Culicoides obsoletus allergens for diagnosis of insect bite hypersensitivity in horses

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Thesis

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CHAPTER

1

General introduction

Immune system

The immune system protects and defends the body against bacteria, viruses and other pathogens to prevent disease. This defence can be divided in reactions of the innate immune system and reactions of the adaptive immune system. The adaptive immune system can be divided by two types: humoral immune responses mediated by antibodies and produced by B lymphocytes and cell mediated immune responses, mediated by T lymphocytes. A proper interaction between cells of the innate immune system (e.g. monocytes/macrophages, dendritic cells, neutrophils, eosinophil's, natural killer (NK) cells and basophils) and cells of the adaptive immune system (lymphocytes: T cells, B cells and NK cells) by communication via direct cell contact or soluble molecules such as cytokines, is essential for an intact, fully effective immune response [1].

There are some major differences between innate immune cells and adaptive immune cells. First of all, the innate immune cells are constitutively present, carry germline-encoded receptors, and are ready for immediate defence, whereas the adaptive immune cells need some time to react to invaders. Furthermore, adaptive immune cells react antigen-specifically because of their presence of rearranged receptors and have memory and can therefore react more rapid and vigorous on a subsequent exposure, while in contrast innate immune cells can react in a generic way to different organisms and have no memory [2].

This thesis is focused on Insect Bite Hypersensitivity, an IgE-mediated skin allergy in horses, which involves several immune mechanisms and thus associated immune cells. The immune cells that are relevant for this thesis are described in more detail in this paragraph of the introduction.

Antigen presenting cells

Antigen presenting cells (APC's) are the initiators of the adaptive immune system and provide a direct link between innate and adaptive immunity. Dendritic cells (DC's), macrophages and B cells are all employed as (professional) APC's, but the DC's are by far the most effective in stimulating naïve T cells.

DC's can efficiently activate T lymphocytes by processing and presenting peptide fragments of a protein antigen to the T cells. Furthermore, DC's express cell surface co-

stimulatory molecules (such as CD80 and CD86), which interact with CD28 on T cells and are critical to for the activation [3].

For the recognition of peptides by T cells, they need to be bound to molecules of the major histocompatibility complex (MHC) on the surface of the DC. There are two different types of MHC molecules, MHC class I and MHC class II, which stimulate cytotoxic CD8+ T cells and helper CD4+ T cells respectively. Endogenous antigens (e.g. viral proteins), cut into fragments in the cytosol of the APC bind to MHC class I molecules and are recognized by the cytotoxic T cells, which can then directly kill these virus-infected target cells. Exogenous antigens (e.g. proteins from foreign micro-organism) are taken up by the APC's, are processed and presented by MHC class II molecules to naïve T helper (Th) cells [1, 4]. After activation, the naïve, activated T cell can differentiate into different T cell subsets, depending on differentiating cytokines that are present in the micro-environment. Interleukin-12 (IL12) produced by DC's, often in association with IFNγ produced by NK cells, usually induces T cell differentiation into Th1 cells. In contrast, Th2 differentiation is usually activated when early IL4, instead of IL12 and/ or IFNγ, production occurs. The source of early IL4 production until now remains unclear but is most likely induced by the presence of thymic stromal lymphopoietin (TSLP), IL25 and IL33 produced by epithelial and endothelial cells [3, 5].

A less well defined approach for particularly DC's is to capture exogenous proteins and process these into the MHC class I pathway. This form of presentation is called cross-presentation and seems to be important for the generation of cytotoxic T cell generation to a variety of cellular antigens, including viral, tumour and DNA-encoded antigens [6].

Since DC's control many T cell responses they have been useful tools to beneficially manipulate the T cell responses to recognize specific antigens (mostly viral or tumour antigens) *in vitro* or *in vivo* [7-9].

Direct isolation of DC's from peripheral blood mononuclear cells results in extremely low yields (0.1%). However large amounts of DC's can be generated from peripheral blood mononuclear cells (PBMC), bone marrow cells or monocytes cultured *in vitro* in the presence of GM-CSF and IL4 [10-12].

Equine DC's from horse peripheral blood monocytes (monocyte-derived DC) were also successfully generated using equine IL4 and human or equine GM-CSF and can be used for clinical research [13-15].

T cells

In mammals, approximately 40-80 % of lymphocytes in the blood account for T cells. The peptide-binding T cell antigen receptor (TCR) complex is an important structure on the surface of the T-lymphocyte that contains many different proteins. These proteins can function in antigen binding for signal transduction and usually have a cluster of differentiation designation (CD). CD3 for example is a signal transducer that acts when an antigen binds the TCR and is therefore found on all T cells. CD4 is expressed on the surface of T helper cells that, as previously described, recognize processed exogenous antigen-derived peptides in combination with major histocompatibility (MHC) class II molecules on the surface of APC's. CD8 is predominantly found on the surface of cytotoxic T cells that recognize processed endogenous antigen-derived peptides in combination with MHC class I molecules [1]. In humans, 60 – 70 % of T cells are CD4+CD8- and 30-40 % are CD4-CD8+. The remaining 5 – 10% of T cells are said to be double negative [16]. Within the CD4+ cell population, regulatory T cells expressing the transcription factor FOXP3 and the surface protein CD25 (IL2 receptor α-chain) are naturally present. CD4+CD25+FOXP3+ Treg cells have a regulatory function by suppressing the activation, proliferation and cytokine production of many immune cells, including CD4+ cells CD8+ and APCs. Their dysfunction can result in autoimmune disease, immunopathology and allergy. In addition, IL10 secreting Tr1 cells, and transforming growth factor beta (TGFβ) secreting T helper 3 cells (Th3) are adaptively regulatory: they acquire regulatory functions after specific antigenic stimulation [17].

Both CD4+ and CD8+ T cells can be differentiated in subpopulations that can be distinguished by their cytokine secretion profile. This is best established for CD4+ T cells. Upon activation by a processed antigen that is presented by APC's, the naïve CD4+ Th cells begin to produce IL-2 and depending on the cytokines present in the environment, they continue to differentiate to designated Th1, Th2 or Th17 cells. Th1 cells express transcription factor t-bet, produce IL2, IFNγ and lymphotoxin and support cell mediated immune responses. Th2 cells express the transcription factor GATA-3, produce IL4, IL5, IL9, IL13 and GM-CSF and support humoral and allergic responses. Th17 cells produce IL17, characterized by the transcription factor RORgT and seem to play a key role in many autoimmune diseases. Although Th1 and Th2 cells often work together in an immune response, the response can become dominantly Th1 or Th2-like [16, 18].

Just as in humans, equine T cells can be divided into equine CD4 and CD8 subpopulations by the use of antibodies against the homologous equivalents of CD4 and CD8 described in man [19]. Within the CD4+ T cells, Th1 and Th2 cells identified by their signature cytokines. have been shown to play a central role within several clinical situations of horses and they can be associated with either protective or pathological responses [20, 21]. T cell responses in horses therefore seem to be similar as those in humans and mice, with respect to surface markers and cytokine production.

B cells

Humoral immunity is part of the adaptive immune system and involves the production of specific antibody molecules (immunoglobulin's (Ig)) in response to an antigen which is mediated by B cells. Naïve B cells produce IgM, but can undergo antibody heavy-chain isotype class switching to IgG, IgE or IgA after activation. Activation of B cells and isotype switching is regulated by Th cells expressing CD40 ligand and by cytokines produced by the Th cells [22]. IL4 and IL13, produced by Th2 cells, besides interaction with up regulated expression of CD40L on these activated T cells mediate isotype switching towards IgE [23]. IgE plays a major role in allergic diseases and is described in more detail later in this introduction. Th2 cells can also induce IgG4 in humans. IgG4 production is initiated by the presence of IL10, which inhibits IgE production [23-25].
Horses have the same immunoglobulin's as humans, but have 7 subclasses of IgG, instead of 4 in humans [26]. Horse IgE, just as IgE in humans, is critically involved in horse allergic diseases [27, 28].

Mast cells and basophils

Basophils and mast cells are important effector cells in response to both parasite infection and allergic inflammation [29]. Basophils account for less than 1 % of human leucocytes, mature in the bone marrow and are released as mature cells in the peripheral blood. They have a life-span of only a few days. Mast cells mature in peripheral tissues, after they are generated by precursor cells in the bone marrow that circulate and migrate into peripheral tissues. The mature mast cells do not circulate in the peripheral blood, but reside in the tissue. Mast cells have a life span for weeks to months [30].
Mast cells and basophils resemble many features. They both have numerous granules that

contain histamine amongst other mediators. They express fully functional high-affinity IgE receptors (FcεRI), which can bind IgE antibodies. Antigen and IgE-induced crosslinking of FcεRI, involving at least 100 to 1000 of these interactions, triggers activation of both cells, leading to degranulation of the mediators. The mediators that are released, including histamine, are associated with immediate hypersensitivity reactions [31].

An important feature that is specific for basophils and which is absent in the mast cells, is their rapid production of IL4 and IL13 after activation, supporting their contribution to allergic inflammation [32]. Recent studies have shown that basophils can also function as antigen-presenting cells with their MHC class II. Therefore basophils can mediate Th2 cell differentiation by their capacity to function as APC's and by their potential to produce IL4 [30, 33, 34].

Due to practical reasons, *in vitro* diagnostic tests that are available to test the allergenicity of candidate allergens in patients with allergies (e.g. the histamine degranulation test) are limited to the activation of basophils, since they are circulating in the blood and therefore more easily to access than tissue-resident mast cells.

Allergy

Although the immune system is primarily associated with the protection against disease, it is possible that an overactive immune response itself can cause damage. An undesirable reaction by the normal immune system causing tissue injury and disease is called hypersensitivity.

Allergic diseases can be classified into two major types of hypersensitivity reactions, Type I and Type IV. The Type I hypersensitivity is caused by the release of mediators from mast cells and mediated by the presence of allergen-specific IgE antibodies. The delayed (Type IV) hypersensitivity is mediated by T cells [35]. The antigen that provokes an allergic reaction is called an allergen.

Similar to humans, horses can suffer from a number of types of allergies with respiratory and skin allergy being the most common clinical features. Insect Bite Hypersensitivity is the most common skin allergy in horses and mainly involves a Type I (IgE mediated) hypersensitivity reaction and will be described in the next paragraph of this chapter.

Sensitisation phase and allergic reaction

The sensitisation phase comprises the induction of Th2 cells and the formation of specific IgE antibodies against specific allergens in individuals that are genetically predisposed to the development of allergy. This is the initial phase in the development of IgE-mediated (Type I) hypersensitivity.

When an allergen is deposited on mucosal tissues (respiratory and gastrointestinal tracts) or skin, it can enter the body where it is taken up by APC's (primarily DC) that process the allergen into smaller peptides. These peptides will be bound to major histocompatibility complex MHC class II on the surface of the APC's and presented to the TCR on naïve $CD4^+$ T cells. This leads to the activation of the selected T cell and results in specific cytokine production profile. In case of an allergic sensitisation the naïve T cell develops into a Th2 cell, leading to a Th2 skewed immune response, which disturbs the Th1/Th2 balance. Th2 cells produce cytokines like IL4, IL5 and IL13, and are involved in IgE-synthesis by inducing isotype class-switching in B cells. Allergen-specific IgE molecules are then secreted by the responding B cells and will subsequently bind to the high-affinity IgE receptors (FcεR1) on mast cells or basophils. The IgE antibody molecules can bind specific amino acids sequences (either continuous of discontinuous sequences) leading to the respective linear or conformational epitopes present on the allergen. The coating of a mast cells or basophil with these allergen-specific IgE antibodies finalizes the sensitisation phase (Figure 1).

When a subsequent exposure to the same allergen occurs, it will cause the binding of the protein to the mast-cell bound IgE. Crosslinking of the IgE bound FcεRI will result in intracellular reactions in the mast cell, leading to degranulation and subsequent release of the vesicle content such as vasoactive amines (histamine and serotonin), cytokines, like TNF-α and other mediators, such as leukotrienes and prostaglandins. This degranulation causes vascular permeability, leading to e.g. swelling and itchiness. These complaints generally occur within minutes and are the first symptoms of an IgE-mediated allergic response. In addition, the released cytokines IL4, IL9 and IL13 will further enhance IgE production and thereby the allergic inflammation in general. Furthermore, released mediators from the mast cells will promote the influx of eosinophils, neutrophils, basophils and macrophages, which after 4-24 hours comprise the late-phase cell-mediated response [35].

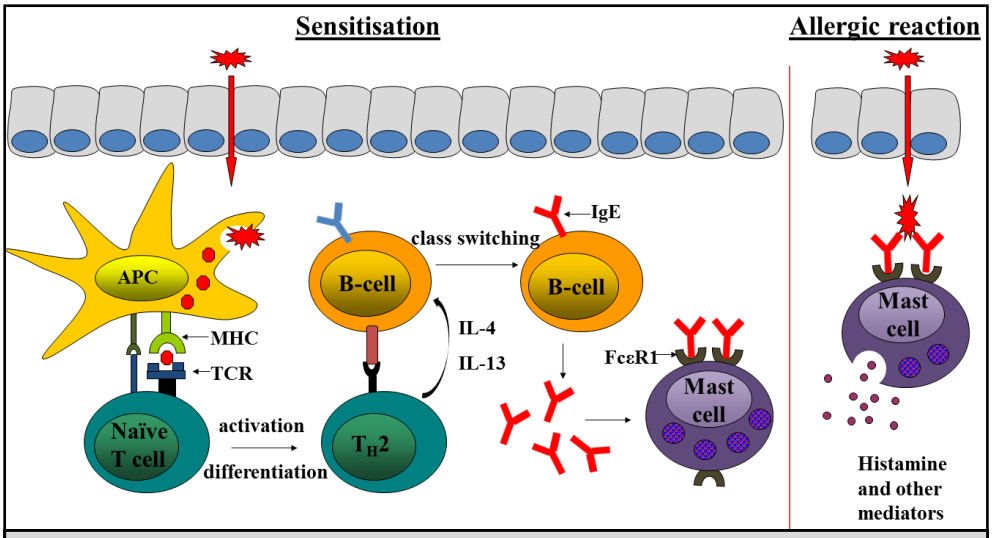

Figure 1: Schematic representation of the sensitisation phase and allergic reaction

Allergic reactions can be expressed in different parts of the body, e.g. itchy swellings on the skin, acute bronchospasm in aeroallergen-challenged asthmatic patients, food-induced diarrhoea and anaphylaxis in the gut, and systemic anaphylactic reactions to insect venoms and drugs [36].

Immunoglobulin E

Immunoglobulin E (IgE) is an immunoglobulin with a four-chain structure (2 identical heavy and 2 identical light chains) with a molecular weight (MW) of around 200 kDa and which is characterized by the presence of 13% of their molecular weight as carbohydrates. In contrast to IgG, IgA and IgD, they have an additional fourth constant domain, which results in a higher MW as a monomer.

Two signals are required for the induction of IgE synthesis. The first signal is provided by IL4 and IL13 produced by T cells, mast cells, and basophils, which activates signal transducer and activator of transcription 6 (STAT 6), which activates transcription at the IgE isotype specific switch region. The second signal involves engagement of the constitutively present B cell antigen CD40 with the induced CD40 ligand (CD154), expressed on activated T cells, which leads to isotype class switching during immunoglobulin synthesis in B cells [32, 37].

In human serum, IgE is the least abundant antibody class with a concentration of only

around 50 ng/ml, in contrast to IgG with serum concentrations of around 5-10 mg/ml. It has a short half-life of around 2 days when circulating in the serum, but when bound to FcεRI on mast cells, the half-life increases to up to months [36].

In horses the total IgE concentration is much higher, reaching the µg/ml range, and variations between individuals are high. These higher IgE levels in horses are most likely caused by generally higher parasitic loads in horses than in humans from western civilizations [38]. The total IgE level of horses with skin allergy is not significantly higher than in healthy horses kept at the same environment, except for Icelandic horses [39, 40]. New-born foals receive high concentrations of maternal IgE through the colostrum, which is suggested to have an immunoregulatory effect on the immune response of the new born. This maternal IgE becomes undetectable at 2-4 months of age [41]. The delayed endogenous IgE production can start as early as 6 months of age [42].

Studies on IgE-mediated hypersensitivity reactions are dependent on the availability of antibodies to detect IgE. The αIgE-134 and αIgE-176 were the first mouse monoclonal antibodies produced against horse IgE [40]. They recognize different epitopes and have shown to be useful for various *in vitro* and *in vivo* immunological applications in horse studies. Some years later other anti-IgE monoclonal antibodies were produced [39].

Allergens

According to the World Health Organization/International Union of Immunological Societies definition, a protein is considered to be an allergen when it elicits a specific IgE antibody response in at least five individuals [43]. Major allergens are defined as allergens to which at least 50 % of the individuals with a particular allergy are sensitised. According to the nomenclature system allergens are named by the first three letters of the genus, the first letter of the species name and a number according to, for example, the order of allergen discovery (http://www.allergen.org). The protein Ara h 1 is for example, a major allergen for people with a peanut (Arachis Hypogaea) allergy.

The capacity of a protein to bind IgE antibodies does not necessarily mean that this protein is allergenic. Allergenicity of an allergen reflects the capacity to induce allergic symptoms and thus refers to the presence of at least two IgE-binding epitopes that can crosslink IgE antibodies bound to FcεRI present on mast cells and basophils leading to degranulation and subsequent mediator release.

Antigenic epitopes can be classified as either B or T cell reactive epitopes. T cell epitopes are linear amino acid sequences that are distributed throughout the primary structure of the allergen. They are recognized by T cell receptors after the whole allergen is processed by APC's and presented as small linear peptides (8 to 18 amino acids) in complex with MHCI or –II molecules. B cell epitopes recognized by IgE antibodies can be either linear or conformational, meaning that the amino acids are not in a linear sequence but brought together by folding of the amino acid chain, thus dependent on the 3-dimensional structure of the protein. Changes in folding of the protein may therefore lead to loss of B-cell epitopes [44, 45].

Since the existence of recombinant technology, many allergens responsible for human allergies have been purified and cloned, revealing a great diversity of proteins based on their sequence similarity, crystal structure or biochemical analysis of their properties. The major allergens that have been identified are almost all proteins present in the environment or diet [46]. Many proteins undergo posttranslational modification, like N- and O-glycosylation. IgE-binding has shown to be dependent on glycosylation sites in several allergens, mainly pollen and insect allergens [47, 48] and can thus be important for the allergenicity of a protein.

Glycosylation of recombinant allergens is dependent on the expression system that is used. The first recombinant allergens were produced in *Escherichia coli* (*E. coli*), which does not facilitate glycosylation. Although this expression system is still frequently used, eukaryotic expression systems can be beneficial when glycosylation and correct folding are desired.

Protein expression in Escherichia coli

The production of recombinant proteins in *E. coli* remains one of the most attractive systems because they offer rapid biomass accumulation, they are easy to handle and relatively inexpensive. If recombinant proteins do not require complex post-translational modification or do not need to be expressed in a soluble form, *E. coli* is usually first selected for large-scale protein production [49].

There are many different types of promoters used for gene expression in *E. coli*. Generally, these promoters are strong, capable of protein production in excess of 10-30% of the total cell protein, they should exhibit a minimal level of basal transcription and they

should be capable of induction in a simple and cost-effective manner. Chemical and thermal induction are widely used for protein production in *E. coli* [50].

pET vectors (commercialized by Novagen) are often used for *E. coli* recombinant protein production. Target genes are positioned downstream of the bacteriophage T7 late promoter on medium copy number plasmids. This promoter is not recognized by *E.coli* RNA polymerase, but only by T7 RNA polymerase. Production hosts typically contain a prophage (ʎDE3) encoding this enzyme under control of the LacUV5 promoter. The LacUV5 promoter can be induced with isopropyl-B-D-1-thiogalactopyranoside (IPTG) [51].

A limitation of high expression levels of eukaryotic genes in *E. coli* is that the target protein is often unable to reach a native conformation and undergoes proteolytic degradation or forms insoluble aggregates of non-native proteins known as inclusion bodies. Inclusion bodies must then be solubilized and refolded into an active conformation, which requires considerable optimization. Refolding of proteins using established strategies usually results in acceptable yields [49, 52].

Protein expression in baculovirus insect cell expression systems

The baculovirus-mediated expression in insect cells is widely used for the production of numerous recombinant proteins. The advantages of this system are the presence of post-translational modification machinery, biosafety, because they are non-infectious to human and a high recombinant protein yield that can be obtained by the use of the strong polyhedrin or p10 promoter [53]. However, insect cells produce structural different N-glycan's than mammalian cells and therefore the bioactivity of recombinant mammalian proteins produced in baculovirus expression system might be reduced or they even can be recognized and induce allergic reactions in mammals [54].

