionality and versatility (Cordeiro 1997), taking an important part in the Portuguese economy. It is mostly raised in an outdoor regimen all year round and subjected to a high insect exposure. Our team's clinical experience in the field revealed a considerable number of IBH cases involving Lusitano horse stud farms, sometimes affecting many animals from the same holding, including different generations of genetically related horses, as previously found in the Dutch Shetland pony population (Schurink et al. 2009). Also, as has been reported in other breeds, the severity of the clinical signs presented by the horses and the obvious negative effects on animal welfare are often reflected in economic losses (Brostrom et al. 1987; Fadok and Greiner 1990).

The prevalence of IBH in Portugal, particularly in Lusitano horses is presently unknown. However, the environmental characteristics of the national territory are favorable to the activity of the hematophagous insects most frequently implicated in this dermatitis. *Culicoides* are present throughout the national territory, especially in the geographical area bellow the Tagus River (Ramilo et al. 2012), where the largest number of stud farms are also located.

Aim of the study

The aim of this study was to assess and characterize 30 IBH-horses and 30 cohabitant controls, lodged in 11 Lusitano Stud farms distributed throughout Portugal mainland, through standardized owner questionnaires, clinical exams and photographic registers, in order to evaluate the existence and severity of the clinical signs and skin lesions and confirm the compliance with inclusion/exclusion criteria. Furthermore, we aimed to characterize the horse’s environmental management, namely the type of housing, in

order to obtain a better epidemiological knowledge of IBH in Lusitano horses living in Portugal mainland.

3. Material and Methods

The study was performed from 2014 to 2016, during early spring, when the *Culicoides* were starting to become active. In total, 11 Lusitano stud farms with a history of IBH were selected from several regions located in the center and south of mainland Portugal. The horses included in this study originated from veterinary referrals and were accompanied with owner reports and full clinical examinations.

3.1. The horses' inclusion and exclusion criteria:

In total, 60 horses were evaluated. Males and females were included, from several age groups.

The horses were divided in two groups:

- Test Group (T) - 30 IBH-affected horses presenting clinical signs at the time of the evaluation.
- Control Group (C) - 30 healthy horses with no clinical signs of IBH, living in the same conditions and in the same stud farm, or very close to the T horses.

The inclusion criteria of the test horses (T) were:

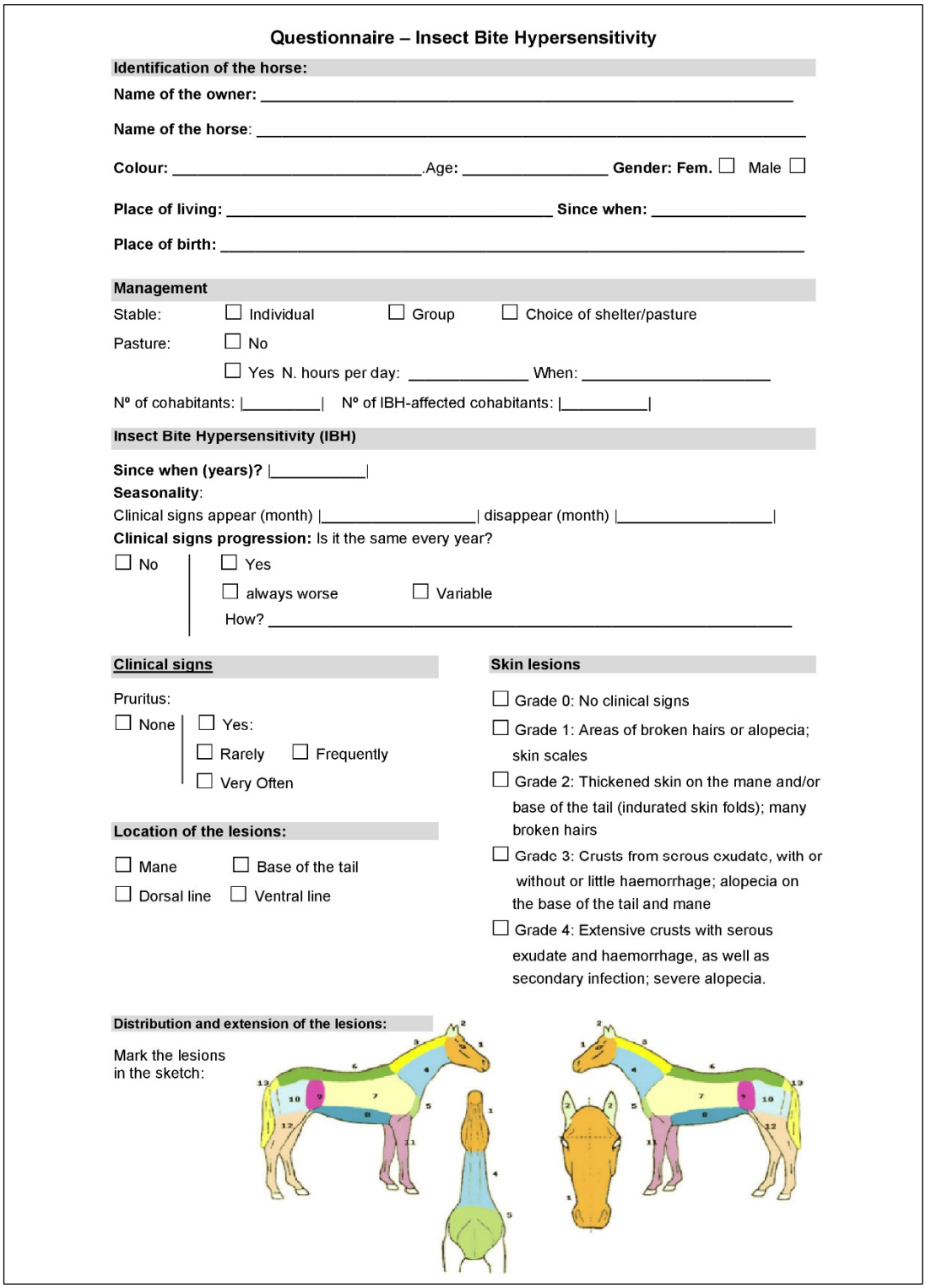
- Must be ≥ 1 year old.
- Must present signs of seasonal pruritic dermatitis.
- Must have ≥ grade 1 lesions: presence of broken hair at the base of the tail and at the base of the mane; skin scales.
- Must have a minimum duration of one year, or from at least the previous equivalent season.

The exclusion criteria were:

- Horse breeds, other than Lusitano.
- Gestating mares.
- Horses that presented other skin diseases.
- Horses that presented systemic signs of other diseases.

3.2. Questionnaires:

A standardized questionnaire was developed by our group, based on previously described questionnaires (Lanz et al., 2017). Different parameters were characterized, namely age, sex, coat colour, clinical signs (pruritus, cutaneous lesions severity and distribution) and housing (management) (Figure 1).



Questionnaire – Insect Bite Hypersensitivity

Identification of the horse:

Name of the owner: _____

Name of the horse: _____

Colour: _____.Age: _____ **Gender: Fem.** ☐ Male ☐

Place of living: _____ **Since when:** _____

Place of birth: _____

Management

Stable: ☐ Individual ☐ Group ☐ Choice of shelter/pasture

Pasture: ☐ No

☐ Yes N. hours per day: _____ When: _____

Nº of cohabitants: |_____| Nº of IBH-affected cohabitants: |_____|

Insect Bite Hypersensitivity (IBH)

Since when (years)? |_____|

Seasonality:

Clinical signs appear (month) |_____| disappear (month) |_____|

Clinical signs progression: Is it the same every year?

☐ No ☐ Yes

☐ always worse ☐ Variable

How? _____

Clinical signs

Pruritus:

☐ None ☐ Yes:

☐ Rarely ☐ Frequently

☐ Very Often

Location of the lesions:

☐ Mane ☐ Base of the tail

☐ Dorsal line ☐ Ventral line

Skin lesions

☐ Grade 0: No clinical signs

☐ Grade 1: Areas of broken hairs or alopecia; skin scales

☐ Grade 2: Thickened skin on the mane and/or base of the tail (indurated skin folds); many broken hairs

☐ Grade 3: Crusts from serous exudate, with or without or little haemorrhage; alopecia on the base of the tail and mane

☐ Grade 4: Extensive crusts with serous exudate and haemorrhage, as well as secondary infection; severe alopecia.

Distribution and extension of the lesions:

Mark the lesions in the sketch:

Figure 1: Individual IBH Questionnaire.

Questionnaires were completed based on the evaluation of the horses' clinical signs (clinical evaluation was performed by the same investigator) and by the information provided by the owners. Owners were also asked if a seasonal pattern consistent with exposure to insects (exacerbation of clinical signs from spring to fall) was observed.

3.2.1. Questionnaries' evaluation: clinical score

The IBH score used in the present study was a severity score defined by the authors and based on a previous IBH scoring system described by Lanz et al. (2017), for each parameter:

a) Pruritus (P) was classified in four grades: (0) never; (1) rarely (2) frequently (3) very often.
b) Severity of cutaneous Lesions (SL) was classified in 5 severity grades: (0) no clinical signs (1) areas of broken hairs or alopecia; skin scales (2) thickened skin on the mane and/or base of the tail (indurated skin folds); many broken hairs (3) crusts from serous exudate, with or without or little haemorrhage; alopecia on the base of the tail and mane (4) extensive crusts with serous exudate and haemorrhage, as well as secondary infection; severe alopecia (figure 2).

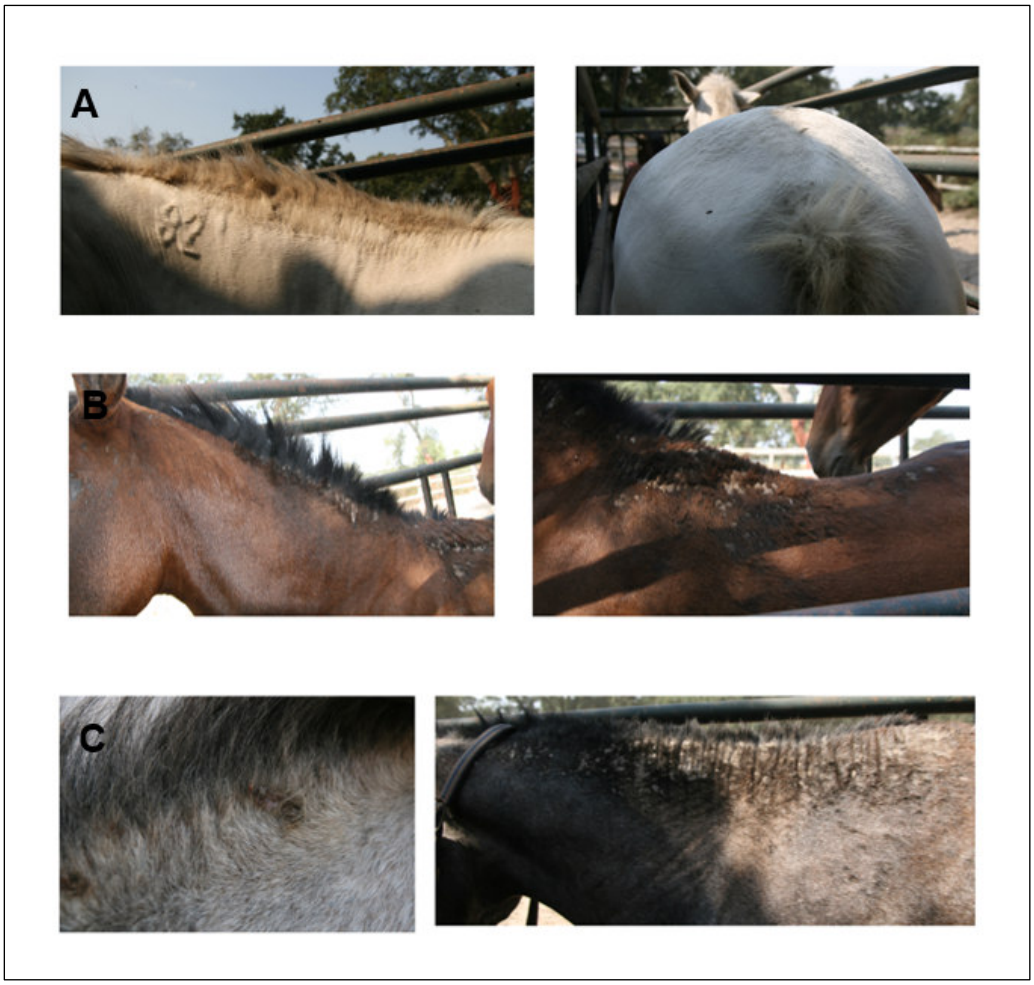


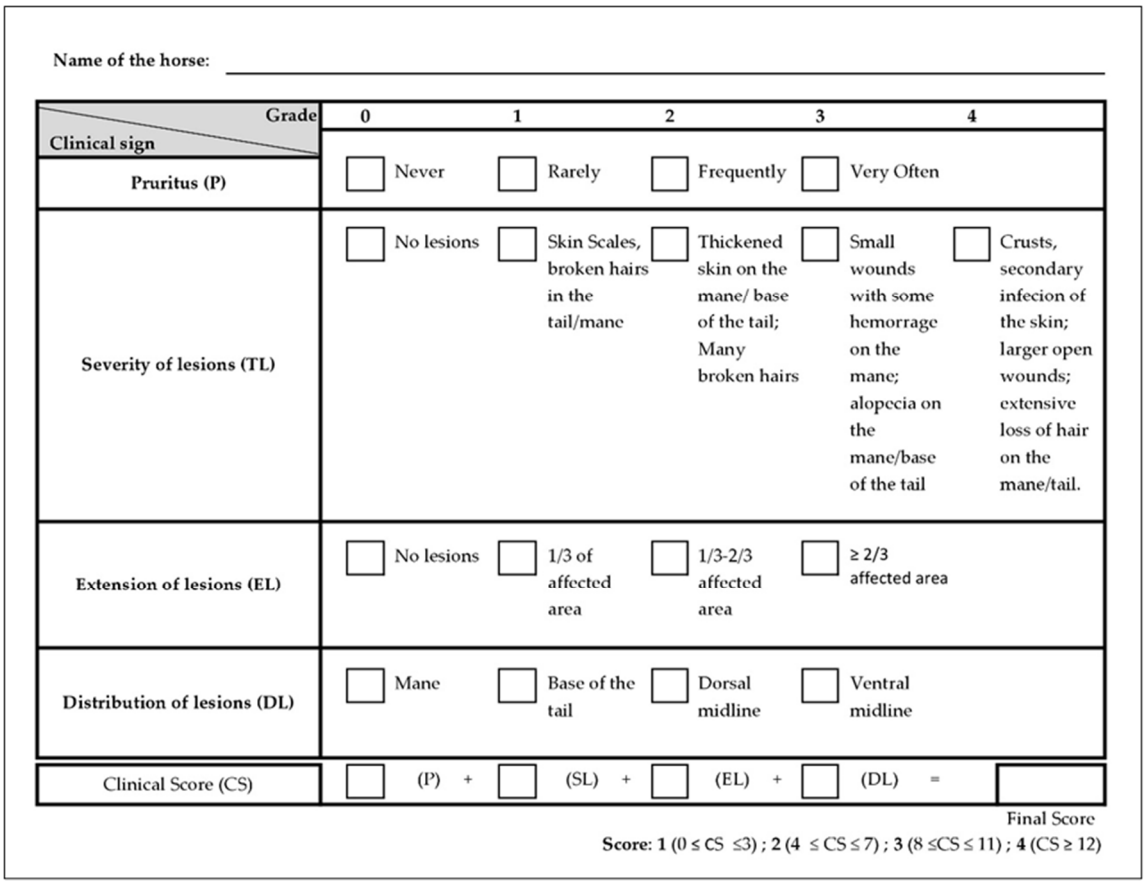
Figure 2: Severity of lesions in IBH-affected horses; (A) grade 1; (B) grade 2; (C) grade 3).

c) Distribution of the lesions (DL was classified in 4 grades (on the mane, base of the tail, dorsal and/or ventral midline, or a combination of these): (0) no lesions; (1) 1 affected area; (2) 2 affected areas (3) >2 affected areas.
d) Extension of lesions (affected body area) (EL): 4 grades were considered: (0) no lesions; (1) 1/3 affected area; (2) 1/3 – 2/3 affected area; (3) ≥ 2/3 of affected area.

An IBH score (S) was given to each horse according to the grade attributed to each evaluated parameter (figure 3).

In total 4 clinical scores were considered: (S1) no signs of IBH; (S2) mild signs of IBH; (S3) moderate signs of IBH; (S4) Severe signs of IBH.

Horses were considered IBH positive (T, test group) if the clinical score was >S1. Horses with score S1 were considered to be healthy horses (C, control group).



Name of the horse: _____

Grade / Clinical sign	0	1	2	3	4
Pruritus (P)	☐ Never	☐ Rarely	☐ Frequently	☐ Very Often	
Severity of lesions (TL)	☐ No lesions	☐ Skin Scales, broken hairs in the tail/mane	☐ Thickened skin on the mane/ base of the tail; Many broken hairs	☐ Small wounds with some hemorrage on the mane; alopecia on the mane/base of the tail	☐ Crusts, secondary infecion of the skin; larger open wounds; extensive loss of hair on the mane/tail.
Extension of lesions (EL)	☐ No lesions	☐ 1/3 of affected area	☐ 1/3-2/3 affected area	☐ ≥ 2/3 affected area	
Distribution of lesions (DL)	☐ Mane	☐ Base of the tail	☐ Dorsal midline	☐ Ventral midline	
Clinical Score (CS)	☐ (P) +	☐ (SL) +	☐ (EL) +	☐ (DL) =	Final Score

Score: 1 (0 ≤ CS ≤3) ; 2 (4 ≤ CS ≤ 7) ; 3 (8 ≤CS ≤ 11) ; 4 (CS ≥ 12)

Figure 3: Clinical score for Test (T) horses.

3.2.2. Questionnaries' statistical evaluation:

Questionnaires were evaluated using descriptive statistics, namely absolute and relative frequencies, means, medians (range) and standard deviation (SD) whenever applicable.

4. Results

4.1. Characterization of the horses and their environment:

The Lusitano horses involved in this study, IBH-affected Test (T) and Control (C) horses, were classified according to sex, age, coat colour, type of housing (mainly pasture or stable) and distribution per region.

All horses were confirmed to comply with the inclusion and exclusion criteria of the group in which they were placed, either T or C.

4.1.1. Total IBH-affected horses and full cohabitants' distribution per stud farm:

Figure 4: Geographical distribution of the Lusitano stud farms with a history of IBH, evaluated in the present study.

The mean of IBH-affected horses per stud farm was 3 with a median of 1 and a standard deviation of 2.

Regarding the number of cohabitant horses per stud farm, the mean was 10, with a median of 5 and a standard deviation of 11.

Furthermore, the percentage of IBH- affected horses was quite variable, ranging from 2-60%, with a mean of 36%, a median of 4%, and a standard deviation of 18%.

Table 1: IBH-affected horses and full cohabitants per stud farm.

Stud farm	Cohab. horses	IBH horses	IBH horses (%)
S1	5	3	60
S2	42	1	2
S3	5	1	20
S4	11	5	45
S5	10	6	60
S6	12	5	42
S7	5	1	20
S8	2	1	50
S9	3	1	33
S10	4	1	25
S11	5	2	40
Mean	**11.29**	**2.45**	**36**
Standard deviation	**2.45**	**1.97**	**18**

4.1.2. General information of C and T horses:

Table 2: Information about age, sex, coat colour, lodging and geographical region of the Control group (C) horses included in this study (n=30).

Horse	Age	Sex	Colour	Lodging	Region
1C	10	F	Grey	Pasture	Cord. (S1)
2C	17	F	Grey	Pasture	Cord. (S1)
3C	10	M	Grey	Stable	Sintra (S2)
4C	6	F	Black	Pasture	Tomar (S3)
5C	2	M	Buckskin	Stable	Pegões (S4)
6C	9	F	Buckskin	Pasture	Pegões (S4)
7C	3	F	Palomine	Pasture	Pegões (S4)
8C	6	F	Bay	Pasture	Pegões (S4)
9C	11	F	Bay	Pasture	Pegões (S4)
10C	21	F	Bay	Pasture	Pegões (S4)
11C	9	F	Buckskin	Pasture	Pegões (S4)
12C	5	F	Bay	Pasture	Pegões (S4)
13C	6	F	Bay	Pasture	Pegões (S4)
14C	6	F	Bay	Pasture	Pegões (S4)
15C	9	F	Grey	Pasture	Beja (S5)
16C	10	F	Grey	Pasture	Beja (S5)
17C	8	F	Grey	Pasture	Beja (S5)
18C	27	F	Black	Pasture	Beja (S5)
19C	13	F	Grey	Pasture	Beja (S5)
20C	2	F	Black	Pasture	Leiria (S6)
21C	2	F	Black	Pasture	Leiria (S6)
22C	2	F	Black	Pasture	Leiria (S6)
23C	7	F	Black	Pasture	Leiria (S6)
24C	2	F	Black	Pasture	Leiria (S6)
25C	5	F	Chesnut	Pasture	Torres Novas (S7)
26C	17	M	Grey	Stable	Caldas (S8)
27C	13	F	Black	Pasture	Torres Novas (S9)
28C	7	F	Grey	Stable	Torres Novas (S9)
29C	19	F	Grey	Pasture	Alcobaça (S10)
30C	6	F	Grey	Pasture	Alcobaça (S10)

Abbreviations: F, female; M, male, S1-10, stud farm 1-10.

Table 3: Information about age, sex, coat colour, lodging and geographical region of the Test group (T) horses included in this study (n=30).

Horse	Age	Sex	Colour	Lodging	Region
1T	11	F	Bay	Pasture	Cord. (S1)
2T	13	F	Bay	Pasture	Cord. (S1)
3T	16	F	Grey	Pasture	Cord. (S1)
4T	25	F	Grey	Stable	Sintra (S2)
5T	11	F	Chesnut	Pasture	Tomar (S3)
6T	2	M	Buckskin	Stable	Pegões (S4)
7T	2	M	Grey	Stable	Pegões (S4)
8T	2	M	Buckskin	Stable	Pegões (S4)
9T	2	M	Bay	Stable	Pegões (S4)
10T	2	M	Grey	Stable	Pegões (S4)
11T	7	F	Grey	Pasture	Pegões (S4)
12T	5	F	Bay	Pasture	Pegões (S4)
13T	6	F	Grey	Pasture	Pegões (S4)
14T	3	F	Bay	Pasture	Pegões (S4)
15T	3	M	Grey	Stable	Beja (S5)
16T	3	M	Grey	Stable	Beja (S5)
17T	10	F	Grey	Pasture	Beja (S5)
18T	14	F	Grey	Pasture	Beja (S5)
19T	7	F	Grey	Pasture	Beja (S5)
20T	4	F	Bay	Pasture	Leiria (S6)
21T	5	F	Black	Pasture	Leiria (S6)
22T	2	F	Black	Pasture	Leiria (S6)
23T	5	F	Black	Pasture	Leiria (S6)
24T	5	M	Black	Stable	Leiria (S6)
25T	5	M	Grey	Stable	Torres Novas (S7)
26T	3	F	Bay	Stable	Caldas (S8)
27T	14	M	Bay	Stable	Leiria (S9)
28T	11	M	Bay	Stable	Alcobaça (S10)
29T	4	M	Bay	Stable	Torres Novas (S11)
30T	8	M	Bay	Stable	Torres Novas (S11)

Abbreviations: F, female; M, male, S1-10, stud farm 1-11.

Ages:

Mean age and standard deviation were 7+/-5.32 in the T group and 9+/-6.06 in the C group. Age range was 2 to 25 years in the T group and 2 to 27 years in the C group.

When the horses were grouped by age ranges, as 2-5 years, 6-9 years, 10-14 years, 15-20 years and over 20 years, the highest percentage of horses belonged to the age range of 2-5 years (57%) in the case of the T group, and to the age range of 6-9 years (37%) in the C group. The lowest percentage of horses, for both groups, were in the ≥20 years range, with 3% of horses in the T group and 7% in the C group.

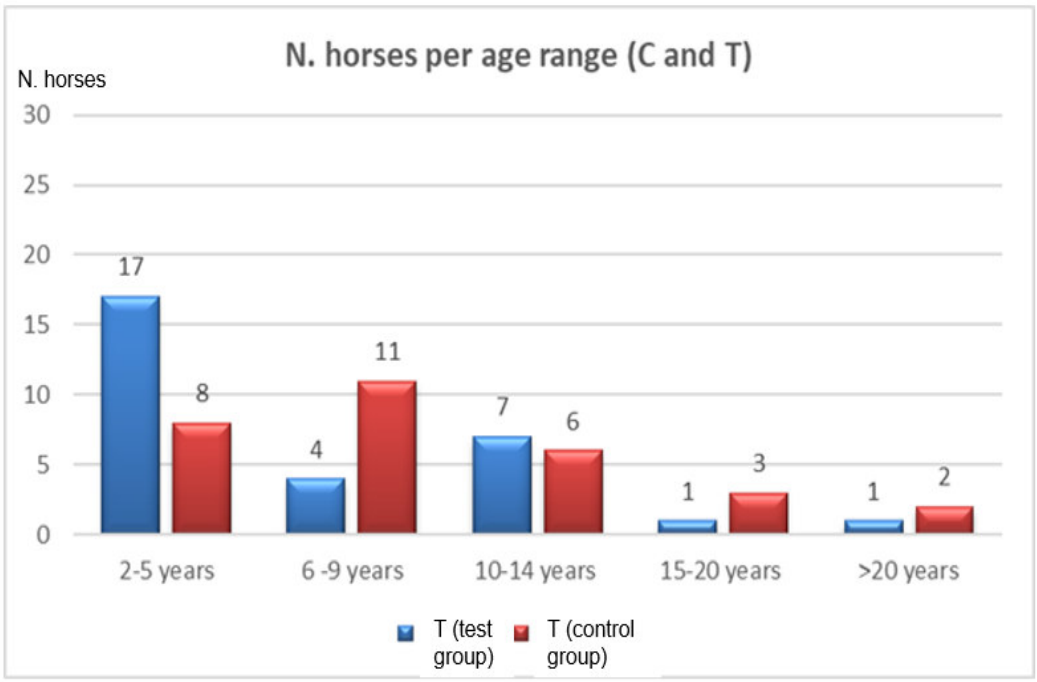


Figure 5: Number of horses, in the C (control group) and T (test group) per age range.

Distribution per sex

Regarding sex, 73% of the animals tested were females, a total of 44 mares, where 27 belonged to the C group and 17 to the T group. Only a third of the tested animals were males (n=16) and most were stallions, a total of 14, only two being gelded (one in the C group and another in the T group).

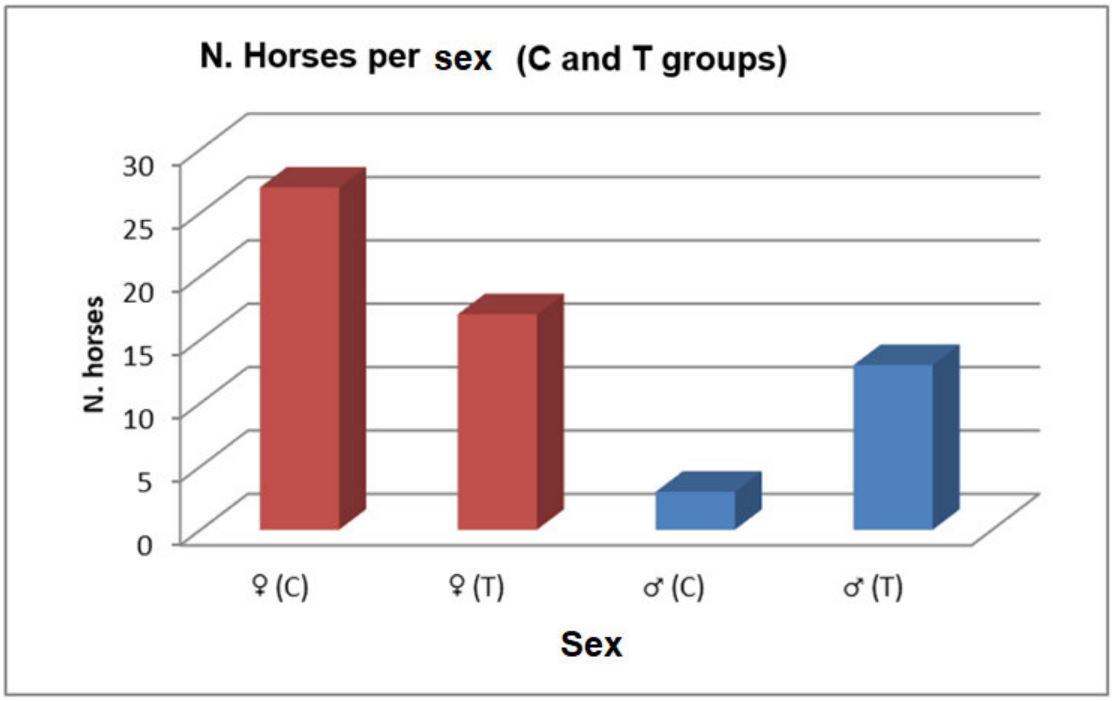


Figure 6: Number of horses, in the Control (C) and Test (T) groups, per sex.