The *Autographa californica* nuclear polyhedrosis virus (AcNPV) is the prototype baculovirus that is used. The system is based on the replacement of the polyhedron gene with foreign DNA and then using these constructed viruses to infect insect cells. The polyhedron promoter is extremely strong and activated during the late stages of infection. Insect cell lines that are generally chosen as host for AcNPV are Sf9 and Sf21 from the fall armyworm, *Spodoptera frugiperda*, and High-5 (TN5B1-4) from the cabbage looper, *Trichoplusia ni* [53].

In the older traditional method recombinant baculovirus expression vectors are isolated using homologous recombination. Transfer plasmid DNA containing the gene to be cloned, along with circular wild-type genomic viral DNA is introduced in insect cells. This results in only about 0.1% recombinant progeny, in which the heterologous gene is inserted in the genome of the parent virus by homologous recombination. This recombinant progeny can be improved when the parent virus is linearized and with the creation of a large deletion that functionally inactivates an essential gene, thus precluding replication of parental virus, increasing the recombinant baculovirus production up to 90%. A time intensive plaque assay however is always required with these methods to purify recombinant virus from the nonrecombinant parental virus [55].

The Bac-to-Bac® expression system is an efficient method to generate recombinant virus via the site-specific transposition of a plasmid containing the gene of interest into a bacterial artificial chromosome (bacmid). The bacmid contains the necessary viral components for infection and replication within the insect cell. After amplification in *E. coli*, purified recombinant viral DNA is transfected into the insect cells. Plaque purification is not necessary using this method and therefore greatly reduces time taken to identify and purify recombinant virus [56].

Allergens for diagnosis and immunotherapy

Allergies can be diagnosed *in vivo* by different skin tests and *in vitro* by basophil degranulation tests or different serological tests. Diagnostic tests based on natural allergen extracts are composed of relatively ill-defined mixtures of non-allergenic materials, major allergens, and cross-reactive allergens, with the result that it is difficult and often impossible to precisely identify the disease-eliciting allergen. Furthermore, natural allergen extracts might contain proteases or other unwanted components, like allergens from other sources which can affect testing for allergenicity [57-59]. Diagnostics with such extracts can therefore result in false negative tests due to insufficient amounts or low quality of disease-eliciting allergens. Advances in allergen characterization (molecular techniques to identify allergens and recombinant DNA technology for the production of recombinant allergen molecules) have provided new tools, such as recombinant allergens, that can improve the diagnosis of allergy.

Immunotherapy is the only causative treatment option for allergies, because it targets the

mechanisms underlying the allergy, e.g. increasing the frequency of regulatory T cells suppressing the allergen-specific Th2-cells and the generation of allergen-specific blocking antibodies. It is based on the administration of increasing doses of allergy-inducing antigens until a state on non-responsiveness to these allergens is reached [60]. However, immunotherapy with natural allergenic extract may result in new IgE sensitisation to other antigens present in the extract [61]. For these reasons, defined (recombinant) allergens may present a better alternative as an individual patient's IgE reactivity profile can be precisely determined with single recombinant allergens or a cocktail of few defined recombinant allergens, thus allowing component resolved diagnostics (CRD). Subsequently, those allergens to which the patient carries specific IgE can be selected for treatment by specific immunotherapy [62].

However, the question arises if the replacement of natural allergenic extracts by recombinant allergens results in similar sensitivity in diagnostic tests. Allergenic extracts contain many different allergens and each of those often exist as multiple isoforms that can differ in their amino acid sequence and post-translational modifications and thus in their allergenicity [63, 64]. Recombinant allergens consist of only one isoform and when produced in a prokaryotic expression system have reported to fail to behave comparably as its native form [65, 66]. However, CRD with recombinant allergens has lately been a major topic for many type I allergy studies and some have demonstrated that recombinant allergens can reproduce comparable or result in even better IgE reactivity as the natural allergen extracts [67-69].

Moreover, vaccination with recombinant allergens for specific immunotherapy has been reported to be successful in inducing tolerance for different type 1 allergies [70, 71].

Insect Bite Hypersensitivity

Insect bite hypersensitivity (IBH) is the most common seasonal recurrent skin allergy in horses caused by the bites of midges of the genus *Culicoides* [72-74], although other insect species, such as *Simulium* and *Stomoxys* species, have also been described to be of importance [73, 75]. IBH is found worldwide and reported under a variety of other names, such as summer eczema [76], sweet itch [77] and Kasen [78]. IBH is clinically characterized by intense itch and irritation caused by hypersensitivity reactions to bites of the insects. Due to scratching and rubbing, hair loss and excoriations can develop (Figure 2), which contribute to the development of secondary infections on the skin. In severe chronic cases the skin might become thick and rigid due to fibrosis [79]. Skin lesions are found predominantly at preferred landing sites of the insects, which are the dorsal midline, main and tail region [80] and along the ventral midline and sometimes at the ears [81]. Welfare of affected horses is seriously reduced and they can become unsuitable for riding or showing purposes, because of the discomfort and disfigurement. Clinical signs are present in the warmer months of the year when *Culicoides* insects are active and regress during the colder months when there is no exposure.

Treatment of IBH is mostly based on insect avoidance by stabling, use of anti-insect blankets or insect repellents and suppression of symptoms by the use of corticosteroids. Some treatment options, like anti-histamines, dietary supplements and ointments are available on the market, but studies on horses using these treatments have shown varying results [82-84]. Immunotherapy could be an interesting curative therapy for horses suffering from IBH, but so far only few trials have been carried out with *Culicoides* whole body extracts and they showed varying results [85, 86]. Anderson et al. [85], however did observe a clear reduction of clinical signs in most and even a complete disappearance in some horses after immunotherapy with *C. variipenis* extract, but more than 10.000 insects were required per horse.

Prevalence

IBH is found all over the world with a prevalence ranging from 3 - 11.6% in areas in the UK [87], 37.7% in parts of Germany [88], 10 - 60 % in areas of Queensland, Australia [89] and 0 -71.4 % in regions of The Netherlands [90]. IBH is not found in Iceland, where *Culicoides* insects are absent. However, IBH has an especially high prevalence of > 50 %

Figure 2: IBH affected main (left) and tail (right) of a Shetland pony

in Icelandic horses exported from Iceland to countries were *Culicoides* insects are present, probably because these horses are not exposed to the antigens of *Culicoides* insects early in life [91, 92]. Furthermore, the risk of IBH for these horses increases with age at import, which suggests that the immune system gradually loses its ability to adapt to new allergens [93]. Icelandic horses born in a *Culicoides* infested country do not seem to be more susceptible to develop IBH compared to other breeds. Furthermore, when Icelandic horses imported from Iceland to Europe, get exposed to allergens around 7-10 months of age they do not develop IBH more frequently than Icelandic horses born in Europe, suggesting that the development of immune tolerance occurs before 10 months of age [93]. Endogenous IgE production in horses starts at an age of 6 months and increases during the next three years [42]. Additionally, the risk of getting IBH for locally born horses is highest in their third year, but earliest symptoms are reported at an age of about 14 months [93].

Although IBH has been described for many horse breeds, e.g. Quarter horses, Arabian horses, Thoroughbreds, Warmbloods, Draft horses, Friesian horses, Shire horses and different pony breeds [27, 72, 73, 88, 90, 94, 95], certain breeds seem to be more often affected, like Shetland ponies and Friesian horses. Based on clinical symptoms, IBH has been shown to be genetically heritable in these breeds [96, 97].

Culicoides biology

Culicoides is the most important insect genus shown to cause IBH in horses. They are also vector for many arboviruses, such as African horse sickness virus (AHSV) and bluetongue

virus (BTV) [98].

Culicoides are small biting insects, measuring 1-3 mm in size, belonging to the family *Ceratopogonidae* and also known as midges, gnats, no-see-ums and punkies. They compromise over 1400 species worldwide of which most are blood suckers, feeding on mammals and birds [98]. Species can be differentiated from each other by their size and wing pattern [99], although some species are very alike.

Culicoides are found on virtually all large land masses, with the exception of Antarctica, New Zealand and Iceland [98]. In the Netherlands about 25 *Culicoides* species can be found, from which the most abundant species seem to be *C. obsoletus, C. impunctatus, C. pulicaris* and *C. dewulfi* [100].

Female *Culicoides* insects need a blood meal to support egg production, whereas males feed on sugars and nectar. Most of these insects are crepuscular (i.e. active during dawn and dusk) that prefer to feed on warm, dry, windless days. *Culicoides* are therefore mainly active around sundown and sunrise in spring and summer months, which explains the alternative name of IBH 'summer eczema'.

Culicoides have a short flight range reaching up to a few hundred meters from their breeding sites [101, 102]. However they can passively reach greater distances when carried by the wind [103-105]. Examples of breeding sites are around puddles, tree forks, drainage canals, streams and swamp, all with organic material [106]. The development from egg to adult can take from a few days up to several months when they overwinter in the larval stage, depending on species and temperature. In general, most adult *Culicoides* survive only between 10-20 days [98].

Many different *Culicoides* species were found to be associated with IBH, including *C. obsoletus, C. pulicaris*, *C. dewulfi*, *C. sonorensis, C. freeborni C. robertsi C. nubeculosus*, *C. insignis,* C. *peregrinus, C imicola* [78, 80, 89, 107-111]. *C. obsoletus* is regularly reported to be the most abundant *Culicoides* species attracted to horses in different countries [78, 107, 108, 110] and thus likely to be an important species responsible for IBH worldwide. *C. obsoletus* is part of the Obsoletus complex, which in central and northern Europe is composed of three species: *C. obsoletus, C. scoticus* and *C. chiopterus* [112]. Females of these species are difficult to distinguish based on morphology. *C. dewulfi* was also considered part of this complex for a long time due to its morphologically similarity, but was recently reported to belong to a different taxonomic

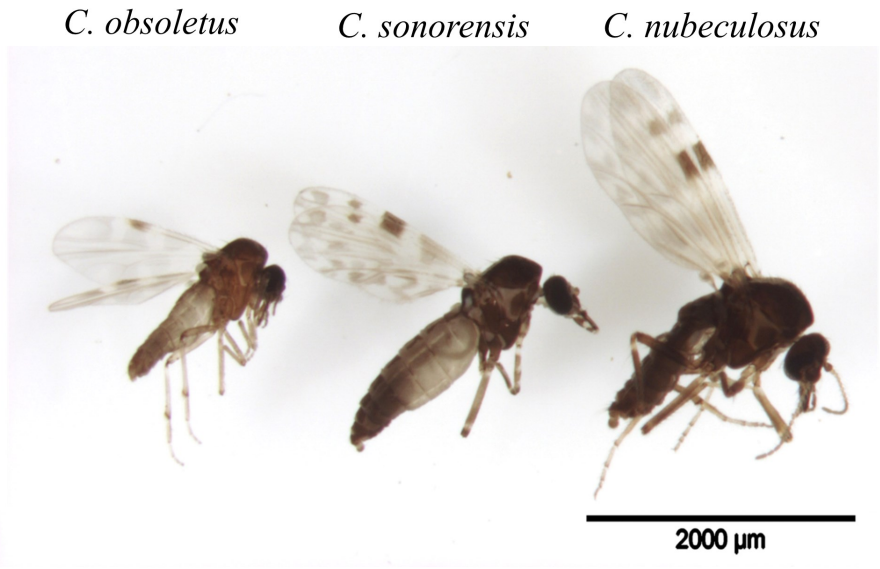

Figure 3: Three *Culicoides* species

group [113]. In this thesis *C. obsoletus* refers to Obsoletus complex, because identification was based on morphologically characteristics. Therefore, *C. dewulfi* in this thesis is also considered part of Obsoletus complex. *Culicoides* species that are relevant for this thesis are presented in figure 3.

Immunopathology

The immunopathogenesis of IBH involves IgE (type I) mediated hypersensitivity, which has been demonstrated by various studies.

The first direct evidence for the role of IgE in the pathogenesis of IBH was provided by a modified Prausnitz-Kustner experiment. IgE was purified from equine serum from IBH-affected horses and transferred into the skin of 4 healthy horses by intradermal injection, followed by a challenge with a commercial available *Culicoides* extract at the same sites. This resulted in an immediate skin reaction at the injection sites in 2 out of 4 horses. These skin reactions were not observed in response to extract alone [28]. Furthermore, significantly more IgE protein and IgE mRNA bearing cells were found in lesional skin of IBH affected horses than in skin of healthy horses [114].

Intradermal skin tests have been used many times over the past years to test *Culicoides*

allergens and extracts for its ability to induce mast cell reactivity in healthy and IBH-affected horses. IBH-affected horses in general show significantly more immediate skin reactions compared to healthy controls and in some horses also late phase reactions are observed [115-117]. Moreover, *in vitro* basophil degranulation in response to *Culicoides* extracts is observed in blood of IBH-affected horses, whereas most control horses do not show reactivity [75, 116, 118]. Finally, horses with IBH have more serum IgE antibodies against *Culicoides* salivary proteins, compared to healthy controls [119-122].

Thus, The IgE-mediated mast cell degranulation is clearly an important reaction in the immunopathology of IBH. However, T cells also play an important role in IBH and the involvement of these cells has been investigated in the skin as well as cultured PBMC's of IBH affected and healthy controls.

The Th1/Th2 polarity was compared between healthy and IBH-affected Icelandic horses, imported from Iceland to Europe (1^{st} generation) or born in Europe (2^{nd} generation) [123]. In summer, but not in winter, IL4 production by PBMC's (stimulated with *Culicoides* extract or polyclonal) from IBH-affected horses was significantly higher than those of healthy controls. Interestingly, 1^{st} generation horses showed higher Th2 cytokine production than the 2^{nd} generation horses with the same disease state. An increased level of IL10 in supernatant from PBMC's of healthy 2^{nd} generation horses was observed upon *Culicoides* or polyclonal stimulation. It was suggested that the suppression of the Th2 type cytokine IL4 in healthy horses, might be due to regulatory cytokines, like IL10. In a follow-up study, adding supernatant of the PBMC's of healthy 2^{nd} generation horses to the cultures of IBH-affected horses 1^{st} generation horses, led to a down-regulation of the IL4 production. This effect could be imitated when adding both IL10 and TGFβ1 to the cultures. In addition, IL4 production by PBMC's of healthy control horses could be increased by adding anti-IL10 and anti- TGFβ1 to the cultures [124]. These results have indicated the importance of regulatory cytokines in horses with IBH. Recently published studies have further investigated the possible role of equine regulatory T cells in IBH using FoxP3 as a marker [125, 126].

FoxP3+ Tcells were detected by immunohistochemical staining in lesional and non-lesional skin of IBH-affected and healthy control horses [125]. Severely IBH affected horses had a lower ratio of Foxp3+ to CD4+ cells in lesions compared to moderately affected horses or healthy horses. At mRNA level the results were more convincing, as the

expression of FoxP3 in lesional skin of IBH affected horses was significantly lower compared to non-lesional and skin of healthy horses. Furthermore non-lesional skin of IBH-affected horses also showed a significant reduced Foxp3 expression compared to skin of healthy horses. There were no differences observed for Foxp3 expression in the blood. However, there was a reduced IL10 expression level in the blood of IBH-affected horses compared to healthy horses and IL10 levels were also lower in lesional skin compared to non-lesional skin of IBH affected horses. Th2 cytokine expression levels were not found to be higher in blood of IBH-affected horses compared to healthy controls, but in the skin of IBH-affected horses a significant higher IL13 level was observed [125].

Hamza et al. [126] focussed on the expression of Foxp3 in $CD4^{+}CD25^{+}$ T cells in PBMC directly after isolation or after *C. nubeculosus* extract stimulation or with a non-relevant control allergen using flow cytometry. They only observed a significant difference in the *C. nubeculosus* stimulated PBMC, which showed a higher expression of Foxp3 by $CD4^{+}CD25^{+high}$ and $CD4^{+}CD25^{+dim}$ cells from healthy horses compared to IBH horses. The expression of FoxP3 within $CD4^{+}CD25^{+high}$ cells of the healthy horses could be significantly decreased by adding IL4 to the cultures stimulated with the *C. nubeculosus* extract.

In conclusion, IBH can be clearly described as a Th2 type, IgE mediated allergic disease and just as in humans, there seems to be a disturbance in the function of the regulatory T cells.

Culicoides allergens and cross-reactivity

Culicoides saliva and whole body extracts consist of many proteins that can bind IgE from IBH affected horses [119-122, 127]. Each IBH-affected horse seems to have its own IgE-affinity pattern for different *Culicoides* proteins, suggesting that multiple allergens play a role in IBH and that they can differ between allergic horses.

Hellberg et al. [120], identified ten different IgE-binding protein bands from *C. nubeculosus* with molecular weights from 12 kDa to 75 kDa with a large variety in IgE-binding pattern between each horse, but the corresponding proteins were not identified in this study. In a different study, western blot analysis revealed a dominant IgE-binding protein band of 65 kDa from a native European species (*C. obsoletus)* for all allergic horses that were tested [115]. A band of similar size (66 kDa), but from a North American

species (*C. sonorensis)*, was identified as a dominant IgE-binding protein band for IBH affected horses in Germany [121]. This band was further characterized by mass spectrometry and bioinformatics and identified to be a maltase and was named Cul s 1. Cul s 1 was expressed in a baculovirus expression system and its clinical relevance was investigated by *in vitro* and *in vivo* diagnostic tests. Most allergic horses showed IgE reactivity against the recombinant Cul s 1 which confirmed its allergenicity. Cul s 1 is the first allergen described to be relevant for IBH.

Candidate allergens were also identified from salivary glands from laboratory bred *C. nubeculosus* [127]. Using a proteomics approach two abundant proteins were identified with a mass of 40-50 kDa that showed sequence similarity to a hyaluronidase and a heavily glycosylated protein of unknown function from an EST library from *C. sonorensis* [128]. The allergenicity of these proteins was not determined in recombinant form, but in 2D blots these antigens had positive reactions with allergic horses.

A different approach to isolate IBH relevant allergens was undertaken by using phage surface display technology with a cDNA library prepared from salivary gland mRNA from either *S. vittatum* or *C. nubeculosus* [129, 130]. Seven IgE-binding proteins from *S. vittatum* were identified and expressed in *E. coli*. Significant higher IgE-levels in IBH-affected horses compared to healthy controls were found against four of them; antigen-5 like protein (Sim V 1), a serine protease inhibitor (Sim V 2) and two α-amylases (Sim V 3 and Sim v 4). Sim V 1 was reported to be an interesting candidate allergen, as the proportion of IBH-affected horses reacting against this allergen was higher than for the other allergens [129].

In a follow-up study, a clear cross-reactivity between the antigen-5 like allergen from *S. vittatum* Sim V 1 and an antigen-5 like protein from *C. nubeculosus* was observed both *in vivo* and *in vitro*. They were found to share 47.7% sequence identity at primary structure level [130]. Cross-reactivity between proteins from these insect species was also observed by Hellberg et al. [131]. However IgE from allergic horses reacted with fewer protein bands from *S. vitattum* than from *C. nubeculosus.* Furthermore a histamine release test gave better results with *C. nubeculosus* extract compared to *Simulium* extract [75].

Cross-reactivity between *Culicoides spp* has also been investigated. Intradermal tests on allergic and healthy control horses in Northern Germany and British Columbia with extracts and saliva of native and exotic *Culicoides* spp, showed no difference between

Culicoides species, indicating the presence of species-shared allergens [95, 116]. This is also confirmed by Hellberg et al. [120] who reports that most IBH-affected horses show IgE-binding to different proteins from *C. nubeculosus*, a species which is rarely found in the country where this study was performed. In contrast, a study in The Netherlands, where *C. obsoletus* was found to be the most common species attracted to horses [107], reported the importance to use a native *Culicoides* species extract for *in vivo* diagnosis, suggesting low *Culicoides* cross-reactivity [117]. Moreover, IBH-affected horses were reported to have stronger IgE responses to proteins from native *C. obsoletus* compared to proteins from *C. nubeculosus* [127].

Recently, ten additional IgE-binding proteins from *C. nubeculosus* were isolated, expressed as recombinant allergens and termed Cul n 2 to Cul n 11 [122] with molecular masses ranging from 15.5- 68.7 kDa. The authors reported that 44 out of 45 tested IBH affected horses reacted with at least one of these allergens in an IgE ELISA.

An overview of all allergens produced as recombinant proteins that have been associated with IBH was recently reviewed and are presented in Table 1 [81].

Thus in summary, many IBH relevant allergens have lately been identified. However these allergens were derived from species that are not native for the countries where they were tested. Although some cross-reactivity between *Culicoides* species has been reported, there is also evidence for the lack of cross-reactivity and the use of recombinant allergens from native species might therefore be important for successful diagnosis and future immunotherapy.

Diagnosis

Observation of clinical symptoms, combined with well documented medical history and ruling out other conditions that may lead to pruritus is often considered as the gold standard for the definitive diagnosis of IBH [75]. However misdiagnosis can occur and because clinical symptoms are absent in colder months the need for a diagnostic test is high. Diagnostic tests have lately been developed and can be divided in 1) intradermal skin testing [115-117, 132], 2) cellular basophil degranulation tests [75, 116, 133] and 3) serological tests that measure allergen-specific IgE in serum [115, 116]. These tests are mostly performed with non-standardized crude allergen extracts from *Culicoides* or

Table 1. Overview of allergens produced as recombinant proteins relevant for Insect Bite Hypersensitivity. Abbreviations: n.s.: not shown, IDT: intradermal test; pos: positive, n.t.: not tested; [a]*C. nubeculosus*; [b]*C. sonorensis;* [c]*S.vittatum [81]*

r-Allergen	Genbank accession nr.	aa	MW	Homology to (NCBI accesion number)	IDT	Reference
Cul s 1	Q66UC5_9DIPT	n.s.	68.6	Maltase (AY603565)[b]	pos	119
Cul n 1	EU978899	209	25.4	Antigen 5 like Protein (ACH56843.1[c]; AAU06471[b])	pos	128
Cul n 2	HM145950	403	46.7	Hyaluronidase (ACM40915.1)[a]	pos	120
Cul n 3	HM145951	391	44.6	Putative cysteine endopeptidase (ACM40897.1)[a]	pos	120
Cul n 4	HM145952	153	17.5	Secreted salivary protein (ACM40874.1)[a]	pos	120
Cul n 5	HM145953	406	45.7	Secreted salivary protein (ACM40903.1)[a]	pos	120
Cul n 6	HM145954	147	16.9	Secreted salivary protein (ACM40905.1)[a]	n.t.	120
Cul n 7	HM145955	186	20.9	Unknown salivary protein (AUU06522.1)[b]	pos	120
Cul n 8	HM145956	603	68.7	Maltase (ACM40914.1)[a]	pos	120
Cul n 9	HM145957	142	15.5	D7-related salivary protein (ACM40878.1)[a]	pos	120
Cul n 10	HM145958	420	47.8	Secreted salivary protein (ACM40903.1)[a]	pos	120
Cul n 11	HM145959	275	30.1	Trypsin (ACM40875.1)[a]	n.t.	120
Sim v 1	EU930215	258	30.1	Antigen 5 like protein (ACH56843.1)[c]	pos	128
Sim v 2	EU930300	83	9.6	Kunitz protease inhibitor (ACH56928.1)[c]	n.t.	127
Sim v 3	EU930236	246	28	a-Amylase (ACH56864.1)[c]	n.t.	127
Sim v 4	EU930294	241	26	a-Amylase (ACH56922.1)[c]	n.t.	127

Figure 4: Intradermal test in a horse. Wheals induced by allergens after 30 min. Upper left wheal is histamine positive control. (Picture made by M. Sloet van Oldruitenborgh-Oosterbaan)

sometimes *Simulium* spp. leading to currently unreliable and/or poorly repeatable diagnostic tests.

Intradermal testing

Intradermal testing has been used in horses for many years to determine sensitisation of skin mast cells with *Culicoides* specific IgE [27, 117, 134]. Mast cell degranulation is evaluated after injection of allergen(s), positive control (histamine) and a negative control (PBS or saline) by measuring the wheal diameters at the injection sites after different time points (Figure 4) [28]. Intradermal testing using *Culicoides* or *Simulium* extracts has been reported to have a high sensitivity and specificity and has therefore potential as a diagnostic test [115-117, 132]. However, low sensitivity and specificity has been reported as well [135] and intradermal testing has some well-known disadvantages. The results can be difficult to interpret and repeatability of the test has reported to be poor [136]. Furthermore, intradermal tests can be irritating and cause false positive reactions when injected to deeply and maybe even lead to sensitisation [137]. Moreover, *in vitro* testing is much more preferable, because it is easier to take a blood sample and more convenient for the veterinarian as well as for the horse.

Basophil degranulation test

The basophil degranulation test, also called histamine release test (HRT), is performed on blood samples and based on mediator release from peripheral blood basophils. Just as the intradermal skin test it determines sensitisation with allergen-specific IgE and can thus be seen as an alternative for skin testing [38].

Two HRT's, the functional *in vitro* test (FIT) and the Cellular Antigen Stimulation Test (CAST), have been tested with *Culicoides* and *Simulium* extracts for diagnosis of IBH [75, 116, 133].

The FIT test has been developed at the University of Hannover [138], is performed on whole blood and monitors the histamine release by sensitised basophils. A schematic set up of this test is shown in Figure 5. The amount of released histamine by horse peripheral blood basophils without stimulation (spontaneous release), after stimulation with anti-horse IgE or IgG (antibody release) or after stimulation with allergen (test release), is measured as a percentage of the total histamine content obtained by cooking (maximum release) or in some cases obtained by anti-IgG or anti-IgE (Figure 5) Supernatant is collected and the histamine content is measured by a competitive Radioimmunoassay (RIA). The allergen induced histamine release is set as a percentage of the maximum release.

The CAST determines the release of sulfidoleukotrienes by peripheral blood leucocytes

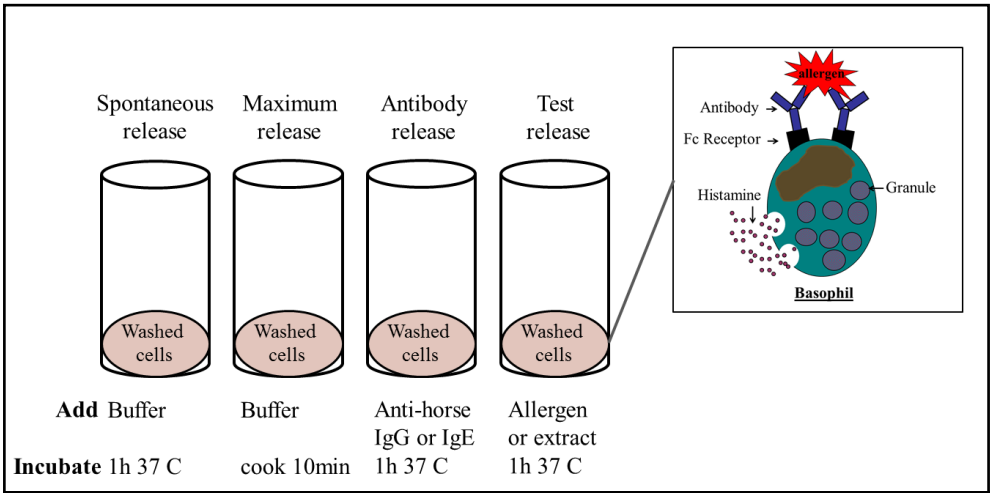

Figure 5: Schematic set up of the functional in vitro test, compromising the spontaneous, maximum, antibody and test histamine release of the basophilic granulocyte for each blood sample (modified from Stuke et al. [143])

(PBL) after incubation with allergens and was developed for the diagnosis of IgE-mediated allergic reactions in human [139]. This test with minor modifications was successfully used with horse PBL for the diagnosis of IBH after stimulation with *C. nubeculosus* or *S. vittatum* extracts. However, when horses were not exposed to insects for several months, the sensitivity of the test decreased [75]. Sulfidoleukotriene production was shown to be highly correlated with histamine release from basophils [133]. Stimulation with *C. nubeculosus* extract resulted in higher sensitivity and specificity compared to *S. vittatum* stimulation, suggesting that horses with IBH are mainly sensitised to *Culicoides* allergens [75].

Although sensitisation of basophils and mast cells with IgE is required for clinical allergy, sensitisation of mast cells and basophils does not necessarily mean that a horse shows clinical symptoms. Clinically healthy horses often show to be clearly sensitised with IgE to *Culicoides* allergens when tested by HRT or intradermal skin testing [117, 118, 135, 136, 140].

Serological test

Serological allergy tests are based on the knowledge that allergic individuals have higher allergen-specific IgE levels in their serum than healthy individuals. The detection of allergen-specific IgE antibodies in serum is often performed by Enzyme Linked Immuno Sorbent Assay (ELISA). Allergen preparations are coated on the plate, serum or purified IgE is added and bound equine IgE is detected by polyclonal reagents, monoclonal antibodies or the human FcεR1α chain, which is then detected by a secondary antibody conjugated with an enzyme, that upon reaction with its substrate can change the colour of this substrate [116, 141, 142]. A schematic set up of such an ELISA is shown in Figure 6.

The FcεR1α-based ELISA test (ALLERCEPT®, Heska) is used to detect the presence of allergen-specific IgE in sera and was evaluated with sera of both IBH-affected and healthy horses [141]. In the ALLERCEPT® IgE test an allergen or extract is bound to the wells of an ELISA plate, serum is added and afterwards recombinant human alpha chain of the high affinity IgE receptor (FcεR1α), known to cross-react with horse IgE, is added that will specifically bind to IgE. Specific IgE against insect (including *C. nubeculosus* extract), mould, mite and pollen allergen was measured, but the test results showed that the ELISA that uses the high-affinity IgE receptor is not a suitable test for diagnosis of

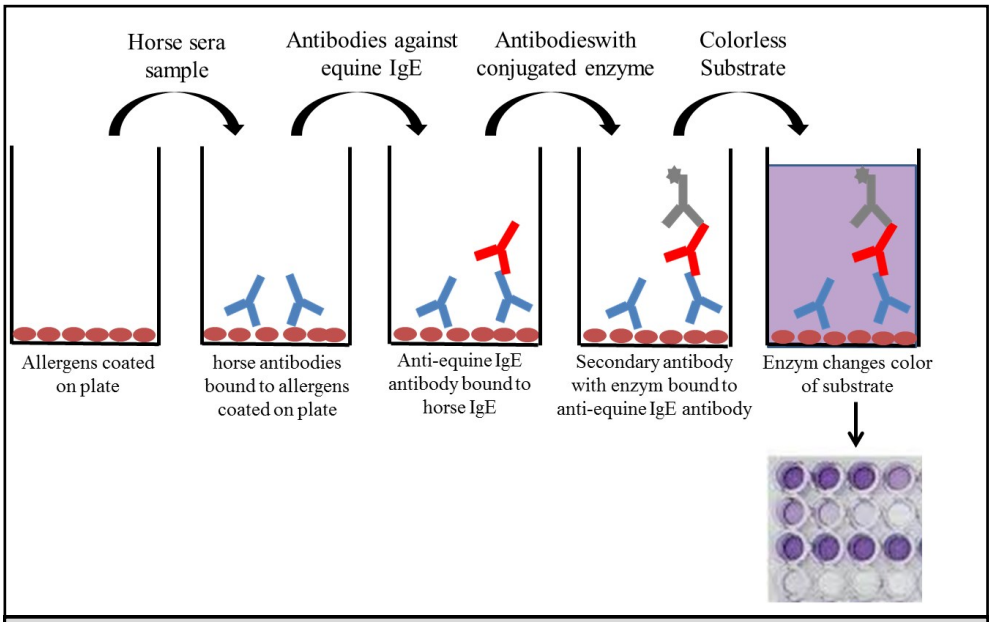

Figure 6: Schematic set up of an IgE ELISA using an anti-equine IgE antibody

IBH, due to high false positive test results with *C. nubeculosus* and other extracts. An IgE ELISA using *C. nubeculosus* and *C. sonorensis* WBE or saliva was reported to have a low sensitivity. Purified IgE increased the sensitivity of the ELISA, but the test was less accurate than HRT [116]. In addition, intradermal skin tests in horses were also reported not to be comparable with results obtained by IgE ELISA using crude allergen extracts and serum [143, 144].

Thus, in conclusion serological testing in horses with IBH with the current available assays seems to perform poorly.

Research aim and thesis outline

Research described in this thesis is part of a bigger project of which the research aim is to improve the understanding of immunologic, epidemiologic and genetic aspects involved in the development of insect bite hypersensitivity (IBH) in horses that can lead to the development of innovative intervention strategies that will effectively decrease the prevalence of IBH in a population.

This thesis focuses on immunological aspects of IBH, with special attention to improving diagnosis by the characterization and production of allergens from the causative agent of IBH. The recombinant allergens produced were studied *in vitro* and *in vivo*, by an IgE ELISA and intradermal tests to assess the potential of these allergens for IBH related research. An IgE ELISA with high sensitivity and specificity for diagnosis of horses with IBH is described.

Before recombinant allergens can be produced it is necessary to know from which species they should be derived. As described in this chapter, *C. obsoletus* is regularly found to be mostly attracted to horses in different countries. However most studies performed on IBH used laboratory available *C. nubeculosus* or *C. sonorensis.* In **chapter 2**, WBE of these two species were compared with WBE of *C. obsoletus* for their applicability in different *in vitro* diagnostic tests on Dutch horses, to assess which species we should further use. A diagnostic ELISA using *C. obsoletus* WBE is presented. In addition, in **chapter 3** seasonal differences in *C. obsoletus* specific IgE levels, cytokine and transcription factor mRNA expression were estimated in lesional and non lesional skin of IBH affected and healthy horses.

Since, *C. obsoletus* was found to be the most important species for diagnosis of IBH in The Netherlands as described in **chapter 2**, mRNA of this *Culicoides* species was sequenced and assembled to create a transcriptome which is presented in **chapter 4.** The transcriptome can be of use for the identification of allergens relevant for IBH. Several allergens from *C. nubeculosus* and *C. sonorensis* were recently described to be associated with IBH (Table 1, [81]). However, allergens from *C. obsoletus* so far have not been described. Using the sequences from described allergens, similarity searches were performed on the transcriptome from *C. obsoletus.* The identification and evaluation of 7 allergens from *C. obsoletus* found by similarity searches with sequences from in literature described allergens is described in **chapter 5**.

The potential of these individual *C. obsoletus* derived recombinant allergens compared with *C. obsoletus* WBE in an IgE ELISA using sera of a large number of horses is evaluated in **chapter 6.** Major allergens among the 7 recombinant proteins were identified and a diagnostic ELISA using a combination of 3 allergens is presented which can be used as a replacement for WBE for robust and large scale testing**.** In addition, various factors associated with the outcome of these ELISA's were evaluated and quantified in **chapter 7.**

Chapter 8 finalizes this thesis with a summarizing general discussion on the implications of the findings of the previous chapters. Additionally, future perspectives for the treatment of IBH are discussed.

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CHAPTER

2

Culicoides obsoletus extract relevant for diagnostics of insect bite hypersensitivity in horses

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Abstract

Insect bite hypersensitivity (IBH) is an allergic dermatitis in horses caused by the bites of *Culicoides* species. The aim of the present study was to evaluate the applicability of whole body extracts of *C. obsoletus* (the main species found feeding on horses in the Netherlands), *C. nubeculosus* (rarely found in The Netherlands) and *C. sonorensis* (typical for North America) for diagnosis of IBH in horses in The Netherlands. Blood and serum samples of 10 clinically confirmed IBH affected and 10 healthy control horses were used to evaluate the IgE titres (ELISA) against the *Culicoides* whole body extracts of the three *Culicoides* species. Basophil degranulation was assessed by histamine release test (HRT) after stimulation with these extracts at 5, 0.5 and 0.05 µg/ml. IBH affected horses had significantly higher IgE titres against *C. obsoletus* than against *C. nubeculosus* and *C. sonorensis*. Furthermore, *C. obsoletus* induced significantly higher histamine release in whole blood of IBH affected horses compared to the other extracts at 0.5 µg/ml. Western blot data revealed IgE-binding to many proteins in *C. obsoletus* extract. This interaction was absent or weak in *C. nubeculosus* and *C. sonorensis* extracts for IBH affected horses. Results on individual level indicate that the HRT is more sensitive than ELISA in diagnosing IBH. However ELISA is more practical as a routine test, therefore the ELISA was further evaluated using *C. obsoletus* extract on 103 IBH affected and 100 healthy horses, which resulted in a test sensitivity and specificity of 93.2% and 90.0%, respectively. The IgE ELISA readings enabled the analysis of the predicted probability of being IBH affected. From an Optical density 450 nm value of 0.33 onwards, the probability of IBH affected was more than 0.9. The results presented in this paper show that the use of native *Culicoides* spp that feed on horse, is important for improved diagnosis and that the described ELISA based on *C. obsoletus* can be used routinely to diagnose IBH in countries where this species is the main *Culicoides* feeding on horses.

Introduction

Insect bite hypersensitivity (IBH), also called 'sweet itch' or 'summer eczema', is a seasonal recurrent allergic dermatitis in horses caused by an allergy against the bites of midges (*Culicoides* spp), or sometimes black flies (*Simulium* spp) and to an even lesser extend other insects [1-5]. IBH is found in many countries of the world with a prevalence ranging from 3 - 11.6% in areas in the UK [6, 7]; 10 – 60% in areas of Queensland, Australia [8] and 0 -71.4% in regions of The Netherlands [9].

Insect Bite Hypersensitivity is clinically characterized by strong pruritus and irritation, leading to alopecia and even secondary lesions due to scratching and rubbing. These symptoms are particularly found along the preferred feeding sites of the insect, which is the ventral midline, mane and tail region of the horse [3, 10]. Several studies indicate that the allergic reaction is predominantly IgE-mediated [11, 12]. However IgG (T) also seems to be involved [13]

Intradermal injections with *Culicoides* extracts often induce immediate and delayed type skin reactions in allergic horses [14, 15]. In Iceland, where *Culicoides* spp do not occur, IBH has never been reported [16, 17]. Several *Culicoides* species have been associated with IBH, including *C. sonorensis*, *C. nubeculosus*, *C. imicola*, *C. obsoletus* and *C. pulicaris* [4, 15, 18-20]. Intradermal tests on allergic and healthy control horses in Northern Germany and British Columbia with extracts and saliva of native and exotic *Culicoides* species, showed no difference between the native and exotic *Culicoides* species, indicating the presence of species-shared allergens [21, 22]. However, intradermal skin tests in The Netherlands, with a commercial extract of *C. nubeculosus* and wild-caught *C. obsoletus,* showed the lack of cross reactivity between these *Culicoides* species [15, 23].

The aim of the present study was to evaluate three *Culicoides* species for their applicability in diagnostic tests for IBH of horses in The Netherlands: *C. obsoletus* which is most frequently found on horses in The Netherlands [24, 25], *C. nubeculosus,* widely distributed in Europe, but only occasionally detected in The Netherlands [26] and not found to be attracted to horses [24] and *C. sonorensis* which is only present in North America. Currently, *C. sonorensis* and *C. nubeculosus* are often used in studies about IBH, because they can be successfully maintained in laboratory bred colonies [27]. *C.*

obsoletus however, are not available from laboratory bred colonies and have to be collected from the wild. An attempt to breed *C. obsoletus* has been made, but was not very efficient [28]. Results presented in this report show that the use of native *Culicoides* spp that feed on horse, is important for improved diagnosis and possibly, for future immunotherapy development. A diagnostic ELISA for IBH based on *C. obsoletus* is described that can be used routinely and has a high specificity and sensitivity.

Material and methods

Animals

A total of 223 horses located in different regions of The Netherlands were included in this study. Pairs of clinically confirmed IBH affected and healthy control horses kept at the same location were formed [29]. Ten clinically confirmed IBH affected and 10 healthy control Shetland ponies were used to compare the different *Culicoides* whole body extracts in different *in vitro* diagnostic tests. The remaining 203 horses (76 Icelandic horses and 127 Shetland ponies) were used to evaluate the predictive value and test sensitivity and specificity of an ELISA using *C. obsoletus* whole body extract.

Blood samples were taken from all horses and serum was frozen in aliquots not later than 24 hours after blood sampling and stored at -20 ^{o} C until use. Blood sampling was approved by the Board on Animal Ethics and Experiments from Wageningen University and Utrecht University.

Collection of Culicoides insects

C. obsoletus insects were captured during spring and summer months using a pooter (aspirator to collect insects) (Supplementary figure 1) or an “Onderstepoort” suction light trap kindly provided by Laboratory of Entomology, Wageningen University.