Coat colour:

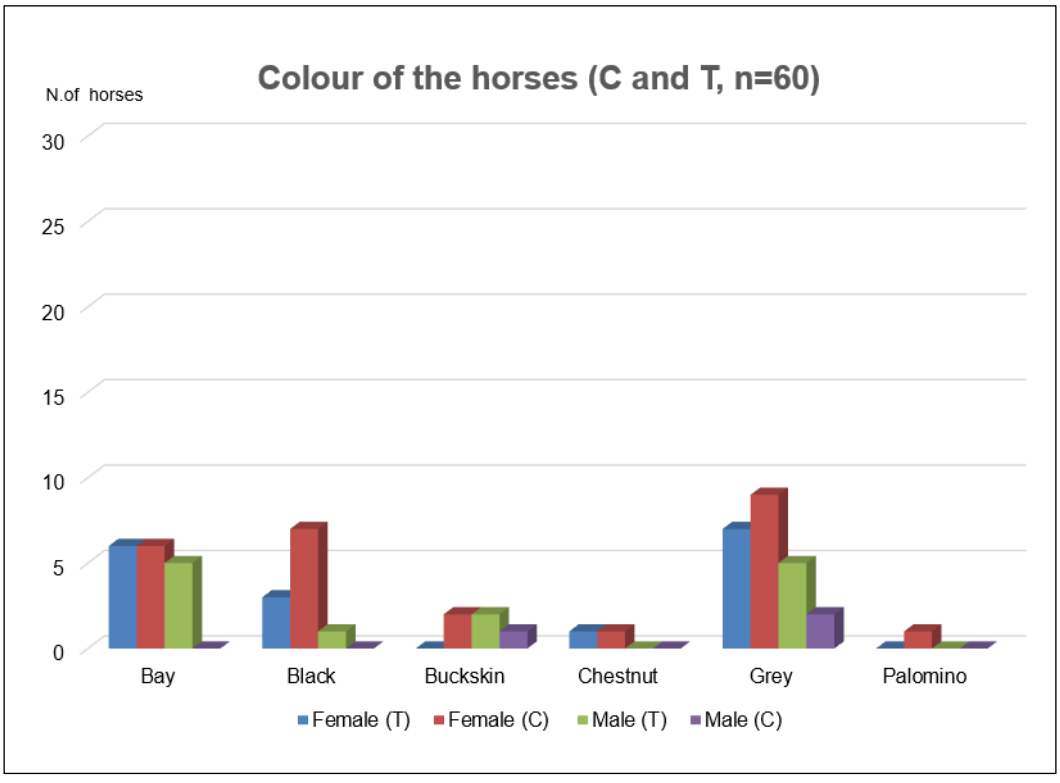


Figure 7: Number of horses, Control (C) and Test (T), per coat colour.

The most frequent colours for females and males from both T and C groups were grey and bay, with a mean of 6 and 4 horses respectively, and a standard deviation of 3 and 2, respectively.

Housing:

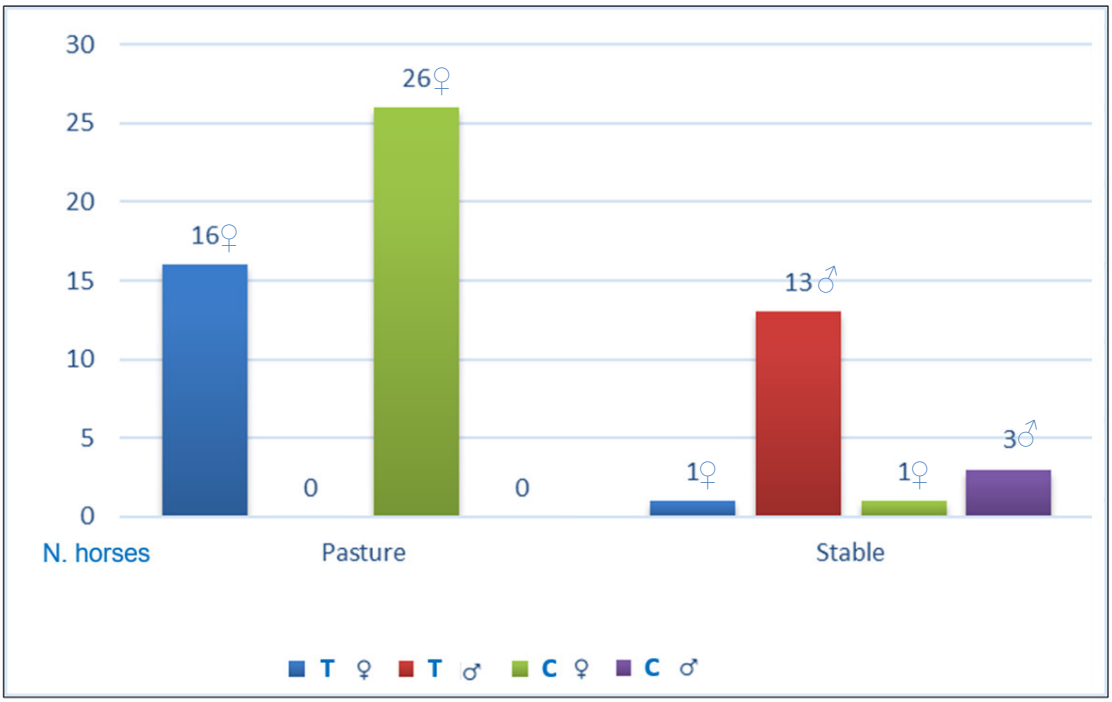


Figure 8: Number of horses per type of housing (stable/pasture), in both Control (C) and Test (T) groups.

As shown in figure 8, most of the females were at pasture all year round, in both groups. Most of the males, both stallions and geldings, were stabled with access to the exterior, in both groups.

4.2. Characterization of clinical signs and lesions

Horses were also characterized based on the clinical signs and on lesion severity, extension, and distribution, using the IBH score previously described in figure 2.

The IBH score was attributed from S1 to S4, where the IBH-affected horses showing the most severe clinical signs and lesions were graded as S4.

Results are displayed bellow in figures 9 (T group) and 10 (comparison of T and C groups).

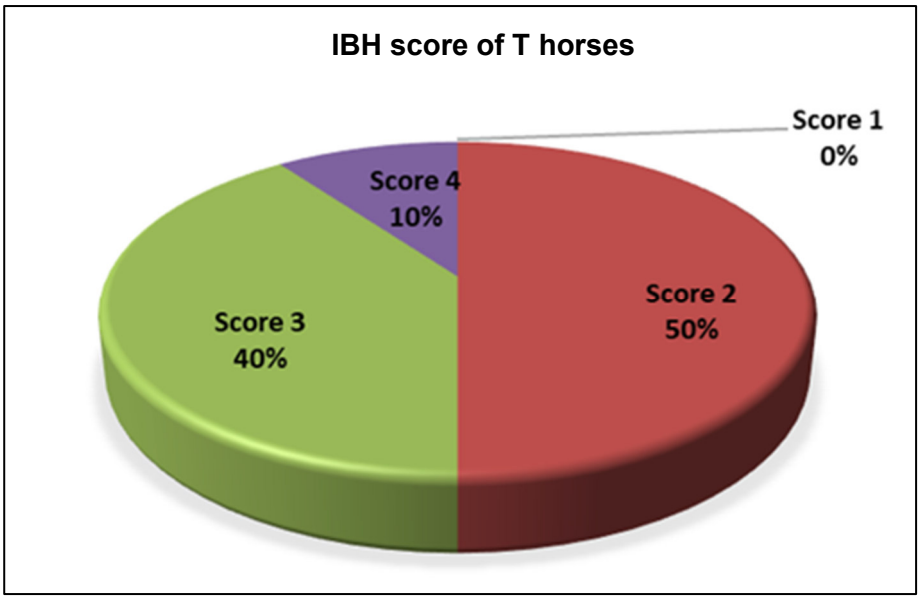


Figure 9: Graphical representation of IBH score for the test group (T).

According to figure 9, most of the IBH-affected horses presented mild to moderate clinical signs and lesions, score 2 (50%) and 3 (40%) respectively.

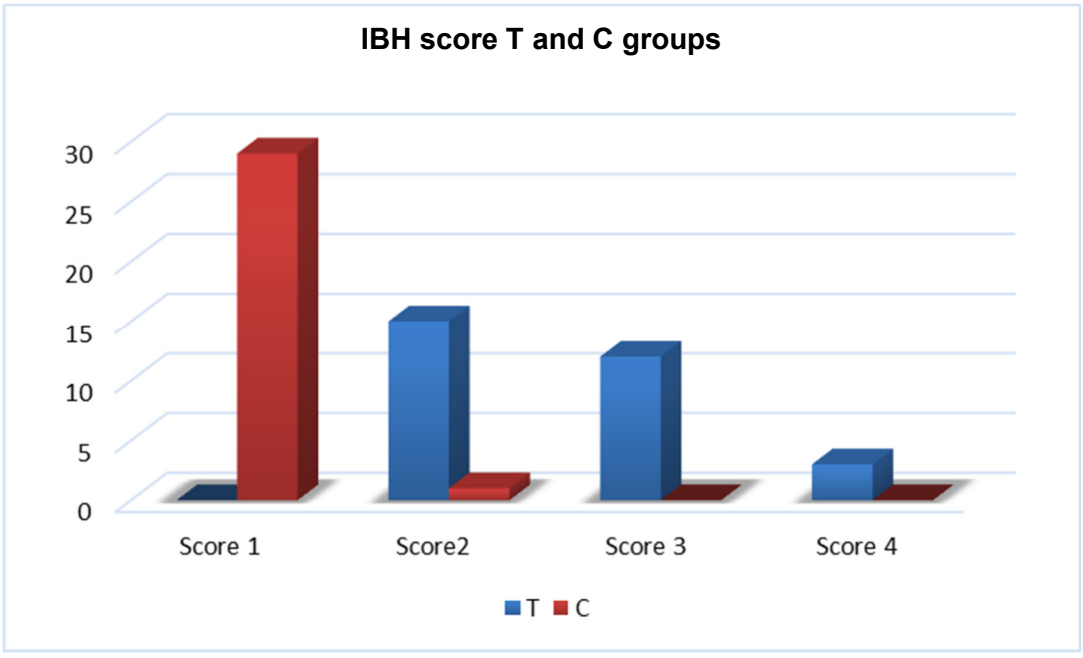


Figure 10: Comparison of IBH Scores for the Test (T) and Control (C) horses.

When compared to the T group horses, which presented mild to moderate clinical signs (score 2 and 3 respectively), the C group horses did not show pruritus nor additional signs or lesions (score 1) except for one horse. The lesions presented by the healthy horse were not related to IBH.

Pruritus:

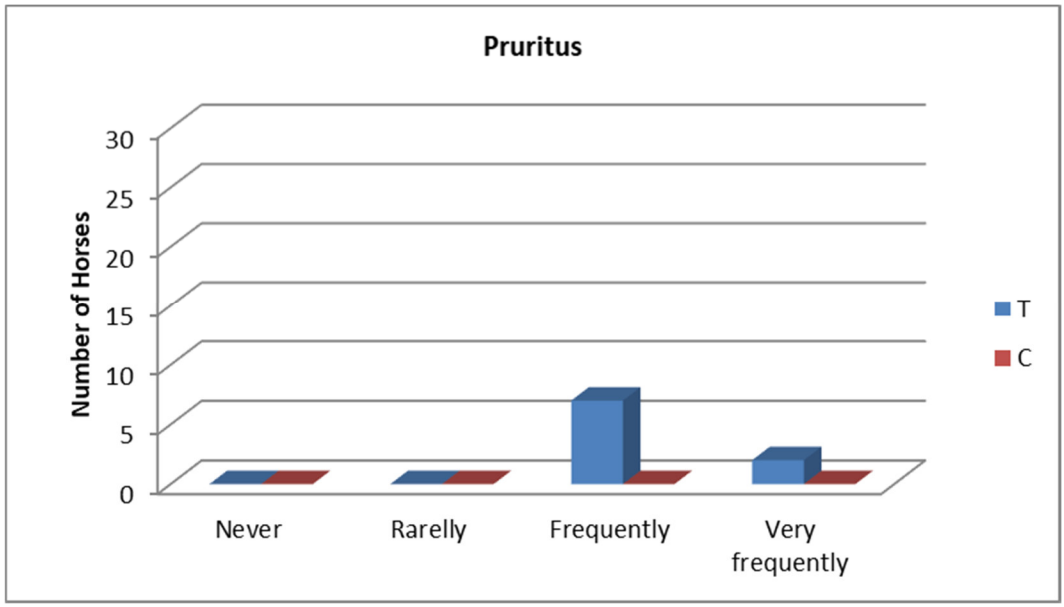


Figure 11: Number of horses, Control (C) and Test (T) groups, that present pruritus.

Most of the T horses presented pruritus frequently. None of the C horses presented pruritus.

Severity of lesions:

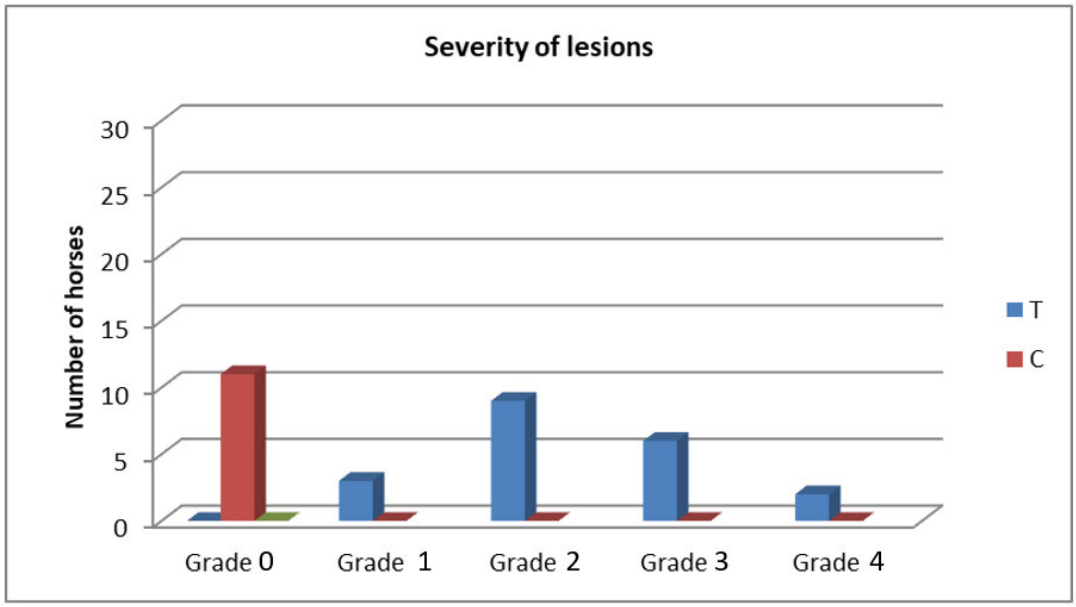


Figure 12: Number of horses, Control (C) and Test (T) groups, per severity of lesions graded from 0-4, 0 being absent and 4 the most severe.

Most of the IBH-affected horses presented grade 2 to 3, i.e., moderate to severe lesions. Regarding C group none presented lesions with any grade of severity.

Extension of lesions:

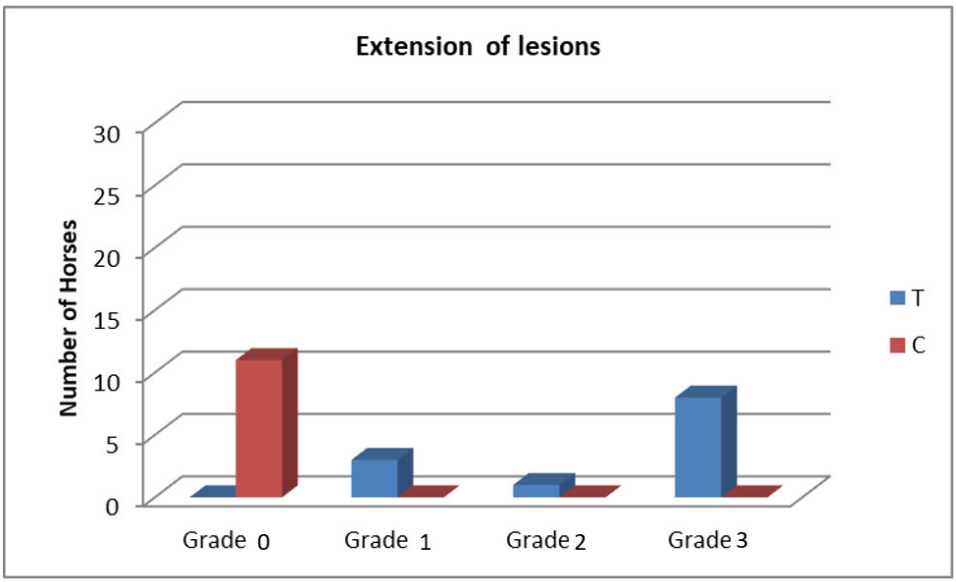


Figure 13: Number of horses, in the Control (C) and Test (T) groups, presenting each grade of lesion extension. Grades 0-3, 1 with 0 - no lesions and 3 presenting very extensive lesions.

Number of horses, in the Control (C) and Test (T) groups, presenting each grade of lesion extension. Grades were attributed from 0 to 3, with 0 showing no lesions and 3 presenting very extensive lesions.

Distribution of Lesions:

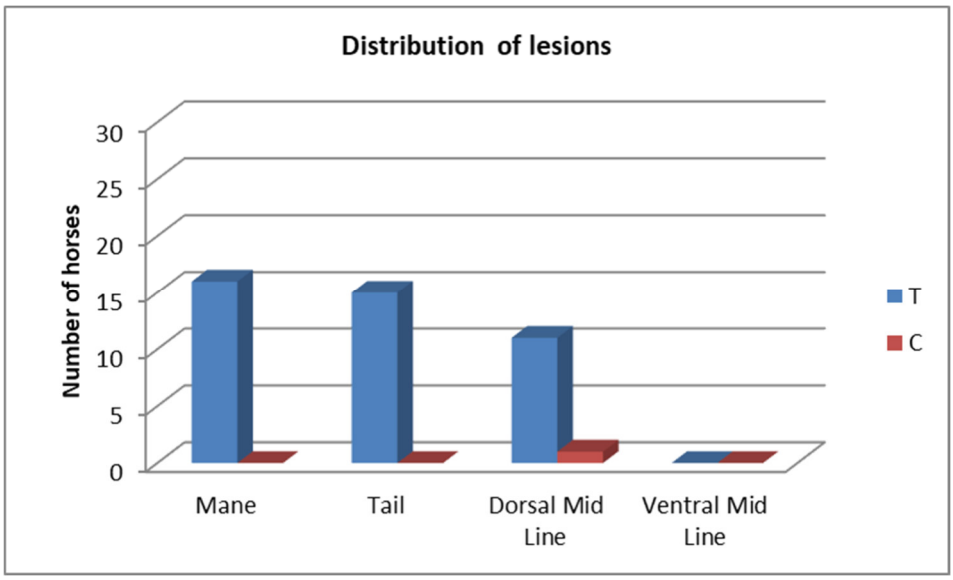


Figure 14: Number of horses, in the Control (C) and Test (T) groups, presenting lesions in each of the considered body areas.

In the T group most horses presented lesions on the tail, mane and dorsal line. In the C group only one horse presented one lesion on the dorsal line, but not related to IBH.

Number of affected areas:

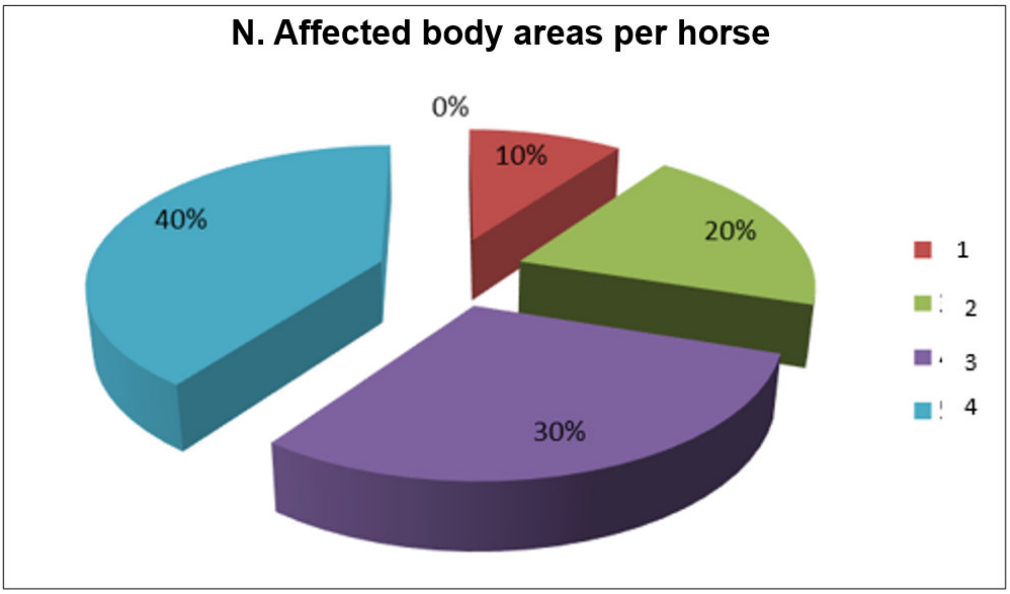


Figure 15: Number of body regions presenting lesions per IBH affected horse.

Most of the IBH-affected horses presented 4 body areas with lesions.

5. Discussion:

The horses involved in this study were characterized, as well as their stud farms environmental management, namely lodging based on descriptive statistics.

Most of the horses were females, around 73%, living in an outdoor regimen all year round which is in accordance with the type of horse breeding in Lusitanos' stud farms in Portugal.

All horses were ≥2 years old at the age of onset of IBH signs. There was a wide age range of IBH-affected horses included in this study (3 to ≥20 years), which is in accordance with other reports that observed the occurrence of this disease in all age groups (Langner et al. 2008). Nevertheless, these previous studies found that animals affected with IBH had an average age of 10 years, but in this study the most frequent age range for IBH-affected horses was 2-5 years (57%).

Regarding coat colours, the more frequent were grey and bay. Nevertheless, according to other authors IBH does not seem to be related to the colour of the coat (Halldórdsóttir & Larsen 1991) nor to the gender (Hallama, 2009; Steinman, Peer & Klement 2003).

Also, IBH does not present any breed predisposition. Nevertheless, there are some breeds that seem to have a higher risk of developing IBH, namely Icelandic horses which are born in Iceland and later exported to countries where *Culicoides* are endemic. This is probably due to the absence of *Culicoides* in Iceland (Wagner 2015).

To our knowledge, there have been no previous studies on the prevalence of IBH in Lusitano horses living in Portugal mainland.

Although it was still not possible to evaluate the prevalence of IBH in the present study, it may be a first contribute to the understanding of IBH in Portugal. The percentage of IBH-affected horses in this study was quite variable, ranging from 2 to 60%, with a mean of 36%, a median of 4%, and a standard deviation of 18%. The number of horses per farm were between 2 and 42, with an average of 9-10 horses per farm, of which an average of 3 horses had IBH, ranging from 1 to 6 IBH-affected horses per farm.

A standardized questionnaire was developed by our research group to record IBH clinical signs and lesions, in order to characterize the studied horses and their management conditions. This questionnaire was based on previously described questionnaires (Lanz et al. 2017; Babel et al. 2014) but adapted to the Lusitano horses and the management conditions in the Portuguese stud farms. The questionnaire allowed us to characterize all horses in the present study by the attribution of an IBH severity score according to the presented clinical signs, such as pruritus, and the type of lesions, their severity, extension and distribution.

An IBH score (S) was attributed to each horse, according to each individual clinical signs and lesions: (S1) no signs of IBH; (S2) mild signs of IBH ;(S3) moderate signs of IBH; (S4) severe signs of IBH. The horses had to have an IBH Score >1 to be included in the IBH-affected T group.

Most of the IBH-affected horses presented mild (S2) to moderate signs (S3). This may be explained by the time at which the animals were tested, at the beginning of spring, when *Culicoides* are not very active yet. Therefore, the allergic horses still hadn't been extensively exposed to the allergens present in the *Culicoides* saliva, thus not presenting severe signs and lesions. Rendle (2014) stated that the reduction of exposure to *Culicoides* may alleviate suffering and optimize the welfare of the IBH-affected horses. Hence, it is predictable that IBH-affected horses that still hadn't been extensively exposed to the *Culicoides* allergens, would not show severe signs or lesions.

Pruritus is the most frequent and characteristic clinical sign of IBH, mainly due to the production of cytokines, namely IL-31, during development of the Th2 regulated and IgE mediated immune response (Jordonstir et al. 2019). By itching and scratching due to pruritus, IBH-affected horses develop lesions mainly on the mane, tail, head, and dorsal/ventral line. In our study, in the T group, most of the IBH-affected horses

presented lesions on the tail, mane and dorsal/ventral line. For C group only one horse presented one lesion on the dorsal line, which could be due to a different etiology, as no pruritus was observed.

Regarding lesion severity, most of the IBH-affected horses presented moderate to severe lesions, distributed mainly on the mane, tail and dorsal line, characteristic of IBH's usual lesion distribution in accordance with other author's observations (Scott and Miller, 2003). As mentioned above, in Portugal, most of the Lusitano females are broodmares and live in an outdoor regimen all year round being more exposed to the *Culicoides spp*. thus presenting more signs of IBH than the males that are kept mostly indoors and less exposed to the *Culicoides spp*. No lesions were observed in the C horses, as pruritus is the main cause of the occurrence of lesions and none of the C horses presented pruritus.

A standardize questionnaire was developed by our study group to record IBH clinical signs, as well as to characterize the studied horses and their management conditions. This questionnaire was based on previously described questionnaires (Lanz et al. 2017) but adapted to the Lusitano horses and the management conditions in the Portuguese stud farms. Currently, there is no standardized method for recording allergy clinical signs and various groups have used different questionnaires to diagnose allergy (Lanz et al. 2017; Olsen et al. 2011; Torsteinsdottir et al. 2018; van der Meide et al. 2012). Miller et al. (2019) has compared three scoring systems to provide a standardized clinical scoring system to quantify disease severity and response to therapeutic intervention, but this still requires further discussion among scientific and clinical communities involved in equine allergy in order to standardize the scoring of IBH.

The questionnaires used in the present study were easy to perform and may be completed in just a brief period of time. In the future they may also have a practical application as a complement for IBH's clinical evaluation by fellow practitioners, but further studies are needed before any recommendations can be made.

6. Conclusion:

All horses included in this study, both IBH-affected T horses and C horses, were characterized based on standardized owner questionnaires, clinical exams and photographic registers, and it was possible to confirm their compliance with the pre-established inclusion and exclusion criteria. In order to be considered IBH positive, horses had to present evidence of seasonal pruritic dermatitis and lesions at the time of evaluation, in this case an IBH score >1. Likewise, C horses, presented no signs or lesions resembling IBH, hence validating the sample and the experimental study.

The questionnaires provided us with complementary information regarding IBH for the horses included in the study. This was especially useful to characterize the IBH-affected horses' environmental management as being mostly in an outdoor regimen all year round, therefore more exposed to the *Culicoides spp*. Furthermore, they served as a first contribute to the understanding of the prevalence of IBH in Portugal, even though the percentage of IBH-affected horses was quite variable.

The questionnaires used in the present study may be a valuable tool to report allergy clinical signs and to quantify several parameters with relevance for IBH.

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Chapter III:

Culicoides spp. found near stud farms in mainland Portugal, which may contribute for IBH studies.

Culicoides spp. found near Lusitano stud farms in mainland Portugal which may contribute for IBH studies.

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Part of this study has been presented at the HavenMeyer congress, 2016, Iceland.

1. Abstract

Insect Bite Hypersensitivity (IBH) is a common cutaneous disease, affecting many horses worldwide. Several studies have identified *Culicoides* spp. saliva as a clinically relevant allergen source. The prevalence of IBH in Portugal, particularly in Lusitano horses, is still not known. However, the environmental characteristics of the national territory are favorable to the activity of *Culicoides*, and several species of this genus can be found, namely *C. imicola* and *C. obsoletus / C. scoticus*. In this study we characterized the *Culicoides spp.* population present in Lusitano stud farms with a history of IBH. Thirteen stud farms with Lusitano horses were selected in several regions of mainland Portugal for having a previous history of IBH-affected horses, with a minimum of 5 affected horses. *Culicoides* were collected in May and June 2016 using OVI traps, placed in these stud farms, and we were able to identify several *Culicoides spp.* We could also verify that *C. obsoletus / C. scoticus*, and *C. imicola* were the ones most frequently found, but other species like *C. policies* were also found.

Key words: IBH*, Culicoides*, Lusitano horses, OVI traps.

2. Introduction

Insect Bite Hypersensitivity (IBH) is a recurrent seasonal pruritic dermatitis and the most common cutaneous disease, affecting many horses worldwide (Scott & Miller 2003). It is a type I and in some cases IV hypersensitivity reaction to allergens present in the saliva of hematophagous insects, mainly belonging to the *Culicoides* genus (Wagner et al. 2009). Several studies have identified *Culicoides* species as a clinically relevant allergen source by demonstration of skin test reactivity, and /or measurement of allergen specific IgE levels in serum of IBH affected horses (Anderson et al. 1993, Ferroglio et al. 2006, Hellberg et al. 2006). It is a multifactorial disease, dependent on genetic factors, environmental exposure, and immune response mechanisms. IBH has limited therapeutical choices, namely insect control and the use of antipruritic and anti-inflammatory agents. Because it is an IgE mediated disease, it is possible to develop specific immunotherapy (Scott & Miller 2003; Wagner et al. 2006).

IBH has a global distribution, but a variable prevalence (Schaffartzik et al. 2012; Scott & Miller 2003; van der Rijt et al. 2008), as it can affect from 5 to 50% of horses depending on multiple factors like breed, family, and environment.

It is thought that the variable prevalence of the disease is related to the influence of environmental factors on the activity of the insect species involved (Schurink et al. 2010; van der Rijt et al. 2008).

The prevalence of IBH in Portugal, particularly in Lusitano horses is presently unknown. However, the environmental characteristics of the national territory are favorable to the activity of the hematophagous insects most frequently implicated in this dermatitis, and they are present throughout the national territory, especially in the geographical area bellow the Tagus River (Ramilo et al. 2012), where the largest number of stud farms are also located.

The Lusitano horse is known for its great functionality and versatility (Cordeiro 1997), taking an important part in the Portuguese economy. It is mostly raised in an outdoor regime all year round and subjected to a high insect exposure. The team's clinical experience in the field revealed a considerable number of IBH cases involving Lusitano horse stud farms, sometimes affecting many animals from the same holding, including different generations of genetically related horses, as previously found in the Dutch Shetland pony population (Schurink et al. 2009). The severity of the clinical signs presented by the horses, and the obvious negative effects on animal welfare is often reflected in economic losses (Brostrom et al. 1987; Fadok & Greiner 1990). These are due to permanent skin lesions and intense itching, which can lead to behavioral changes

such as anxiety, nervousness, restlessness or even aggression, making the horses unfit to ride (Gortel 1998), increasing owners' concern about this disease.

Several *Culicoides* species seem to have a high impact in IBH (Wagner et al. 2009), in many regions of the world. Nevertheless, the histopathology and epidemiology of IBH seem to be identical worldwide. *Culicoides obsoletus* (Meigen) was incriminated as the major cause of IBH in British Columbia (Anderson et al. 1991) and maybe in Japan (Yamashita et al. 1957). In England (Mellor & McCaig 1974) and probably in Denmark (Hesselholt & Agger 1977) *C. pulicaris* (Linneaus) has been reported. *Culicoides punctatus* (Meigen)*, C. nubeculosus* (Meigen) and *C. pulicaris* are the main suspects in Ireland (Townley et al. 1984). *Culicoides imicola (*Kieffer) plays a role in Israel (Braverman et al. 1983), and *C. circumscriptus (*Austen) in China (Baker & Collins 1984). When horses are submitted to intradermal tests (IDT) with different *Culicoides spp.* extracts, they exhibit immediate (type I) and delayed (type IV) skin test reactions (Anderson et al. 1993; Coombs & Gell 1975; Katz 1978; Pepys 1975), suggesting that some allergens may be common to several species of biting midges (Langner et al. 2008).

In Portugal, different species of *Culicoides* can frequently be found (Ribeiro et al. 2015), the most common being *C. imicola* and *C. obsoletus / C. scoticus* (Ramilo et al. 2012). The complete list of *Culicoides* species present in mainland Portugal can be seen in Ramilo et al. (2018) but up to the present study there was only very little reference to the *Culicoides* species present specifically near horses in Portugal.

Aim of the study

In the present study, we characterized the *Culicoides* population present in Lusitano stud farms with a history of IBH-affected horses, with the aim of contributing to future studies of IBH in this breed.

3. Material and Methods

3.1. Stud Farms

In the present study, we identified Lusitano stud farms with a history of IBH, each having at least five horses showing clinical signs of the disease. Thirteen stud farms with Lusitano horses were selected in several regions of mainland Portugal. Distribution of Lusitano stud farms per region of mainland Portugal, as well as their location, is represented in Table 1.

Table 1: Distribution of stud farms per region in mainland Portugal, and number of IBH- affected horses.

Region	Latitude	Longitude	N. stud farms per region	N. Equines per stud farm (mean interval)	N. IBH-affected horses per Stud farms (Mean)
Alenquer	39.056633	9.0076057	1	>10 - 20	5
Benedita	39.4257219	8.9800984	1	>20	5
Carregado	39.0230782	8.9768885	2	>10 – 20	5
Castelo Branco	39.8289712	7.4919318	2	≤10	5.5
Leiria	39.8289712	8.8394053	2	>20	5.5
Pegões	38.6837874	8.6115414	1	>20	5
Sintra	38.79846	9.3881	1	≤10	6
Tomar	39.6036596	8.4150492	1	≤10	5
Torres Novas	38.736946	9.142685	2	>20	5
Total			**13**	**----**	**68**

3.2. Inclusion criteria for IBH-affected horses

The horses had to show evidence of seasonal pruritic dermatitis and lesions ≥ grade 1 according to the authors scoring evaluation (presence of broken hair at the base of the tail and at the base of the mane), with a minimum of one year duration, or since the previous equivalent season. Also, complete remission of injury marks between seasons could occur.

The IBH score used in the present study was a severity grade score based on previous descriptions by Lanz et al. (2017), where the owners completed a questionnaire, based on the clinical signs presented by the IBH-affected horses. These signs included pruritus, cutaneous lesions observed on the mane, tail head, ventral midline, or a combination of these. Grade (0) corresponded to no signs of IBH; grade (1) included areas of broken hairs or alopecia in the base of the tail and mane, as described above; grade (2) presented indurate skin folds; grade (3) showed obvious crusts from serous exudates, but without bleeding; and grade (4) revealed bleeding from self-inflicted abrasions.

Furthermore, males and females were included, in similar proportions, of several age groups.

3.3. Further characterization of IBH

A questionnaire was filled for each horse, where different parameters were characterized, namely environmental conditions, previous history, family connections, cutaneous lesions, and their distribution. The characterization of cutaneous lesions and

their distribution was also performed by direct clinical observation and photographic record, as seen in Figure 1.

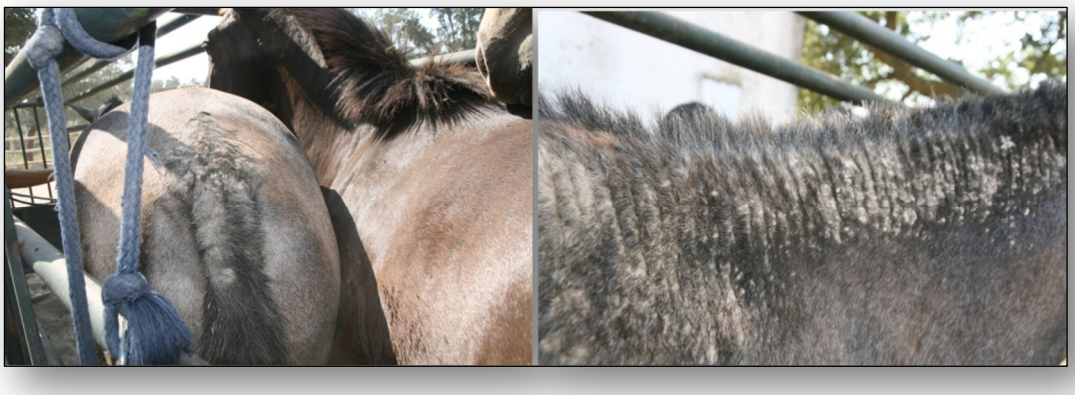

Figure 1: Photographic record of lesions found in IHB-affected horses, mainly on the main and the base of the tail areas.

3.4. *Culicoides* capture and identification:

3.4.1. Insect collection

Culicoides were collected from May to June 2016 using Onderstepoort Veterinary Institute (OVI) traps fitted with 8W UV black light bulbs and a polyester netting (mesh size 2 mm) placed around the light source of the trap, to avoid bigger insects (Figure 2).

Figure 2: Onderstepoort Veterinary institute – OVI trap.

The OVI traps were placed overnight, from dawn to dusk as the majority of *Culicoides spp*. are known to be most active during the sunset period (van der Rijt et al. 2008), in the selected horse farms, located at least 2.5 km from the coastline and a minimum of 10 km from each other. No insecticides were allowed during the study. Note that only a single collection per farm was made during this period.

In each horse farm, OVI traps were placed within 30 m of animal enclosures and 1.70 m above ground. The LCS-2 Photo switch system automatically switched the trap on at dusk and off after dawn, and they were left over night. Insects were collected to 500 ml flasks containing 300 ml of 70% ethanol.

3.4.2. Insect identification

Culicoides female specimens were first identified by their wing pattern using stereoscope microscopy and appropriate identification keys (Delécolle, 1985; Mathieu, 2010; Ramilo, 2016). When *Culicoides* specimens could not be identified by their wing pattern, each specimen was separated into different body parts (head, thorax, abdomen, and wings) using 26-gauge (0.404 mm diameter) needles, mounted on glass slides using Hoyer's medium and dried in an incubator at 37 °C, for 3-4 days.

Afterwards, slides were observed with composed optical microscopy and the specimens were identified based on different morphological characteristics, using identification keys (Delécolle 1985; Mathieu 2010; Ramilo 2016). Some examples of *Culicoides* wing patterns are shown on Figure 3.

Figure 3: Different aspects of wing pattern in *Culicoides* female species (Adapted from: Ramilo et al. 2016). A – *Culicoides paradoxalis (*Ramilo & Delécolle 2013) ; B– *Culicoides newsteadi, (*Austen 1921) ; C – *Culicoides pulicaris (*Downes & Kettle 1952).

4. Results

A total of 13 Lusitano stud farms were studied, with a total of 68 horses IBH-affected, according to the inclusion criteria defined in the present study. At least five horses showing evidence of IBH were identified in each stud farm, as represented in Table 1 and Figure 4.

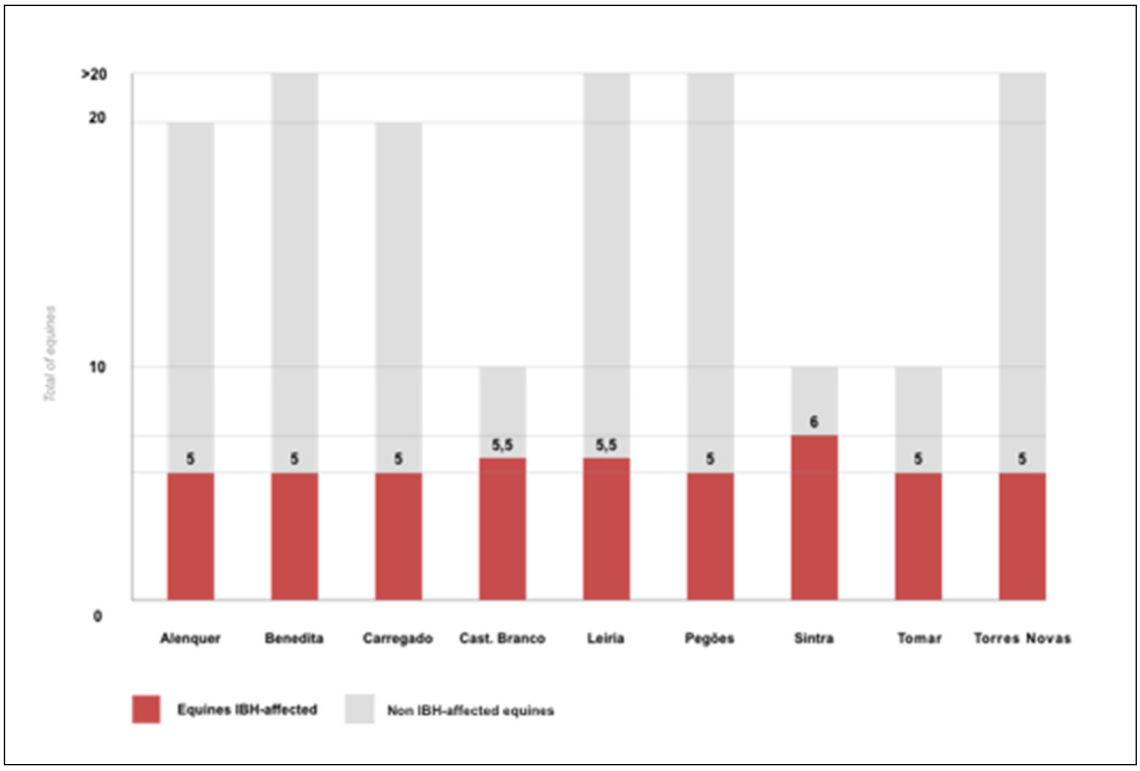

Figure 4: IBH-affected Equines per stud farm.

During the period of the study, several *Culicoides* species were captured, and the distribution per stud farm is represented in Figure 5 and Table 2.

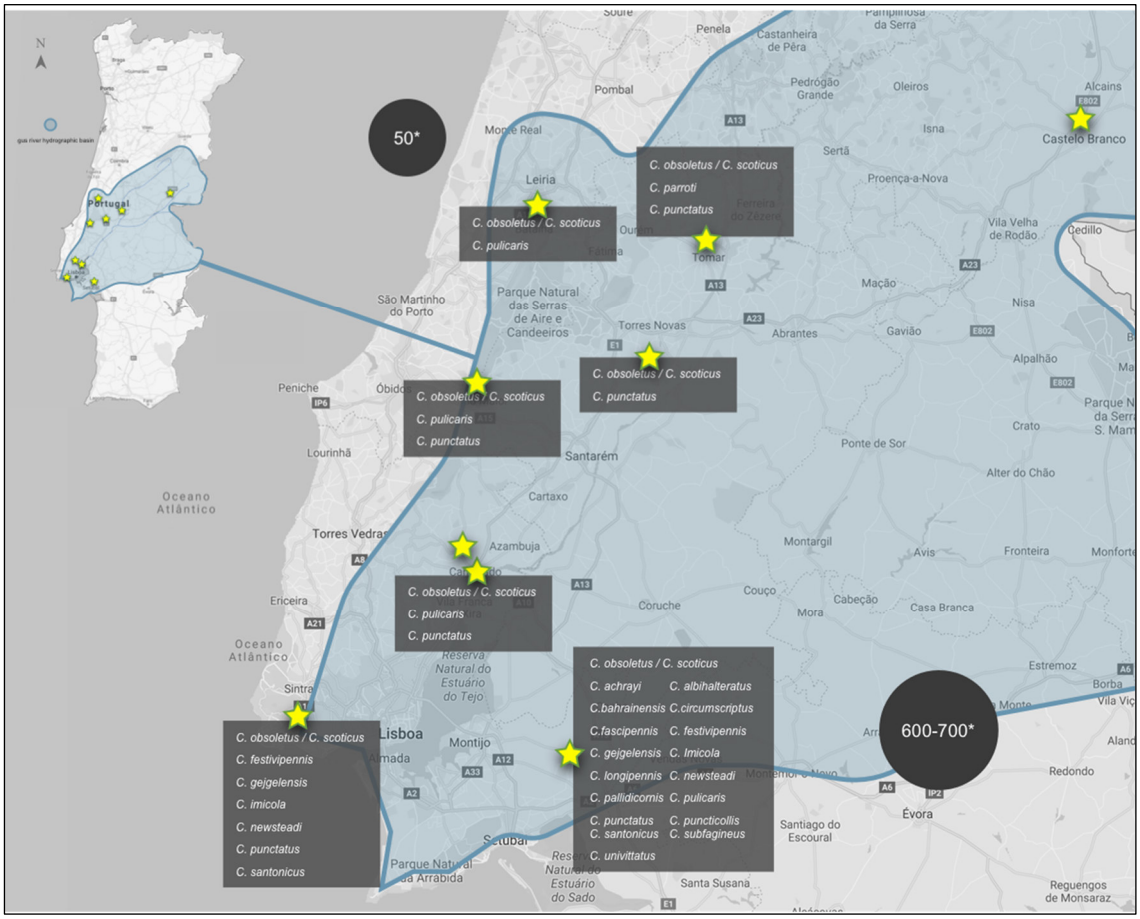

Figure. 5: Distribution of studied Lusitano stud farms and of the *Culicoides spp*. in mainland Portugal. *Minimum and maximum number of *Culicoides* specimens found in each sample found in the samples collected north *vs* south of the river Tagus, respectively.

Table 2: Insects collected in the present study with OVI traps, from May to June 2016.
F - Females. The entire sample was analyzed, unless otherwise noted. **C. nubeculosus* morphologically identical species; the difference lies only on the spermathecae conformation. **Obsoletus* Group refers to *C. obsoletus / C. scoticus*

Place of Harvest	**Capture date**	**Total of *Culicoides* (F)**	**Species, Number**
Quintas, Carregado	18-05-2016	3	**Obsoletus* G., 1 *C. newsteadi,1* *C. circumscriptus*,1
Alvorão, Torres Novas	07-06-2016	5	**Obsoletus* G., 1 *C. punctatus, 4*
Tomar	07-06-2016	43	**Obsoletus* G., 7 *C. punctatus34* *C. parroti, 2*
N. Sra. Do Rosário – Torres Novas	14-06-2016	2	*C. punctatus, 2*
Fernando Fonseca Benedita	14-06-2016	50	**Obsoletus* G., 48 *C. pulicaris,1* *C. punctatus, 1*
Sintra	15-06-2016	33	*C. imicola, 1* **Obsoletus* G., 31 *C. punctatus, 1*
Sintra	15-06-2016	35	*C. imicola, 1* **Obsoletus* G.,27 *C. newsteadi, 4* *C. festivipennis, 1* *C. santonicus, 1* *C. gejgelensis, 1*
Alenquer	16-06-2016	6	**Obsoletus* G., 6
Pegões	16-06-2016	670	*C. imicola ,43* **Obsoletus* G., 10 *C. newsteadi, 536* *C. circumscriptus,14* *C. punctatus, 15* *C. subfagineus, 2* *C. fascipennis, 3* *C. festivipennis,18* *C. bahrainensis,1* *C. longipennis, 1* *C. puncticollis*, 1* *C. santonicus, 14* *C. gejgelensis, 7* *C. albihalteratus.,1* *C. pallidicornis, 4*

The species of female *Culicoides* and total specimens (Log number) harvested in Lusitano stud farms are also represented in Figure 6.

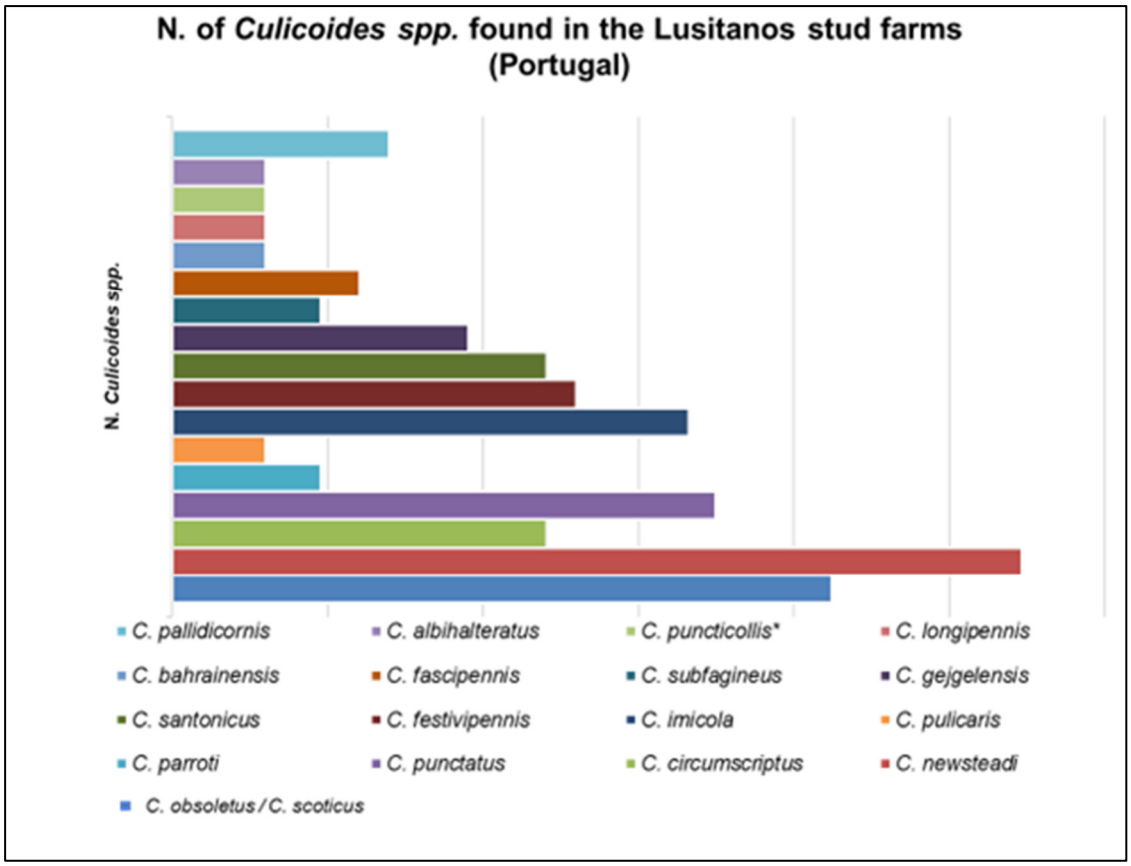

Figure 6: Number of *Culicoides* per species, found in Lusitano Stud farms.

Furthermore, most of these species have been identified in previous studies carried out in Portugal and *C. santonicus* was referred for the first time in our country, by Ramilo et al. (2016).

5. Discussion

The main goal of this study was to identify and improve our knowledge of the *Culicoides spp.* present in Lusitano Stud farms and in their surroundings, which could have an impact in IBH studies.

We were able to verify that, although during the present study a significant amount of different *Culicoides* species were captured (n= 17), *C. newsteadi*, *C. obsoletus / C. scoticus (Obsoletus* group) and *C. imicola* were the ones most frequently found near IBH-affected horses. This observation agrees to prior observations made by Ramilo et al. (2012), already stated above.

Ramilo (2016) also observed that, comparing to cattle, seven *Culicoides* species were captured only in stud farms, such as *C. corsicus, C. fascipennis, C. harati, C.*

odiatus, C. santonicus and *C. tbiliscus*. From those, only *C. fascipennis* and *C. santonicus* were found in the present study. This is probably due to the small period of performed captures, comparing to the prior study.

However, other species referred in the literature as being responsible for IBH in horses, such as *C. pulicaris* (Hesselholt & Agger 1977; Mellor & McCaig 1974), *C. punctatus* (Townley et al. 1984) and *C. circumscriptus* (Baker & Collins 1984), were also caught in the traps which were placed in the present study, but in smaller quantities.

These findings are partially in agreement with other authors' findings, referred by van der Rijt et al. (2008), in which *C. obsoletus* was the most common species found, followed by *C. pulicaris*.

Regarding our study, in one capture *C. puncticollis* was also found. Since this species is very close to *C. nubeculosus*, a species known to be responsible for IBH cases (Hellberg et al. 2006, Schaffartzik et al. 2012), it may have a role in IBH as well. As referred by van der Rijt et al. (2008), the authors also believe that the great variation in the different *Culicoides* species trapped in the various locations may be explained by environmental conditions and availability of breeding sites.

Temperature is of great importance to the activity, longevity, and survival of *Culicoides* to adulthood (Wittmann & Baylis 2000). Ortega et al. (1999), determined minimum and maximum air temperatures for adult *Culicoides* activity for a few different species in Spain, 18º–38º C for *C. imicola*, a minimum of 12º C for *C. newsteadi*, a minimum of 14º C for *C. circumscriptus* and 14º–32º C for all *Culicoides* species (van der Rijt et al. 2008). Since environmental conditions in the whole of the Iberian Peninsula are similar in terms of humidity and temperature, the authors find that this can explain the similarity of the species captured in Spain, and the difference seen from other related studies, namely van der Rijt et al. (2008). In fact, the authors strongly believe that the present study is of major importance for further IBH studies, because of the lack of knowledge of which *Culicoides spp*. may have an impact in IBH studies in Lusitano horses living in Portugal mainland.

C. obsoletus / C. scoticus and *C. imicola* are also implied in other diseases, such as Bluetongue, which affects small ruminants in mainland Portugal and several other European countries, and in African Horse Sickness (AHS) (Ramilo et al. 2012) which causes a high rate of horse mortality. The last AHS outbreak in the Iberian Peninsula occurred in 1989 and killed around 200 horses (Portas et al. 1999). Also, for this reason, studies implicating *Culicoides spp.* identification close to stud farms in mainland Portugal, and in other places of the world, are of major importance, in the authors' opinion. Furthermore, in accordance with Hadje-Henni et al. (2015) it is critical to study the host

preferences of the native population of hematophagous *Culicoides* spp, in order to take action for the prevention of infectious disease outbreaks.

The most cited *Culicoides* to have an impact in IBH, especially for diagnostic purposes, are *Culicoides nubeculosus*, *C. obsoletus* and *C. pulicaris* (Van Oldruittenborgh-Oosterbaan et al. 2009). Even though these species were not the most representative in the present study, the authors strongly believe that the *Culicoides* captured may be relevant for IBH in the Lusitano breed mainland in Portugal. To evaluate the role of the species captured in the present study, the use of an extract derived from these *Culicoides spp*. saliva would be of great importance to determine their allergic properties, for further knowledge concerning IBH in Lusitano horses.

Therefore, these preliminary results should be complemented with further captures in several stud farms in mainland Portugal, to establish consistent data concerning *Culicoides* species occurring near Lusitano horses.

For some of the captured species it is already known that they can feed on horses, namely *C. obsoletus / C. scoticus, C. imicola, C. circumsriptus, C. newsteadi, C. punctatus, C. pulicaris, C. paroti, C. gejgelensis* and *C. puncticolis* (Ramilo 2016). Hadje-Henni et al. (2015) also observed that *C. subfasciipennis* can feed on horses. For *C. festivipennis* although some work has been done, there are still no references about them feeding on horses (Ramilo 2016). Additionally, it is also necessary to characterize the feeding preferences of some other captured species, namely *C. bahrainensis* and *C. albihalteratus* whose feeding preferences are still not well known, as their presence in traps does not necessarily mean that all of them perform their blood meal on the nearest host.

Furthermore, these species' saliva must then be inoculated in horses to understand if they have some role in IBH.

Although the prevalence of IBH in Lusitano horses is still unknown, we can say that in mainland Portugal, it seems to be very high according to owner's complaints and to the observations of veterinarian clinicians working in stud farms throughout the country. IBH has also been increasing through the years, having a negative economic impact in stud farms. This may have to do with the environmental changes, with an increased temperature all year round, but predominantly from spring to fall. Another explanation could be the fact that stud farm owners are now more aware and concerned about IBH and the effects it can have on their horses not only economically, but also at the welfare level.

In agreement with other similar studies previously performed, namely by van der Rijt et al. (2008) the authors strongly believe that the present study can be very relevant for further studies of IBH disease in Lusitano horses and in other breeds, especially those

already identified and/or referred in other studies of IBH, such as Icelandic horses, Thoroughbreds, Arabians, Warm bloods, Draft horses, Quarter horses and ponies (Wagner et al. 2006). Also, the authors would like to emphasize that this study can be an important tool for further research regarding the future use of immunotherapy in IBH-horses.

6. Conclusion:

In the present study the most frequent *Culicoides spp.* found near IBH-affected horses were *C. obsoletus / C. scoticus* and *C. imicola*. Other species like *C. pulicaris*, previously described by van der Rijt et al. (2008) in a similar study, was also found. Because of the lack of knowledge of which *Culicoides spp*. may have an impact in IBH studies in Lusitano horses living in mainland Portugal, the authors strongly believe that the present study is of major importance for further IBH studies.

Furthermore, the authors believe that to evaluate the role of the species captured in the present study, it is mandatory to do *in vivo* testing of a specific extract derived from the Portuguese *Culicoides spp.* to determine their allergy inducing properties, which would be of great importance for further knowledge concerning IBH in Lusitano horses, and other breeds that cohabitate with them.

Concerning feeding preferences, further studies should be performed to confirm on which hosts the above *Culicoides* species feed.

Conflict of interest

None declared.

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Chapter IV:

Comparison of Skin Prick tests (SPT), Intradermal tests (IDT) and *in vitro* tests in the characterization of Insect Bite Hypersensitivity (IBH) in a population of Lusitano horses: contribution for future implementation of SPT in IBH diagnosis

Comparison of Skin Prick tests (SPT), Intradermal tests (IDT) and *in vitro* tests in the characterization of Insect Bite Hypersensitivity (IBH) in a population of Lusitano horses: contribution for future implementation of SPT in IBH diagnosis

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1. Simple Summary | Abstract:

Insect Bite Hypersensitivity (IBH) prevalence in Portugal and in Lusitano horses is not known, but environmental characteristics are favorable to the activity of *Culicoides* and common knowledge shows high occurrence in Lusitano stud farms. This study aimed to compare skin tests and in vitro allergy tests for the diagnosis of IBH in Lusitano horses. Results of our study showed that skin prick tests presented a higher discriminatory diagnostic potential in IBH diagnosis. This increases our knowledge about IBH in Lusitano horses and could represent a step forward in the future development of specific immunotherapy.

Abstract: 30 controls (C) and 30 IBH-affected (T) Lusitano horses were evaluated. T horses were included based on anamnesis and physical examination, supported by questionnaires. All horses were submitted to skin tests, Intradermal (IDT) and Skin Prick Tests (SPT), on the neck with 14 specific allergens, 13 recombinant proteins (r-proteins) from C*ulicoides nubeculosus (*Cul n) and *Culicoides obsoletus* (Cul o) salivary glands,

and *Culicoides nubeculosus* Whole Body Extract (Cul n WBE). Additionally, a cluster of 6 T and 6 C horses were also tested with Cul n 3 and Cul n 4 produced in insect cells and barley, as well as *E. coli* produced Cul o 3 and Cul o WBE. Allergen concentrations were 10µg/ml for IDT and 100µg/ml for SPT and wheal diameters assessed at 20 min, 6 and 48 hours. IDTs were considered positive when wheal diameter was ≥50% of the histamine wheal and SPTs ≥0.9cm. *In vitro* tests, allergen-specific serum IgE and sulfidoleukotriene (sLT) release assay were also carried out. Results showed that Cul n WBE, Cul n 7, 8, 9, Cul o1P and Cul o 2P were the best performing allergens for SPTs ($p \leq 0.0001$) for the 1st allergen panel and Cul o WBE, Cul n 3 Bar and Cul n 4 Bac ($p \leq 0.05$) for the 2nd, presenting a higher discriminatory diagnostic potential than IDTs, at a concentration of 100µg/ml, with readings assessed at 20 minutes. Regarding i*n vitro* tests overall sLT release assay performed best.