Horses wearing an anti-insect blanket were put outside around dawn hours on warm (> 20 °C), dry and low wind days and *Culicoides* insects were collected directly from the horses using the pooter. The insects were collected and completely frozen alive at -80 °C and stored at that temperature until preparation of the extracts. A small fraction (5%) of the insects collected with the pooter was checked under a stereo microscope to confirm the species. Identification of *C. obsoletus* was based on size and wing patterns [30]

(Supplementary figure 2). The "Onderstepoort" suction light trap was operated from before dusk to far after dawn near a horse stable for 19 days at different locations in The Netherlands in the summer months of 2009. Insects were captured in 100% alcohol and frozen the next day in alcohol at -80 ^{o}C until determination. *C. obsoletus* insects were selected and separated from the other captured insects using a stereo microscope as described above. Separated *C. obsoletus* insects were used for the preparation of extracts. Three-day-old laboratory bred *C. sonorensis* were a kind gift from Arthropod Borne Animal Diseases Research Unit Centre for Grain and Animal Health, Manhattan, US. *C. nubeculosus* insects were kindly donated by the Institute for Animal Health, Pirbright, UK. All insects were kept frozen (without alcohol) at -80 °C until preparation of the extracts.

Preparation of Culicoides protein extracts

Whole body extracts (WBE) were prepared from about 300 insects that were transferred to a 2 ml Eppendorf tube with 1 ml of PBS containing a protease inhibitor cocktail (Sigma-Aldrich, P8849) and crushed with a micro pestle. The insoluble material was removed by centrifugation at 13000 x g for 10 min at 4 °C. Supernatant was collected and filtered through sterile Millex-GV filters (Millipore) with a pore diameter of 0.22 µm and protein content of the filtrate was determined by OD_{280nm} measurement on a Nanodrop spectrophotometer (NanoDrop 1000, Thermo Scientific). Samples were aliquoted, directly frozen in liquid nitrogen and stored at -80 °C until use. Quality of the protein WBE was checked by protein staining with Gelcode Coomassie blue staining (Thermo Scientific) after proteins were separated by 15% SDS-PAGE.

SDS-PAGE and Western blotting

Whole body protein extract samples, 20 µg/lane (Western blotting) or 60 µg/lane (Coomassie staining) were heated at 96 °C for 5 min with sample buffer containing dithiotreitol (DTT) and separated by SDS-PAGE (15% gel). These separated proteins were transferred to a nitrocellulose membrane (Protrans, Schleicher & Schuell, Bioscience GmbH) by means of electrophoresis. Membranes were blocked with 5% non-fat cow's milk in Tris buffered saline (TBS)-Tween (10 mM Tris, 150 mM NaCl, pH 7.5, 0.05% (v/v) Tween 20) for 1 h at room temperature (RT) and then incubated overnight with horse

sera from allergic or control horses diluted 1:10 in 5% non-fat cow's milk in TBS-Tween. Membranes were then incubated for 1.5 hour with a mAb against horse IgE (αIgE-176) [31] followed by goat anti-mouse IgG horseradish peroxidase (Dako, 1:1000 in milk powder/TBS-Tween). Between each incubation step, membranes were washed three times with TBS-Tween.

Signal was detected by development with an enhanced chemiluminescence (ECL) western blotting detection reagent (Amersham, GE Healthcare) according to the manufacturer's protocol and visualized by the use of Lumni-fil chemiluminescent Detection Film (Roche, Woerden, The Netherlands).

Histamine release test (HRT)

The histamine release by basophils was determined by a modified method of Kaul [32]. Blood samples were collected in anticoagulant tubes (EDTA) and kept at RT in the dark until further use within 24 hours. The total blood cells were washed twice (500 x g for 10 min) with PBS to remove non-cell bound antibodies. Supernatant was discarded and the cell pellet was resuspended in PBS to its original blood sample volume. Endogenous histamine from whole body *Culicoides* extracts was removed by PD-10 Desalting columns (GE Healthcare) according to manufacturer's recommendations. The antigen induced histamine release was obtained by incubating 250 µl of washed blood cells with 250 µl of PIPES buffer (110 mM NaCl, 5 mM KCl, 40 mM NaOH, 2 mM $CaCl_2$, 25 mM PIPES, 2 mM $MgCl_2$) containing the histamine depleted *Culicoides* whole body extracts at final concentrations of 5 µg/ml, 0.5 µg/ml and 0.05 µg/ml at 37 °C for 60 min. Spontaneous release was obtained by incubating 250 µl of PIPES B buffer with 250 µl of washed blood cells at 37 °C for 60 min. Physical maximum release was obtained by boiling 200 µl of washed blood cells with 800 µl of PIPES buffer for 10 minutes. After incubation, all samples were chilled on ice for 5 min and pelleted by spinning down at 700 x g for 10 min. The cell-free supernatants were collected and stored at -20 °C. Subsequently, competitive RIA was carried out as per the manufacturer's instructions (LDN Nordhorn, Germany) to determine the histamine content of the supernatants.

The maximum amount of histamine obtained by boiling was set to 100%. The histamine content of each test sample was calculated from this maximum

histamine release. The allergen-specific release (ASR) is calculated as: ASR = (sample induced release – spontaneous release)/ (maximum release – spontaneous release) x 100%. Net-histamine releases that were equal or greater than 10% of the maximum release were considered as positive.

Culicoides-specific IgE ELISA

Specific IgE levels in sera of 10 IBH affected and 10 healthy control Shetland ponies, binding the different *Culicoides* WBE, were measured by ELISA. Optimal coating concentration, serum dilution and antibody concentrations were determined prior to the experiment by titration of the different components. Costar 96-well microtitre plates were coated with 100 µl/well of 10 µg/ml *C. obsoletus*, *C. nubeculosus* or *C. sonorensis* extract, diluted in PBS, and incubated overnight at 4 °C and afterwards blocked with 150 µl of a 1.5% casein buffer (SDT, Germany) for 1.5 hour at RT. Plates were washed 5 times with PBS containing 0.05% Tween20, followed by incubation for 1.5 h at RT with 100 µl of horse serum samples diluted 1:5 in a 1.5% casein buffer. After washing, wells were incubated for 1 hour at RT with 100 µl of 2.5 µg/ml mouse monoclonal anti-equine IgE-176 [31] diluted in casein buffer. After washing the plates 5 times with PBS containing 0.05% Tween20, goat anti-mouse peroxidase conjugate (multispecies adsorbed, Serotec) diluted 1000 times in casein buffer, was applied to the wells and incubated for 1 h at RT. After 5 washes with PBS/0.05% Tween20, 100 µl tetramethylbenzidine (high sensitivity, SDT, Germany) was added to the wells and incubated for 10 min at room temperature. The reaction was stopped with 100 µl/well of 1% HCl. Absorbance was measured with a multi-mode microplate reader (SpectraMax M5, Molecular Devices) at a wave length of 450 nm corrected for 650 nm. Based on the preliminary experiments a standard serum dilution of 1:5 was selected as suitable for comparison of OD_{450nm} values in the IgE ELISA. The cut off level was assigned as the mean + 3 times the standard deviation (SD) of the IgE levels of the healthy control horses.

An additional 203 horse serum samples, 103 IBH affected and 100 healthy control horses, were evaluated for *C. obsoletus* specific IgE (OD_{450nm}) values in a 384 wells plate to determine the sensitivity and specificity of this *Culicoides*-specific ELISA. The same conditions as described for the 96-wells plate were used, with 20 µl volumes per well. Distribution plots of the healthy and IBH affected horses were obtained by categorizing

horses according to their OD_{450nm} values. The first category ranged from 0 to 0.01, the second from 0.01-0.02 and the following categories each increased with 0.02 up to 0.32. Then the categories ranged from 0.32-0.35, 0.35-0.4 and subsequent categories each increased with 0.2 until the maximum OD_{450nm} of 2.2 was reached.

Accuracy of diagnostic tests is often determined from so-called receiver-operating characteristic (ROC) curves. ROC-curves represent the trade-off between sensitivity (i.e. true positive rate) of a test and (1-specificity) (i.e. false positive rate) at all possible positivity cut-off points. The area under the curve summarizes the overall diagnostic accuracy. It takes values from 0 to 1, where a value of 0 indicates a perfectly inaccurate test and a value of 1 reflects a perfectly accurate test. A good first choice for a test cut-off value that results in a balanced optimal sensitivity and specificity is that value which corresponds to a point on the ROC-curve nearest to the upper left corner of the ROC graph.

Statistical analysis

Analysis of variance was performed on log transformed data obtained by either HRT or ELISA to determine influence of *Culicoides* species on the outcome. Factors included in the model were, *Culicoides*-species (*C. nubeculosus*, *C. sonorensis* and *C. obsoletus*), IBH -status of the horse (yes/no) and interaction between these two factors. Additionally, WBE concentration (0.05, 0.5 and 5 µg/ml) was included in the analysis of HRT results. IBH-status within individual horses was included as random factor since the same set of horses were used in testing the three *Culicoides* types. Analysis was performed using PROC MIXED of SAS (SAS Inc, V9.2).

The relation of OD_{450nm} value in the IgE ELISA to the IBH-status (negative or positive) was analysed with a logistic regression. The analysis was performed with the PROC LOGISTIC of SAS (SAS Inc, V9.2).

Results

Collection of C. obsoletus

Two different collection methods ("Onderstepoort" light trap and pooter) were used to determine the most selective and efficient way of collecting *C. obsoletes* from the wild.

Determination of insects collected with the "Onderstepoort" light trap revealed that many different insect species were collected in this manner, from which only a small fraction (<1%) belonged to *Culicoides* species. A total of 766 *Culicoides* midges were collected during these 19 days. The large majority of these *Culicoides* were identified as *C. obsoletus* (82%), followed by *C. dewulfii* (6.5%) and *C. punctatus* (5.0%).

Using the pooter hundreds of *Culicoides* insects were easily collected within an hour from a horse wearing an anti-insect blanket. A small part (± 5%) of the collected insects was used for identification and these were all identified as *C. obsoletus* based on size and wing patterns.

Quality of extracts of Culicoides captures by different methods

WBE were prepared from *C. obsoletus* insects collected alive with the pooter system, as well as insects collected in alcohol with the Onderstepoort light trap. Insects that were stored in alcohol were excluded in the following experiments due to substantial degradation of the proteins revealed on SDS-PAGE (Supplementary figure 3).

The extracts from laboratory bred *C. sonorensis* and *C. nubeculosus* and wild-caught *C. obsoletus* prepared in the same manner from freshly frozen insects, showed some differences in the lower molecular weight regions (indicated by arrows in Figure 1) on SDS-PAGE (15% gel), but overall pattern and intensity of the protein bands were similar, with no obvious degradation, indicating similar quality of the different extracts.

IgE-specific antibodies in horse sera specific for Culicoides proteins as determined by Western blotting

Western blotting was performed to evaluate sera of clinically confirmed IBH affected and healthy control Shetland ponies for the presence of IgE specific for proteins from *C. obsoletus*, *C. sonorensis* and *C. nubeculosus*. Typical examples of 5 allergic (upper panel) and 5 healthy horses (lower panel) are shown (Figure 2).

The IgE in sera of all allergic horses reacted strongly to a number of proteins from *C. obsoletus* extract, but much weaker with proteins from *C. sonorensis* and C. *nubeculosus* extract despite the similar quality of the extracts. The antigen recognition pattern for each individual horse was different, but most IBH affected horses reacted with a protein(s) around 20 kDa. IgE from the healthy horses, except for one, hardly recognized any

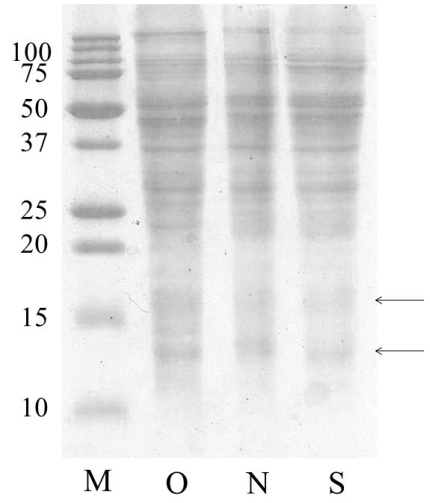

Figure 1: Coomassie staining of proteins from *C. obsoletus* extract (O), *C. nubeculosus* (N) and *C. sonorensis* (S) whole body extracts demonstrating similar quality of the extracts. M represents the molecular weight marker.

proteins from any of the 3 *Culicoides* species. Proteins that did bind to the IgE in serum of healthy horses all had a Mw of 25 kDa or higher (Figure 2).

Basophil degranulation induced by Culicoides whole body extracts determined by histamine release test

For each of the 3 different *Culicoides* species the allergen-specific release (ASR) of histamine from basophils was tested at 3 WBE concentrations on whole blood samples of 10 IBH affected and 10 healthy control Shetland ponies (Fig. 3), including the horses that were used for the Western blot analysis.

At the highest WBE concentration of 5 µg/ml 10 out of 10 (100%) of the IBH affected horses scored positive on the *C. obsoletus* WBE, while this was 8 out of 10 (80%) for *C. nubeculosus* and *C. sonorensis*. However, at this highest WBE concentration some of the healthy horses also scored positive on all *Culicoides* species. At a concentration of 0.5 µg/ml none of the healthy control horses scored positive on any of the extracts, but at this concentration only 20% of the IBH affected horses scored positive with *C. nubeculosus* and *C. sonorensis* extract. In contrast, 100% of the IBH affected horses scored positive when the *C. obsoletus* extract was used. At this concentration the reactivity towards *C. obsoletus* was significantly higher than to *C. nubeculosus* ($p< 0.01$) and *C. sonorensis* ($p<$

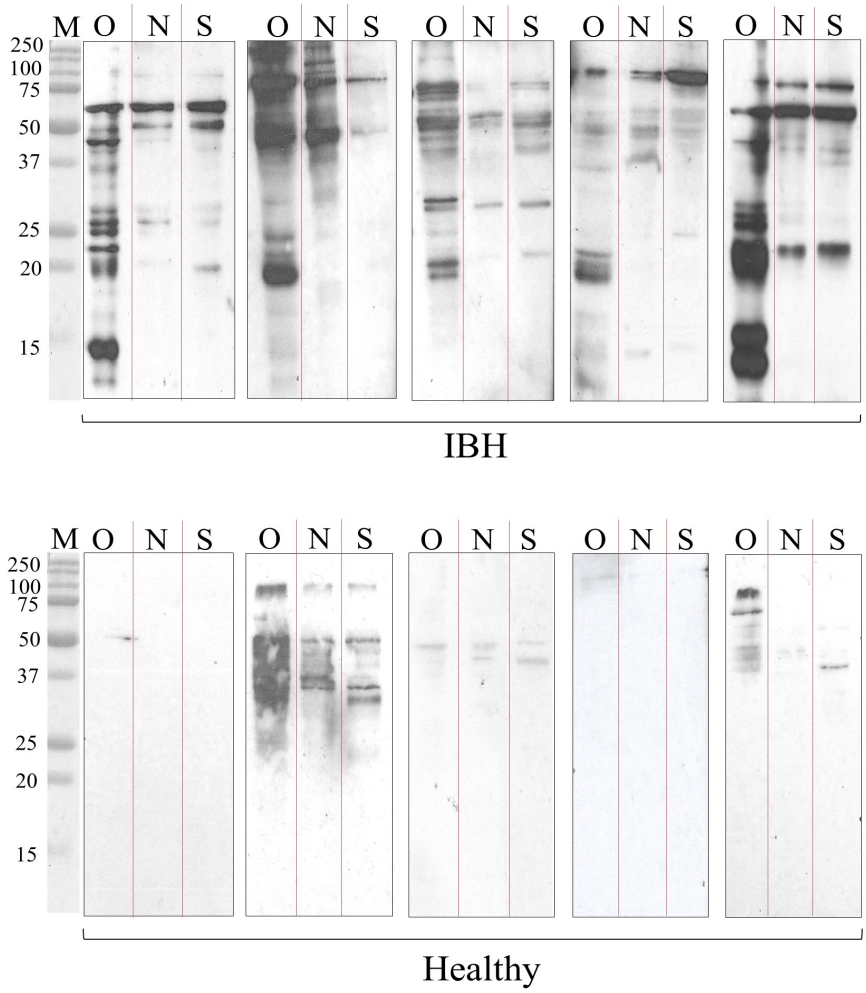

Figure 2: Immunoblot analysis of 5 IBH affected and 5 healthy horses using whole body extracts of *C. obsoletus* (O), *C. nubeculosus* (N) and *C. sonorensis* (S). Proteins were separated on 15 % SDS-PAGE gels and transferred to nitrocellulose membranes. Binding of IgE from horse sera was detected with an anti-equine IgE mouse mAb and HRP goat anti-mouse IgG. The molecular weight marker (M) is indicated on the left in kDa.

0.001) (Figure 3). At the lowest WBE concentration of 0.05 µg/ml 40% of the IBH affected horses were still found to be positive with C. *obsoletus*, whereas for *C. nubeculosus* and *C. sonorensis* this was only 10% of the IBH affected horses. Also at this concentration all healthy control horses had a negative test results for all *Culicoides* extracts.

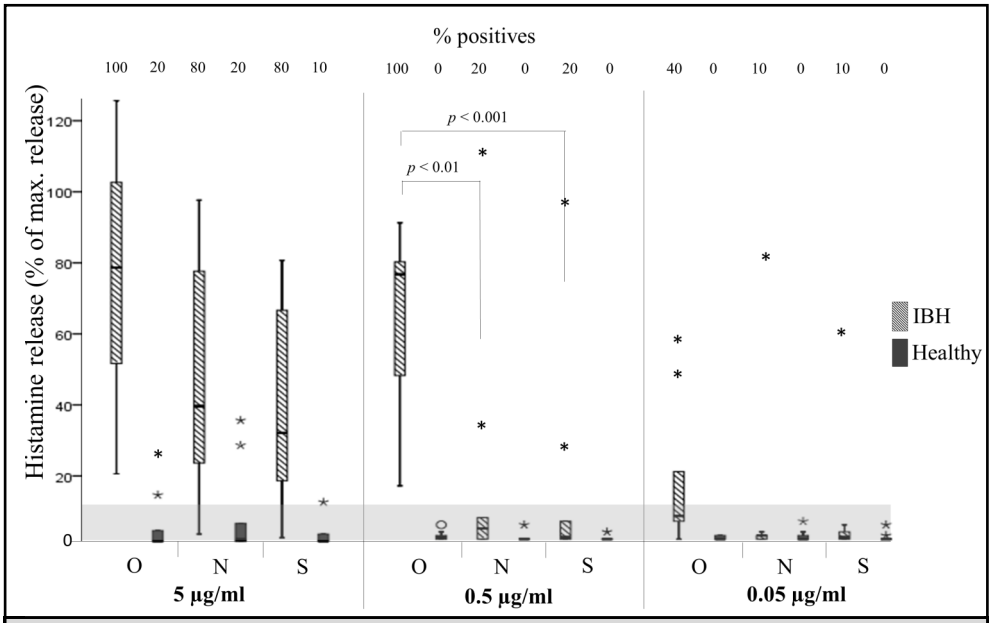

Figure 3: *Culicoides* induced histamine release as percentage of maximum release of 10 IBH affected horses and 10 healthy control horses. Whole blood samples were analysed after stimulation with three different *Culicoides* whole body extracts (O = *C. obsoletus*, N = *C. nubeculosus*, S = *C. sonorensis)* tested at three different concentrations (µg/ml, x-axis) by a histamine release test (HRT). Results are presented in box plots. The horizontal line near the middle of the box is the median of the measurements. The bottom and top of the box are the 25th and 75th percentile, respectively. The end of the whiskers represent the minimum and maximum value. The stars represent the outliers. Horses with histamine release above the cut off value of 10 % (highlighted in gray) were considered positive (Kaul et al., 1998). Statistical analysis was performed on log transformed data. The p values account for unequal variance.

IgE measurements in horse sera against Culicoides whole body extracts by ELISA

Specific IgE serum levels against WBE of the three different *Culicoides* species were determined in an indirect ELISA (Figure 4)

IgE levels expressed as OD_{450nm} values of the clinically confirmed IBH affected Shetland ponies against *C. obsoletus* extract were significantly higher compared to OD_{450nm} values of the same IBH affected horses against the other extracts (both $p < 0.0001$). With *C. obsoletus* extract only, specific IgE serum levels of IBH affected horses were significantly higher than specific IgE levels of healthy control horses ($p < 0.0001$).