Keywords: Horses, Insect bite hypersensitivity (IBH), Allergens, Intra dermal Tests (IDT), Skin Prick tests (SPT), i*n vitro* tests.

2. Introduction

The best characterized allergic disease in horses (Fadok 2013) is a type I hypersensitivity, IgE-mediated dermatitis caused by bites of hematophagous insects belonging to the genus *Culicoides* (Wagner et al. 2009). It is most frequently known as insect bite hypersensitivity (IBH) but can also be called *Culicoides* hypersensitivity, summer eczema or Queensland itch (Mueller et al. 2016). Insect Bite Hypersensitivity (IBH) is a recurrent, seasonal, pruritic dermatitis that affects many horses worldwide (Scott & Miller 2003) and clinically manifests as a chronic relapsing seasonal dermatitis (Jonsdottir 2019; Scott & Miller 2003). The prevalence of IBH varies between 3 to 60% depending on the environment and on the genetic background of the horse (Mueller et al. 2016; Schaffartzik 2012). The prevalence of IBH in Portugal is not yet known. However, the environmental characteristics are favourable to the activity of the hematophagous insects most frequently involved in this dermatitis, which are present throughout the national territory (Pessoa et al. 2020; Ramilo et al. 2016). As it is a multifactorial disease, involving genetic and environmental factors, as well as immune response mechanisms, a very variable prevalence is observed (Schaffartzik, et al. 2011; Scott & Miller 2003; van der Rijt 2008). IBH diagnosis is still based on expert assessment comparing the presence of the representative lesions with the history of seasonal pruritus, mainly in the summer, and improvement of clinical signs in response to environmental management (Miller et al. 2019; Scott & Miller 2003; Van Damme et al. 2020). To date an effective treatment for this disease remains elusive (Mueller et al.

2016) and is based on insect control in the environment and use of topical and systemic antipruritic and anti-inflammatory agents (glucocorticoids, antihistamines, omega-6 / omega-3 fatty acids). However, as it is an allergic disease, mostly IgE mediated, there is the possibility of developing specific immunotherapy (Scott & Miller 2003; Wagner et al. 2006). Various techniques are available to detect allergic sensitization, commonly designated as allergy tests. Since several years allergy diagnostic tests included Intradermal tests (IDT) and serological assays to determine allergen specific IgE (Wagner et al. 2009), the later using mostly ELISA techniques. IDT using multiple allergens have been used as a diagnostic tool in IBH-affected horses, for over 20 years (Anderson et al. 1993; Wagner et al. 2009). A few studies have shown that IBH-affected horses more frequently have positive IDT results with *Culicoides* extract, and sometimes also with other insect extracts, than healthy control horses (Mueller et al. 2016; Scott & Miller 2003). The involvement of *Culicoides* allergens in IBH has also been demonstrated with functional *in vitro* tests such as sulfidoleukotriene (sLT) release (CAST, Bühlmann laboratories AG) (Baselgia et al. 2006) or histamine release tests, using *C. nubeculosus, C. sonorensis* or *C. obsoletus* extracts (Baselgia et al. 2006; Langner et al. 2008), as sLT and histamine are more frequently released in IBH-affected horses than in healthy controls (Mueller et al. 2016). A previous study had shown a high correlation between *in vitro* sLT and histamine release in peripheral blood leukocytes from horses after a 40 min stimulation with *Culicoides* allergens (Marti et al. 1999), indicating that the sLT release can be used to detect immediate type reaction *in vitro* to given allergens. However, as stated above, even though intradermal skin testing is still currently used to identify the allergens responsible for the disease, sensitization to a particular allergen does not necessarily mean that the individual is allergic or will develop clinical signs of allergy (Wagner et al. 2009). Other authors (Tilley et al. 2012) previously published a study introducing Skin Prick Tests (SPT) as a valid diagnostic tool for detection of positive reactions to environmental allergens in horses showing clinical signs of Equine Asthma Syndrome, as an alternative and/or complement to IDTs. Also, despite *in vitro* measurement of specific IgEs present in general a high specificity, they often present a lower sensitivity than SPTs, in the diagnosis of IgE mediated reactions (Carrapatoso et al. 2005; Homburger et al. 1998). Advances in the identification of the allergens involved in IBH provide the basis for both improved diagnostic methods with specific allergens and for new promising tools for targeted allergen immunotherapy (AIT) using the major allergens of *Culicoides spp* (Meulenbroeks et al. 2015; Miller et al. 2019; Novotny et al. 2020; Peeters et al. 2013; Scott & Miller 2011; Van Damme et al. 2020).

In the present study, we evaluated the use of *in vivo* Skin tests, SPT and IDT, and *in vitro* allergy tests, namely allergen-specific serum IgEs and sulfidoleukotriene

(sLT) release assay in the diagnosis of IBH in Lusitano horses, using recombinant *Culicoides* allergens (r-*Culicoides* allergens) and *Culicoides* extracts. The present study contributes to increase our knowledge about IBH in Lusitano horses and could represent a step forward in the future development of specific immunotherapy.

Relevance of the study:

To the authors' best knowledge there are no studies about the impact of IBH in Lusitano horses, neither in mainland Portugal, nor outside. Nevertheless, Portugal presents all the environmental conditions for *Culicoides spp.* activity and in the last few years, there has been an increase in IBH cases. Lusitano horse breeding plays an important role in the Portuguese economy and riders are making increasing use of the Lusitanos around the world (Cordeiro Raposo 2002). IBH can result in reduced commercial value of the affected horses, as well as extra costs to control the disease symptoms and altered behaviour due to discomfort. Moreover, euthanasia may be considered when the symptoms are too severe and there is no way of controlling their impact on animal welfare. Furthermore, the results of this study may have an important contribution to future implementation of locally relevant SPT allergen panels for IBH diagnosis and eventually for specific immunotherapy.

Aim of the study:

The present observational cross-sectional study aimed at characterizing and comparing the results of skin allergy tests, both IDTs and SPTs, and *in vitro* allergy tests (sLT release assay and serum specific IgEs), in Lusitano horses from stud farms in Portugal with a clinical diagnosis of IBH, as compared to healthy controls living on the same farms.

3. Materials and Methods

3.1. Horses (sample)

In total 60 horses were tested, divided in two groups for the 1st allergen panel:

- Test Group (T) - 30 IBH-affected horses presenting symptoms at the time of the tests.
- Control Group (C) - 30 healthy horses.

For the 2nd allergen panel, a cluster of these horses, in total 12 animals (6 T and 6 C horses), were simultaneously (1st and 2nd panels) tested in the same conditions, as described above.

3.1.1. Characterization of the C and T horses:

a) Ages

The mean age of the T and C groups was 7 and 9 years with a standard deviation of 5.32 and 6.06, respectively. The horses presented an age range that varied between 2 and 25 years and 2 and 27 years, in groups T and C respectively. They were grouped into several age ranges namely, 2-5 years, 6-9 years, 10-14 years, 15-20 years and over 20 years, with the highest rate of horses occurring, in the case of the T group in the age range of 2-5 years (57%) and 6-9 years (37%) in group C. The lowest rate of horses tested was, for both groups, ≥20 years with 3 and 7% in groups T and C respectively.

b) Sex

Regarding sex, 73% of the tested animals were females, a total of 44 mares, 27 and 17 from C and T groups respectively. Only a third of the tested animals were males (n=16) and mostly were stallions, a total of 14, and only two geldings (1 each C and T groups). In Portugal most of the Lusitano females are breeders and live in an outdoor regimen all year round being more exposed to the *Culicoides spp*. thus presenting more signs of IBH, than the males that are kept mostly indoors, and less exposed to the *Culicoides spp*.

3.1.2. Inclusion criteria for the horses in the T group:

- Must be ≥ 1 year old.
- Living predominantly outdoor.
- Must present a seasonal pruritic dermatitis.
- The lesions must be no less than grade I (broken hair on the mane and/or base of the tail), from at least the previous equivalent season.
- No glucocorticoid or antihistamine therapy within the two weeks prior to the tests was allowed.

3.1.3. Exclusion criteria for the horses:

- Horse breeds, other than Lusitano.
- Gestational mares.
- Horses that presented other skin diseases.
- Horses that presented systemic signs of other diseases.

3.2. Skin allergy tests:

Two different types of skin allergy tests were performed in all animals: SPTs and IDTs. These were mainly performed during springtime (from March to June, between

2013 and 2016), corresponding to the onset of the symptoms for the IBH-affected horses. The readings of the papules' diameters were assessed at 20 min, 6 and 48 hours.

3.2.1. *Culicoides* allergens:

In total, in a first stage, 14 specific allergens were tested on all horses, which included 13 recombinant proteins, all expressed in *E.coli*) (Peeters et al. 2013; Schaffartzik et al. 2011) from C*ulicoides nubeculosus* and *Culicoides obsoletus* salivary glands (Cul n 1 to Cul n 11, Cul o 1P and Cul o 2P), as shown in table 1, and *Culicoides nubeculosus* (Cul n) whole body extract (WBE).

Table 1: Allergens used in skin tests and in vitro testing of IBH-affected horses (T) and control horses (C).

Allergen	Buffer	Skin Test (ST)/ Serology (S)	References
Cul n 3	PBS	S/ST	Schaffartzik et al 2011
Cul o 1P	20mM Tris 0.5M NaCl	S/ST	Peeters et al. 2013
Cul o 2P	20mM Tris 0.5M NaCl	S/ST	Peeters et al. 2013
Cul o 3	20mM Tris 0.5M NaCl	S	Van der Meide et al 2013
Cul o WBE	0.9% NaCl	ST	Peeters et al. 2013
Cul n 1	H2O **(Solvent)**	ST	Schaffartzik et al 2011
Cul n 2	PBS	ST	Schaffartzik et al 2011
Cul n 3	H2O **(Solvent)**	S/ST	Schaffartzik et al 2011
Cul n 4	H2O **(Solvent)**	S/ST	Schaffartzik et al 2011
Cul n 5	H2O **(Solvent)**	ST	Schaffartzik et al 2011
Cul n 6	H2O **(Solvent)**	ST	Schaffartzik et al 2011
Cul n 7	H2O **(Solvent)**	ST	Schaffartzik et al 2011
Cul n 8	H2O **(Solvent)**	ST	Schaffartzik et al 2011
Cul n 9	PBS	ST	Schaffartzik et al 2011
Cul n 10	H2O **(Solvent)**	S/ST	Schaffartzik et al 2011
Cul n 11	H2O **(Solvent)**	ST	Schaffartzik et al 2011
Cul n WBE	0.9% NaCl	ST	Ziegler et al 2018
Cul n 4 Bar	PBS	ST	Jonsdottir et al 2018
Culn 4 Bac	PBS	ST	Jonsdottir et al 2018
Cul n 3 Bac	PBS	ST	Jonsdottir et al 2018
Cul n 3 Bar	PBS	ST	Jonsdottir et al 2018

In a second stage of the study, six other allergens were tested, but only on 12 horses, 6 IBH-affected and 6 C-group: they included the allergens Cul n 3 and Cul n 4, expressed both in insect cells and barley (Cul n 3Bac, Cul n 3Bar, Cul n 4Bac, Cul n 4Bar) (Jonsdottir et al. 2019) and the Cul o recombinant allergen, Cul o 3, as well as a

Cul o WBE. The *Culicoides* extracts (Peeters et al. 2013) and all recombinant allergens had been produced and purified as described previously (Jonsdottir et al. 2019; Schaffartzik et al. 2011).

3.2.2. Performing the tests:

Horses were sedated with intravenous detomidine hydrochloride 0.01mg/kg (Domosedan® 10 mg/ml, Orion Corporation; Espoo, Finland) 5 min before testing.

A 20 x 40cm rectangle was then clipped on the left side of the neck, to allow 4 lines with 8 inoculation points each, for SPTs. On the right side of the neck a rectangle of 20 x 30cm was clipped, allowing 3 lines with 6 inoculation points each, for the IDTs, as seen in figure 1 (A and B).

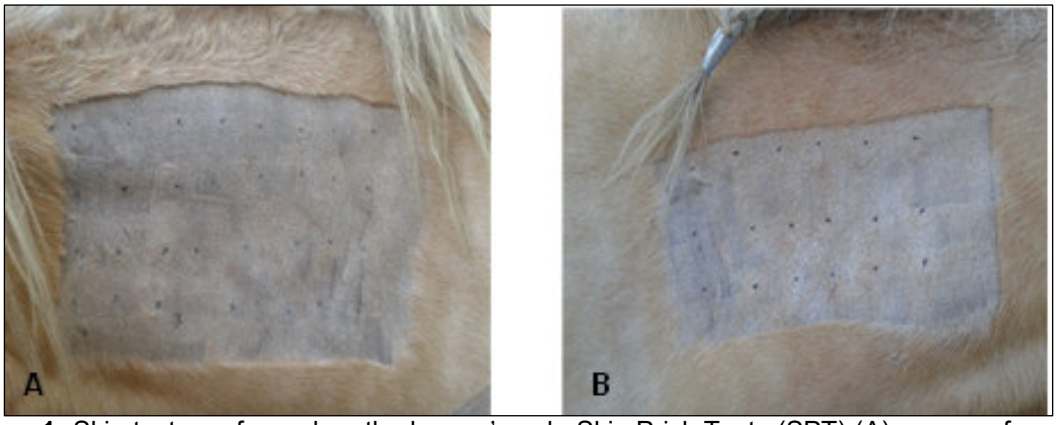

Figure 1: Skin tests performed on the horses' neck; Skin Prick Tests (SPT) (A) were performed on the left side and Intradermal Test (IDT) on the right side (B).

Five cm distance was allowed between inoculation points. For the IDTs, 0.1 ml of each allergen was injected at a concentration of 10µg/ml, using a 25GX5/8 needle. For the SPTs, a single drop of each of two concentrations (10 and 100µg/ml) was inoculated per allergen (figure 2).

Figure 2: Skin Prick tests were performed by using a single drop of the allergen on the marked site, and a lancet was used for inoculation.

Papule diameters were measured 20 min after inoculation and at 6 and 48 hours. Saline, PBS, or the buffer in which the recombinant allergens were soluble (table 1) were used as negative controls and histamine chlorhydrate (10 mg/ml) as the positive control. To avoid technical errors performing IDTs, all syringes were filled with the predetermined volume before starting the tests. All administrations were performed by the same investigator.

3.2.3. Readings

Skin test results, for both IDTs and SPTs, were evaluated by directly reading the papules' diameter. The mean of two orthogonal diameters (mm) was then calculated. For the IDTs, results were considered positive when the papule size was at least half the size of the histamine wheal (positive control), as reported previously (Tahon et al. 2019). For the SPTs results, a statistical analysis was performed to find the cut-off value for the wheal diameter to be considered positive. Cut-off values were evaluated from 0.8 to 1.2 cm, as is explained later in the results, and were considered as positive when the mean of the papules' orthogonal diameters was ≥ 0.9 cm, with readings assessed at 20 min after inoculation.

3.3. Sulfidoleukotriene (sLT) release assay

To perform the *in vitro* sLT release assay, blood samples were collected into vacuette containing ACD-B as anticoagulant (Greiner Bio One®) and shipped to the laboratory at the Vetsuisse Faculty, University of Bern, Switzerland within 24 h. The cellular antigen stimulation test (CAST, Bühlmann laboratories, Allschwil, Switzerland) was performed as described previously (Baselgia et al. 2006). To briefly describe the technique: leucocyte-rich plasma was collected, transferred into a propylene tube, and then centrifuged. The supernatant was removed, and the pelleted cells resuspended in stimulation buffer (Bühlmann laboratories) containing heparin. Cells were then incubated in buffer only to determined spontaneous sLT release or with anti-IgE (0.75µg/ml) as stimulation control, and with whole body extracts (WBE) from *Culicoides* nubeculosus (Cul n WBE) (2 µg/ml) and *Culicoides obsoletus* (2 µg/ml). After 40 min, plates were centrifuged, and the supernatants transferred into 96-well microtiter tissue culture plate to be kept at -20°C until assayed. Released sLTs were measured using the CAST ELISA (Bühlmann laboratories) following the manufacturers' instructions. For all further evaluations values of the net stimulation were used, i.e., the spontaneous sLT release was subtracted from values obtained with the anti-IgE or *Culicoides* WBEs. Data from horses with sLT values <250 pg/ml after stimulation with the anti-IgE were considered as non-responders) (Marti et al. 1999) and their results were not included for the analysis of the data obtained with the *Culicoides* WBEs. Three out of the 25 IBH horses and 4 out of the 24 controls tested in CAST were non-responders.

3.4. IgE serology by ELISA

For the serological tests, jugular blood was collected from all horses into 9 ml vacutainer serum tubes, about 10 minutes before the onset of the skin tests, and serum was then separated by centrifugation. In agreement with previous studies (Frey et al. 2008; Marti et al. 1999; Peeters et al. 2012; Peeters et al. 2013; van der Meide et al. 2012; Ziegler et al. 2018), our serum samples were collected during *Culicoides* exposure season, so the serological responses were evaluated after the IBH-affected horses started showing clinical signs. Frozen serum samples were then sent to the laboratory of the Clinical Immunology Group, Vetsuisse Faculty of the University of Bern. Allergen-specific IgE was measured by ELISA as described previously (Peeters et al. 2013; Schaffartzik et al. 2011), for the allergens listed in table 1. ELISA plates were coated with *Culicoides* recombinant allergens at a final concentration of 2 ug/ml in 0.2 M Carbonate–Bicarbonate buffer, pH 9.4 (Thermo scientific™) for 2h at 37°C. After washing in 0.9% NaCl, 0.05% Tween 20, non-specific binding sites were blocked with blocking buffer

(PBS- 5% dried milk powder and 0.05%Tween® 20, pH 7.4) for 1h at 37°C. Plates were then washed twice with wash buffer and the sera, previously diluted 1:5 in blocking buffer, added in duplicates to the ELISA plate and incubated overnight at 4°C. After washing, a monoclonal antibody specific for equine IgE (3H10, diluted in blocking buffer to a final concentration 1 mg/ml) was added to the plates and incubated for 2h at room temperature (RT) on a shaker. After a further washing step an alkaline-phosphatase-conjugated goat anti mouse IgG with minimal cross reactivity to horse serum proteins (Jackson ImmunoResearch™; https://www.jacksonimmuno.com) was added and incubated for 1.5h at RT on a shaker. After final washes, plates were developed with 1.5 mg/ml phosphatase substrate (Sigma™; https://www.sigmaaldrich.com/CH) in 10% diethanolamine (Fluka™; https://www.sigmaaldrich.com/CH), pH 9.8 and absorption measured at 405 nm after 2 h. After subtraction of the blank, OD values were corrected for differences between the plates coated with the same allergen. The correction factor for each plate was calculated based on the average of the OD values of the positive controls included on all plates (Jonsdottir et al. 2019). Results are shown as corrected OD values.

3.5. Statistical Analysis

Statistical analysis was carried out using SPSS program and different statistical tests were used to evaluate the skin allergy tests' data (size of the papule), namely ANOVA Repeated Measures and Discriminant Analysis with a classification matrix ≥70% for SPTs and ≥60% for IDTs, testing three independent variables: Exposure– two groups (control - C and IBH-affected - T), Time – over three time points (20min, 6 and 48h), and Type of test – Skin Prick Test (SPT) and Intradermal Test (IDT). To determine cut-off values, non-parametric tests were used, namely Kruskal-Wallis's test to determine statistically significant differences between groups (T and C) at each time, and Friedman's test to compare the evolution over time, considering p-value ($p \leq 0.05$). Cut-off values were selected at a given specificity and at the highest accuracy possible.

As to the sLT release assay and the IgE serology, medians were used as the results were not normally distributed. The Mann-Whitney U test was used to compare median values of the IBH and control groups. To evaluate the performance of the tests, non-parametric Receiver Operating Curve (ROC) analysis was performed and calculations of the area under the empirical curve (AUC) were performed NCSS version 11. ROC Curves' analysis was used to select the best cut-off values in the sLT release assay. Cut-off values were selected at the highest accuracy possible. The level of significance was set at $p \leq 0.05$ for all comparisons performed.

4. Results

All horses showed a positive response to histamine (positive control), in both skin tests performed, as shown in figure 3.

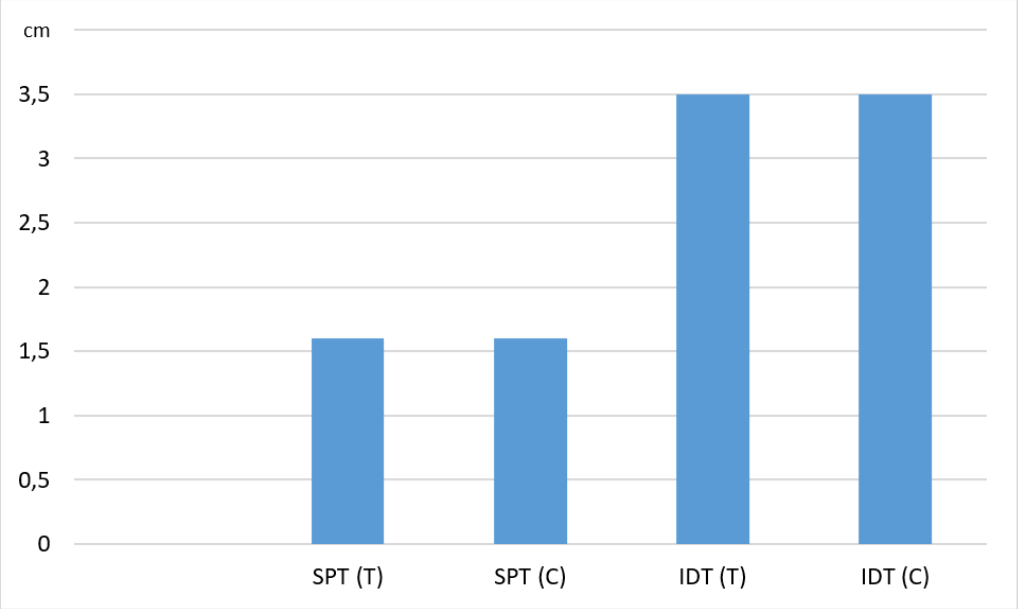

Figure 3: Absolute mean value of papules' diameter (cm) for test (T, n=30) and control (C, n=30) groups evaluated in both skin tests (SPT and IDT) after inoculation of histamine (positive control). (σ: C=1,2017; T= 1,1199, for IDTs). Abbreviations: T, test group; C, control group; IDT, Intradermal Test; SPT, Skin Prick Test; σ, Standard deviation.

Regarding negative controls, in case of PBS there were some positive reactions in both T and C groups. In case of SPTs we had a total of nine horses that showed positivity from T group and 2 from C group. The papules' mean diameter was 1,021 cm (T) and 0,886 cm (C) with a standard deviation of 0,8110 and 0,8636 for T and C groups respectively, with a significance of 0,31 ($p>0.05$). Regarding IDTs we had a total of 16 IBH-affected horses and 18 from C group that showed positivity to PBS, with the papules' mean diameter of 1,6 cm (T) and 1,56 cm (C), for $p>0.05$. Despite having positive values in T and C groups for both SPTs and IDTs, no statistically significant differences were detected, hence no interference in the results of the significant allergens diluted in PBS, was considered.

Hypersensitivity reactions to the *Culicoides* allergens were evaluated immediately by means of both methods to estimate possible significant differences between T and C groups and contribute to the identification of the specific allergens involved in IBH.

4.1. Skin Tests:

4.1.1 Skin prick test (SPT)

For the first panel of allergens, after a discriminant analysis with a classification matrix ≥70, significant differences between population of C and T horses were found for Cul n WBE, Cul n 7, Cul n 8, Cul n 9, Cul o1P and Cul o2P (table 2).

Table 2: Papules' diameters mean and standard deviation (σ) for the statistically significant allergens ($p<0.05$), for IDT and SPTs and both control (C, n=30) and IBH-affected (T, n=30) groups, for the 1st allergen panel and readings assessed at 20 min. Abbreviations: ^{NS} Non-significant allergens for IDTs; IDT, Intradermal Test; SPTs, Skin Prick Test. Significant differences are indicated by * ($p<0.05$).

Allergen (1st panel)	Test	Group	Mean (cm)	Standard deviation (cm)	95% Confidence Limits	
					Lower	Upper
Cul n WBE	IDT*	C	1,47	0,45	1.308	1.631
		T	1,65	0,47	1.481	1.818
	SPT*	C	0,33	0,42	0.179	0.480
		T	0,78	0,54	0.586	0.973
Cul n7	IDT^{NS}	C	1.12	0.55	0.923	1.310
		T	1.01	0.50	0.831	1.188
	SPT*	C	0,21	0,4	0.066	0.353
		T	0,48	0,5	0.301	0.658
Cul n8	IDT*	C	0.87	0,57	0.666	1.074
		T	0.97	0,77	0.694	1.245
	SPT*	C	0,31	0,42	0.159	0.460
		T	0,39	0,45	0.228	0.551
Cul n9	IDT*	C	1,21	0,58	1.002	1.417
		T	1,37	0,35	1.244	1.495
	SPT*	C	0,32	0,4	0.176	0.463
		T	0,66	0,57	0.456	0.863
Cul o1P	IDT^{NS}	C	1.33	0.60	1.115	1.541
		T	1.30	0.62	0.803	1.521
	SPT*	C	0.30	0.40	0.156	0.443
		T	0.61	0.54	0.416	0.803
Cul o2P	IDT*	C	1,21	0,73	0.948	1.471
		T	1,26	0,72	1.002	1.517
	SPT*	C	0,46	0,48	0.288	0.631
		T	0,69	0,45	0.529	0.851

Differences between C (n=30) and T (n=30) groups for these allergens are shown in figure 4 in the graphic representation of Estimated Marginal Means.

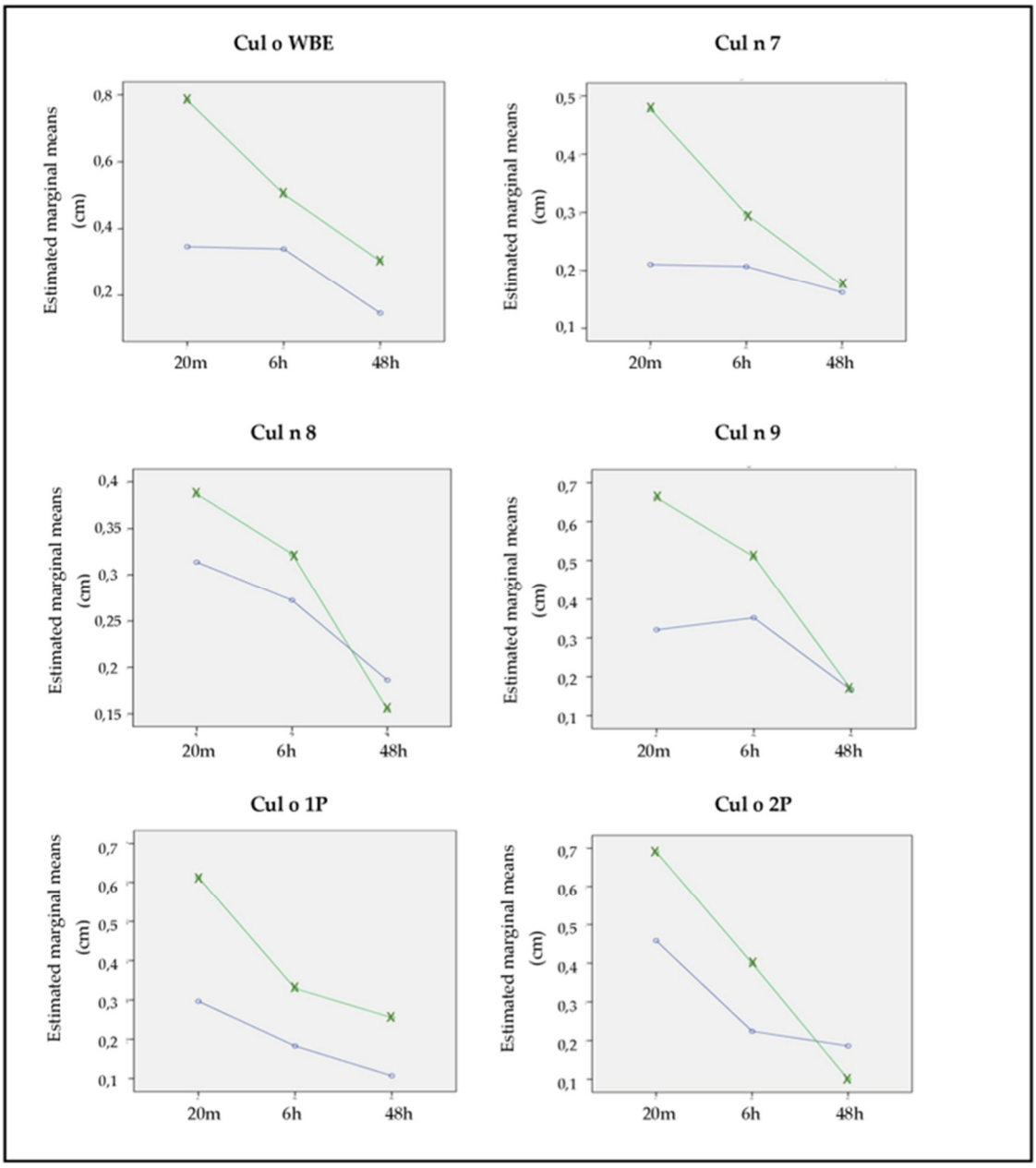

Figure 4: Estimated Marginal Papules' Means Measures (cm) for the statistically significant allergens, over time, between IBH-affected (T, n=30) and healthy (C, n=30) horses tested, for the 1st allergen panel (SPTs, at a concentration of 100µg/ml), with a classification matrix of 70%, ($p<0.05$). Abbreviations: SPT, Skin Prick Test; T, test group; C, control group. In the graphic representation of estimated marginal means, T group is indicated with a green line T —x— and C group with a blue line —o—.

For the second allergen panel, the allergens that presented statistically significant differences and better discriminated between groups C and T for both IDTs and SPTs,

were Cul o WBE, Cul n 3Bar and Cul n 4Bac (p≤0.05). However, the number of horses tested was quite small. Papule's diameters' mean and standard deviation (σ) for these allergens are shown in table 3.

Table 3: Papules' diameter means and standard deviation (σ) for the statistically significant allergens* (p≤0.05), for IDT and SPTs, and both control (C=6) and IBH-affected horses (T=6) groups for the 2nd allergen panel, with readings assessed at 20 min.

Allergen (2nd panel)	Test	Group	Mean (cm)	Standard deviation (cm)	95% Confidence Limits	
					Lower	Upper
Cul o WBE	IDT	C	1.46	0.33	1.195	1.724
		T	1.53	0.33	1.265	1.794
	SPT	C	0.13	0.33	-0.134	0.394
		T	0.65	0.51	0.217	1.082
Cul n 3 Bar	IDT	C	0.39	0.22	0.213	0.566
		T	1.56	0.29	1.327	1.792
	SPT	C	0	0	0	0
		T	0.48	0.54	0.0479	0.912
Cul n 4 Bac	IDT	C	0.53	0.29	0.340	0.762
		T	0.83	0.29	0.597	1.062
	SPT	C	0.23	0.36	- 0.058	0.518
		T	0.27	0.47	- 0.106	0.646

Abbreviations: IDT, Intradermal Test; SPTs, Skin Prick Test.

In general, and for both allergen panels, the papules' diameters of the IBH-affected horses (T) were statistically larger when compared to the control group (C) as seen in tables 2 and 3.

On the other hand, statistically significant differences between C and T groups were only observed when a concentration of 100µg/ml (p≤0.05) was used, with readings assessed at 20 min.

Regarding the cut off value for SPTs, evaluations were made from 0.8 to 1.2 cm. For a cut-off of 0.8 cm, there was a high variability and no statistically significant differences were found between groups. Considering a cut-off of 0.9 cm, it was possible to determine statistically significant differences between test and control groups, at 20 min.

Overtime there was a decrease in papules' diameter, with the maximum mean value occurring at 20 minutes, and no papules were present at 48 hours. This is shown in figure 5, for Cul n WBE, comparing SPTs and IDTs, for the T group.

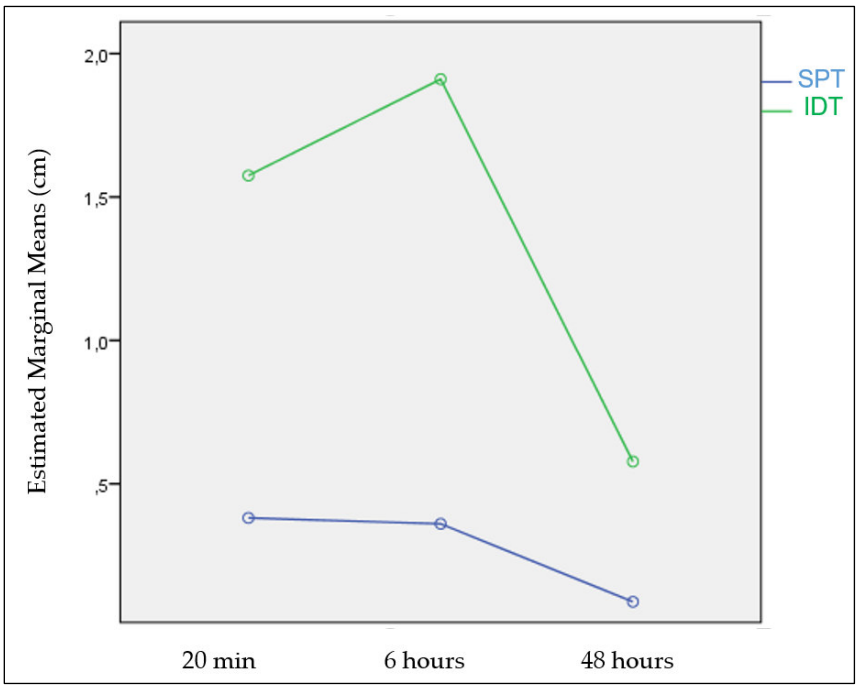

Figure 5: Estimated Marginal Papules' Means Measures (cm), over time, for both Skin Prick Test (SPT) and Intradermal Test (IDT), for IBH-affected horses (test group), for Cul n WBE.

Estimated Marginal Papules' Means Measures (cm), over time, for both Skin Prick Test (SPT) and Intradermal Test (IDT), for IBH-affected horses (test group), for Cul n WBE.

For the rest of the significant allergens the diameter of the papules performed identical over time for both skin tests, IDTs and SPTs, reflected in identical and therefore overlapping graphs.

Regarding the 1st allergen panel, it was also possible to determine that all the IBH-affected horses showed positivity for 1 to ≥4 of the statistically significant allergens. Furthermore, 70% of these T group horses presented positivity to at least 4 allergens (figure 6).

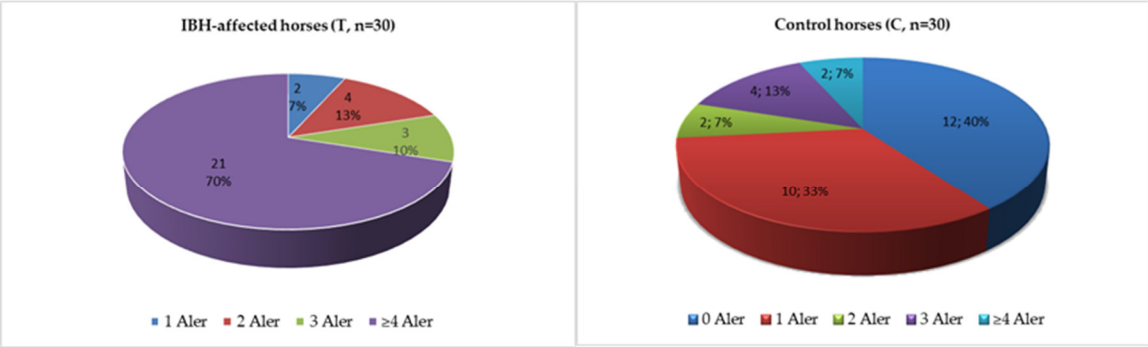

Figure 6: Total number of statistically significant allergens' positive reactions for the 1st allergen panel, for both control (C) and test (T) groups, for SPTs, at a concentration of 100µg/ml. Abbreviations: control (C); test (T); SPT, Skin Prick Test; Aler, allergens.

Regarding the control group, 10 horses showed positivity to just one of the statistically significant allergens, and only 2 horses presented positivity to ≥4 of the statistically significant allergens, but the majority, 40%, presented no positivity to any of the allergens (figure 6). In addition, all IBH-affected horses, without exception, were positive for the Cul n WBE allergen, and only six horses from C group were positive to Cul n WBE (figure 7). The second highest frequency of positive results in the T group occurred for Cul n 9 (figure 7).

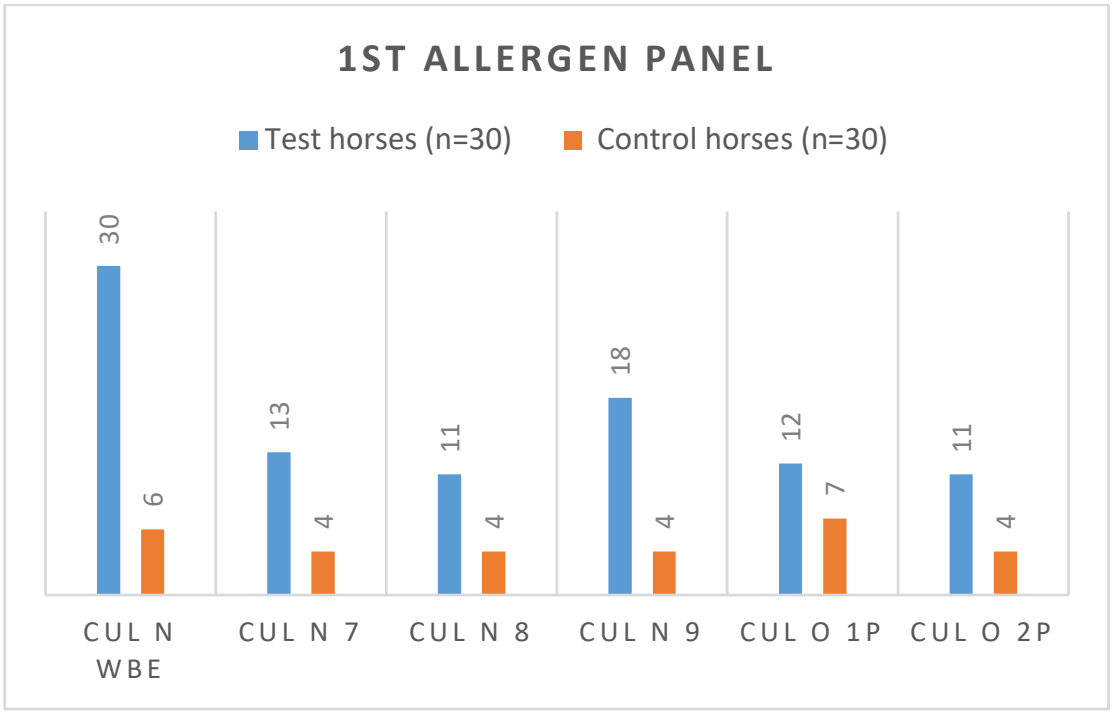

Figure 7: Number of horses with positive reactions for each statistically significant allergen tested SPT at a concentration of 100µg/ml, for both control (C) and IBH-affected horses (T), for the 1st allergen panel. Abbreviations: Control, C; Test, T, SPT, Skin Prick Test.

Regarding the 2nd allergen panel results are shown in figure 7A. At least 1 of the IBH-affected horse were positive to each statistically significant allergen, and Cul o WBE presented 2 IBH-affected positive reaction. No positive reactions were seen in C group for any of the statistically significant allergen.

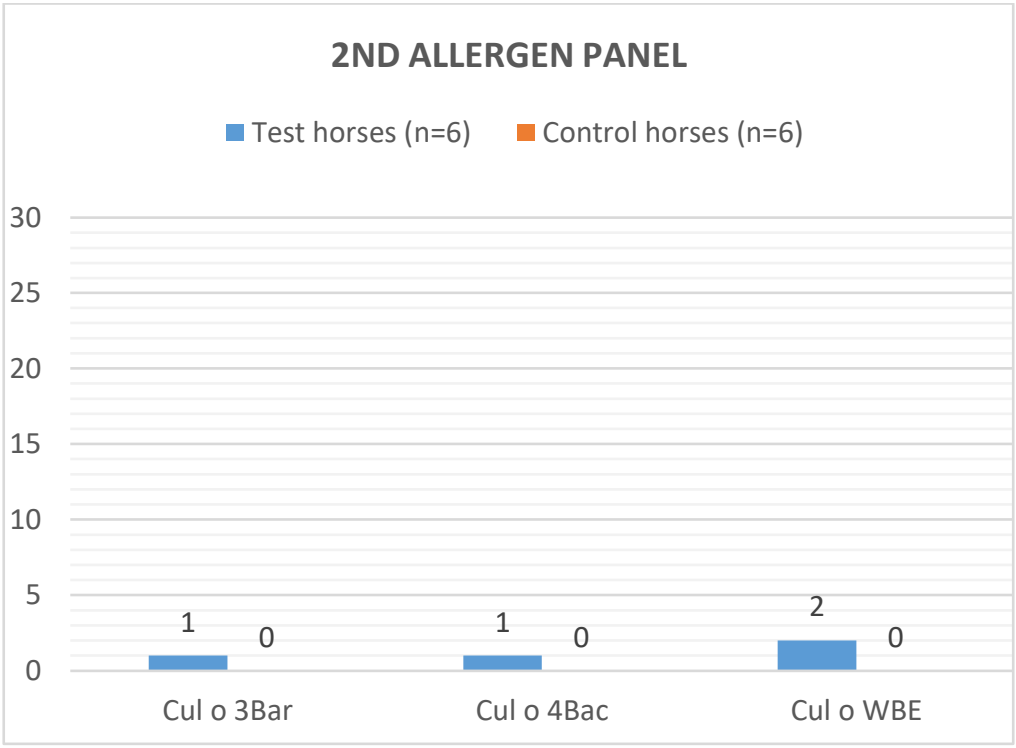

Figure 8: Number of horses with positive reactions for each statistically significant allergen tested (SPTs) at a concentration of 100µg/ml, for both Control (C) and IBH-affected (T) groups, for the 2nd allergen panel. Abbreviations: Control (C); Test (T); Skin Prick Test (SPT).

4.1.2. Intradermal Tests:

In case of IDTs a classification matrix of 60% was determined and statistically significant differences were found for Cul n WBE, Cul n 8, Cul n 9 and Cul o2P (table 2).

In comparison with SPTs' regarding the second allergen panel, the allergens that presented statistically significant differences and better discriminated between groups C and T, were Cul o WBE, Cul n 3Bar and Cul n 4Bac ($p \leq 0.05$). Papule's diameters' mean and standard deviation (σ) for these allergens are shown in table 3.

For both allergen panels, the papules' diameters of the IBH-affected horses (T) were statistically larger when compared to the control group (C) as seen in tables 2A and 2B.

The authors also observed that for most of the allergens tested, the papules' diameters in the IDTs were always greater than those observed in SPTs (Tables 2 and

3), but we must acknowledge that the volume that was inoculated in each site for the IDTs, was by far higher than the volume used for the SPTs.

Overtime there was an increase in papules' diameter, with the maximum mean value occurring at 6 hours, and a decrease at 48 hours (figure 5).

Regarding IDTs, readings at 20 min, 6 and 48 hours are shown in figure 9. There was an increase at 6 hours with a significant decrease at 48 hours after inoculation. This observation may also be seen in figure 5.

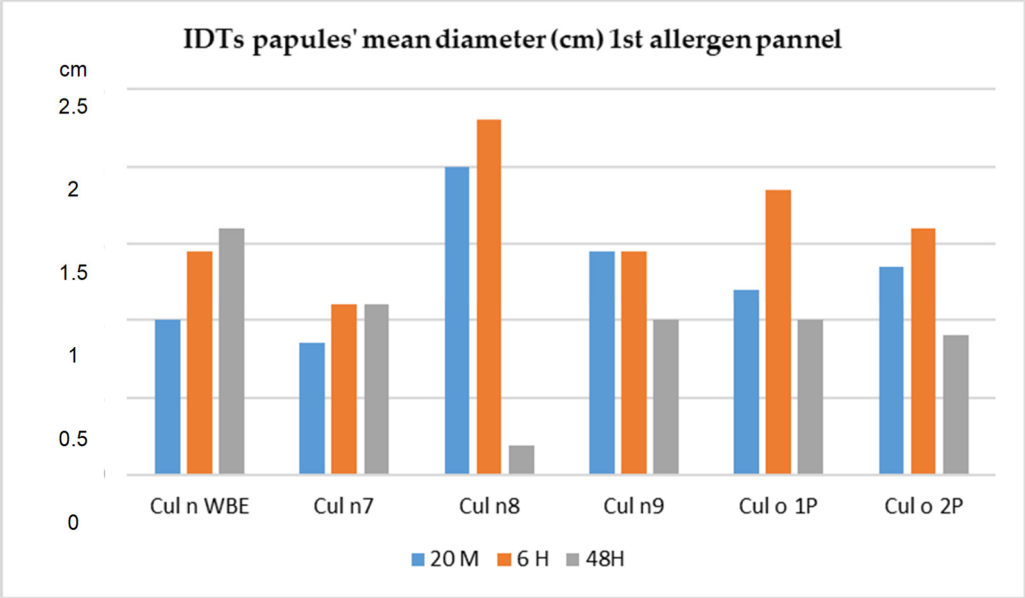

Figure 9: Intradermal test papules' mean diameter (cm) overtime (20 min. 6 and 48 hours) for the test group (T, n=30), 1st allergen panel.

4.2. sLT Release Assay

The median sLT release was significantly higher in IBH horses compared to controls with Cul n WBE (142 vs 84 pg/ml, $p<0.05$) and more clearly, with Cul o WBE (445 vs 12 pg/ml, $p<0.0001$) as shown in figure 10.

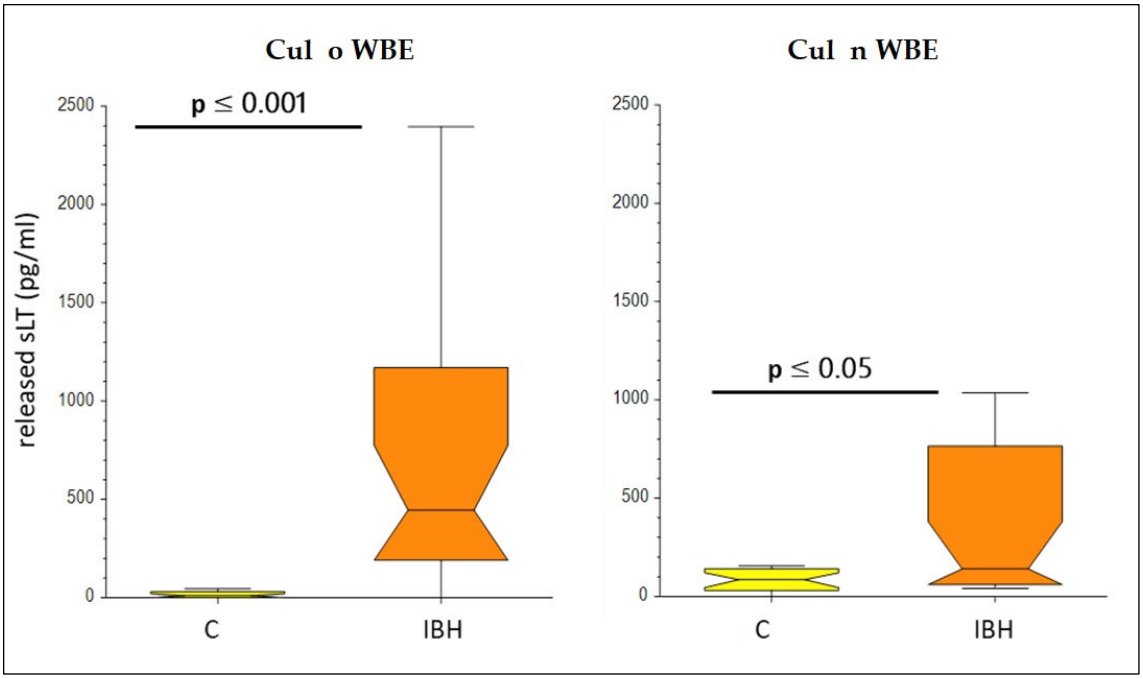

Figure 10: Sulfidoleukotriene (sLts) release assay with *Culicoides obsoletus* WBE (Cul o WBE) and *Culicoides nubeculosus* WBE (Cul n WBE). A total of 20 control (C) and 22 test (IBH) horses were tested. Results are presented as box plots, whereby, the center horizontal line of the box plot marks the median of the sample. The edges of the box mark the first and third quartiles and the whiskers define the upper adjacent value which is the largest observation that is less than or equal to the 75th percentile plus 1.5 times the interquartile range (IQR) and the lower adjacent value which is the smallest observation that is greater than or equal to the 25th percentile minus 1.5 times IQR. P-values were calculated using the non-parametric Mann-Whitney U test ($0.001<p<0.05$). Abbreviations: Control (c, n=20); test (IBH, n=22).

However, when using the cut-off value of 340 pg/ml that had been established in an earlier study (Baselgia et al. 2006) the sensitivity with Cul n WBE was very low (36%) with a moderate specificity of 85%. The use of Cul o WBE resulted in a better sensitivity (64%) and specificity (90%), but the performance of the test was still lower than previously published (Baselgia et al. 2006). A ROC analysis was thus performed as seen in table 4.

Table 4: ROC analyses of the sulfidoleukotriene (sLTs) release assay with Cul o WBE and Cul n WBE (0.001<p<0.01).

			Z-Value to test	Upper 1-Sided	95% Confidence Limits	
Allergen	**AUC**	**Standard Error**	**AUC>0,5**	**P-value**	**Lower**	**Upper**
Cul_n WBE	0.6986	0.0835	2.377	≤0.01*	0.4965	0.8288
Cul_o WBE	0.8923	0.0533	7.363	≤0.0001*	0.7262	0.9600

Abbreviations: AUC, area under curve; Control (C, n=20); IBH-affected (T, n=22) horses describing non-responders.

It confirmed the better performance of the assay when using Cul o WBE instead of Cul n WBE, as shown by the higher area under curve (AUC) of 0.892 for Cul o WBE compared to Cul n WBE (AUC 0.699). Decreasing the cut-off for Cul o WBE from 340 pg/ml to 250 pg/ml allowed an increase of the sensitivity to 73% at the same specificity of 90%. The best accuracy of the test was obtained using a cut-off value of 70 pg/ml and resulted in a sensitivity of 86% and a specificity of 85%.

4.3. IgE serology

The results from the IgE serology are summarized in table 5.

Table 5: Results of allergen specific IgE levels (OD Values) in horses' sera determined by ELISA for both IBH-affected (T) and control (C) horses, and differences found for specific IgE levels between groups, T and C, using ROC Analysis (0.001<p<0.05).

Specific IgE Levels (ELISA)				ROC Analysis (n=59)					
Allergen	C (N= 29)	T (N=30)	P Value (U-Test)	Significance	AUC	Standard Error	Z-Value to Test	Upper 1-Sided P-Value	95% Confidence Limits
	Median (Min-Max Values)	Median (Min-Max Values)					AUC > 0.5		Lower/Upper
Cul n 3	0,106(0,052-0,171)	0,293 (0,152-0,454)	p ≤ 0,001	Significant	0.7621	0.0616	4.251	≤0.0001	0.612/0.859
Cul n 4	0,016 (0-0,06)	0,119(0,02-0,18)	p≤ 0,05	Significant	0.6707	0.0702	2.432	≤0.0175	0.509/0.786
Cul n 10	0,035 (0-0,086)	0,09 (0,07-0,195)	p≤ 0,05	Significant	0.7063	0.0674	3.060	≤0.01	0.548/0.815
Cul o 2	0,391 (0,323-0,444)	0,573 (0,439-0,776)	p≤ 0,05	Significant	0.6747	0.0714	2.446	≤0.01	0.509/0.791
Cul o 1P	0,201 (0,167-0,217)	0,564 (0,26-0,82)	p ≤ 0.01	Significant	0.7730	0.0626	4.361	≤0.0001	0.495/0.778
Cul o 2P	0,216 (0,132-0,275)	0,36 (0,223-0,467)	p ≤ 0.05	Significant	0.7184	0.0672	3.249	≤0.001	0.559/0.826
Cul o 3	0,159 (0,114-0,201)	0,323 (0,159-0,389)	p ≤ 0.05	Significant	0.6598	0.0719	2.221	≤0.01	0.495/0.778

Statistically significant differences were found between T and C groups (p≤0.05) for the following allergens: Cul n 3, Cul n 4, Cul n 10, Cul o 2, Cul o 1P, Cul o 2P and Cul o 3 (figure 11).

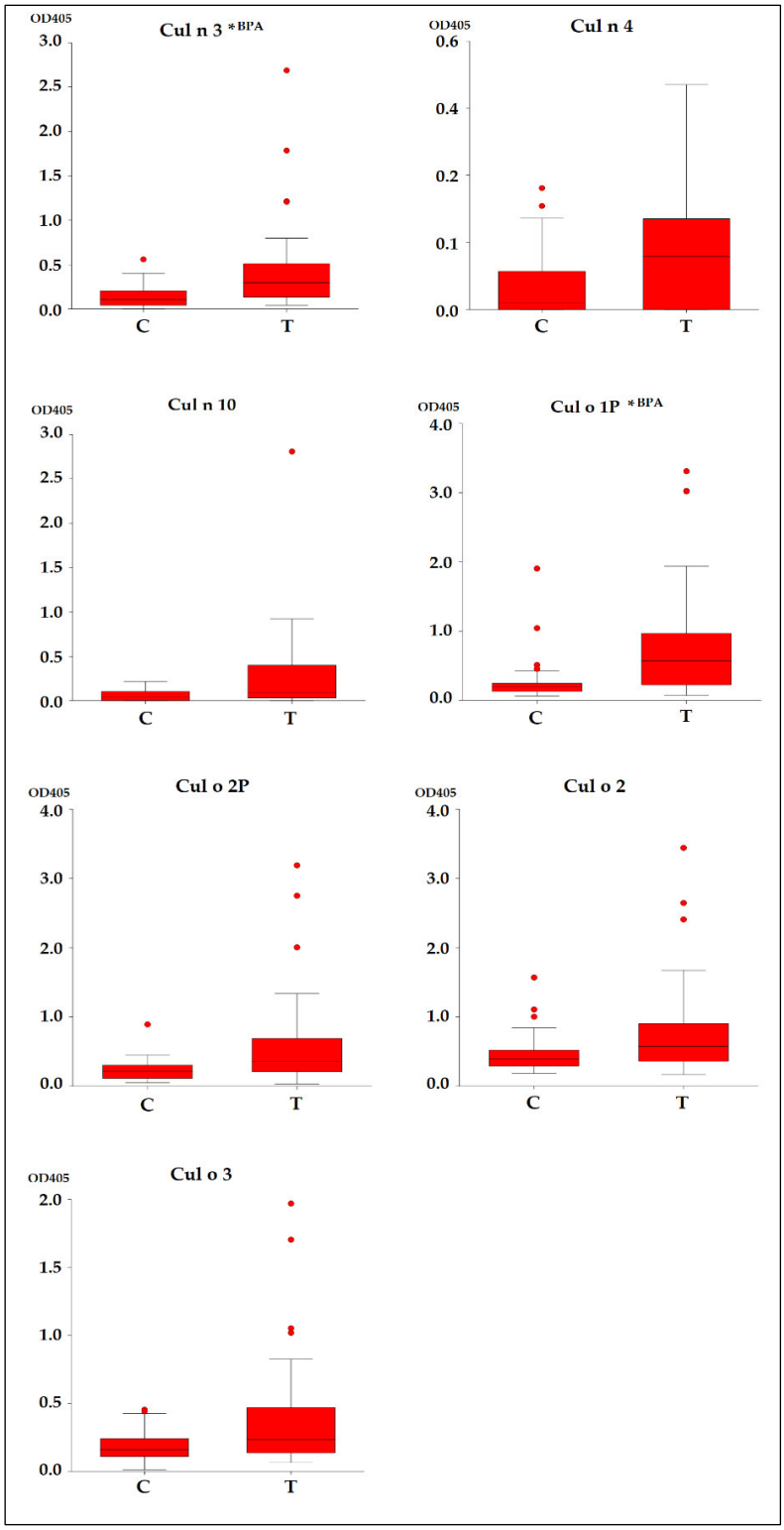

Figure 11: Boxplot representation for specific IgEs (OD405) that presented statistically significant differences, detected by ELISA in horses' serum (n=59) (p<0.05). Abbreviations: C, Control Group, n=29; T, Test Group, n=30. *BPA Best Performing Allergens

The area under curve for the *Culicoides* allergens ranged from 0.659 to 0.773 and shows that Cul n3 and Cul o1P are the best performing allergens (BPA).

5. Discussion

Our experimental observational cross-sectional study allowed us to analyze if IgE mediated reactions to certain *Culicoides* allergen could distinguish between allergic and non-allergic horses. Based on the results obtained in the skin tests of the present study, Cul n WBE, Cul n 7, Cul n 8, Cul n 9, Cul o 1P and Cul o 2P may potentially be the most relevant of the tested allergens for the study of IBH in Lusitano horses. These seem to be promising candidates for an SPT diagnostic test panel, involving allergens which were chosen based on the geographical area. To determine which combination of specific allergens would result in the best panel, discriminant analysis by the stepwise method was used with IBH status as the outcome, as also used in another recent study (Novotny et al. 2020). This showed that the allergens described above, Cul n WBE, Cul n 7, 8, 9, Cul o 1P and Cul o 2P, were the minimum number of allergens that truly discriminated between the Test (T) and Control (C) groups, regarding SPTs, with a classification matrix of the discriminant analysis ≥70%, as stated above. If some of these allergens were not included in the panel, the classification matrix would lower. In case of IDTs, Cul n WBE, Cul n 8, Cul n 9 and Cul o 2P were the allergens that discriminated between the Test (T) and Control (C) groups, with a lower classification matrix of the discriminant analysis (≥60%). Cul o WBE, Cul n 3Bar and Cul n 4Bac for both SPTs and IDTs, although tested on a smaller number of horses, were apparently also relevant, and the authors believe that they should be tested on a larger number of horses before any recommendations are made. Interestingly, Cul n3 and Cul n4 did not seem relevant in the first group of horses. This could be explained by the fact that Cul n 3 and Cul n 4 expressed in *E. coli* were used in the first group of horses, while the same allergens expressed in insect cells and barley (Jonsdottir et al. 2019) were tested in the second group. Proteins expressed in *E. coli* are often found in inclusion bodies and need a refolding step. As they lack posttranslational modifications, because eukaryotic proteins expressed in *E. coli* often form inclusion bodies and cannot always be refolded correctly, it may decrease allergen's functionality (Curin et al. 2016). In the present study, 70% of the IBH horses seem to be allergic to ≥4 of these allergens, in case of SPTs. The allergen that all IBH- affected horses were positive to was Cul n WBE. Although there are also some positive results for Cul n WBE in the C group (6 animals), the wheal diameters for Cul n WBE were larger in the IBH- affected horses. Still, from our results we could assess that Cul n WBE presented a specificity of 80% It is known from previous studies (van der Meide et al. 2012) that IDTs using *Culicoides* whole body extract (WBE) often result in a positive

reaction, even in clinically healthy horses. Natural allergenic extracts are heterogeneous and may contain non-allergenic components, in addition to allergens (Arruda et al. 2013). These extracts are susceptible to contamination with allergens from other sources and may contain enzymes proteolytic with the ability to reduce the concentration of allergen in the extract (Arruda et al. 2013). The associated problems to the use of natural extracts include difficulties with the assessment of potency and inconsistencies inherent in the production of extracts with equivalent content of allergen (Arruda et al. 2013). Almost all allergen sources contain multiple major and minor allergens, and even with the use of modern techniques, it is difficult to standardize these mixtures of different proteins (Chapman et al. 2000). As the Cul n WBE used in our study, in both IDT and SPT, was a whole-body extract (WBE) allergen containing in principle all allergens, this may explain why most of our IBH-affected horses and some horses from the C group showed a positive reaction to it.