Comparison of ELISA and HRT data for individual horses

Individual HRT (Figure 5 upper panel) and ELISA (Figure 5 lower panel) responses to the WBE of the different *Culicoides* species were compared for the same Shetland ponies

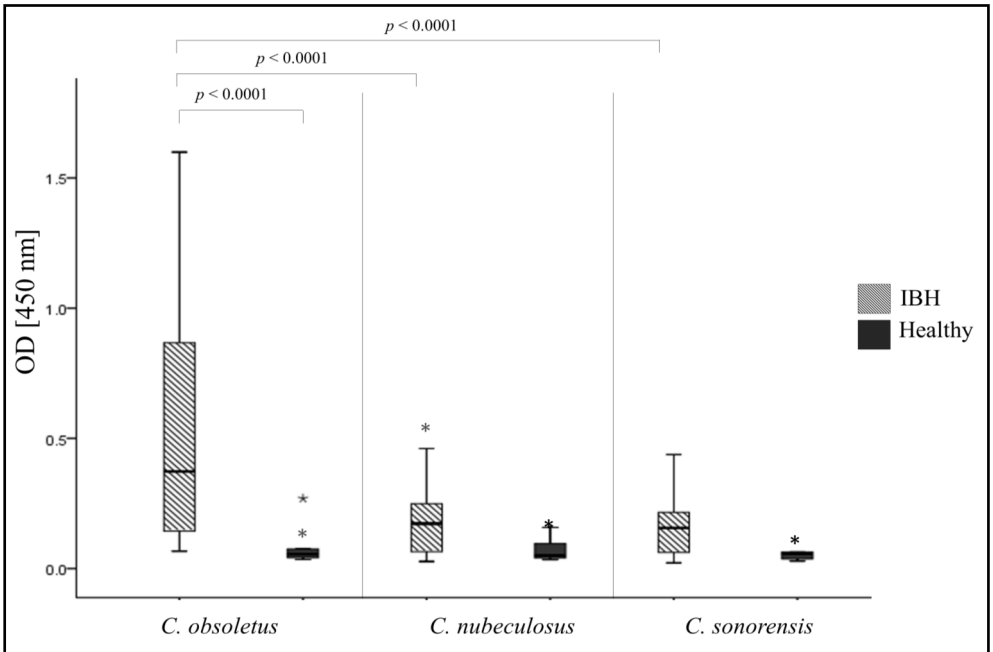

Figure 4: IgE levels presented as OD_{450nm} values against three different *Culicoides* extracts sera diluted 1:5 of 10 IBH affected horses and 10 healthy control horses. Results are presented in box plots, for a definition see legend of Fig. 3. Statistical analysis was performed on log transformed data. The p values account for unequal variance.

used in Figure 3 and 4. The values at a concentration of 0.5 µg/ml per extract were chosen to analyse the horses on individual level for the HRT, because at this concentration the best distinction could be made between IBH affected and healthy control horses. On individual level, for the *C. obsoletus* extract the histamine release of all IBH affected horses was higher than 10% and therefore positive [49], whereas the maximum histamine release of all healthy control horses was below 10% of the maximum histamine release. For the ELISA 6 out of 10 IBH affected horses had OD_{450nm} values against *C. obsoletus* extract above the set cut-off level (mean + 3 times the standard deviation SD of the OD_{450nm} values of the healthy control horses). With this cut-off level, all healthy horses were negative. In 6 out of 10 IBH cases (horses 2, 3, 4, 5, 6, and 7) the HRT and ELISA values against *C. obsoletus* WBE correlated with each other. Interestingly, the IBH affected horses with OD_{450nm} values below the cut-off level in the ELISA (horses 1, 4, 8 and 10) did have a high positive histamine release with the HRT using *C. obsoletus* WBE. One horse had a higher histamine release after stimulation with *C. nubeculosus* and *C.*

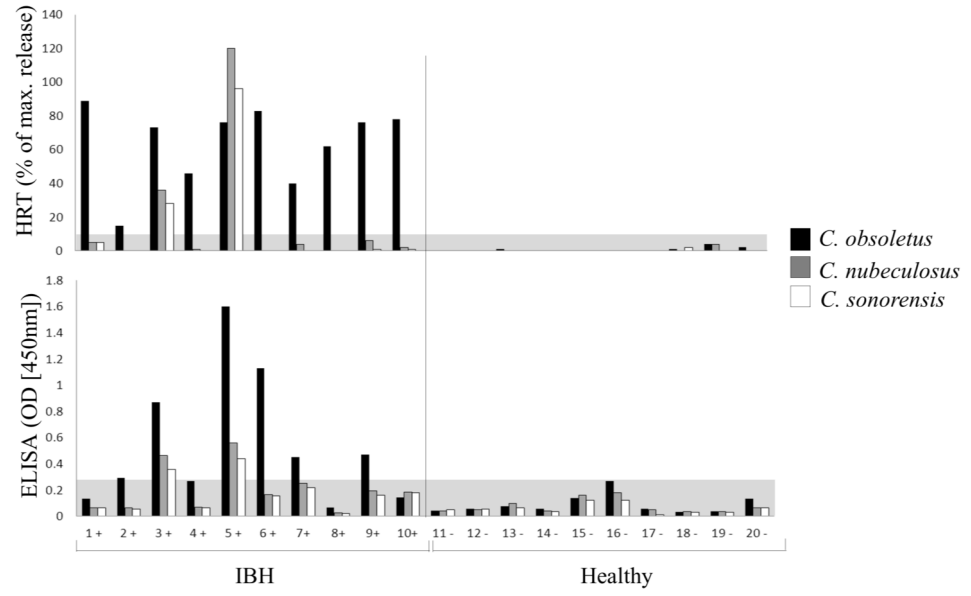

Figure 5: Comparison between histamine release test (HRT) and ELISA of individual horses. Numbers 1-10 represent IBH affected horses; horses 11 – 20 represent healthy control horses. IgE levels (ELISA) are presented as OD_{450nm} values; Histamine release after stimulation with whole body extract at a concentration of 0.5 µg/ml is presented as percentage of maximum release. The cut-off of each test is highlighted in light gray and corresponds to 10 % for the HRT and mean + 3 times standard deviation of the *C. obsoletus* values of the healthy control horses for the ELISA.

sonorensis WBE compared to *C. obsoletus*, but did have a higher IgE level against *C. obsoletus* when measured by ELISA.

ELISA test sensitivity and specificity

The results described in section 3.6 indicate that the HRT outperforms the ELISA as a diagnostic test for IBH, but the ELISA is more practical as a routine test. It is much less laborious and can be performed on serum samples, while the HRT requires fresh full blood. Therefore the ELISA using *C. obsoletus* extract was further evaluated with sera of 103 clinically confirmed IBH affected horses and 100 healthy control horses (76 Icelandic horses and 127 Shetland ponies).

When categorizing the healthy and IBH affected horses according to their OD_{450nm} values from the IgE ELISA, two distributions with equal variance were observed which are nearly baseline-separated (Figure 6a). Although the distribution curve of healthy and IBH affected horses overlapped somewhat, the IBH-affected horses had higher serum IgE

levels against *C. obsoletus* WBE compared to healthy control horses ($p < 0.0001$) (Figure 6b).

The pattern of the ROC-curve (Figure 6c) indicates that the sensitivity sharply increases already at low false positive rates. The sensitivity of the test is therefore high over a large range of cut-off points. The accuracy of the test as evaluated by the area under the curve (AUC) is high and amounted to 0.97, indicating that high sensitivity is achieved with a high specificity. The point on the ROC-curve nearest to the upper left corner of the curve corresponds with a sensitivity of 93.2% and a specificity of 90.0% and is obtained at an OD_{450nm} cut-off value of 0.2.

Logistic regression was performed to analyse the relation of IgE OD_{450nm} values to the IBH-status. The response IBH-affected or healthy was regressed on OD_{450nm} values.

Figure 6d shows the predicted probabilities of both IBH-outcomes (i.e. affected or healthy) related to OD_{450nm} values. With IgE-values close to zero the probability of being healthy is much higher than the probability of having IBH; up to an IgE value (OD_{450nm} value) of 0.07 (true for 70% of the healthy horses) the probability of being IBH negative is approximately 10 times higher than being IBH positive and 5 times higher for a value of 0.12 (true for 85% of the healthy horses). At the inflection point at an OD_{450nm} value of approximately 0.2 the probability being IBH-positive or negative is equal. From an OD_{450nm} value of 0.33 onwards (true for 75% of the IBH affected horses) the probability of IBH-positive is more than 0.9.

Discussion

In this study we evaluated three different *Culicoides* whole body extracts for their applicability for *in vitro* diagnosis of IBH horses in The Netherlands. We show that *C. obsoletus* (a species found feeding on horses in The Netherlands) whole body extract is much better for *in vitro* diagnosis of IBH by ELISA and HRT, than *C. nubeculosus* and *C. sonorensis* (not found feeding on horses in the Netherlands) whole body extracts. An IgE ELISA with *C. obsoletus* whole body extract performed with 103 IBH affected horses and 100 healthy horses located in the Netherlands demonstrated a high sensitivity and specificity (93.2% and 90.0%, respectively) and can thus be used as a valuable test to diagnose horses for IBH in countries where *C. obsoletus* is the main species found feeding

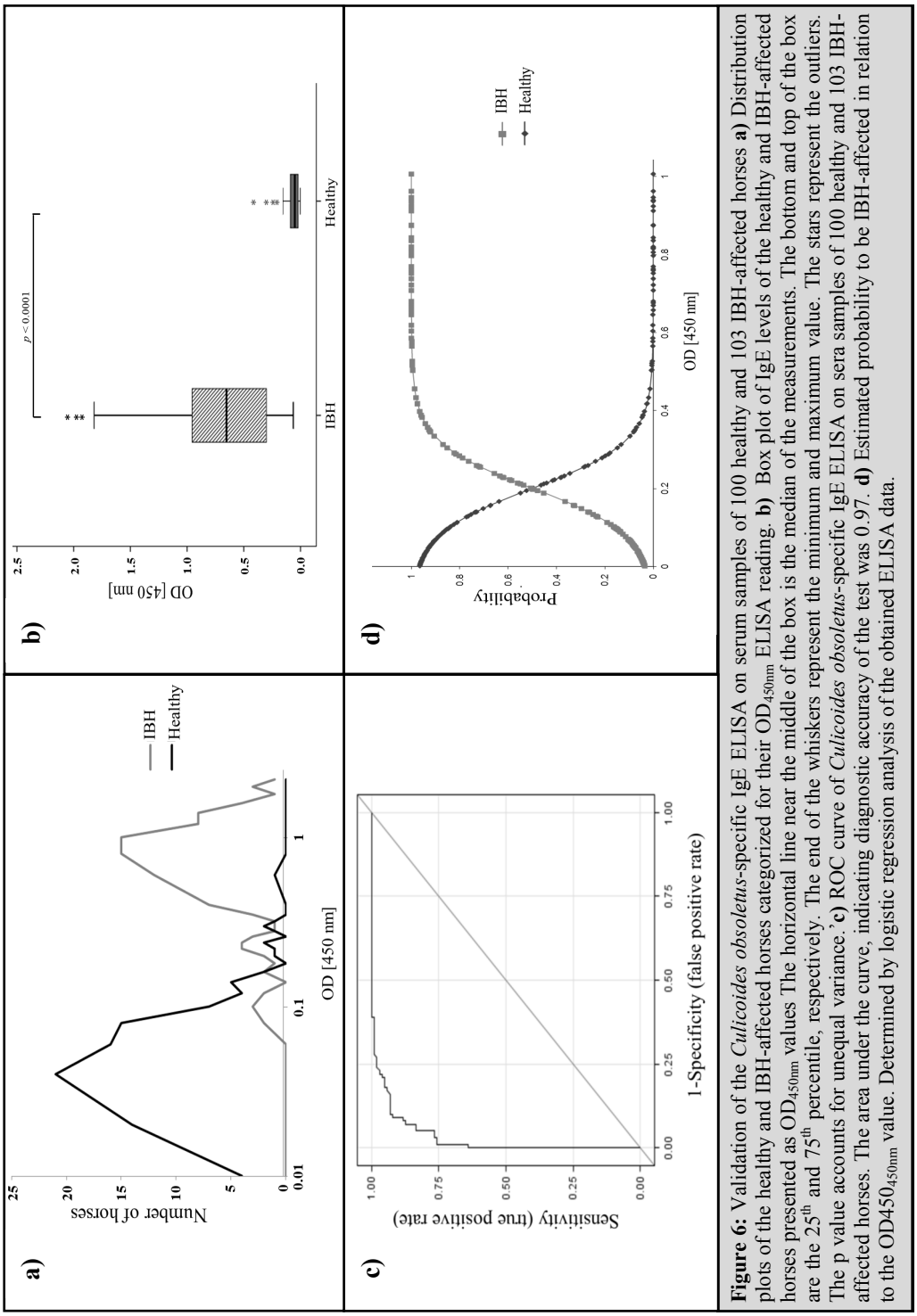

Figure 6: Validation of the *Culicoides obsoletus*-specific IgE ELISA on serum samples of 100 healthy and 103 IBH-affected horses **a)** Distribution plots of the healthy and IBH-affected horses categorized for their OD_{450nm} ELISA reading. **b)** Box plot of IgE levels of the healthy and IBH-affected horses presented as OD_{450nm} values The horizontal line near the middle of the box is the median of the measurements. The bottom and top of the box are the 25th and 75th percentile, respectively. The end of the whiskers represent the minimum and maximum value. The stars represent the outliers. The p value accounts for unequal variance.' **c)** ROC curve of *Culicoides obsoletus*-specific IgE ELISA on sera samples of 100 healthy and 103 IBH-affected horses. The area under the curve, indicating diagnostic accuracy of the test was 0.97. **d)** Estimated probability to be IBH-affected in relation to the $OD450_{450nm}$ value. Determined by logistic regression analysis of the obtained ELISA data.

on horses.

This study describes an easy and selective method of catching *Culicoides* spp. attracted to horses by using a pooter. Although this method is initially more labour intensive than an "Onderstepoort" suction trap, it is a selective way of collecting preferentially those *Culicoides* species attracted to horses. This obviates the need for the labour intensive selection of the desired *Culicoides* species out of a large majority of unwanted insects that is required when using a light trap. The biggest advantage of the pooter method is that the insects are caught in a gentle way that keeps them alive, preventing substantial protein degradation as is observed for insects captured in alcohol by the "Onderstepoort" light trap.

The majority of *Culicoides* spp that were caught by both capturing methods were found to be *C. obsoletus:* over 80% when using the light trap and nearly 100% when directly collected from the horse by the pooter. This is in agreement with earlier studies performed in The Netherlands that also found *C. obsoletus* to be the main *Culicoides* species attracted to horses [24, 25]. In other countries, such as England [5] Ireland [19], Japan [33] and United States [4], *C. obsoletus* was also found to be the most important *Culicoides* species attracted to horses.

Comparison of three *Culicoides* extracts demonstrates that in The Netherlands horses with IBH have more specific IgE directed against whole body extracts of *C. obsoletus* than against *Culicoides* species that do not feed on these horses. Although cross-reactivity between different *Culicoides* species has been reported [21, 22] our study shows weaker IgE-binding in Western blot and ELISA to proteins of non-indigenous *Culicoides* species compared to native *C. obsoletus*, which was also observed before [5]. This indicates the importance of using extracts from native *Culicoides* species feeding on horses for reliable diagnostics of IBH.

The binding of IgE from allergic horses with *C. sonorensis* and *C. nubeculosus* proteins might be due to cross reactivity between these proteins and the *C. obsoletus* antigens and maybe also proteins of other insect species that the horses were exposed to. The horses in this study might have been exposed to the native *C. nubeculosus*, but our as well as another study performed in The Netherlands [24] did not find any *C. nubeculosus* insects to be attracted to horses. Although *C. sonorensis* and *C. nubeculosus* antigens have

previously been successfully used in different diagnostic tests [34-36] our study clearly shows the importance of using an extract of a *Culicoides* species to which horses have been actually exposed to for diagnosis. Western blot data revealed many IgE-binding proteins in *C. obsoletus* extract that were absent in *C. nubeculosus* and *C. sonorensis* extracts. Interestingly, a protein of around 20 kDa from *C. obsoletus* extract was found to be bound by IgE from almost all clinically confirmed IBH affected horses, whereas this was not observed for *C. nubeculosus* and *C. sonorensis* extracts and also not for IgE from healthy horses. This makes this protein an interesting candidate allergen for further characterization.

The *C. obsoletus* insects collected from the wild using the pooter were all female species, since females need blood to reproduce and were trying to feed on the horse when captured. Although WBE of laboratory-bred insects were of both sexes, it is unlikely that this explains the lower allergen-reactivity by *C. nubeculosus* and *C. sonorensis* in this study. Other studies found *Culicoides* extracts made from males only, to be just as effective in stimulating horse basophils [37] and non-salivary antigens from the thorax of *Culicoides* spp. have shown IgE reactivity with IBH-affected horses [11]. One of the IBH affected horses reacted even slightly stronger in the HRT with *C. nubeculosus* and *C. sonorensis* than with *C. obsoletus* WBE, and therefore rules out a lack of antigens of lower protein quality in the *C. nubeculosus* and *C. sonorensis* WBE. This was also demonstrated by the similar pattern and intensity of the protein bands of all three WBE on SDS-PAGE.

Surprisingly, some horses had low IgE-binding in the ELISA but a high histamine release in response to *Culicoides* extract stimulation in the HRT. IgG (T) has also been observed to bind to skin mast cells and therefore might play an important role in the histamine release reaction in IBH [13]. Therefore, the observation that some horses have low IgE-binding in ELISA but a high histamine release in response to *Culicoides* extract stimulation, might be due to cross-linking of allergen-specific IgG(T) instead of IgE.

The results in this study indicate that the histamine release test (HRT) might be more sensitive and reliable for diagnosis of IBH than the ELISA. However, this comparison was made on a relatively small number of horses (10 IBH affected and 10 healthy control horses). When tested with a large number of horses, the IgE ELISA, resulted in a high specificity (90.0) and sensitivity (93.2) and proved to be the method of choice for routine

screening, because it is more robust, easier to perform and more economical than the HRT. The test clearly discriminates between IBH-affected and healthy controls such that there is little overlap of distributions. The accuracy of the test as evaluated by the area under the curve (AUC) of the ROC-graph is high (0.97), indicating that high sensitivity is achieved with a high specificity. At an OD_{450nm} cut-off value for positivity of 0.2 the test has a sensitivity of 93.2% and a specificity of 90.0%. However, this cut-off value does not take the actual OD_{450nm} value of an individual horse into account, apart from being lower or higher than the determined cut-off value for positivity. Therefore logistic regression analysis was performed to determine the relation of the IgE OD_{450nm} values to the IBH-status. For most horses the ELISA can determine with 90-100% probability the correct IBH-status of the individual tested horse. For those OD_{450nm} values where reliability is less, e.g. around the inflection point at OD_{450nm} value of 0.2, the horse owner can choose for an additional HRT to obtain a more conclusive diagnosis. Western blot analysis on *C. obsoletus* whole body extracts using serum of healthy horses showed some IgE reactivity of these horses against proteins with Mw above 25 kDa. Therefore, the use of selected recombinant proteins from *C. obsoletus* might further improve the sensitivity of the ELISA described in this study.

At present, treatment of IBH is based on insect avoidance by stabling, use of anti-midge blankets (pyjamas) or insect repellents and suppression of symptoms by the use of corticosteroids. Specific immunotherapy might be possible and some immunotherapy trials have been carried out with *Culicoides* whole body extracts, but with varying results [38, 39]. The use of purified allergens could further improve diagnostics, but could also be a benefit for immunotherapy. Collecting insects is very time consuming and for immunotherapy over 10,000 insects were necessary per horse [38], therefore recombinant allergens from an infinite source would be a sensible alternative for whole body extracts. Currently, allergens have been identified and produced from species that are not present or common in The Netherlands, e.g. *C. sonorensis* and *C. nubeculosus* [34-36]. Implementation of future immunotherapy in horses will depend on the availability of correct allergens and therefore the use of allergens from *Culicoides* spp to which horses have been exposed, which for The Netherlands is mostly *C. obsoletus*, might be crucial.

In conclusion, our results show that horses with IBH in the Netherlands have much more

IgE antibodies against *C. obsoletus* proteins compared to *C. sonorensis* and *C. nubeculosus* proteins which can be routinely detected in different diagnostic tests. The developed ELISA to identify sensitisation against *C. obsoletus* allergens provides a valuable diagnostic test to discriminate IBH affected from healthy control horses in The Netherlands, but will also be valuable in other countries were *C. obsoletus* is mostly found feeding on horses.