Nevertheless, the performance of the SPT with *Culicoides nubeculosus* WBE (Cul n WBE) was rather good. The specificity with some of the recombinant allergens was better, reaching 87%, but the sensitivity was usually much lower, at best 60% with Cul n 9. On the other hand, the use of recombinant allergens (r-allergens) may have an advantage compared to natural allergen sources, as they provide specific allergen products for the diagnosis and treatment of allergic diseases (Jutel et al. 2012). Recombinant allergens are produced with a higher degree of purity, in larger quantities, either with a similar capacity to bind IgEs when compared to their natural counterparts or with modifications to reduce the reactivity to IgEs (hypoallergens) (Linhart, Valenta 2012). However, as mentioned above, depending on the expression system used their functionality may be reduced (Curin et al. 2016). In humans, studies performed using r-allergens in skin tests revealed that their use is not only safe, but also has a good diagnostic efficacy when compared to natural extracts for diagnostic purposes (Jutel et al. 2012). Some previous studies in horses (Jonsdottir et al. 2019; van der Meide et al. 2012; van der Meide et al. 2013) suggested that the use of recombinant *Culicoides* allergens (r- Cul) may provide a more specific diagnosis of IBH in sensitized horses, decreasing the number of false positive reactions. Hence the use of r-Cul allergens may allow better diagnosis and better allergen immunotherapy (Jonsdottir et al. 2009).

Our results showed that at least 5 r-allergens Cul n 7, 8, 9, Cul o 1P and Cul o 2P, presented relevance for the study of IBH in Lusitano horses, as they have a considerably elevated diagnostic discriminant potential (≥70%) and seem to be a good choice to include in an SPT diagnostic test panel. New *Culicoides obsoletus* allergens (Novotny et al. 2020), were discovered and produced after this study had been carried out, which may further improve the discriminant potential of the SPT for IBH. To the

authors' best knowledge this was the first study comparing two different skin tests, IDTs and SPTs, *Culicoides* allergen specific IgE determination and a sLT release assay in horses with a history of IBH, and specifically in Lusitanos. IDTs were until now considered the most sensitive and confirmatory skin tests for horses (Langner et al. 2008; Novotny et al. 2021; Sloet van Oldruitenborgh-Oosterbaan et al. 2009; Van der Meide et al. 2013), and although some authors still argue in favour of IDTs (Jose-Cunilleras et al. 2001), according to others (Wagner et al. 2009), allergic horses may sometimes present several positive reactions to multiple allergens in IDTs, even to those allergens that are unlikely to cause allergies. Multiple studies over the years have shown that even healthy horses can react to IDTs (Jose-Cunilleras et al. 2001; Lane et al. 2017; Sloet van Oldruitenborgh-Oosterbaan et al. 2009; van Damme et al. 2020), which can frequently induce false positive reactions in clinically healthy individuals (DeBoer, Hillier 2001; O'Driscoll et al. 2009). This may represent an additional challenge to identify the allergens that truly induce disease. Also, IDTs may require a more specialized technique and interpretation of results may be more time consuming (Wagner et al. 2006). IBH is mainly an IgE-mediated immediate type I reaction (van der Haegen et al. 2001; Wilson et al. 2001) and skin test readings should thus be assessed at 20 min and at 6 hours, when binding of allergen to mast cells, already loaded with specific IgE's, occur. However, readings may also be assessed at 48 hours, considering that later reactions may also occur (Lomas, Robinson 2018), if there is cell involvement, or other late reactions due to the release of non-histaminic factors such as sulphidoleukotrienes (sLT) (Bagelsia et al. 2006). In SPTs, the amount of inoculated allergen is much smaller, when compared to IDTs. Nevertheless, extracts utilized for intradermal skin testing are less concentrated (for example 1:10–1:1000; 0.00001 µg/ml up to 1 µg/ml) than those used for SPTs (Heinzerling et al. 2013; Wood et al. 1999). It is usual to use this difference in concentrations between IDT and SPT in human medicine, where SPT is indicated if a type I (immediate type) allergy is suspected, based on the medical history and clinical signs (Heinzerling et al. 2013). Furthermore, previous work published by our group also used this higher concentration is SPTs in horses with asthma (Tilley et al. 2012). The technique used to perform SPTs is minimally invasive (Heinzerling et al. 2013) and the probability of SPTs inducing false positive reactions is lower when compared to IDTs (Heinzerling et al. 2013). Hence, IDTs are considered less specific than SPTs according to other authors (Heinzerling et al. 2013; Wood et al. 1999). This observation is in accordance with our results, considering the classification matrix (≥70% for SPTs and ≥60 % for IDTs) and C/T ratio determined for each allergen and test (IDT and SPT). For Cul n 7, Cul o 1P C/T ratio was ≥1 only for the IDTs, and no statistically significant differences were found between C and T groups. Hence according to our results, SPTs

presented a higher discriminatory diagnostic potential than IDTs in this population of studied horses and for the allergens tested. Furthermore, SPT results are immediately available and when carried out by trained professionals can be interpreted in 20 minutes with no need for later readings. SPTs are easily reproducible at a relatively small cost allowing the clinician to show a cutaneous reaction to a hard-to-convince owner (Jensen-Jarolim et al. 2015; Tilley et al. 2012). Also, many different allergens can be tested simultaneously because the reaction to a specific allergen is localized to the immediate area of the SPT and systemic anaphylactic reactions are rare (Heinzerling et al. 2013). In our case, during the experiment, no anaphylactic reactions occurred. Furthermore, in human allergology, SPTs are an important cornerstone for standard diagnosis of type I, IgE-mediated, immediate allergic diseases (Heinzerling et al. 2013; Jensen-Jarolim et al. 2015) and wheal diameters above the positive control or ≥0.3 cm are considered positive (Jensen-Jarolim et al. 2015). In our case a cutoff value of 0.9 cm was determined, and as seen in a previous study (Tilley et al. 2012), where the cutoff value estimated was 1 cm, positive control values (histamine) were also much higher than those that normally occur in the human species. In the present study, results showed that SPTs performed at a concentration of 100µg/ml, with readings assessed at 20min, with a cutoff value of 0.9 cm, presented better results than IDTs (10µg/ml) in terms of discriminatory diagnostic potential. As IBH is known as a seasonal skin disease associated with a type I, IgE mediated, hypersensitivity reaction to *Culicoides spp*. (Fadok & Greiner 1990), the authors believe that further studies should be carried out to include SPTs in its diagnostic procedures. This would represent a step forward in the establishment of IBH diagnostic tools and allow an accurate determination of the allergens to which IBH-affected horses are sensitized. Nevertheless, even though the results found in the present study are quite promising, the clinical significance of specific IgEs and sLTs release assay in the diagnosis of IBH or any other allergic disease, shown by SPTs, should be interpreted according to the patient's history and physical examination (Lorch et al. 2001; O□Driscoll et al. 2009) and seen within the clinical context. In human allergy diagnostics i*n-vitro* measurement of allergen specific IgEs (Liccardi et al. 2002; Ricci et al. 2003) remains an important complementary tool to diagnose type I, IgE-mediated, allergic diseases (Heinzerling et al. 2013). Skin tests may not be feasible, particularly if there are widespread skin lesions, even being contraindicated in situations involving the risk of anaphylactic shock. They may also sometimes lead to false negative or positive results (Crockard, Ennis 2001[a, b]). The measurement of specific IgE, despite presenting, in general, a relatively high specificity in the diagnosis of type I allergic reactions IgE mediated, often has a lower sensitivity than that of skin prick tests (Homburger 1998). According to other authors (Peeters et al. 2013), the diagnostic value of commercially

available serological IgE tests for equine IBH is questionable (Frey et al. 2008). Nevertheless, a previous study using ELISA has shown that IBH-affected horses have significantly higher serum IgE levels against recombinant *Culicoides* allergens than healthy control horses (Novotny et al. 2021; Schaffartzik et al. 2011; Van der Meide 2013). Hence, IgE levels to recombinant *Culicoides* allergens were determined in the sera of the C and T Lusitano horses. The T horses had significantly higher serum allergen specific IgEs than the C group for Cul n 3, Cul n 4, Cul n 10, Cul o 2, Cul o 1P, Cul o 2P and Cul o 3. However, there was some overlap between the groups, meaning that some of the C horses also had serum specific IgE for *Culicoides* r-allergens. This might be explained by a high degree of exposure to *Culicoides* bites resulting in some degree of asymptomatic sensitization (Lam et al. 2020). A high exposure to allergens can lead to IgE sensitization to asymptomatic individuals due to some feedback mechanisms (for example IgGs) that maintain the homeostasis of the immune system. Asymptomatic sensitization is defined as the presence of positive skin prick test (SPT) and/or positive serum allergen specific IgE in the absence of clinical allergic symptoms (XU et al. 2021). In humans there are at least about 10 to 20% of the population who exhibit evidence of IgE mediated sensitization and who have never had relevant symptoms of allergic disease. Currently, there is no convincing explanation why some people with positive allergen tests do not show symptoms (XU et al. 2021). Cul n 3 and Cul o 1P were the allergens that better detected differences in allergen specific-IgE values between the C and T groups. Cul o1P also showed significant differences between T and C horses in the SPTs. Similarly, Cul o2P also showed significant differences between groups, in both SPTs and IgEs ELISA. For Cul n 3, Cul n 4 and Cul n 10 there was no significant difference in SPT between T and C groups, although the T horses had significantly higher serum IgE in ELISA than the C group (Table 5). These discrepancies might be due to a not correct refolding of the *E. coli* expressed allergens, which is more important for functional tests such as SPT or cellular allergy tests than for binding of free serum IgE. Interestingly, barley or insect cell expressed Cul n 3 and Cul n 4 led to significantly higher SPT reactivity in the T compared to the C group (Table 4). This illustrates the relevance of posttranslational modification and folding of the allergens for functional tests. Unfortunately, because of lack of availability of the recombinant allergens not all allergens could be evaluated both in skin tests and IgE ELISA. It has been previously stated by Wilkołek et al. (2019) that *in vitro* measurement of allergen s-IgEs is the laboratory equivalent of clinical skin testing**.** It is also said that *in vitro* measurement of allergen s-IgEs could be an alternative to IDTs in horses and equine practitioners often prefer to use only serological IgE assays to identify significant allergens (van Damme et al. 2020). The authors find that, and in accordance with Lorch et al. (2001) and O'Driscoll

et al. (2009), to determine the clinical significance of specific IgEs in IBH diagnosis, it is necessary to undertake SPTs and serum allergen specific IgE testing, as well as considering the patient's history and physical examination. This agrees with previous studies in humans (O'Driscoll et al. 2009), and with our group's study on equine asthma syndrome (Tilley et al. 2019).

Importantly, serological IgE assays only show sensitization to an allergen, which does not necessarily reflect clinical allergy. Functional tests such as SPT are usually considered to be closer to the clinical expression of allergy. The same is true for functional *in vitro* assays. They are based on the *in vitro* activation of blood basophils in the presence of the allergen (Wilkołek et al. 2019). Some are based on the liberation of inflammatory mediators, such as histamine and sulfidoleukotrienes (sLts) (CAST – Cellular Allergen Stimulation Test) (Crockard, Ennis[a, b] 2001; De Weck et al. 2002). In some cases, the sulfidoleukotrienes (sLT) release assay by leukocyte stimulation when exposed to the allergens (CAST), demonstrated greater sensitivity and specificity compared to other tests, including the determination of specific IgE (De Weck et al. 2002). For equine IBH CAST can so far only be performed with *Culicoides* extracts, as recombinant *Culicoides* allergens did not or only very weakly induce sLT release. Studies have shown that the CAST with *Culicoides nubeculosus* extract is useful to confirm the clinical diagnosis of IBH in horses from Switzerland (Bagelsia et al. 2006). The CAST results confirm the relevance of *Culicoides* allergens and of *Culicoides obsoletus*, for IBH in Lusitano horses. From all *in vitro* assays (IgE serology and CAST), the highest AUC was obtained for the CAST with *Culicoides obsoletus* extract (Cul o WBE) (AUC=0.897, 95% CI =0.735-0.962), when compared with Cul n WBE. Recombinant *Culicoides* allergens were not used in the CAST because the *E. coli* expressed allergen did not or only weakly induce sLT release (Eliane Marti, personal communication). Another limitation of the CAST is that fresh blood samples are needed. In conclusion based on the results of this study for the SPTs, Cul n WBE, Cul n 7, Cul n 8, Cul n 9, Cul o 1P and Cul o 2P, were the best performing allergens. Regarding *in vitro* assays sLT release essay Cul o WBE was the best performing allergen, and even with a lower sensitivity and specificity, the serum allergen specific IgEs, Cul n 3, Cul n 4, Cul n 10, Cul o 1P, Cul o 2P, Cul o 3 and Cul o 2 were determined as the best performing allergens in Lusitano horses.

6. Conclusions

The results of this study support the use of SPTs as a major contribute to the diagnosis of IBH and may represent a step forward as an IBH diagnostic tool and eventually in the establishment of patient tailored, component resolved specific immunotherapy. Moreover, the identification of relevant *Culicoides* allergens involved in IBH in Lusitano horses using the allergen specific IgE ELISA and the sLT release assay suggests that it will be worthwhile to test further *Culicoides obsoletus* recombinant allergens in SPT, probably increasing the performance of this test for the diagnosis of IBH. However, in agreement with other authors (Carrapatoso et al. 2005), even though conducting objective tests, both *in vivo* and/or *in vitro* is important to confirm the clinical suspicion of allergy, a thorough anamnesis and clinical examination remains essential for diagnosis of allergy. Finally, the evaluation of new pharmacological or immunotherapeutic options for IBH will benefit from valid skin testing with a specific allergen panel which may also allow for the comparison of results among study populations.

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equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice.

Informed Consent Statement: Written informed consent was obtained from the legal owners of the horses.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author. The data are not publicly available due to a privacy request from the horse legal owners.

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Chapter V:

General discussion, general conclusions, future perspectives, general limitations, human and animals' rights

"An itchy horse is an unhappy horse…"

1. General Discussion:

Insect Bite Hypersensitivity (IBH) is the most common skin allergy in horses and involves mainly a Type I (IgE mediated) hypersensitivity reaction. It is clinically characterized by intense pruritus, areas with alopecia and/or broken hairs, skin irritation and other skin lesions. Clinical signs are caused mainly by an enhanced immune reaction to allergens present in the salivary glands of biting midges belonging mainly to the *Culicoides* genus.

Welfare of affected horses is seriously reduced, and they can become unsuitable for riding or showing purposes, due to discomfort and disfigurement. No fully curative treatment is yet available for IBH, and current diagnostic tests still lack in accuracy.

IBH's prevalence in Lusitano horses in Portugal is still unknown. Nevertheless, there seems to be a high prevalence of the disease, according to owner's complaints and the observations of veterinary clinicians working in stud farms throughout the country, leading to a negative economic impact in Lusitano stud farms. These increased reports of IBH in Lusitano horses may have to do with the environmental changes, especially the increase in temperature, predominantly from spring to fall. At present, stud farm owners are more aware and concerned about IBH and the effects it can have on their horses, not just economically, but also at the welfare level.

Although it was still not possible to evaluate the prevalence of IBH in the present study, it may be a first contribute to the understanding of IBH in Portugal. The percentage of IBH-affected horses in this study was quite variable, ranging from 2 to 60%, with a mean of 36%, a median of 4%, and a standard deviation of 18%. The number of horses per farm were between 2 and 42, with an average of 10 horses per farm, of which an average of 3 horses had IBH, ranging from 1 to 6 horses IBH-affected horses per farm.

The goal of the study described in this thesis was to increase our understanding of IBH in Lusitano horses living in Portugal mainland. It aimed mostly at identifying the *Culicoides* species more frequently detected in Lusitanos' stud farms that may have relevance in IBH, and improving diagnostic methods using skin prick tests, with a panel of different *Culicoides* allergens (extracts and r-allergens).

In order to characterize the studied population, questionnaires were made, along with physical exams and photographic records. General information about the horses, stud farms and housing, was obtained. More than seventy percent of the horses were females (73%), living in an outdoor regimen all year round. This is in accordance with the type of horse breeding in Lusitanos' stud farms in Portugal. All horses were ≥2-year-old, at the age of the onset of IBH's signs.

Furthermore, most of the IBH-affected horses presented frequent pruritus, as expected, since it is the most frequent and characteristic clinical sign of IBH. Pruritus occurs due to the production of cytokines, namely IL-31, during the development of the immune response Th2 regulated and IgE mediated (Jonsdottir et al. 2019).

Moreover, most of the IBH-affected horses presented lesions mainly on the mane, base of the tail, head, and dorsal line. The distribution of lesions is in accordance with previous descriptions (Marsella et al. 2023).

The severity of the lesions for IBH-affected horses was mostly mild to moderate. This observation may be explained by the period of the year that the animals were tested. The tests were performed mainly in the beginning of spring when *Culicoides* are not very active yet. Rendle (2014) stated that the reduced exposure to the *Culicoides* may alleviate suffering and optimize the welfare of the IBH-affected horses. Hence, it is predictable that IBH-affected horses that still hadn't been extensively exposed to the *Culicoides* allergens, will not present severe signs or lesions.

Therefore, the IBH-affected horses included in this study complied with the pre-established inclusion and exclusion criteria, as they presented evidence of seasonal pruritic dermatitis and lesions at the time of the evaluation. Regarding C horses, no clinical signs, or lesions of IBH were present at the time of the evaluation. These observations validate the sample of the horses included in both T and C groups.

Regarding the identification of *Culicoides spp.* in the Lusitanos' stud farms, a significant amount of different *Culicoides* species were captured. *Culicoides* from *Obsoletus* group (*C. obsoletus / C. scoticus), C. newsteadi* and *C. imicola* were the ones most frequently found near Lusitano stud farm with a history of IBH.

Other species referred in the literature as being responsible for IBH in horses, such as *C. pulicaris (*Mellor & McCaig 1974, Hesselholt & Agger 1977), *C. punctatus* (Townley et al. 1984) and *C. circumscriptus* (Baker & Collins 1984), were also caught in the traps, but in smaller quantities. In one capture, *C. puncticollis* was also found. This species is very close to *C. nubeculosus*, known to be responsible for IBH cases (Hellberg et al. 2006, Schaffartzik et al. 2012, van der Meide et al. 2013), suggesting that C*. puncticollis* may also have a role in IBH in Lusitanos in Portugal mainland.

The *Culicoides* that are mainly associated with IBH are *C. nubeculosus, C. obsoletus* and with less expression *C. sonorensis.* Losson et al. (2017) described that in Belgium *C. obsoletus* was found the most important *Culicoides spp.* attracted to horses.

In the present study the variation in the *Culicoides* species captured in the different stud farms may be explained by environmental conditions and availability of breeding sites (van der Rijt et al. 2008). Regarding the environmental conditions, temperature is of great importance to the activity, longevity, and survival of *Culicoides*

(Wittmann & Baylis 2000). In fact, van der Rijt et al. (2008) stated that the ideal temperature range for all *Culicoides spp*. was 14º–32º C and, since environmental conditions in the Iberian Peninsula are similar, this can explain the similarity to the species that were captured in Spain, and the different scenario seen in other related studies (van der Rijt et al. 2008).

It is of major importance to identify *Culicoides spp*. close to stud farms in Portugal and in other countries that may have an impact in IBH, to understand the host preferences of the native population of *Culicoides spp.* (Hadje-Henni et al. 2015)

Nevertheless, IBH-affected horses may react to both native and exotic species of *Culicoides*, even though, according to van der Meide et al. (2012), native species of *Culicoides* may induce a stronger IgE-mediated immune response and better sensitivity assays are achieved.

In order to evaluate the role of the species identified in the present study, testing saliva extracts from these *Culicoides* would be of great importance. This may contribute to determine their allergic properties, not only for further knowledge of IBH in Lusitano horses, but to enhance future research. The results obtained in this study may help to establish a local and relevant allergen panel for IBH diagnostic and to implement future immunotherapy (ASIT) in IBH-horses.

Hence, further captures in Lusitano stud farms in Portugal should be performed to complement our results and to establish consistent data concerning *Culicoides* species occurring near these horses.

In order to contribute to IBH diagnosis in this geographical region, this was the first study to compare both *in vivo* allergy skin tests, intradermal tests (IDT) and skin prick tests (SPT), and *in vitro* tests, allergen specific-IgE determination and sLT release assay, in Lusitano horses with a history of IBH. These tests allowed us to analyse if IgE mediated reactions to a given *Culicoides'* allergen panel could distinguish between allergic (T group) and non-allergic horses (C group).

Regarding skin prick tests (SPTs) Cul n WBE, Cul n 7, Cul n 8, Cul n 9, Cul o 1P and Cul o 2P seemed to be the most relevant allergens, that truly discriminated between Test (T) and Control (C) groups. These allergens presented a classification matrix of the discriminant analysis ≥70% and seem to be promising candidates to be included in a diagnostic allergen panel for SPTs.

In case of IDTs, Cul n WBE, Cul n 8, Cul n 9 and Cul o 2P were the allergens that discriminated between the Test (T) and Control (C) groups, with a lower classification matrix of the discriminant analysis (≥60%).

For the second allergen panel which was tested in a smaller number of horses from the T and C groups (n=12, 6T and 6C), Cul o WBE, Cul n 3Bar and Cul n 4Bac

were apparently also relevant, for both SPTs and IDTs. Nevertheless, they should be tested on a larger number of horses before further recommendations are made.

The expression system used to produce the allergens may be relevant to explain some differences found in the same allergens, when expressed with different systems. For example, Cul n 3 and Cul n 4 expressed in *E. coli* did not seem relevant, but the same allergens expressed in insect cells and barley (Jondonstir et al. 2019) were considered relevant. Proteins expressed in *E. coli* are often found in inclusion bodies and need a refolding step. As they lack post translational modifications, some may not refold properly, which can be critical for the functionality of the allergen (Curin et al. 2016), decreasing their allergenicity.

Cul n WBE was the allergen that all IBH- affected horses were positive to in both IDT and SPT, with a specificity of 80%. This result is in accordance with a previous study (Langner et al. 2008), where IDTs, using *Culicoides spp.* whole body extract (WBE) or saliva extract, were performed in IBH-affected horses presenting a sensitivity of 78% and specificity of 100%. However, intradermal tests have limited use as a routine clinical test for IBH diagnostic (Hensel et al. 2004; Peeters et al. 2013). Also, it is known from previous studies (van der Meide et al. 2012) that IDTs using *Culicoides* whole body extract (WBE) often result in a positive reaction, even in clinically healthy horses. In the present study some C horses presented positive reactions to Cul n WBE, but with no statistical significance. These were considered false positives to non-allergic substances present in the extracts, or even due to some degree of sensitization as reported by other authors (van der Meide et al. 2012).

The results of the present study showed that SPTs not only presented a higher discriminant diagnostic potential but are also more specific than IDTs (Heinzerling et al. 2013) with the possibility to be easily adapted as a routine clinical test. Even though in SPTs the amount of inoculated allergen is much smaller when compared to IDTs, the extracts used for SPTs are more concentrated (for example 1:1000–1:10; 1µg/ml up to 0.00001 µg/ml) (Heinzerlign et al. 2013; Wood et al. 1999). This difference in concentrations between IDTs and SPTs is commonly used in human medicine. A previous study published by our work group also reported to have used a higher concentration for the allergens used in SPTs, in horses with asthma (Tilley et al., 2012).

Allergic whole body/salivary extracts (WBE) are heterogeneous mixtures that contain multiple major and minor allergens mixed (Arruda et al. 2013; Chapman et al. 2000) allowing estimating the reactivity of a patient's serum toward all potentially allergenic components. And, even though some authors say that the use of recombinant allergens (r-allergens) may provide more specific allergens for the diagnosis of IBH (Jutel et al. 2012) and to implement targeted immunotherapy (Jonsdottir et al. 2019; van der

Meide et al. 2012) their performance may vary, depending on the expression system used (Curin et al. 2016). Also, recombinant allergens do not cover the entire spectrum of potentially allergenic proteins present in extracts and may give false-negative results (Smoldovskaya et al. 2016).

The Cul n WBE used in our study, for both IDT and SPT, was a whole-body extract (WBE). This allergen contained in principle all allergens, which may explain why most of our IBH-affected horses and a few horses from the C group showed a positive reaction to it.

Our results showed that at least 5 r-allergens Cul n 7, 8, 9, Cul o 1P and Cul o 2P, presented relevance for the study of IBH in Lusitano horses, as they have a considerably high diagnostic discriminant potential (≥70%) and seem to be a good choice to include in an SPT diagnostic test panel.

IDTs were until now considered the most sensitive and confirmatory biological *in vivo* assays for horses (Langner et al. 2008; Sloet van Oldruitenborgh-Oosterbaan et al. 2009). Nevertheless, allergic horses may sometimes present several positive reactions to multiple allergens in IDTs, and even healthy horses can react to IDTs (Jose-Cunilleras et al. 2001; Lane et al. 2017; Sloet van Oldruitenborgh-Oosterbaan et al. 2009; van Damme et al. 2020). As IDTs can induce false positive reactions even in clinically healthy individuals (DeBoer et al. 2001; O'Driscoll et al. 2009), it may be challenging to identify the allergens that truly induce disease.

Also, IDTs are more useful to assess cell involvement in delayed hypersensitivity reactions (Heinzerling et al. 2013). As a practical example, in human medicine IDTs are standard of care in the study of drug allergies, as they evaluate cell involvement in delayed hypersensitivity reactions, either in the late phase of IgE mediated reactions or non-IgE mediated allergic reactions (Torres et al. 2017). Moreover, in practice, IDTs may require a more specialized technique and the interpretation of results may be more time consuming, when compared to SPTs (Wagner et al. 2006).

SPTs are considered more specific than IDTs, as the probability of inducing false positive reactions is lower. (Heinzerling et al. 2013; Wood et al. 1999). This was confirmed in the present study, with SPTs presenting a higher discriminatory diagnostic potential than IDTs in these horses regarding the allergens tested. Furthermore, SPT results are immediately available and when carried out by trained professionals can be interpreted in 20 minutes. Also, they are easy to reproduce at a relatively small cost allowing the clinician to show a skin reaction to a hard-to-convince owner (Jensen-Jarolim et al. 2015; Tilley et al. 2012), by testing different allergens simultaneously.

For all that was stated above, there are clear advantages in using SPTs instead of IDTs in IBH diagnostics and further studies should be carried out to include SPTs as

a standard diagnostic test. SPTs can be easily performed in ambulatory clinic in the horse's own environment, with proper training by experienced professionals. Currently this training is being provided in Portugal by our study group through postgraduate workshops organised within the interest group in Compared and Veterinary Immunology of the Portuguese Society of Allergology and Clinical Immunology (SPAIC). These workshops have reinforced the use of SPTs by clinicians for the diagnosis of equine asthma. In a near future, it is our goal to extend SPTs for IBH's diagnosis as well. Presently, the panel can be used at the Equine Hospital of the Faculty of the Veterinary Medicine of the University of Lisbon in collaboration with the University of Bern. In the future, it is expected to be available for outpatient use. We believe this represents a step forward in the establishment of IBH diagnostic tools and can allow clinicians to determine more accurately the allergens to which IBH-affected horses are sensitized.

The results found in the present study, regarding the use of SPTs in the diagnosis of IBH in Lusitano horses in Portugal, are quite promising. SPTs revealed to be clinically relevant in the diagnosis of IBH, when used at a concentration of 100µg/ml, with readings assessed at 20min and a cut off value of 0.9 cm. Nevertheless, it should be noted that results need to be interpreted according to the patient's history and physical examination and seen within the clinical context.

Regarding *in vitro* tests, the measurement of specific IgEs present in general a relatively high specificity in the diagnosis of IgE mediated type I allergic reactions (Homburger et al. 1998). Nevertheless, Peeters et al. (2013) stated that no reliable serological diagnostic tests for equine IBH are commercially available. Also, according to Homburger et al. (1998) the measurement of specific IgEs often presents a lower sensitivity when compared to skin prick tests. Serological tests may sometimes present low sensitivity probably due to low IgE serum levels since most of the allergen s-IgE are bound to the surface of mast cells and basophils. Nevertheless, previous studies using ELISA showed that IBH-affected horses have significantly higher serum IgE levels against recombinant *Culicoides* allergens than healthy horses (Schaffartzik et al. 2011). A study in the Netherlands showed that IBH-affected horses presented higher specific IgE levels for the Cul o 1 allergen deriving from the local *C. obsoletus*, than for the Cul s 1 allergen from the less common *C. sonorensis*, even though they were the same protein, a maltase (van der Meide et al. 2013). It is therefore crucial for IBH-affected Lusitano horses that further allergens are isolated from the species that were identified to be most prevalent in the Portuguese Lusitano stud farms, in order to further develop diagnostic tests with an adequate allergen panel. This is particularly true if we want to complete the allergen panel that was advanced by the present study, in order to develop specific immunotherapy.

In the present study specific IgE levels to r-allergens were determined in the sera of the C and T Lusitano horses. T horses had significantly higher serum allergen specific IgEs than the C group for the significant allergens, Cul n 3, Cul n 4, Cul n 10, Cul o 2, Cul o 1P, Cul o 2P and Cul o 3, but some of the C horses also had serum allergen s-IgE for *Culicoides* r-allergens. This might be explained by a high degree of exposure to *Culicoides* bites resulting in some degree of asymptomatic sensitization (Lam et al. 2020). A high exposure to allergens can lead to IgE sensitization in asymptomatic individuals due to some feedback mechanisms (for example IgGs) that maintain the homeostasis of the immune system. Asymptomatic sensitization is defined as the presence of positive SPTs and/or positive serum allergen specific IgE in the absence of clinical allergic symptoms (Xu et al. 2021). Lam et al. (2020) suggested that the allergic mechanisms of IBH are influenced by the duration of the allergen exposure and, somehow, it is likely that allergic horses when chronically exposed to the allergens may also experience a shift in their immune response. The early onset of IBH is classified as an IgE-mediated or type I allergy. During the development of the allergy, chronic allergen exposure might shift the differentiation of cTh2 into pathogenic effector (peTh2) cells, which are highly positive for IL-5 and also a major source for eosinophil production, recruitment, and activation.

It was previously stated by Wilkołek et al. (2019) that the *in vitro* measurement of allergen s-IgEs is the laboratory equivalent of clinical skin testing and could be an alternative to IDTs in horses. In fact, equine practitioners often prefer to use only serological IgE assays to identify significant allergens (van Damme et al. 2020), but serological IgE assays only show sensitization to an allergen, which does not necessarily reflect clinical allergy. Hence, it is my believe and it is also in agreement with of our group's study on equine asthma syndrome (Tilley et al. 2012) that, to determine the clinical significance of specific IgEs in IBH diagnosis, it is necessary to undertake a multiple approach. This should include functional tests, such as SPTs, which are closer to the clinical expression of allergy, serum allergen specific IgE testing and the patient's history and clinical examination.

Functional *in vitro* assays, histamine and sLT release tests, are based on the *in vitro* activation of leukocytes in the presence of the allergen. These tests are also considered to be closer to the clinical expression of allergy, when compared to serological IgE's determination (Wilkołek et al. 2019). In one example, the sulfidoleukotrienes (sLTs) release assay to determine allergen-induced leukocyte stimulation (Cellular Antigen Stimulation Test - based on the measurement of sulfidoleukotriene secretion by peripheral blood leukocytes stimulated with specific

allergens), demonstrated greater sensitivity and specificity when compared to the determination of specific IgE (De Weck et al. 2002).

In this study the CAST could only be performed with *Culicoides* full body extracts, as r-*Culicoides* allergens which were *E. coli* expressed, induced very weak sLT release, or no release at all. Nevertheless, previous studies showed that the CAST with *Culicoides nubeculosus* extract was useful to confirm the clinical diagnosis of IBH in horses from Switzerland. Baselgia et al. (2006) evaluated a sLT release test (cellular antigen stimulation test, CAST) based on test results from 314 horses. With *C. nubeculosus* whole body extract (WBE) a high diagnostic sensitivity and specificity, was attained. These previous results agree with the CAST results attained in the present study, confirming the relevance of *Culicoides* full body extract allergens, more specifically *Culicoides obsoletus*, for IBH in Lusitano horses.

When comparing both *in vitro* assays, IgE serology and CAST, the CAST presented a better performance with *Culicoides obsoletus* extract (Cul o WBE) with the highest Area Under Curve (AUC) of 0.897 with a 95% CI (confidence interval).

Even though the current tendency is to use *Culicoides* r-allergens in IBH diagnostic test, the results of this study showed that *Culicoides* extracts performed better overall in the IBH diagnostic tests. This observation is in accordance with the previous studies above referred, validating the use of *Culicoides* full body extracts as a valid and reliable tool in both *in vitro* and *in vivo* IBH diagnostic tests.

Based on the results of this study, it is suggested that Cul n WBE, Cul n 7, Cul n 8, Cul n 9, Cul o 1P and Cul o 2P, are strong candidates for inclusion in an SPT panel for the diagnosis of IBH.

Regarding *in vitro* assays, sLT release essay determined that Cul o WBE was the best performing allergen which should also be considered as a candidate to include in a future diagnostic panel.

Also, to be included in this panel are the serum allergen specific IgEs, Cul n 3, Cul n 4, Cul n 10, Cul o 1P, Cul o 2P, Cul o 3 and Cul o 2 determined as the best performing allergens in Lusitano horses, even though with a lower sensitivity and sensibility. Furthermore, Cul o 1 and Cul o 2 were shown to be major allergens (more than 50 % of the IBH affected horses tested had IgE response to them with a specificity of 95 %).

Lam et al. (2020) suggested that different treatment methods e.g., antihistamines and allergen specific immunotherapy (ASIT) will be most beneficial when conducted in young animals with a short history of allergy, which emphasizes the relevance of an early diagnosis with the identification of the relevant allergens. In Lam's study it was also suggested that the more severe cases, already showing chronic features, should be

treated with the goal of decreasing numbers of eosinophils, with other approaches such as corticosteroids or the immunotherapy against interleukins (IL-5 and 31).

Future management of allergic diseases might be determined considering the current dominating allergic response of each allergic individual, and the improvement of more suitable therapies will lead to more satisfying effects.

The available tests so far, appear to present low sensitivity due to the diversity of the etiological agents that can trigger IBH in horses (Langner et al. 2008; Sloet van Oldruitenborgh-Oosterbaan et al. 2009). This, in addition to the nonexistence of specific commercial diagnostic tests has made IBH difficult to diagnose in some parts of the world, with no more than history and clinical examination. Hence, I would like to emphasize the importance of the results obtained in this study, highlighting the better performance of the SPTs with the determined specific allergen panel as an important tool for future implementation of a standard diagnostic exam for IBH.

Also, it is of utmost importance to be able to implement ASIT for each IBH-affected horse in a near future, based on the results of this specific allergen panel. The main goal of ASIT is to be able to desensitize these animals, reducing the clinical signs and contributing to the increase of animal welfare and decrease economical losses.

It is my belief that this study is of major importance in IBH, not only in Lusitano horses but in other breeds around the globe, with relevant future implications for IBH diagnosis and immunotherapy. It may be a precedent to standardizing a valid diagnostic test which is easy to perform and can also contribute to implementing tailored immunotherapy, as well as to evaluate the results of its use, in the horses' own environment.

Lastly, this study may also have practical applications in human medicine. Einhorn et al. *(*2018) has previously described the importance of the study of animal allergic diseases and their causative allergens, in a compared and veterinary immunology perspective.

To summarize:

- Both C and T horses included in this study complied with the established inclusion and exclusion criteria.
- The *Obsoletus* group was one of most representative of the *Culicoides spp.* detected in Lusitano stud farms in Portugal.
- *Culicoides* extracts are valid allergens and may present better performances in both *in vitro* and *in vivo* diagnostic tests.

- SPTs are a valid diagnostic tool and presented a better discriminant diagnostic potential than IDTs.
- The amount of inoculated allergen in SPTs is smaller, but more concentrated than IDTs.
- The use of SPTs as a routine clinical test in the diagnostic of IBH seems to be an important step forward for IBH diagnosis in a near future.
- Regarding *in vitro* tests, sLT release test (CAST) performed better than serological determination of allergen specific IgEs, and Cul o WBE was the best performing allergen.
- The use of a specific diagnostic panel including both *Culicoides* WBE and *Culicoides* r-allergens is recommended to increase the performance of IBH diagnostic tests.
- IBH diagnosis should be carried out considering clinical history and the results of *in vivo* and/or *in vitro* allergy tests.

2. General conclusions:

Several salivary proteins from different *Culicoides* species, such as *C. nubeculosus*, *C. obsoletus*, have been identified, though the classification of these proteins as major and minor allergens of IBH still needs to be confirmed for different regions and environments around the world.

Until now IBH□s diagnosis is still mainly based on anamnesis, clinical examination, and response to insect control measures. Positive allergen specific IgE test results, based on serological or intradermal testing, were considered minor criteria, and used to support a clinically established diagnosis.

There is general agreement that SPTs are the core diagnostic test for type I immediate allergy and should be applied as standardized diagnostic exams with adequate allergen panels (Heinzerling et al. 2013). Regarding the results of this study SPTs, seem to be a valid diagnostic method for IBH. Hence, this study presents the possibility to include SPTs as a major diagnostic criterion to support the clinical diagnosis and help to establish better evidence-based recommendations on the treatment of IBH.

The determined allergen panel seems to be a good candidate for further development of a standardized diagnostic tool for Lusitano IBH-affected horses living in Portugal mainland. IBH-affected horses seem to react to both native and exotic species of *Culicoides* but local species may induce a stronger IgE-mediated immune response. It is therefore crucial in the case of Lusitano IBH-affected horses, in Portugal mainland,

to isolate allergens from the *Culicoides* species that were identified to be most prevalent in the stud farms, in order to develop a more specific allergen panel. Future large-scale studies are needed to define more precisely which allergens are important in different geographical locations and make further recommendations on the diagnosis of IBH.

The use of a valid and standardized skin testing system, in this case SPTs as they presented better diagnostic potential, with a specific allergen panel, is an important diagnostic tool for IBH, and can also be helpful in monitoring the response to therapeutic interventions, either pharmacological or immunotherapeutic.

So far, IBH therapy is still based on allergen avoidance and symptomatic treatment. Many attempts to achieve an effective immunotherapy treatment are being done with promising results, but not yet broadly effective for all. Evidence is still lacking to recommend allergen specific immunotherapy (ASIT) as a treatment for IBH and further studies are needed to explore its benefits.

Knowing each individual significant allergen sensitizations is of major importance to implement a more effective targeted immunotherapy as a treatment for IBH. Having this in mind, a more specific diagnostic approach is needed, such as the use of SPTs as a standard protocol to diagnose IBH and the individual significant allergen sensitizations.

In conclusion management and treatment of IBH is challenging. All available approaches to IBH diagnosis and control have limitations, emphasizing that control plans must be tailored to the individual situation, and may involve some adaptation.

Future management of IBH and other allergic diseases might be determined by considering the current dominating allergic response of each allergic individual. This way, more suitable and improved therapies may be applied, leading to more satisfying effects and better owner adherence to treatment.

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3. General limitations:

In general, awareness of the importance of allergic diseases in animals has been increasing, but there are still several limitations. Many times, the complete diagnostic methodology available in human allergology often cannot be transferred to horses, without adjustments. For example, the exact interaction of the immunoglobulin isotypes with the cells' affinity receptors of the horses immune system is still not as elucidated as in humans (Muller et al. 2016), which makes it sometimes difficult to understand the mechanisms underlying allergic diseases in animals. This may also be a limitation regarding the application of a successful immunotherapy protocol, which is the only treatment option that can modulate IBH s process (Marsella et al. 2023).

In the present study some difficulties/limitations were found, namely:

- Regarding Skin tests, intradermal tests are time consuming, requiring a specialized technician to properly perform the tests and interpret the results.
- For aesthetic reasons and other “owners’ issues” it was sometimes hard to find horses “available” for the skin tests.
- Sometimes poor working conditions (skin tests were all performed in the horses own environment).
- Following-up the tested animals was difficult. Some horses were sold, moved out of the country, or were even deceased.
- Regarding the *in vitro* tests, one limitation of the CAST was that fresh blood samples were needed. CAST had to be performed in less than 24 h after the blood was collected. Since the horses were living in Portugal and the tests were performed at the Vet Suisse Fakultat of the University of Bern, to ensure that the blood arrived in good conditions and in time to be processed, was sometimes a real challenge, which could only be overcome with the help of a friend who is a frequent traveller.

This was a complex and time-consuming experimental work, but in the end quite rewarding, because even with all the limitations and difficulties found, the main objectives were achieved.

4. Limitations of the study

The present study has some limitations, mostly regarding the allergens. For example, the production of the whole-body extracts may vary depending on the manufacturer and the same extract may present different allergenicity. Also, it may be sometimes challenging to standardize the extracts, due to some “disparities” inherent to their production. Natural allergenic extracts are heterogeneous substances, sometimes susceptible to contamination from other sources or may even contain proteolytic enzymes that can reduce the concentration of the allergen in the extract. Therefore, the assessment of their allergenicity may sometimes be difficult to achieve.

Regarding the recombinant allergens depending on the expression system used, their functionality may be reduced. For example, an allergen expressed in *E. coli* may present a decreased functionality when compared with the same allergen expressed in baculovirus or even barley.

Skin tests may also present some limitations. Sometimes they may not be feasible, particularly if there are widespread skin lesions, for example. They can even be

contraindicated in some situations that may involve the risk of anaphylactic shock, especially with IDTs.

Regarding *in vitro* tests for IBH diagnosis there are also some limitations. Serological IgE assays only show sensitization to an allergen, which does not necessarily reflect clinical allergy. In our study serological determination of specific IgEs revealed that T horses presented a higher concentration of specific IgEs when compared with C horses, but there was some overlap between the groups. This was attributed mainly to the exposure to the *Culicoides* bites resulting in some degree of asymptomatic sensitization.

In vitro functional tests namely CAST are considered to be closer to the clinical expression of allergy and present a higher sensitivity and specificity than serological tests. Nevertheless, equine IBH sLT's CAST so far can only be performed with *Culicoides* whole body extract (WBE). Another limitation is that fresh blood samples (<24hours) are needed to perform the CAST.

Regarding the *Culicoides spp*. that were identified near Lusitanos stud farms, testing all the allergens, whole-body extract and recombinant *Culicoides* allergens, is still required to assess their relevance in IBH in Lusitano horses in Portugal mainland.

Furthermore, the second allergen panel should still be tested in more horses before any recommendations can be made regarding these allergens.

Lastly, IBH is a very complex allergic disease, and a multiple approach is required to manage the disease, in terms of diagnosis and therapeutical approach. Future studies in larger populations will be valuable to achieve a better therapeutical approach, namely allergen targeted immunotherapy (ASIT), using the allergen panel determined in this study.

5. Future perspectives:

As priorly said, IBH in Lusitano horses is increasing. This study may contribute to the improvement of IBH diagnosis, with the standardization use of SPTs and the establishment of an allergen panel that may be more adequate to the studied population, and in accordance with the native *Culicoides* species found.

Further studies are needed to further evaluate IBH□s prevalence, distribution, and evolution over time, to have a better understanding of the disease in Lusitano horses living in Portugal.

Also, understanding the genetics and transmission mechanism of the disease is particularly important to improve our knowledge of IBH in Lusitano horses. Due to the negative aspects of the disease, it is of paramount importance to decrease vertical

transmission of IBH, and therefore the negative impact that IBH plays in Lusitano breeding, and in animal welfare.

We hope to contribute to the development of IBH-specific immunotherapy in horses. As several studies have been conducted with the aim of establishing equine IBH specific immunotherapy, results are quite different and still not very conclusive.

The results of this work may also have practical application in human medicine, as adverse reactions to insects can occur in both human and veterinary patients. Human's allergy may also benefit from the veterinary field, as allergic diseases in domestic species can represent natural translational models in the study of allergies in humans. The establishment of a comparative and veterinary immunoallergology special interest group within the EAACI and in the Portuguese Society of Allergology and Clinical Immunology (SPAIC), is giving new impetus to the field of veterinary allergy. There is great potential in the collaboration of human and veterinary allergology. Systematic comparison may lead to improved recommendations for prevention and treatment in both species (Pali-Scholl et al. 2019). In fact, there is now broad consensus that comparative allergology fits into the "One Health Concept" of human, animal, and environmental health, facilitating interaction of specialists in both fields.

Also, it is an important future goal to explore the IBH therapeutic options, knowing that better choices for therapies will improve the owner's compliance leading to more satisfying therapeutic effects.

6. Human and Animal Rights

This was a study involving animal subjects, which was performed upon request of an informed consent form signed by the horses' owners.

Also, according to the directive 2010/63/EU of the European Parliament and of the council, of 22 September 2010, on the protection of animals used for scientific purposes (Text with EEA relevance), in particular page 39, point 5 (f), "… during this study no procedures were likely to cause pain, suffering, distress, or lasting harm equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice..."

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Appendix

Supplementary Files

1. Consent Forms and participant study presentation

1.1 Study presentation to participants (owners):

Estudo de Hipersensibilidade à Picada de Insetos (HPI) em cavalos Lusitanos

No âmbito de uma tese de doutoramento a desenvolver na Faculdade de Medicina Veterinária da Universidade de Lisboa (Departamento de Clínica de Equinos), está-se a proceder ao estudo da Hipersensibilidade à Picada de Insetos (HPI) na população de cavalos Lusitanos. Este estudo está a ser desenvolvido no âmbito de um projeto estabelecido em colaboração com a Faculdade de Medicina Veterinária da Universidade de Berna (Vetsuisse-Fakultät, Universität Bern), na Suíça.

A HPI é uma doença do foro alérgico (alergia à picada de insetos), que se caracteriza por uma dermatite pruriginosa crónica sazonal e recorrente que se estende da Primavera ao Outono. A progressão da doença para além de potenciar infeções secundárias e desconforto animal podendo até levar ao aparecimento de lesões cutâneas permanentes, pode estar na base de um prejuízo económico importante para as coudelarias.

De momento o tratamento é limitado, mas as características imunológicas da doença parecem permitir o desenvolvimento de uma imunização eficaz no tratamento e prevenção da doença.

Com este estudo pretende-se caracterizar a HPI no cavalo P.S. Lusitano em Portugal continental, com identificação de fatores de risco, melhorar as técnicas de diagnóstico para a HPI e contribuir para o desenvolvimento de tratamento alternativo: a imunoterapia específica.

O estudo envolve a resposta a um questionário (para determinar a prevalência e eventuais fatores de risco), a colheita de sangue para diagnóstico laboratorial e a execução de testes cutâneos para identificação dos alergénios implicados. Dependendo dos resultados, proceder-se-á à imunoterapia específica para alguns dos cavalos afetados, e por isso pertencentes ao grupo teste.

Neste âmbito seria importantíssimo obter o apoio de todas as Coudelarias e/ou proprietários de P.S. Lusitano.

Este estudo é toda a importância uma vez que o tipo de clima em Portugal e o maneio em que os cavalos são habitualmente criados, torna a raça muito exposta aos insetos, logo potencialmente em risco de desenvolver HPI.

1.2 Study of Insect Sting Hypersensitivity (IPH) in Lusitano horses

As part of a doctoral thesis to be developed at the Faculty of Veterinary Medicine of the University of Lisbon (Department of Equine Clinic), a study is being carried out on Insect Bite Hypersensitivity (IBH) in a population of Lusitano horses. This study is being carried out within a project established in collaboration with the Faculty of Veterinary Medicine of the University of Bern (Vetsuisse-Fakultät, Universität Bern), Switzerland.

IBH is an allergic disease (allergy to insect bites), characterized as a seasonal and recurrent chronic pruritic dermatitis that occurs mainly from spring to autumn. The progression of the disease, occurrence of skin lesions with the potential appearance of secondary skin infections and animal discomfort, can be the basis of a significant economic loss for stud farms.

Until now treatment is limited, but the immunological characteristics of the disease seem to allow the development of an effective immunization in the treatment and prevention of the disease.

With this study we intend to characterize IBH in the Lusitano horse in Portugal mainland, identifying risk factors, improving diagnostic techniques for IBH and contributing to develop an alternative treatment: specific immunotherapy.

The study involves answering a questionnaire (to determine the prevalence and possible risk factors), collecting blood for laboratory diagnosis, and carrying out skin tests to identify the allergens involved. Depending on the results, specific immunotherapy will be carried out for some of the affected horses, and therefore belonging to the test group.

In this context, it would be extremely important to obtain the support of all Lusitano Stud Farms.

This study is very important since the type of climate in Portugal and the management in which the horses are usually raised, makes the breed very exposed to insects, therefore potentially at risk of developing IBH.

2. Consent Form:

FORM OF CONSENT FOR ALL PROCEDURES INVOLVED IN THE PHD STUDY
"CHARACTERIZATION OF INSECT BITE HYPERSENSITIVITY (IBH) IN A POPULATION OF LUSITANO HORSES: CONTRIBUTION FOR FUTURE IMPLEMENTATION OF SPT IN IBH DIAGNOSIS**".**

Owner's Name _____

Address _____

_____

Telephone: Home _____ Work_____

Mobile _____

NB: Please complete the section below if you have authority to act on behalf of the owner.

Name _____

Address _____

_____

Telephone: Home _____ Work_____

Mobile _____

Species and Breed _____

Identification of the animal:

Name _____

Colour _____

Sex: ☐ M ☐ F | Neutered: ☐ M ☐ F | Age: _____

Microchip/Tattoo/Brand _____

_____

Details of the Operation/Procedure:

_____

_____

_____

_____

_____

_____

_____

_____

- I hereby give permission for the administration of standing sedation to the above animal and to the procedures detailed in this form together with any other procedures which may prove necessary.
- The nature of these procedures and of other such procedures as might prove necessary has been explained to me and are the following:
  - Skin Prick Tests (SPT)
  - Intradermal Tests (IDT)
  - Blood Sample collection from the Jugular Vein
  - Photographic capture of lesions, as well as SPT and IDT inoculation areas
- I understand that there are some risks involved in all procedures.
- I have been informed that there will be no costs for me. In the event of further treatment being required or of complications occurring, I shall be contacted as soon as practicable so that my consent to such additional treatment may be obtained and these additional costs will be discussed with me.
- In the event that the veterinary surgeon is unable to contact me on the numbers provided, I understand the veterinary surgeon will act in the best interests of my animal.
- In order to protect the welfare of my animal, in the unlikely event of an emergency, or where additional pain relief or sedation may be required, I understand the veterinary surgeon may decide to use medicines that are not authorised for use in horses.

Notes and Instructions: _____

_____

_____

_____

☐ If you are NOT the owner, please tick the box to confirm you have the authority to act on behalf of the owner of the animal described above.

☐ Please tick the box if you are UNDER the age of 18.

Date _____/_____/20____.

*Signature _____

*A copy of the form should be provided to the person signing and the original retained by the hospital

3. Stud farm Owner questionnaire:

Inquérito - Hipersensibilidade à Picada de Insetos (HPI)
(Coudelaria)

Data: ____/____/20____

Identificação do Proprietário:

Nome: _____

Contacto telefónico: _____

Localização da coudelaria:

☐ Rural ☐ Urbano

Concelho:_____ Distrito:_____

A coudelaria distribui-se por outras propriedades:

☐ Não ☐ Sim Como ocorre essa circulação _____

_____

Número total de animais: |_____|

Caracterização dos Grupos de animais:

Animais estão todos juntos

☐ Não ☐ Sim Nº de grupos? |_____|

Grupo I	Nº animais	Idades	Sexos	Alojamento	Pastagem	Estábulo	Misto
Grupo II	Nº animais	Idades	Sexos	Alojamento	Pastagem	Estábulo	Misto
Grupo III	Nº animais	Idades	Sexos	Alojamento	Pastagem	Estábulo	Misto
Grupo IV	Nº animais	Idades	Sexos	Alojamento	Pastagem	Estábulo	Misto

Caracterização do Alojamento:

<u>Estábulo</u>

☐ Não

☐ Sim Nº do Grupo(s): |_____| |_____| |_____| |_____|

Tipo instalações: _____

Proximidade de água |_____|(m)

Tipo: ☐ Lago ☐ Charco ☐ Ribeira ☐ Rio ☐ Arrozal ☐ Zona Alagada ☐ Outro

Inquérito - Hipersensibilidade à Picada de Insetos (HPI)

(Coudelaria)

Pastagem

☐ Não

☐ Sim Nº do Grupo(s): |_____| |_____| |_____| |_____|

Tipo de pastagem e tamanho: _____

Proximidade de água |_____|(m)

Tipo: ☐ Lago ☐ Charco ☐ Ribeira ☐ Rio ☐ Arrozal ☐ Zona Alagada ☐ Outro

Misto

☐ Não

☐ Sim Nº do Grupo(s): |_____| |_____| |_____| |_____|

Proximidade de água |_____|(m)

Tipo: ☐ Lago ☐ Charco ☐ Ribeira ☐ Rio ☐ Arrozal ☐ Zona Alagada ☐ Outro

Caracterização de HPI:

Sabe o que é a Hipersensibilidade à Picada de Inseto?

☐ Não

☐ Sim

Tem animais identificados com HPI?

☐ Não

☐ Sim

De que grupo(s): |_____| |_____| |_____| |_____|

Nº de animais: |_____| |_____| |_____| |_____|

As lesões são Sazonais?

☐ Não

☐ Sim

Época: ☐ Primavera ☐ Verão ☐ Outono ☐ Inverno

Duração: |_____| (dias/meses)

Já fez:

Testes Cutâneos por Picada ☐ Não ☐ Sim

Testes Intradérmicos ☐ Não ☐ Sim

Imunoterapia ☐ Não ☐ Sim

Muito Obrigado pela Colaboração!

4. Provided material for the present IBH study:

4.1. Allergens related material:

Material provided from Vetsuisse Bern for study on insect bite hypersensitivity in horses in Portugal

- **Culicoides nubeculosus extract** (ca. 500 microlitre of 2.5 mg/ml in 0.9% NaCl).
  Make aliquots and keep at -20°C or better -80°C.

  For IDT we use it at a concentration of 10 microgr/ml (diluted in 0.9% NaCl).
  For CAST see CAST protocol.
  Negative control for IDT or skin prick test would thus be 0.9% NaCl

- **Recombinant allergens:**
  Lyophilized (500 microgr each): keep at -20°C in lyophilized form, after reconstitution keep at 4°C!

  Culicoides nubeculosus recombinant allergens:
  - Cul n 1 = Ag5
  - Cul n 2 = e16
  - Cul n 3 = d2
  - Cul n 4 = b2
  - Cul n 5 = a21
  - Cul n 6 = e8
  - Cul n 7 = g18
  - Cul n 8 = g13
  - Cul n 9 = d4
  - Cul n 10= c4
  - Cul n 11= a12

  Culicoides obsoletus recombinant allergens
  - CO23 already reconstitute in water. Keep at 4°C
  - CO110 already reconstitute in water. Keep at 4°C

Reconstitute lyophilized recombinant allergens with **sterile water** (at least 1ml, i.e. 500 microgr per ml, but you can also have then in a more diluted form, for example 200 microgr per ml) , **except Cul n 9 (D4) which needs to be reconstituted with sterile PBS**. **(Use of different buffers results in precipitation of the recombinant proteins!!!)**.

For prick test or IDT use sterile water as negative control for recombinant allergens!

- **Equine Interleukin-3 (100 microgr/ml) for CAST**
  Keep at -20°C or -80°C.
  Needs to be diluted 1:10 to reach a concentration of 10 microgram/ml. Can be used in the CAST.

- **Anti-horse IgE 134 as positive control in CAST**
  Keep at -20°C or -80°C until use. When used keep at 4°C to avoid too many freeze/thawing.
  Use a 1:500 dilution in CAST, i.e. dilute previously 1:500 and then final dilution in CAST is 1:2500.