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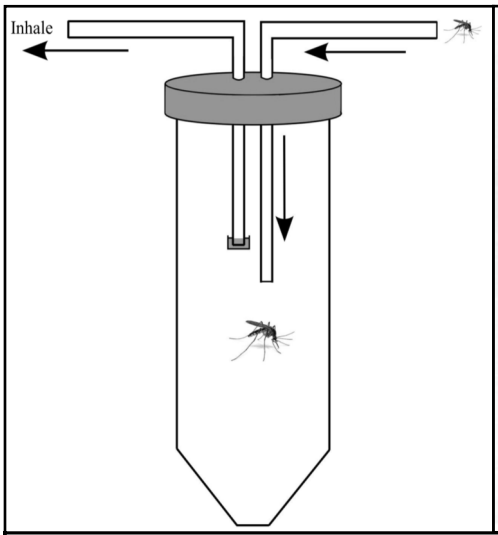
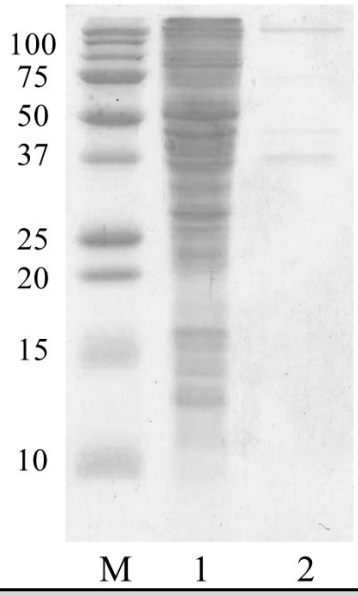
Supplementary data



Supplementary figure 1 (left): Pooter used to collect *Culicoides* from horses. Two separate straws are inserted in two holes into a 50 ml tube. One straw has a fine mesh covering the end inside of the tube. This straw is used to inhale while aiming the other one at the insect to suck it into the tube.
Supplementary figure 2 (right): Individual example of a female *C. obsoletus* used in this study and identified on size and wing pattern according to Campbell and Pelham-Clinton [30]



Supplementary figure 3: Coomassie staining of proteins from *C. obsoletus* extract (50 µg), from insects collected alive with the pooter (1) and from insects collected in alcohol with the "Onderstepoort" light trap (2). M represents the molecular weight marker.

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CHAPTER

3

Seasonal differences in cytokine expression in the skin of Shetland ponies suffering from insect bite hypersensitivity

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Abstract

Insect bite hypersensitivity (IBH) in horses is a seasonal, IgE-mediated, pruritic skin disorder primarily caused by *Culicoides spp*. We hypothesize that a mixed Th2/Th1-type immune status, off season, alters into Th2-dominated immune reactivity in the skin of IBH-affected ponies in the IBH season. To study these immune response patterns *Culicoides*-specific IgE levels, skin histopathology and cytokine and transcription factor mRNA expression (IL4, IL10, IL13, IFNγ, FoxP3 and CD3$_\zeta$) in lesional and non-lesional skin of ponies affected by IBH in the IBH season were compared with those of the same animals off season and those in skin of healthy ponies in both seasons. The present study revealed a significantly higher histopathology score in lesional skin of affected ponies than in non-lesional skin and skin of healthy ponies in the IBH season. *C. obsoletus*-specific IgE serum levels of ponies with IBH were significantly higher than those in healthy ponies in both seasons. Interestingly, *C. obsoletus*-specific IgE serum levels within each group were the same in the IBH season and off season. The expression of IL4, IL13 and IFNγ mRNA in skin biopsies in the IBH season showed a significant increase compared to off season in both skin derived from healthy control ponies (n=14) as well as in lesional and in non-lesional skin from IBH-affected animals (n=17). This apparently general up-regulation of cytokine expression during the IBH season directly correlated with an increased CD3ζ mRNA expression in the skin, indicating an overall increased T cell influx during the summer months. The only significant difference observed between lesional skin from IBH-affected animals as compared to skin from healthy control animals in the IBH season was a lower expression of IL13/CD3$_\zeta$ in the affected animals. FoxP3 and IL10 levels were unaffected, except for a lower expression of FoxP3 in healthy control skin in the IBH season as compared to off season, In addition, the increased level of *C. obsoletus*-specific IgE did not correlate with higher histological scores in LE skin. In summary, our data indicate a general immune activation in the skin of both healthy and IBH-affected ponies during the IBH season that potentially obscures the Culicoides-specific immune reaction pattern, even in lesional skin of IBH-affected animals.

Introduction

Equine insect bite hypersensitivity (IBH) is a seasonally recurrent, pruritic skin disorder mainly caused by the bites of female *Culicoides* spp. [1-5]. In Europe all horses are exposed to *Culicoides* bites and develop IgG antibodies to salivary antigens. About 5 percent of horses develop clinical signs of allergy with elevated *Culicoides*-specific IgE [6 -8]. Approximately 95 percent of the *Culicoides spp*. found around IBH horses in the Netherlands and other European countries, such as Sweden is *C. obsoletus* [2, 4, 9]. Clinically, at onset IBH is characterized by intense pruritus with papules and wheals, which develop into serous crusts, scaling and alopecia on the mane, tail and in some cases the ventral midline, when the disease becomes chronic [10-12]. Lesions are only observed during the *Culicoides* season and are absent off season. The diagnosis of IBH is based on these characteristic seasonal clinical manifestations as well as skin test reactivity to the geographically relevant *Culicoides* antigen [13]. Histologically, IBH lesions are characterized by a mixed perivascular to diffuse cellular infiltrate consisting of mainly CD4+ T cells [14] and, especially in acute lesions, eosinophil's [15, 16].

Expression levels of IL13 mRNA, but not of IL4 or IL5 mRNA, have been described to be significantly elevated in lesional and non-lesional skin of IBH-affected horses [17]. On the other hand, IL10 mRNA levels were lower in lesional compared to non-lesional skin, but this difference was not statistically significant [17]. An increase in IL4 mRNA production with a concomitant decrease in IFNγ mRNA production by cultured equine PBMC was observed during the IBH season [18-20].

No differences in total serum IgE levels were found between healthy horses and animals affected by IBH [21]. However, *Culicoides*-specific IgE serum titres have been reported to be higher in horses with IBH as compared to healthy horses [6-8, 17]. Correlations between *Culicoides*-specific IgE titres and histological scores for IBH have not been reported so far, nor have differences in these disease parameters between the IBH season and off season been investigated. In order to better understand the pathogenesis of IBH we investigated the differences in skin pathology and cytokine expression between IBH "on and off" season. We hypothesize that the immune balance (a mixed Th2/Th1-type) off season alters into a Th2-dominated immune response in the skin of ponies with clinical manifestations of IBH, due to exposure to *C. obsoletus.* Therefore we have compared

Culicoides-specific IgE serum levels, parameters of dermatopathology, the mRNA expression of cytokines (IL4, IL10, IL13, IFNγ), the transcription factor FoxP3 and the T cell receptor linked $CD3_{\zeta}$ in lesional and non-lesional skin of ponies affected by IBH during the IBH season with those of the same animals off season, as well as of healthy control ponies in both seasons.

Our results show significantly higher histopathology scores in lesional skin of affected ponies than in non-lesional skin in the IBH season. The same result was observed when comparing lesional skin of IBH ponies and skin from control ponies in the IBH season. *Culicoides*-specific IgE serum levels were higher in IBH-affected ponies compared to control animals, but within the group of IBH-affected ponies they were equally high in each season. In addition, in both IBH skin and control skin there was an up regulation of $CD3_{\zeta}$, IL4, IL13 and IFNγ during the IBH season compared to the situation off season. Except for a down regulation of FoxP3 in healthy control skin in the IBH season compared to off season, FoxP3 and IL10 levels were unaffected.

Material and Methods

Animals

A total of 31 privately-owned Shetland ponies were included in the study. Ponies with IBH (n=17, age range 3-19 years) were selected on the basis of clinical symptoms and history, i.e. recurrent, seasonal, pruritic skin lesions located at the mane, tail and ventral midline with remission off season. Control ponies (n=14, age range 2-20 years) were randomly chosen from the same stables as the ponies with IBH and had no clinical symptoms or history of IBH. Ponies in both groups were dewormed and vaccinated at regular intervals and did not receive any corticosteroid treatment.

All animal experiments were approved by the Animal Ethics Committee of the Utrecht University.

Collection and processing of blood and skin samples

To ensure that ponies were not exposed to *Culicoides spp*. recently, off season samples were taken in March 2011 before the start of the IBH season when the average maximum temperature was 11.1 °C and midnight temperatures were down to -2.5°C. During the

Culicoides season in August 2011 (with an average maximum temperature of 20.2°C) to insure maximum exposure to *Culicoides* the same ponies were sampled again.
Skin biopsies of 4 mm were taken under local anaesthesia with 2% lidocaine (B. Braun, AG Melsungen, Deutschland). Biopsies of healthy and non-lesional skin were collected from the dorsolateral neck, whereas chronic lesional skin biopsies were taken from the crest. There were no acute lesions present at the time of sampling. Three biopsies were taken per sampling site: the first two were snap-frozen in liquid nitrogen and stored at -70°C until used for RNA isolation. The third biopsy was fixed in 4% neutral buffered formaldehyde for 24-48 h and paraffin-embedded for histopathology. Blood samples (20 ml) were collected from the jugular vein of each pony in off and IBH seasons into heparin tubes. Serum was separated after centrifugation and frozen at -20°C until analysed for *Culicoides*-specific IgE levels.

C. obsoletus whole body extract preparation

Whole body extract (WBE) was prepared from about three hundred female *C. obsoletus* insects, which were frozen at -80 °C without any liquid. They were suspended in a 2ml Eppendorf tube with 1ml of PBS containing protease inhibitor cocktail according to the manufacturer's specification (Sigma-Aldrich, St. Louis, MO, USA) and crushed with a micro pestle. The sample was centrifuged at 14000 rpm for 10 min at 4°C to remove any insoluble material, the supernatant was filtered through a sterile 0.22µm pore diameter Millex-GV filter (Millipore, Amsterdam, NL) and the protein content of the filtrate was measured at an OD of 280nm with a Nanodrop ND-1000 (Thermo Scientific, Etten-Leur, NL). This WBE was snap-frozen in liquid nitrogen en stored at -80°C [22].

Culicoides-specific serum IgE levels

According to van der Meide et al. [22] a costar 96-well microtitre plate was coated with 100µl per well of 10µg/ml *C. obsoletus* WBE in PBS and incubated overnight at 4°C. The next day, the plate was washed 3 times with 0.05% Tween20 in PBS and each well was incubated with 150µl blocking buffer containing 1.5% casein (SDT, Baesweiler, Germany) for 1.5 hours at RT. The wells were washed 5 times with 0.05% Tween20 in PBS and subsequently incubated with the serum samples diluted 1:5 in 1.5% casein buffer in duplicate. After washing, wells were incubated with 100µl (2.5µg/ml) mouse

monoclonal anti-equine IgE-176 [21] for 1 hour at RT. Again the wells were washed 5 times. Subsequently, wells were incubated for 1 hour at RT with 1:1000 goat anti-mouse IgG peroxidase conjugate (AbD Serotec, Düsseldorf, Germany) in casein buffer. After washing 5 times wells were incubated with 100µl high sensitivity tetramethylbenzidine (SDT, Baesweiler, Germany) for 10 min at RT. The reaction was stopped by adding 100µl of 1% HCL per well. Absorbance was measured with a SpectraMax M5 multi-mode microplate reader (Molecular Devices, Berkshire, UK) at a wavelength of 450nm corrected for the OD measured at 650nm.

Histological examination of skin samples

Paraffin-embedded biopsies were cut in 4 µm sections and stained with haematoxylin-eosin (HE) for routine histopathology. A semi-quantitative grading system (0 = absent, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe) was used to assess the degree of epidermal (both hyperplasia and hyperkeratosis) and dermal (infiltration of both mononuclear cells and eosinophil's) changes. The overall histological grade, defined as the sum of the grades for each of these parameters, was determined for each biopsy. All slides were analysed by the same European College of Veterinary Pathologists-certified pathologist, who was blind with regard to the group and time assignment of biopsies.

Quantitative Real-time reverse transcription-polymerase chain reactions

Frozen skin samples were disrupted and homogenized in TRIzol reagent (Invitrogen, Breda, NL) using a Biopulverizer (Biospec #59012N, Biospec Inc., Bartlesville, OK) and polytron (PT 1200 E, Kinematica AG, Lucerne, CH). TRIzol manufacturer's instructions were followed until the water-phase was obtained after the chloroform step. Subsequently, total RNA was extracted using RNeasy columns (Qiagen, Venlo, NL) and eluted with 30 µl of RNase free water. The RNA was quantified spectrophotometrically using a Nanodrop ND-1000 (Thermo Scientific, Etten-Leur, NL). One µg of total RNA was used to produce cDNA with an iScript cDNA Synthesis Kit (Bio-Rad laboratories, Veenendaal, NL) according to manufacturer's instructions. A 5′ nuclease assay using TaqMan probes was employed for qRT-PCR amplification. Expression of the following genes was assessed: IL4, IL10, IL13, IFNγ, FoxP3 and CD3ζ. Reactions were performed in 25 µl

volumes containing 5 µl cDNA, 12.5 µl TaqMan Universal PCR Mastermix (Applied Biosystems, Austin, TX, USA), 0.9 µM relevant primers and 0.25µM fluorescence-labelled probes and milliQ. The qRT-PCR were performed in an iCycler (Bio-Rad laboratories, Veenendaal, NL) with amplification conditions of 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 62, 60 or 57 °C (for details see Table 1). Each reaction was run in duplicate and a no-template control was included to exclude contamination of reagents with cDNA on every plate. PCR efficiencies were calculated using a relative standard curve derived from a cDNA pool of equine PBMC stimulated with pokeweed mitogen (Sigma–Aldrich, St. Louis, MO, USA) for 24h. Relative expression of IL4, IL10, IL13, IFNγ, FoxP3 and $CD3_\zeta$ was calculated using the Pfaffl method [23] using the housekeeping gene 18s ribosomal RNA (18s rRNA) as a reference gene. To assess the role of T cells in IBH the ratios of IL4, IL10, IL13, IFNγ, FoxP3 with $CD3_\zeta$ were determined.

Statistical analyses

Statistical analyses were carried using GraphPad Prism 4.00 (Graphpad Software, San Diego, CA). As our data were not normally distributed, correlation between *C. obsoletus*-

Table 1: Primers and probes and the PCR conditions used for qRT-PCR

Gene	cDNA (µl)	Ta	reference	Sequences (5'-3')
IL4	2.5	60	(Ainsworth et al., 2003)	
IL10	5	60	Primers: (Swiderski et al., 1999)	Probe: 6FAM-GGCTGAGAACCACGGCCCAG-MGBNFQ
IL13	2.5	60	(Heimann et al., 2011)	
FoxP3	2.5	59	NM_001163272.1	Forward: CCAGCTATGATCAGCCTTCC Reverse: GAAGACCTTCTCGCATCCAG Probe: 6FAM-GCTGGCAAATGGCGTCTGCA-MGBNFQ
IFNγ	2.5	60	Ec03468605_g1; Applied Biosytems	
$CD3_\zeta$	2.5	60	(Debrue et al., 2005)	
18S	0.25	60	4352930E; Applied	

specific IgE levels and total LE skin histological scores was examined using a non-parametric correlation (Spearman) test. The Mann–Whitney *U* test (+) was used to compare values from IBH-affected ponies with those from healthy controls. The non-parametric paired Wilcoxon Signed-Rank Test (WSR Test) (*) was used for comparison of paired data (lesional/non-lesional and IBH season/off season). Results were considered significant at $p \leq 0.05$.

Results

C. obsoletes-specific IgE levels

C. obsoletus-specific IgE serum levels of ponies with IBH were significantly higher than those in control ponies in both seasons (P=0.0001 and P= 0.0002, respectively) (Figure 1A). However, within each group there was no significant difference between the levels in the IBH season and off season (IBH vs. IBH P=0.2676 and Co vs. Co P=0.0537).

Histopathology

HE-stained sections of 14 control ponies (Co) and non-lesional skin (NL) of 16 IBH ponies, sampled off season, were examined by light microscopy according to the semi-quantitative grading system. Since some of the ponies were sold during the summer and one died of an unrelated cause, 11 of the control and 15 IBH-affected ponies were available for examination during the IBH season. Control skin histology scores did not differ between the seasons, nor did control skin and NL skin (Tables 2 and 3). The average total histological score was significantly higher (P=0.0039) in LE skin compared to NL skin from the same ponies as well as compared to that of the control animals (P=0.0006) (Figure 1B and D). More specifically, LE skin had higher numbers of infiltrating mononuclear cells and eosinophil's (Tables 2 and 3). In addition, acanthosis and hyperkeratosis were found only in LE skin.

The total histological scores of LE skin of the IBH ponies did not correlate with their *Culicoides*-specific IgE values (P=0.4366) (Figure 1C).

Cytokine expression levels in the skin

The mRNA expression levels of CD3ζ, a marker for T cells, normalized against 18s

Table 2: Off season semi-quantitative histological scores (average and range between brackets) from healthy ponies and ponies with IBH.

	Healthy (n=14)	Non-lesional (n = 16)
Acanthosis	0 (0-0)	0 (0-1)
Hyperkeratosis	0 (0-1)	0 (0-1)
Upper dermis: lymphocytes	1 (1-2)	1 (1-2)
Upper dermis: eosinophilic granulocytes	1 (1-2)	0 (0-1)
Middle dermis: lymphocytes	1 (0-2)	1 (0-2)
Middle dermis: eosinophilic granulocytes	0 (0-0)	0 (0-1)
Deep dermis: lymphocytes	0 (0-0)	0 (0-0)
Deep dermis: eosinophilic granulocytes	0 (0-0)	0 (0-0)
Average total histological score	3 (2-5)	3 (1-5)

0=absent, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe

Table 3: IBH season semi-quantitative histological scores (average and range between brackets) from healthy ponies and ponies with IBH.

	Healthy (n=11)	Non-lesional (n = 15)	Lesional (n = 10)
Acanthosis	0 (0-0)	0 (0-1)	2 (1-2)
Hyperkeratosis	0 (0-0)	0 (0-0)	1 (0-3)
Upper dermis: lymphocytes	1 (1-2)	1 (1-2)	2 (1-4)
Upper dermis: eosinophilic granulocytes	0 (0-1)	0 (0-1)	1 (0-3)
Middle dermis: lymphocytes	1 (0-2)	1 (0-1)	2 (0-3)
Middle dermis: eosinophilic granulocytes	0 (0-1)	0 (0-1)	1 (0-3)
Deep dermis: lymphocytes	0 (0-1)	0 (0-1)	2 (0-3)
Deep dermis: eosinophilic granulocytes	0 (0-1)	0 (0-1)	1 (0-2)
Average total histological score	3 (1-6)	3 (1-6)	14 (2-18)

0=absent, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe

(CD3ζ/18s) were significantly increased in the IBH season in healthy control skin (P=0.0039) compared to off season; the same observation was made in NL skin of ponies with IBH (P=0.0001) (Figure 2). However, there was no difference between NL and LE

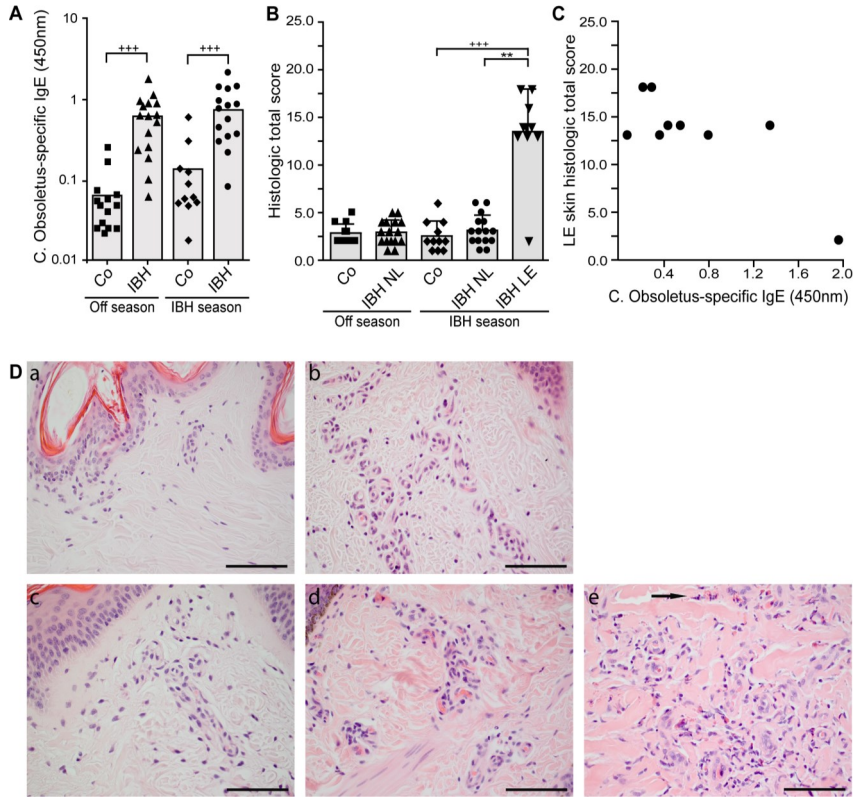

Figure 1: *C. obsoletus*-specific IgE serum levels and histology in the off season and in the IBH season. IgE serum levels and histological scores of skin of 14 control and 16 IBH ponies (off season) and 11 control and 15 IBH ponies (IBH season) were examined. Histology scores of lesional skin of 9/15 IBH ponies were determined. **(A)** *C. obsoletus*-specific IgE serum levels as determined by ELISA assay. The bars represent the mean of all samples in the group. **(B)** Mean histological scores were determined according to a semi-quantitative grading system (0-4) for acanthosis, hyperkeratosis, lymphocyte infiltration and eosinophilic granulocytes **(C)** *C. obsoletus*-specific IgE serum levels plotted against the lesional skin's total histological sores (P=0.4366). **(D)** Representative examples of HE stained section of: a) Off season control skin with few perivascular mononuclear cells in the superficial dermis (average total histological grade 3). b) Off season non-lesional (NL) skin with mild perivascular inflammation (average total histological grade 3). c) IBH season control skin (Co) with minimal perivascular inflammation of the superficial dermis (average total histological grade 3). d) IBH season non-lesional skin (NL) with mild perivascular inflammation (average total histological grade 3). e) IBH season non-lesional skin (NL) with pronounced dermal inflammation and moderate numbers of eosinophil's (arrow) (average total histological grade 14). Original magnification 400x. The bar corresponds to 50 µm.

skin in the IBH season or between Co and NL skin in the off season (Figure 2).