4.2 Alergen List

Name Allergen	Name Genbank	New name	Function	Homology/ Identity	GenBank
Cul n 2	Cul n 2	Cul nu 2	Hyaluronidase	Cul o 2 (75%)	HM145950
Cul n 3	Cul n 3	Cul nu 3	Cysteine Protease	Cul n 5 (57%	
Cul n 4	Cul n 4	Cul nu 4	unknown	Cul o 7 (33%)	HM145952
Cul n 5	Cul n 5	Cul nu 12	unknown		HM145953
Cul n 6	Cul n 6	Cul nu 17	Glycosyl hydrolase C-term	Cul n 4 (40%)	HM145954
Cul n 7	Cul n 7	Cul nu 7	unknown		HM145955
Cul n 8	Cul n 8	Cul nu 1	Maltase	Cul s 1 (92%)/ Cul o1 (78%)	HM145956
Cul n 9	Cul n 9	Cul nu 9	D7-related/OBP	Cul o 6 (40%)	HM145957
Cul n 10	Cul n 10	Cul nu 10	unknown		HM145958
Cul n 11	Cul n 11	Cul nu 11	Serine Protease	Cul o 4 (51%)	HM145959
Cul n 12	Cul n 1	Cul nu 5	Antigen 5like	Cul o 3 (70%) Sim v 1 (47%)	EU978899
Cul so 8	Cul s 1	Cul so 1	Maltase	Cul o 1 (78%)/ Cul n 8 (92%)	Q66UC5_9DIPT
Cul ob 2	Cul o 2	Cul ob 2	hyaluronidase	Cul n 2 (75%)	KC339672
Cul ob 4	Cul o 7	Cul ob 7	unknown	Cul n 4 (33%)	KC339677
Cul ob 7	Cul o 5	Cul ob 15	unknown		KC339675
Cul ob 8	Cul o 1	Cul ob 1	Maltase	Cul s 1 (78%) /Cul n 8 (78%)	KC339671
Cul ob 9	Cul o 6	Cul ob 6.01	D7-related / OBP	Cul n 9 (40%) /Cul o3* (73%)	KC339676
Cul ob 11	Cul o 4	Cul ob 1 4	Serine protease/trypsin	Cul n 11 (51%)	KC339674
Cul ob 12	Cul o 3	Cul ob 5	Antigen-5 like	Cul n 1 (70%)	KC339673
missing	Cul o1*	Cul ob 18	Kunitz protease inhibitor		JX512273
missing	Cul o2*	Cul ob 16	D7-related/OBP		JX512274
missing	Cul o3*	Cul ob6.02	D7-related/OBP	Cul o 6 (73%)	JX512275

5. Test Report Sheets

5.1. Intra dermal Test. Table report sheet per horse

TESTES INTRADÉRMICOS (TID)

ID: DATA: HORAS: PROPRIEDADE: (Alergeno)=10ug/ml) Volume=100up=0.1ml

(+) Histamina	(-) PBS Esteril	(-) Água Estéril	(-) NaCl 0,9	(-) TRIS	CN 10µg/ml
Cul1 10µg/ml	Cul2 10µg/ml	Cul3 10µg/ml	Cul4 10µg/ml	Cul5 10µg/ml	Cul6 10µg/ml
Cul7 10µg/ml	Cul8 10µg/ml	Cul9 10µg/ml	Cul10 10µg/ml	Cul11 10µg/ml	CO23 10µg/ml
	CO142 10µg/ml	COext 10µg/ml	Cul O3 10µg/ml	Cul 3 Bac 10µg/ml	Cul 3 Barley 10µg/ml
Cul 4 Bac 10µg/ml	Cul4 Barley10µg/ml				

5.2. Skin Prick. Table Report Sheet per horse

TESTES CUTÂNEOS POR PICADA (TCP)

ID: DATA: FOLHA 1 PROPRIEDADE:

HORAS:

(+) Histamina	(-) PBS Esteril	(-) Água Estéril	(-) NaCl 0,9	(-) TRIS	CN 10µg/ml	Cul1 10µg/ml	Cul1 100µg/ml
Cul2 10µg/ml	Cul2 100µg/ml	Cul3 10µg/ml	Cul3 100µg/ml	Cul4 10µg/ml	Cul4 100µg/ml	Cul5 10µg/ml	Cul5 10µg/ml
Cul5 100µg/ml	Cul6 10µg/ml	Cul6 100µg/ml	Cul7 10µg/ml	Cul7 100µg/ml	Cul8 10µg/ml	Cul8 100µg/ml	Cul9 10µg/ml
Cul9 100µg/ml	Cul10 10µg/ml	Cul10 100µg/ml	Cul11 10µg/ml	Cul11 100µg/ml	C023 10µg/ml	CO23 100µg/ml	CO110 10µg/ml
CO110 100µg/ml	CO142 10µg/ml	CO142 100µg/ml	COext 10µg/ml	COext 100µg/ml	Cul O3 10µg/ml	Cul O3 100µg/ml	Cul 3 Bac 10µg/ml
Cul 3 Bac 100µg/ml	Cul 3 Barley 10µg/ml	Cul3Barley 100µg/ml	Cul 4 Bac 10µg/ml	Cul 4 Bac 100µg/ml	Cul4 Barley10µg/ml	Cul4 Barley100µg/ml	

6. Research Communications and Publications

6.1. Communications:

This work was presented in the following events:

Poster presentation at the IV Conference of the Research Group on Equine Studies, on November 11, 2015, at EqusPolis congress center, Golegã, during the National Horse Fair, entitled "Allergic Hypersensitivity to *Culicoides* Sting on Lusitano Horse".

Oral communication at the Royal College of London, 4th of February of 2016, entitled "Equine Insect (*Cullicoides spp.*) Bite Hypersensitivity - A Portuguese perspective".

Oral presentation at the "IV Workshop of the HPI Research Group" of the Havenmeyer Foundation, entitled "Preliminary Results in a study of insect bite hypersensitivity (IBH) in Lusitano horses" 3, held at the Faculty of Agricultural Sciences of Iceland from June 22 to 26, 2016, with publication in the book of "Abstracts", and later participation in a summary communication of the "Workshop" in the journal Equine Veterinary Journal.

Co-author in poster presentation at the ESOVE congress, to be held in Lisbon from October 3 to 7, 2016, entitled "*Culicoides* species found near horses in Portugal which could be related to Insect Bite Hypersensitivity (IBH)".

Presentation of oral communication at the II Conference of the PhD Program in Veterinary Sciences, ICBAS-FF, UPorto, on 28 September 2018, entitled "Study of Insect Bite Hypersensitivity (IBH) in Lusitano horses - Contribution to the development of Skin Allergy Tests ", with publication in the book of abstracts.

Presentation in the form of oral communication at the International Congress of CIISA - 2018 to be held on 16 and 17 November at FMV-ULisboa, entitled "Study of insect bite hypersensitivity (IBH) in Lusitano horses in Portugal - preliminary contribution to the development of immunotherapy ".

Presentation in the form of oral communication at the International Congress of CIISA - 2022 to be held on 11 and 12 November of 2022 at FMV-ULisboa, entitled: "Comparison of Skin Allergy tests, Intradermal tests (IDTs) and Skin Prick tests (SPTs) in the characterization of Insect Bite Hypersensitivity (IBH) in a population of Lusitano horses: contribution for future implementation of SPT in IBH diagnosis".

Presentation in the form of oral communication the National Congress of Veterinary Medicine Students on the 12th of March 2023, with the lecture “Study of allergic hypersensitivity to *Culicoides* in the Lusitano horse in Portugal”, which took place at the Vasco da Gama University School – Coimbra.

Presentation of a Mini-Talk promoted by the Centre of Investigation of Vasco da Gama University School (CIVG) of the EUVG - Coimbra, on the 14th of June of 2023, with the theme: “Study of allergic hypersensitivity to *Culicoides* in the Lusitano horse in Portugal – contribution to the development of diagnostic methods”.

6.2. Publications:

Pessoa VP, Ferreira MB, Tilley P. 2015. Estudo da hipersensibilidade à picada de insetos (HPI) em equinos da raça Puro-Sangue Lusitano – Contribuição para o desenvolvimento de imunoterapia específica. Proceedings of the IV Jornadas do Grupo de Trabalho e Investigação em Equinos, Golegã-Portugal, 11th of november of 2015.

Pessoa VP, Marti, E, Branco Ferreira M, Tilley P. 2016. Preliminary Results in a study of insect bite hypersensitivity (IBH) in Lusitano horses – Contribution to the development of specific immunotherapy. Proceedings of the 4th International Workshop of Equine Allergy, Hvannery-Iceland, 22-26 June 2016.

Tilley P, Ramilo DW, Madeira S, Pessoa VP, Branco Ferreira M, Pereira da Fonseca I. 2016. *Culicoides* species found near horses, in Portugal, that could be related to Insect Bite Hypersensitivity (IBH). Proceedings of the 20th E-Sove Conference, Lisbon-Portugal, 3-7 October 2016.

Pessoa VP, Marti E, Branco Ferreira M, Tilley P. 2018. Study of Insect Bite Hypersensivity (IBH) in Lusitano horses – Contribution to the development of Skin Allergy Tests. Proceedings of the II Jornadas do Programa de Doutoramento em Ciências Veterinárias, ICBAS-FF, Uporto, 28 September 2018.

Pessoa VP, Marti E, Branco Ferreira M, Tilley P. 2018. “Study of insect bite hypersensitivity (IBH) in Lusitano horses in Portugal – preliminary contribution to the development of immunotherapy”. Proceedings of the CIISA 2018 Congress, FMV-ULisboa, 16, 17th of November 2018.

Pessoa V, Ramilo DW, Pereira da Fonseca I, Ferreira MP, Marti E, Tilley P. 2020. *Culicoides* spp. found near Lusitano stud farms in mainland Portugal which may contribute for IBH studies. Veterinary Parasitology: Regional studies and reports. 20: 100385. https://doi.org/10.1016/j.vprsr.2020.100385

Tilley P, Simões J, Pessoa V, Fonseca R, Sales-Luís JP. 2020. No Room to Breathe: Airway Conditions Affecting the Equine Athlete. In: Freitas Duarte A., Lopes da Costa L. (eds) Advances in Animal Health, Medicine and Production. Springer, Cham. https://doi.org/10.1007/978-3-030-61981-7_27.

Pessoa, V., Ferreira, M.B, Jónsdóttir S, Marti E, Tilley P. 2023. Comparison of Skin Prick tests (SPT), Intradermal tests (IDT) and in vitro tests in the characterization of Insect Bite Hypersensitivity (IBH) in a population of Lusitano horses: contribution for future implementation of SPT in IBH diagnosis Animals. 13: 2733. doi.org/10.3390/ani13172733

Pessoa, V., Ferreira, M.B, Jónsdóttir, S. Marti, E., Tilley, P. 2023. Comparison of Skin Prick tests (SPT), Intradermal tests IDT) and in vitro tests in the characterization of Insect Bite Hypersensitivity (IBH) in a population of Lusitano horses: contribution for future implementation of SPT in IBH diagnosis." Proceedings of the 2023 CIISA Congress, 29-30th of November 2023, FMV-ULisboa.

Pessoa, V., Ferreira, M.B, Jónsdóttir, S. Marti, E., Tilley, P. 2023. "Comparação de Testes Cutâneos por Picada (TCP), Testes Intradérmicos (TID) e Testes In Vitro na Caracterização da Hipersensibilidade à Picada de insetos (HPI) em cavalos Puro-sangue Lusitano: Contribuição para Futura Implementação dos TCP no Diagnóstico da HPI". Livro de resumos das VII Jornadas do GTIE, 16 de dezembro de 2023 FMV-ULisboa.

6.3. Abstracts

6.3.1. Estudo da hipersensibilidade à picada de insetos (HPI) em equinos da raça puro-sangue Lusitano – contribuição para o desenvolvimento de imunoterapia específica

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Hipersensibilidade à Picada de Insetos (HPI) é uma dermatite pruriginosa sazonal recorrente, afetando inúmeros equinos em todo o mundo[1], tendo por base reações de hipersensibilidade (I e IV) a alergénios presentes na saliva de insetos hematófagos, nomeadamente *Culicoides* spp.[2]. É uma doença multifatorial, com tratamento limitado ao controlo de insetos, uso de agentes antipruriginosos e anti-inflamatórios. Por ser uma alergia imunomediada (IgE's), é possível desenvolver-se imunoterapia específica.[1 e 3]

Foi objetivo deste trabalho melhorar o diagnóstico da HPI, através da aplicação de testes cutâneos, e desenvolver imunoterapia específica.

Preencheram-se questionários relativamente à história clínica, ambiente e sintomatologia, e realizaram-se exames clínicos e registo fotográfico das lesões. Aplicaram-se testes de diagnóstico *in vitro* para a quantificação de sulfidoleucotrienos (sLT) produzidos por leucócitos do sangue periférico[4]. Determinaram-se as imunoglobulinas presentes no soro equino, por micro-ensaio ELISA[5]. Executaram-se testes cutâneos por picada (TCP) e intradérmicos (TID). Implementar-se-á Imunoterapia Específica, em ensaio experimental tipo "double blinding", para posterior avaliação da sua eficácia.

Até à data testaram-se 48 cavalos de várias coudelarias dispersas por Portugal (24 do grupo teste e 24 do controlo), com preenchimento dos questionários e observação clínica das lesões. Colheram-se e analisaram-se as amostras sanguíneas (no Departamento de Imunologia da Faculdade de Veterinária de Berna) para a quantificação dos sLT, para avaliar as diferenças entre grupos. Foram realizados os testes cutâneos (TID e TCP) aos cavalos, no local de habitação, estimando *in vivo* a reação de hipersensibilidade direta, e as diferentes respostas aos alergénios de *Cullicoides spp.*[6].

Este estudo inovador em Portugal, permite caraterizar a ocorrência da HPI em cavalos Lusitanos, pela avaliação dos questionários e observação clínica das lesões. Possibilita a aplicação de testes cutâneos de diagnóstico da HPI, contribuindo para a identificação dos alergénios envolvidos. Possibilitará avaliar a eficácia clínica da imunoterapia no tratamento e prevenção do aparecimento de sintomas.

6.3.2. Preliminary Results in a study of insect bite hypersensitivity (IBH) in Lusitano horses – Contribution to the development of specific immunotherapy

Pessoa, VP.[1], Marti, E.[2], Branco Ferreira, M.[3], Tilley, P.[1]

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Introduction: IBH is mainly a type I hypersensitivity reaction (IgE mediated) to salivary allergens from *Culicoides spp.* The diagnostic is based on clinical history and examination, and response to culicid control in the environment. The aim of this study is to describe the results of skin allergy tests, Intradermal (IDT) and Skin Prick Tests (SPT), in Lusitano horses with clinical diagnosis of IBH.

Material and Methods: 18 healthy controls and 20 IBH horses were tested. The IBH horses were included based on medical history and physical examination supported by questionnaires and standardized photos. They were submitted to skin tests on the neck (a rectangle of hair was trimmed) with 14 with specific allergens, 13 different recombinant proteins from C*ulicoides spp.* salivary glands (termed Cul n 1 to 11, Cul o1 and Cul o2) and *Culicoides nubeculosus* whole salivary extract; IDT-10µg/ml, SPT-100µg/ml concentration. Wheals' diameters were assessed at 20 min (SPT/IDT), 6 hours (IDT) and 48 hours (IDT). SPT's were considered positive when wheal diameter ≥0.8 cm, and IDT's when wheal diameter was ≥50% of histamine's wheal and at least ≥0,8 cm. SPSS was used for descriptive statistics and mixed factorial ANOVA was used to compare differences between control and test groups at different readings.

Results: Between test and control groups statistical differences were observed for skin tests to Cul n WBE, Cul n 10 and Cul n 11 allergens ($p<0.05$).

Discussion/Conclusions: Our preliminary results indicate that Cul n WBE, Cul n 10 and Cul n 11 may potentially be the more relevant allergens in IBH in the population of horses studied. Further studies are needed to identify best cut-off values for skin tests, as well as a minimum set of *Culicoides* allergens for complementary IBH diagnosis. SPT may be useful in IBH diagnosis, at a concentration of 100µg/ml, and readings assessed at 20 min.

6.3.3. *Culicoides* species found near horses in Portugal which could be related to Insect Bite Hypersensitivity (IBH)

Tilley, P.[1a], Ramilo, D.W.[1a], Madeira, S.[1], Pessoa, V.P.[1], Branco Ferreira, M.[2], Pereira da Fonseca, I.[1]

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Introduction: Insect bite hypersensitivity (IBH) is an allergic disease in horses caused by species of *Obsoletus* group, *C. Nubeculosus* and *C. imicola.* The knowledge of *Culicoides* fauna present in each country and their ecological preferences can help in the establishment of control strategies for this and other diseases transmitted by this vector species. A study on IBH in Lusitano horses is being carried out, focusing on the *Culicoides* distribution near horse farms in Portugal.

Material/Methods: *Culicoides* were captured with 3 OVI traps in 13 horse farms in 9 regions of Portugal, including those where the IBH study is being carried out. *Culicoides* were identified to species by their wing pattern and when species identification was not possible, specimens were separated into different body parts followed by composed optical microscopy examination.

Results: Per night, an average of 50 specimens was collected in the trap sites localized above Tagus River and an average of 650 specimens was collected in the trap sites below Tagus River. A total of 29 different *Culicoides* species were captured near horse farms.

Discussion/Conclusions: Despite the high number of *Culicoides* species captured, the number of collected specimens depended on the location of the capture point in relation to Tagus River, being significantly higher in captures performed below that site. Although *C. nubeculosus* has not been found in the samples collected up to now, *C. puncticollis*, a very similar species, was found. As other species can also be involved in horse IBH, these preliminary results must be complemented with further captures and laboratorial studies on feeding preferences, since the presence of different *Culicoides* species in traps located in horse farms does not necessarily mean that they feed on horses.

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6.3.4. Study of insect bite hypersensitivity (IBH) in Lusitano horses in Portugal – preliminary contribution to the development of immunotherapy

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Background: Insect Bite Hypersensitivity (IBH) is a recurrent, seasonal pruritic dermatitis, and mainly a type I hypersensitivity reaction (IgE mediated) to salivary allergens from *Culicoides spp,* that affects many horses worldwide. Though prevalence of HPI in Portugal is not yet known, the environmental characteristics are favourable to the activity of *Culicoides spp,* and common knowledge shows high occurrence in valuable Lusitano stud farms. This study describes the results of skin allergy tests, Intradermal (IDT) and Skin Prick Tests (SPT), performed in Lusitano horses with clinical diagnosis of IBH.

Methods: 30 healthy controls and 30 IBH horses were tested. IBH horses were included based on medical history and physical examination supported by questionnaires and photos. They were all submitted to skin tests on the neck with 14 specific allergens, 13 different recombinant proteins from C*ulicoides spp.* salivary glands (termed Cul1 to 11, Cul O1 and Cul O2) and *C. nubeculosus (CN)* whole salivary extract. Furthermore, 12 horses were also tested with another 6 allergens, 4 allergens from recombinant *C. nubeculosus* and 2 from recombinant *C. obsoletus*. The allergen concentrations used were 10µg/ml for IDT and 100µg/ml for SPT. Wheal diameters were assessed at 20 min (SPT/IDT), 6 hours (IDT) and 48 hours (IDT). SPT were considered positive when wheal diameter ≥0.9 cm, and IDT when wheal diameter was ≥50% of the histamine wheal.

Results: Statistical differences were observed, between test and control groups, for CN, Cul 1, 7, 8, 9, CO23, CO110 ($p<0.05$); in the second allergen panel, statistical differences were observed for Cul 4 Bac, Cul 3 Bar and Cul o WBE ($p<0.05$). Most animals studied seemed to be allergic to at least 4 of these allergens.CN was the allergen that all IBH horses were positive to, Cul9 being the second most frequent.

Conclusions: Our results indicate that Cul n WBE, Cul1, 7, 8 and 9 and Cul o 2P and Cul o 1P may potentially be the more relevant allergens in IBH in the population of horses studied. For the new allergen panel, although tested only in a reduced number of horses, we consider that Cul o WBE, Cul o 3 Bar and Cul o 4 Bac, can potentially be relevant for IBH. SPT may be useful in IBH diagnosis, at a concentration of 100µg/ml, and readings assessed at 20 min.

Key Words: Insect bite hypersensitivity, Allergens.

6.3.5. *Culicoides spp.* found near Lusitano stud farms in mainland Portugal which may contribute for IBH studies.

Pessoa, V.[1§], Ramilo, D.W.[1], Pereira da Fonseca, I.[1], Ferreira, M.B[2], Marti, E.[3] Tilley, P[1]

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Abstract: Insect Bite Hypersensitivity (IBH) is a common cutaneous disease, affecting a large number of horses worldwide. Several studies have identified *Culicoides* spp. saliva as a clinically relevant allergen source. The prevalence of IBH in Portugal, particularly in Lusitano horses, is still not known. However, the environmental characteristics of the national territory are favorable to the activity of *Culicoides*, and several species of this genus can be found, namely *C. imicola* and *C. obsoletus / C. scoticus*. In this study we characterized the *Culicoides* population present in Lusitano stud farms with a history of IBH. Thirteen stud farms with Lusitano horses were selected in several regions of mainland Portugal for having a previous history of IBH-affected horses, with a minimum of 5 affected horses. *Culicoides* were collected in May and June 2016 using OVI traps, placed in these stud farms, and we were able to identify several *Culicoides* species. We could also verify that *C. obsoletus / C. scoticus*, and *C. imicola* were the ones most frequently found, but other species like *C. pulicaris* were also found.

Key words: IBH, *Culicoides*, Lusitano horses, OVI traps.

6.4.6. No Room to Breathe: Airway Conditions Affecting the Equine Athlete

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Abstract: During exercising endoscopy, head flexion has been shown to be an important predisposing factor for upper respiratory tract collapse and is associated with conflict behavior. Based on the substantial number of studies on the impact of hyper flexed postures on horse welfare, it was recently suggested for further research to be done on the physiological/psychological effects of a lesser degree of flexion. Our group evaluated horses ridden in two very close head positions and were able to identify significant differences for various parameters.

Inflammatory airway disease (IAD) could be the effect of repeated episodes of nasopharyngeal asphyxia, its sequel being exercise induced pulmonary hemorrhage (EIPH). EIPH and IAD account for a wide number of horses failing to perform to their potential. The American College of Veterinary Internal Medicine consensus statement proposed equine asthma syndrome (EAS) to describe horses with mild or moderate (IAD) to severe (RAO) airway disease.

Insect bite hypersensitivity has been associated with airway hyperreactivity, suggesting a probable link with EAS, and multiple hypersensitivities are significantly associated with the absence of nematode eggs in feces.

Because severe EAS is a chronic disease with significant impact on the equine population, the development of staging methods for this disease by our group became essential to optimize equine medical care.

Key words: Endoscopy URT, Collapse, Head hyperflexion, Equine Asthma Syndrome (EAS) Staging, Hypersensitivity.

6.4.7. Comparison of Skin Prick Tests (SPT), Intradermal Tests (IDT) and *In Vitro* Tests in the Characterization of Insect Bite Hypersensitivity (IBH) in a Population of Lusitano Horses: "Contribution for Future Implementation of SPT in IBH Diagnosis"

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Introduction: Insect Bite Hypersensitivity (IBH) prevalence in Portugal, in Lusitano horses, is not known, but environmental characteristics are favourable to the activity of *Culicoides* and common knowledge shows high occurrence in Lusitano stud farms.

Aim of the study: This study aimed at comparing skin tests and in vitro allergy tests for the diagnosis of IBH in Lusitano horses.

Material and Methods: Thirty controls (C) and 30 IBH-affected (T) Lusitano horses were evaluated. T horses were included based on anamnesis and physical examination, supported by questionnaires. All horses were submitted to skin tests, Intradermal (IDT) and Skin Prick Tests (SPT), on the neck with 14 specific allergens, 13 recombinant proteins (r-proteins) from *Culicoides nubeculosus (Cul n)* and *Culicoides obsoletus (Cul o)* salivary glands and *Culicoides nubeculosus* Whole Body Extract (Cul n WBE). Additionally, a cluster of six T and six C horses were also tested with Cul n 3 and Cul n 4 produced in insect cells and barley, as well as *E. coli* produced Cul o 3 and Cul o WBE. Allergen concentrations were 10 µg/mL for IDT and 100 µg/mL for SPT, and wheal diameters were assessed at 20 min, 6 and 48 h. IDTs were considered positive when wheal diameter was ≥50% of the histamine wheal and SPTs ≥ 0.9 cm. *In vitro* tests, allergen-specific serum IgE evaluation and sulfidoleukotriene (sLT) release assay were also carried out.

Results: Results showed that Cul n WBE, Cul n 7, 8, 9, Cul o1P and Cul o 2P were the best performing allergens for SPTs ($p \leq 0.0001$) in the 1st allergen panel and Cul o WBE, Cul n 3 Bar and Cul n 4 Bac ($p \leq 0.05$) in the 2nd, presenting a higher discriminatory diagnostic potential than IDTs, at a concentration of 100 µg/mL, with readings assessed at 20 min. Regarding *in vitro* tests overall, the sLT release assay performed best.

Conclusion: Our study showed that skin prick tests presented the highest discriminatory diagnostic potential in IBH diagnosis. This increases our knowledge about IBH in Lusitano horses and could represent a step forward in the future development of specific immunotherapy.

Keywords: Insect Bite Hypersensitivity (IBH); Allergens; Intradermal tests (IDT); Skin Prick tests (SPT); *In vitro* tests.

6.4.8. Comparação de Testes Cutâneos por Picada (TCP), Testes Intradérmicos (TID) e Testes *In Vitro* na Caracterização da Hipersensibilidade à Picada de insetos (HPI) em cavalos Puro-sangue Lusitano: "Contribuição para Futura Implementação dos TCP no Diagnóstico da HPI"

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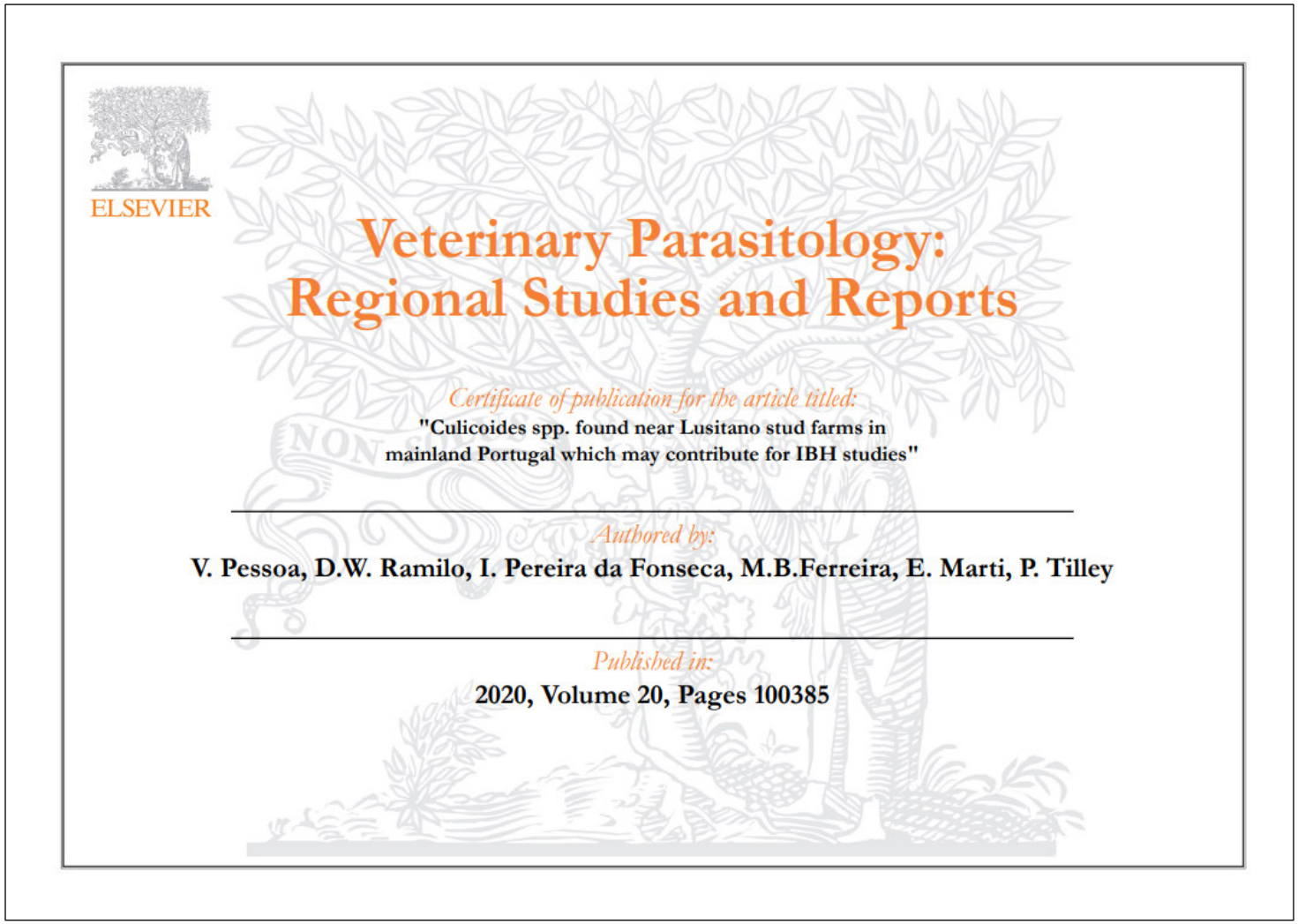
A prevalência da HPI nos cavalos Lusitanos em Portugal não é conhecida, mas as características ambientais são favoráveis à atividade do *Culicoides spp.* O objetivo do estudo foi a comparação de testes *in vivo* e *in vitro* no diagnóstico da HPI no cavalo Lusitano. No total foram avaliados 30 cavalos controlo (C) e 30 teste (T), incluídos com base na anamnese, exame físico e questionários individuais. Foram submetidos a testes Intradérmicos (TID) e Cutâneos por Picada (TCP), efetuados no pescoço. O painel de alérgenos incluiu 13 proteínas recombinantes de *Culicoides nubeculosus, C.obsoletus* (Cul n, Cul o) e extrato de *C. nubeculosus* (Cul n WBE). Adicionalmente 6 cavalos T e C foram testados com Cul n 3 e 4 produzidos em células de insetos e cevada, e Cul o 3 e Cul o WBE em *E. coli*. As concentrações foram 10 µg/ml para os TID e 100 µg/ml para os TCP e o diâmetro das pápulas avaliado aos 20 min, 6 e 48h. Os TID foram considerados positivos quando o diâmetro era ≥50% que o diâmetro da histamina e os TCP ≥0.9 cm. Realizou-se a determinação sorológica de IgE específicas e teste de libertação de sulfidoleucotrienos (sLT). Os resultados mostraram que, para os TCP o Cul n WBE, Cul n 7, 8, 9, Cul o1P e Cul o 2P foram os alérgenos com melhor desempenho no 1º painel, e Cul o WBE, Cul n 3 Bar e Cul n 4 Bac ($0.0001 \leq p \leq 0.05$) no segundo, com uma matriz discriminante superior aos TID, à concentração de 100 µg/ml aos 20 min. Nos testes *in vitro* o de libertação de sLT apresentou melhor desempenho. O maior potencial diagnóstico dos TCP representa um avanço importante no diagnóstico da HPI e implementação de imunoterapia específica.

Palavras-chave: HPI; Alergénios; ID; TCP; Testes i*n vitro.*

7. Certificates



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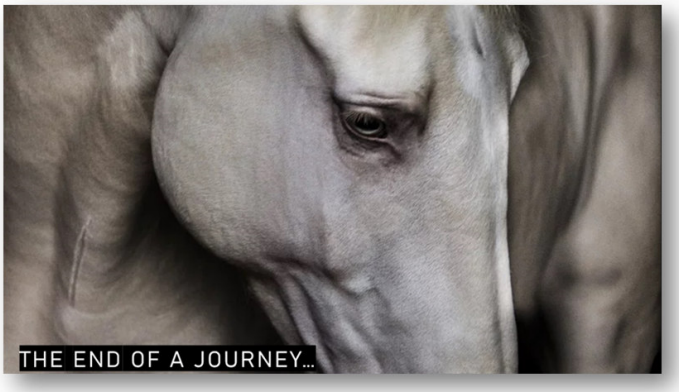
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