The ratio IL4/CD3ζ, as determined by IL4/18s divided by its corresponding CD3ζ/18s value, both in control and affected ponies showed no difference between seasons (Figure 3). There was also no difference between Co and NL skin in the off season and Co, NL

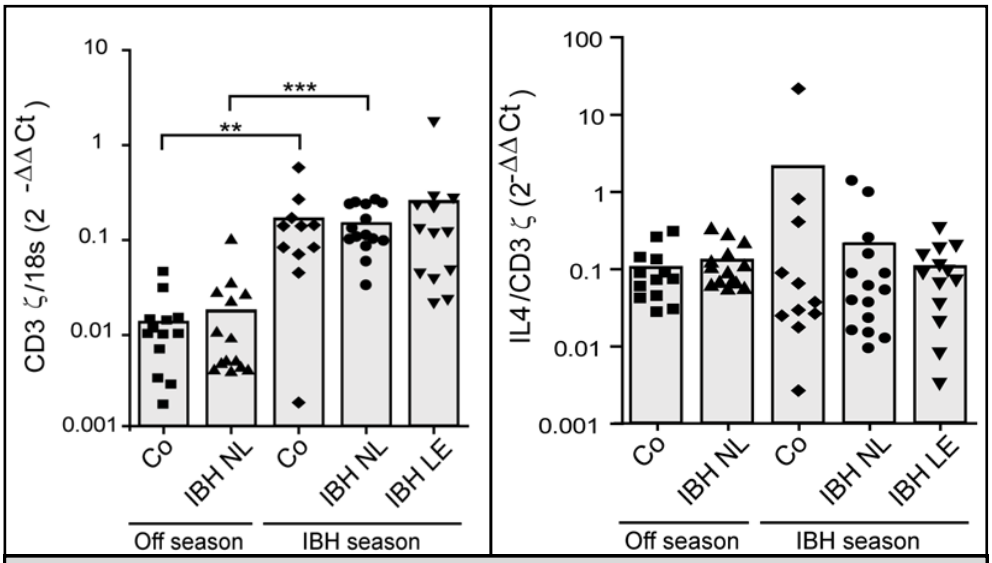

Figure 2 (Left): Expression of $CD3_{\zeta}$ mRNA, a T cell receptor marker. Off season 13 control and 14 IBH ponies were used. In the IBH season 11 of the 13 control ponies were examined and all 14 IBH ponies were examined of which 13 had lesional skin 1 IBH ponies was added in the course of the experiment. The mRNA expression of $CD3_{\zeta}$ was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method [23]. The bars represent the mean of all samples in the group.

Figure 3 (Right): Expression of IL4/$CD3_{\zeta}$ mRNA, a Th 2 cytokine. The skin of 13 control and 13 IBH ponies were used during off season. In the IBH season 11 of the 13 control ponies were examined and all 13 plus 2 extra IBH ponies were examined of which 13 had lesional skin. The mRNA expression of IL4 was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method [23] and subsequently divided by the $CD3_{\zeta}$/18s value of the same sample. The bars represent the mean of all samples in the group.

and LE skin in the IBH season. When comparing only IL4/18s expression an increase was observed when comparing NL skin as well as control skin off season and during the IBH season (respectively P=0.004 and P=0.0273). In the IBH season no difference was found between healthy, NL and LE skin (Supplementary figure 1). IL13 mRNA expression off season was not detectable, whereas in the IBH season IL13/$CD3_{\zeta}$ was lower in LE skin compared to healthy control skin (P=0.0239), but not compared to NL skin (Figure 4). The same results were obtained when only 18s was used for normalization (Supplementary figure 2).

The IFNγ/$CD3_{\zeta}$ analysis showed a significant increase when comparing Co off season and Co IBH season (P=0.002), NL off season versus NL IBH season (P=0.0002). However, there was no difference between Co and NL skin in the off season and Co, NL and LE

skin in the IBH season (Figure 5). The same results were observed when IFNγ/18s levels were compared (Supplementary figure 3).

IL10/CD3$_\zeta$ mRNA expression levels did not differ between off season and the IBH season (Figure 6). Moreover, there was no difference between Co and NL skin in the off season and Co, NL and LE skin in the IBH season. Analysis of IL10/18s shows a tendency for increased IL10 expression in the IBH season (Supplementary figure 4).

Analysis of FoxP3/CD3$_\zeta$ mRNA expression showed a significant down regulation between Co off season and Co skin in the IBH season (P=0.0078) (Figure 7). However, there was no difference between NL off season and NL skin in the IBH season. The same was observed for NL and LE skin in the IBH season. Finally, there was no difference in FoxP3 expression levels between on and off season normalized against only 18s. There

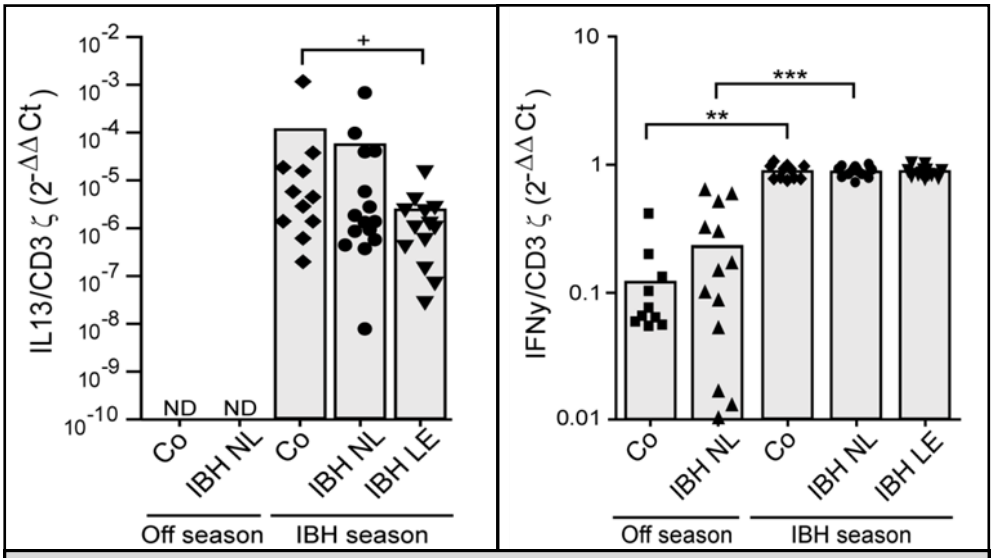

Figure 4 (Left): Expression of IL13/CD3$_\zeta$ mRNA, a Th 2 cytokine. The skin of 13 control and 13 IBH ponies were used during off season. In the IBH season 11 of the 13 control ponies were examined and all 13 plus 2 extra IBH ponies were examined of which 13 had lesional skin. The mRNA expression of IL13 was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method [23] and subsequently divided by the CD3$_\zeta$/18s value of the same sample. The bars represent the mean of all samples in the group.

Figure 5 (Right): Expression of IFNγ/CD3$_\zeta$ mRNA, a Th 1 cytokine. The skin of 10 control and 13 IBH ponies were used during off season. In the IBH season all 10 control ponies plus 1 extra were examined and all 13 plus 2 extra IBH ponies were examined of which 13 had lesional skin. The mRNA expression of IFNγ was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method [23] and subsequently divided by the CD3$_\zeta$/18s value of the same sample. The bars represent the mean of all samples in the group.

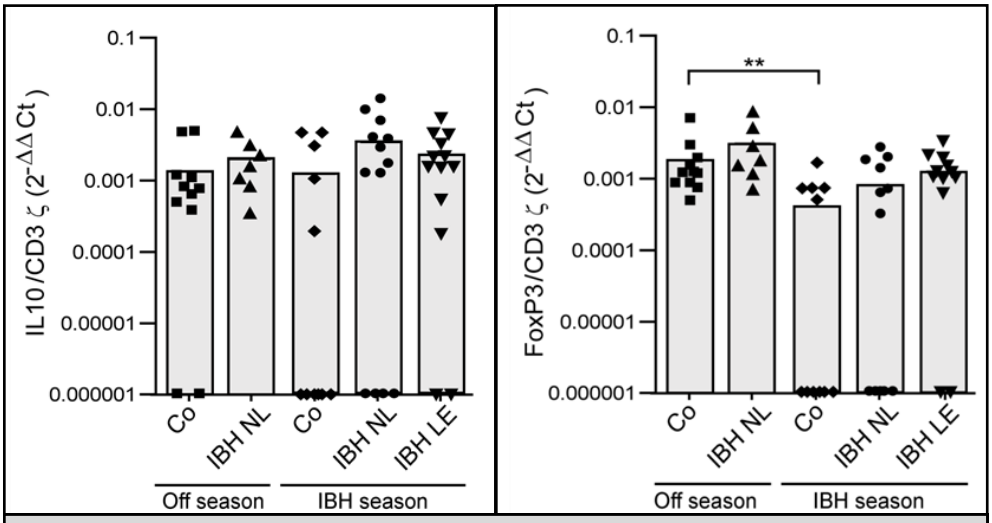

Figure 6 (Left): Expression of IL10/CD3$_\zeta$ mRNA, an immune suppression cytokine. The skin of 11 control and 7 IBH ponies were used during off season. In the IBH season all control ponies were examined and all 7 plus 6 extra IBH ponies were examined, all having lesional skin. The mRNA expression of IL10 was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method [23] and subsequently divided by the CD3$_\zeta$/18s value of the same sample. The bars represent the mean of all samples in that group.
Figure 7 (Right): Expression of FoxP3/CD3$_\zeta$ mRNA, a Treg cell marker. The skin of 11 control and 7 IBH ponies were used during off season. In the IBH season all control ponies were examined and all 7 plus 6 extra IBH ponies were examined, all having lesional skin. The mRNA expression of FoxP3 was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method [23] and subsequently divided by the CD3$_\zeta$/18s value of the same sample. The bars represent the mean of all samples in the group.

was also no difference between Co and NL skin in the off season and Co, NL and LE skin in the IBH season (Supplementary figure 5).

Discussion

IBH is an IgE-mediated, pruritic skin disorder caused primarily by *Culicoides spp*. Differences in cell types present in the skin and as a potential consequence in functional immunological aspects between IBH "on and off" season may be indicative for processes underlying the disease. In general, IBH is thought to be a Type-1 hypersensitivity with a potential Th2 skewed immune responsiveness [17, 18].

In the present study, we examined the skin-infiltrating cells by histopathological analysis as well as cytokine expression by PCR in the off and IBH season in the same pony to determine Th1 versus Th2 immune responsiveness. Additionally, specific IgE serum

levels were measured to determine correlation between allergen-specific IgE levels and the presence of histological manifestations.

A study by Wagner et al. [7] provided the first direct evidence that IgE mediates classical Type-I hypersensitivity in horses and plays a major role in the pathogenesis of IBH. In addition, *C. nubeculosus*-specific IgE serum levels were found in IBH-affected horses [17]. Such a finding is comparable with the situation in other animals such as the dog, where significantly higher flea-specific IgE levels where found in cases of flea allergy dermatitis (FAD) than in healthy dogs [24, 25].

The *C. obsoletus* WBE-specific IgE titres in IBH ponies are higher than those in the control ponies in the off season as well as in the IBH season, suggesting that in IBH there is a Th2 skewing of the immune system. There was no difference between the specific IgE serum titres off season and in the IBH season within each group. IgE titres being stable throughout the year indicate continuous production of IgE by plasma cells, which instead of memory B cells do not need to be reactivated to produce IgE.

Next to clinical symptoms, there are currently two major tests to diagnose IBH in ponies and horses i) the intradermal skin test using WBE, ii) a Culicoides-specific serum IgE ELISA. The intradermal skin allergy test is based on degranulation of mast cells in vivo, causing tissue swelling, due to cross-linking of *Culicoides*-specific IgE by the allergen and the ELISA assay is based on the binding of *Culicoides* specific serum IgE to *Culicoides* extract coated plates. Since Culicoides-specific IgE is the most important factor in both these tests and IgE titres seem to stay stable between seasons it is likely that these tests can be done off season as well as in the IBH season.

The higher histological scores for LE skin suggests a Th2 response of the immune system. There was however, no difference between healthy control skin and NL skin of IBH ponies in the IBH season. This might be explained by the fact that only mild clinical symptoms were found in all IBH ponies potentially due to the relatively cold IBH season, and consequently less active *C. obsoletus* midges than in an average season.

Remarkably, there was no correlation between the allergen specific IgE titre and the total histological score of LE skin from the same pony. This indicates that levels of free serum IgE cannot be used as markers for the severity of IBH. An explanation for the discrepancy may be that circulating allergen-specific IgE levels do not reflect the amount of mast cell- or eosinophil-bound IgE, which more likely contributes to the inflammatory response. In

addition, Wagner et al. [7] suggest that IgG(T), which can also activate skin mast cells, may play a role in IBH. Also in dogs with FAD, flea-specific IgG levels are higher than in healthy dogs [24].

To further assess our hypothesis that a mixed Th2/Th1-type immune status, off season, alters to a Th2-dominated immune response in the skin of IBH-affected ponies, hence local immune response parameters, skin mRNA expression levels of IL4, IL10, IL13, $CD3_{\zeta}$, FoxP3 and IFNγ were determined.

The $CD3_{\zeta}$ mRNA expression in skin of IBH-affected and control ponies was determined to assess differential T cells presence. A significant increase in T cells was observed in Co skin in the IBH season as compared to off season. The same was seen in NL skin. However, there was no significant difference between Co and NL skin in the off season, or in all types of skin in the IBH season, which suggests that the immune system in the skin is activated. This activation might be due to other seasonal factors such as bites of mosquitoes and warble flies. It might even be preferential to perform experiments during the off season to exclude additional effects from other insect bites, which might interfere with the assays.

To address Th2 skewing of the immune response in IBH, we examined mRNA expression of the Th1 type cytokine IFNγ and of the Th2 type cytokines IL4 and IL13. These mRNA levels were subsequently assessed relative to the corresponding $CD3_{\zeta}$ mRNA levels since these cytokines are mainly secreted by T cells. There was no difference between NL and LE skin in the IBH season in expression of IL4, which was increased in the IBH season in both controls and ponies with IBH when only normalised with 18s (Supplementary figure 1). Correcting for the number of T cells (IL4/$CD3_{\zeta}$) abolished the significant increase of IL4 in Co and IBH skin (Figure 2). This indicates that the increase in IL4 mRNA expression is due to increased numbers of T cells, which is in line with the study of Heimann et al. [17] where difference in IL4/18s expression between healthy horses and horses with IBH were observed neither. Similar observations have been reported for example in healthy dogs and dogs with FAD [26]. The lack of altered IL4 expression by T cells and the general increase of IL4/18s suggests that other factors may contribute to the development of clinical manifestations.

In the present study, a substantial expression of IL13 mRNA was observed during the IBH season suggesting again a general immune activation in all types of skin. In the IBH

season a significant lower expression of IL13/CD3$_\zeta$ was observed in chronic lesional skin as compared to control skin. IL13 as a Th2 cytokine plays a role in the effector phase of allergic reactions [27]. Hence, IL13 mRNA levels are expected to increase in acute lesional skin as shown by [17], but maybe not in chronic lesional skin, which was confirmed by our results. A similar situation has been described for FAD dogs [26]. A down regulation of IL13 was observed after *in vitro* stimulation of allergic skin exposed to fleas for 4 days with flea antigen [26] which is coherent with our observation *in vivo*. Hence, T cells do not seem to alter IL13 production in our study. However, we do need to take into account that chronic lesional skin was examined and that Heimann et al. (2011) [17] did find a significant increase in IL13 in more acute lesional skin indicating a Th2 skewing of the immune response.

Our study showed an overall significant elevation of IFNγ in the IBH season as compared to off season confirming a general activation of the immune system in the skin. Contrary to elevated IFNγ levels in human chronic AD lesions [28], there were no differences observed in IFNγ mRNA expression between control, non-lesional and chronic lesional skin of ponies in the IBH season (Figure 5), which is in line with earlier reports by Heimann et al. [17]. Our combined results indicated a mixed Th2/Th1 cytokine expression in both seasons, but at a higher level during the IBH season.

In addition, to the Th1 and Th2 cytokines, Treg cells have been suggested to play a major role in allergies in humans [29]. We therefore decided to examine FoxP3 and IL10 mRNA expression. FoxP3 expression relative to that of CD3$_\zeta$ only showed a significant decrease in healthy skin in IBH season compared to the off season. This suggests that there is a decrease of Treg cells in the skin of healthy ponies in the IBH season. This yet remains to be explained. There was no difference in FoxP3 expression between healthy and NL skin off season. Furthermore, there was no difference between healthy, NL and LE skin in the IBH season (Figure 7). When examining the FoxP3/18s data no significant differences could be found. This suggests that numbers of FoxP3 positive cells in the skin do not differ between healthy and IBH-affected ponies. This is in contrast with the study of Heimann et al. [17], which reported a significant decrease in FoxP3 in NL and LE skin compared to healthy skin suggesting a decrease in Tregs. The reason for these differences might be that they have omitted horses with low histological scores from further analysis. In line with our results a study by Hamza et al. [30] showed that the number of CD4$^+$

$CD25^{+hi}$ $FoxP3^{+}$ T cells levels in PBMC of healthy horses was similar to that in IBH-affected horses. In addition, when $CD4^{+}$ $CD25^{+}$ T cells were stimulated with *C. nubeculosus* extract, expression of FoxP3 was significantly higher in healthy controls compared to IBH-affected horses [30]. Our results do not address the reactivity of Treg cells in the skin of IBH-affected ponies compared to healthy ponies.

There was no difference in IL10/$CD3_{\zeta}$ between seasons. Moreover, there was also no difference between control, non-lesional and chronic lesional skin in the IBH season (Figure 6), which is similar to the observations of Heimann et al. [17]. Our current approach does not confirm a regulatory role for IL10, a longitudinal study may give more inside into the kinetics.

In summary, our data of the same ponies in the off season versus the IBH season showed a general increase of $CD3_{\zeta}$ expression in the IBH season. IL4/18s, IL13 and IFNγ levels were also higher in the IBH season compared to the off season. The increased level of *C. obsoletus*-specific IgE and numbers of cutaneous eosinophils, although not correlated in LE skin, both suggest a Th2 type reactivity of the immune system of IBH-affected ponies. However, based on the IL4, IL13 and IFNγ parameters the Th2/Th1 balance seems to be maintained. Moreover, the role of regulatory T cells (FoxP3 and IL10) could not be confirmed in the present study. These overall results suggest that the immune system in the skin for healthy and IBH-affected ponies is activated in the IBH season in general.

Our data warrant a careful re-evaluation of studies describing the immune status of the skin, taking general seasonal influences in account.

Acknowledgments

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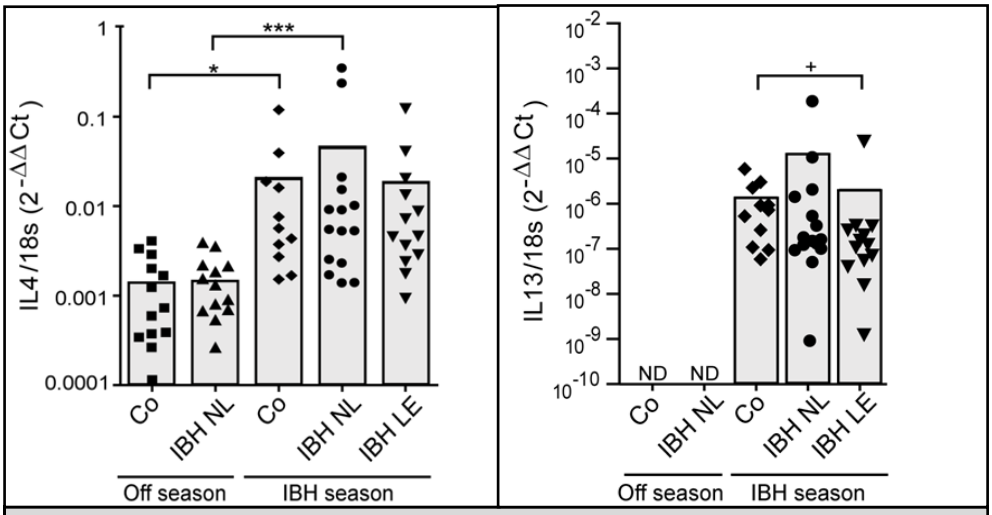
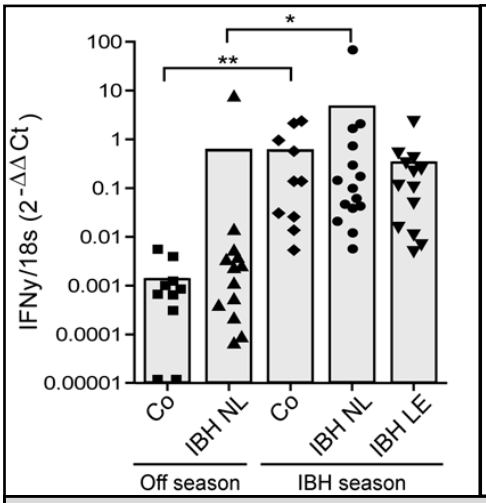
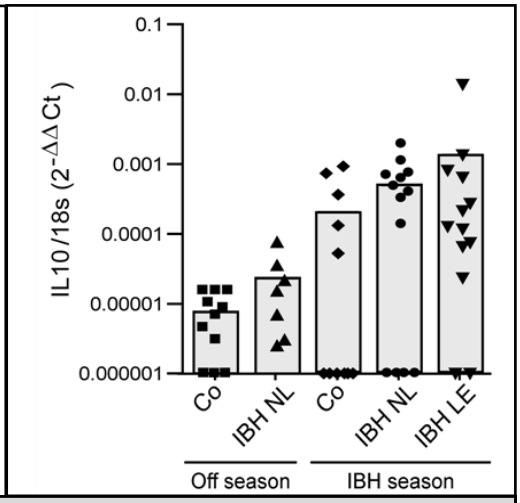
Supplementary data



Supplementary figure 1 (Left): Expression of IL4 mRNA, a Th2 cytokine. The skin of 13 control and 13 IBH ponies were used during off season. In the IBH season 11 of the 13 control ponies were examined and all 13 plus 2 extra IBH ponies were examined of which 13 had lesional skin. The mRNA expression of IL4 was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method [23]. The bars represent the mean of all samples in the group.

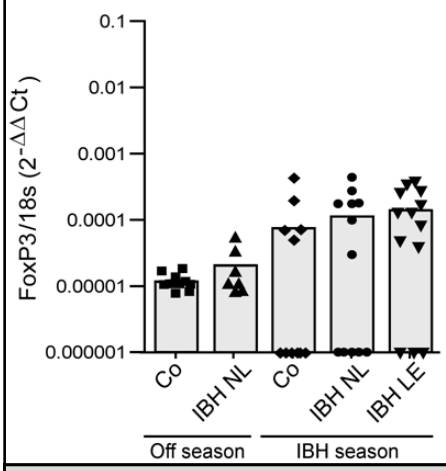
Supplementary figure 2 (Right): Expression of IL13 mRNA, a Th2 cytokine. The skin of 13 control and 13 IBH ponies were used during off season. In the IBH season 11 of the 13 control ponies were examined and all 13 plus 2 extra IBH ponies were examined of which 13 had lesional skin. The mRNA expression of IL13 was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method [23]. The bars represent the mean of all samples in the group.





Supplementary figure 3 (Left): Expression of IFNγ mRNA, a Th 1 cytokine. The skin of 10 control and 13 IBH ponies were used during off season. In the IBH season all 10 control ponies plus 1 extra were examined and all 13 plus 2 extra IBH ponies were examined of which 13 had lesional skin. The mRNA expression of IFNγ was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method [23]. The bars represent the mean of all samples in the group.

Supplementary figure 4 (Right): Expression of IL10 mRNA, an immune suppression cytokine. The skin of 11 control and 7 IBH ponies were used during off season. In the IBH season all control ponies were examined and all 7 plus 6 extra IBH ponies were examined, all having lesional skin. The mRNA expression of IL10 was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method [23]. The bars represent the mean of all samples in the



Supplementary figure 5: Expression of FoxP3/CD3ζ mRNA, a Treg cell marker. The skin of 11 control and 7 IBH ponies were used during off season. In the IBH season all control ponies were examined and all 7 plus 6 extra IBH ponies were examined, all having lesional skin. The mRNA expression of FoxP3 was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method [23]. The bars represent the mean of all samples in the group.

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CHAPTER

4

De novo assembly and transcriptome characterization of Obsoletus complex *Culicoides* insects: vector for arboviruses and causative agent of a skin allergy in horses

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* equal contribution

In preparation

Abstract

Culicoides midges are vectors for numerous important viruses, including African horse sickness virus (AHSV) and bluetongue virus (BTV). Furthermore, they are the primary cause of Insect Bite Hypersensitivity (IBH), a skin allergy in horses. Only limited sequence data of *Culicoides* species is publicly available. In this study, a transcriptome database was constructed of female *Culicoides* Obsoletus complex insects.

Approximately 91 million raw reads were generated by Illumina paired-end RNA sequencing technology. These reads were assembled *de novo* into 41,223 contigs using the assembler IDBA-UD. The estimated open reading frames (ORFs) were extracted from the contigs using QUAST, which resulted in 28,519 unique candidate genes. Contigs were compared with *Nematocera* proteome by BLAST-X searches and resulted in the highest number of matches with proteins from *Aedes aegypti*. Functional annotation using gene ontology (GO) resulted in similar distribution of the functions between *Obsoletus* complex and the top 4 matching species, indicating that Obsoletus complex transciptome is complete. In addition, contigs were compared with publically available transcriptome sequences of *C. nubeculosus* (salivary gland) and *C. sonorensis* (midgut and salivary gland). Only a quarter of salivary gland proteins of *C. nubeculosus and C. sonorensis* was found to match with contigs of Obsoletus complex insects. Thus, there appears to be a lot sequence differences between salivary proteins of the different *Culicoides* species.

This is the first report of a transcriptome database for insect of the Obsoletus complex. This transcriptome provides a valuable tool for vector biology studies and identification of allergens relevant for IBH in horses.

Introduction

Culicoides midges belong to the family Ceratopogonidae (Diptera) and comprise over 1400 different species from which about 96 % are blood suckers feeding on mammals and birds. With only 1-3 mm in size they are among the world's smallest haematophagous flies and can be found in almost all parts of the world with some exceptions like Antarctica, New Zealand and Iceland [1]. *Culicoides* midges are vectors for numerous important viruses affecting both domestic and wild animals, of which African horses sickness virus (AHSV) and bluetongue virus (BTV) are of most international and economical significance and therefore formally classified by the World Organisation for Animal Health (OIE) on the list of notifiable diseases [2]. Furthermore, a recently discovered animal virus emerging in Europe, named "Schmallenberg Virus (SBV)", also appears to be transmitted by *Culicoides* insects [3]. Moreover, *Culicoides* insects can transmit protozoa and filarial worms affecting birds and mammals and are the primary cause of a skin allergy in horses, known as Insect Bite Hypersensitivity (IBH) [4-6]. IBH is a serious welfare problem for affected horses and results in economic losses, because horses can become unsuitable for riding or showing purposes. Severely affected horses might even be euthanized [7].

Within the different *Culicoides* species, members of the Obsoletus complex (subgenus *Avaritia* Fox) are among the most important species associated with BTV in different parts of Europe [8-12]. Members of this complex are also suspected of being vector of AHSV and SBV [13, 14]. The Obsoletus complex comprises about 30 species in the Palearctic region [15]. In central and northern Europe the Obsoletus complex is composed of three species: *C. obsoletus, C. scoticus* and *C. chiopterus* [16]. Females midges of the Obsoletus complex are morphologically very difficult to distinguish from each other. Furthermore, *C. dewulfi* is morphologically similar as the members of the Obsoletus complex, but was recently reported to be treated as a separate group [17]. Several PCR based methods have been described for the identification of species from the Obsoletus complex and *C. dewulfi* [16, 18, 19].

No genome of any *Culicoides* species, including members of the Obsoletus complex, is available in the Genbank database. However, salivary gland cDNA libraries of *C. sonorensis* and *C. nubeculosus* are available [20, 21]. These libraries have been useful for

the characterisation of allergens from these species relevant for IBH in horses [22, 23]. However, *C. sonorensis* and *C. nubeculosus* are less relevant for IBH than *C. obsoletus*, at least in countries were *C. obsoletus* is the main species attracted to horses [24]. Genomic or transcriptome sequence data of Obsoletus complex *Culicoides* insects will provide a valuable source of information for research on IBH and on the transmission of important viruses, like BTV, AHSV and SBV.

Next generation sequencing technologies, such as the the Roche 454 platform and Illumina platform resulting in short read sequences, have made it possible to gather large amount of sequence data of an organism at affordable costs [25]. The Roche 454 platform results in longer reads than Illumina platform and is therefore easier for *de novo* assembly, but is also much more expensive. Illumina sequencing is thus an interesting choice and has lately been successfully used for *de novo* transcriptome assembly of different organisms [26-28].

The aim of this work was to sequence RNA from adult female Obsoletus complex insects, using Illumina paired-end sequencing and to assemble a reliable transcriptome database of these species. This database will be a valuable tool for gene expression analyses, gene discovery and for future genome projects of Obsoletus complex and other *Culicoides* species.

Additionally we analysed salivary proteins and compared their similarity with the salivary proteins of *C. sonorensis* and *C. nubeculosus*. These salivary protein sequences will be valuable for the identification of candidate allergens relevant for IBH.

Material and methods

Collection of Obsoletus complex insects

Female *Culicoides* insects of the Obsoletus complex were collected alive directly from a horse wearing a protective blanket in the east of the Netherlands using a pooter system as previously described [24]. About 5% was separated for morphologically determination and all were determined to be insects of the Obsoletus complex by an experienced entomologist. Because *C. dewulfi* is hard to distinguish from insects of the Obsoletus complex, *C. dewulfi* is considered part of the Obsoletus complex in this paper. The remaining *Culicoides* insects were frozen alive at -80 °C until use.

RNA extraction

RNA was isolated from the wild-caught female Obsoletus complex insects by homogenization in TRIzol® (Invitrogen, Paisley, UK) according to the instructions of the manufacturer. The concentration was measured on a spectrophotometer (Nanodrop Technologies) at OD_{260nm}. The integrity of RNA was analysed by electrophoresis on a 1% agarose gel. Total RNA was stored at -80 °C until use.

RNA sequencing and assembly

RNA sequencing was performed at the Genome Analysis Facility of the Universitair Medical Center Groningen (UMCG), The Netherlands. The method used was deep sequencing RNA with the Illumina HiSeq2000, 2x100 bp paired-end technology. The RNA-sequence data were treated with IDBA-UD [29] for a *de novo* assembly without genome of reference. The quality of the assembly was evaluated by the 'quality assessment tool for genome assemblies' (QUAST) [30].

Functional annotation of C. obsoletus transcriptome

Contigs generated by the assembly were compared with *Nematocera* proteome by Blast-X search using NCBI Genome Workbench version 2.6.0. Protein sequences minimum alignment sequence of at least 75 amino acids and identical for at least 40 % were considered as a significant match.

From the alignment results, proteins were linked to Uniprot and each protein's associated Gene Ontology (GO) terms were retrieved in order to observe the functional categories within the biological process, cellular component and molecular function. To gain more information about the completeness of the transcriptome, the functional annotated transcriptome of the *Obsoletus* complex was compared with the functional annotation of the genomes from the four best matching *Nematocera* species.

Detection of other species sequences

Because we performed the transcriptome assembly with whole female Obsoletus complex insects collected from the wild, there might be some unwanted transcribed sequences present. Therefore we performed BLAST-n alignment with our contigs over different organisms likely to be present. To perform this analysis, we retrieved gene sequences

from horse (*Equus caballus*) and viruses from the NCBI website (using respectively the taxonomy Ids :txid9796, txid10239). We also specifically looked for the presence of sequences from BTV, AHSV and SVBV.

Relation of Obsoletus complex contigs with C. sonorensis and C. nubeculosus salivary proteins

A possible relation between salivary proteins of *C. nubeculosus* and salivary and proteins of *C. sonorensis* with contigs within the Obsoletus complex transcriptome was analysed by inhouse programs. Sequences of at least 75 amino acid matching length and an identity of at least 40 % were considered a match.

Results and discussion

Illumina paired-end sequencing and de novo assembly

A total of 91,462,723 raw reads were generated from the paired-end Illumina HiSeq2000 sequencing. The assembler IDBA-UD was used for *de novo* assembly of the full dataset of short-read sequences of Obsoletus complex. A total of 41,223 contigs were assembled with a total length of 64,668,537 nucleotides. The maximum contig length was 15,641 bp (data not shown).

The estimated open reading frames (ORFs) were extracted from the contigs using QUAST. This *de novo* assembly and ORFs estimation yielded 28,519 unique candidate genes of which more than 70 % had a length of >1000 bp (Figure 1). No genome or full transcriptome of any other *Culicoides* species is available to compare the number of candidate genes. As it has been estimated for *Drosophila melanogaster* that the overall transcriptome represents a coverage of nearly 75 % of the genome [31]. The whole assembly of the Obsoletus complex transcriptome counts 64,668,537 nucleotides. From this basis and considering that Obsoletus complex and *D. melanogaster* are close enough to be compared in that matter, we can assume that the genome size of Obsoletus complex insects is larger than 85 million nucleotides. On the other hand, the ORFs (representing candidate proteins) extracted from QUAST counts 27,952,064 nucleotides representing about half of the assembled transcriptome.

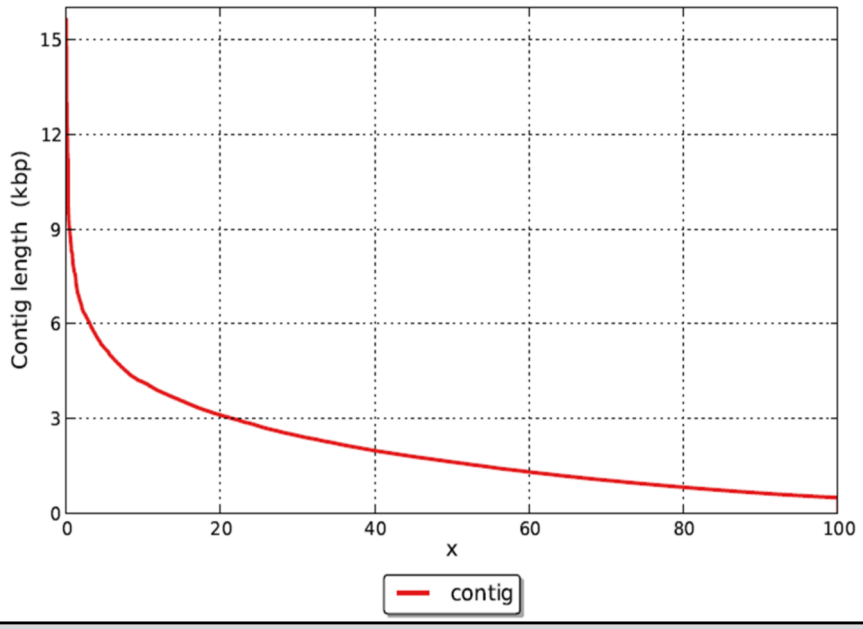


Figure 1: Distribution of the candidate genes over their length. The y-axis refers to the length of the candidate genes (contigs) in kbp and the x-axis the percentage of candidate genes corresponding to that length.

Detection of other species sequences

The whole assembly of the Obsoletus complex transcriptome includes all mRNA present in the entire organism. Therefore we also checked for viral and horse sequences, because the insects might have sucked blood. However, the contribution of virus sequences in the obtained ORFs sequences were limited. With low filtering (≥ 80 % identity and ≥ 100 nucleotides), only 581 genes were found to match virus gene sequences, representing 2.2% of the transcriptome. Two ORFs were found to match one untranslated region (UTR) from the BTV, but no other significant alignments were found for AHSV and SBV.

The presence of horse sequences is very low, as only 12 ORFs have been found significantly (95 % of identity and minimum size of 100 nucleotides) similar to 11 horse genes (data not shown). This suggests that most Obsoletus complex insects did not feed on the horses, which were protected by an anti-insect blanket, when they were caught.

Functional annotation of Obsoletus complex transcriptome

Contigs generated by the assembly were compared with *Nematocera* proteome by Blast-X search using NCBI Genome Workbench version 2.6.0. The *Nematocera* proteome was retrieved directly from the NCBI website *(using the taxonomy ID : txid7148*).

The top eight organisms having the highest number of identified proteins best matching with Obsoletus complex contigs are presented in Figure 2. The organism with most matching proteins was found to be *Aedes aegypti*, followed by three other mosquito species. A whole genome is available for these four organisms. For *C. sonorensis* only limited sequence data is publicly available and the number of matches therefore only account for a relatively small number of genes [20]. It is for this reason why *C. sonorensis* is not located at the first position in spite of its much closer phylogenetic relationship with insects from the Obsoletus complex than *Aedes aegypti*.

The distribution of proteins to the GO terms indicates that Obsoletus complex transcriptome contains proteins from a diverse range of functional classes (Figure 3).

The functional annotation of the four best matching *Nematocera organisms* was compared with the functional annotation of the transcriptome to evaluate the completeness of the transcriptome. The GO terms showed a similar distribution of the functions between *Obsoletus* complex and the top four species (indicated by similar colour pattern, Figure 4). Furthermore, no class is missing within the transcriptome which indicates that all

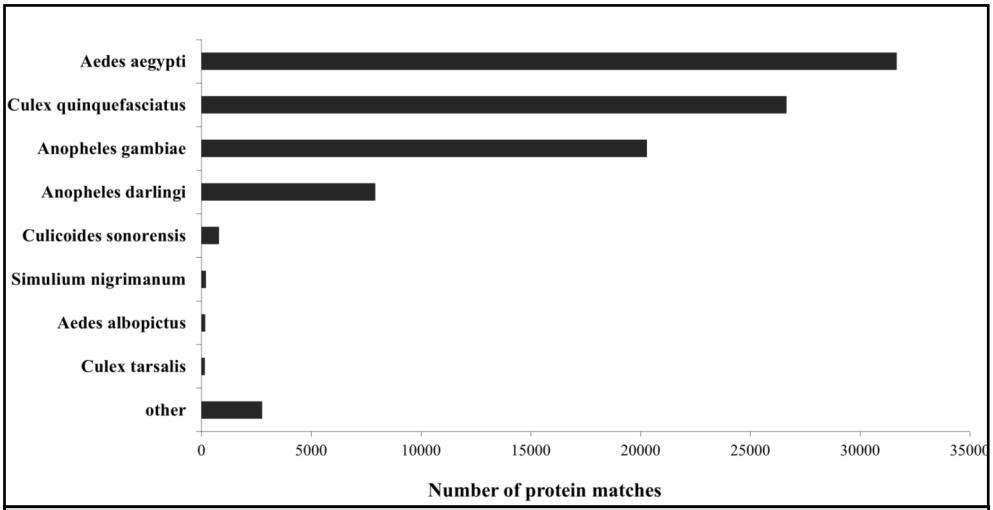


Figure 2: Top eight organisms having the highest number of identified proteins matching with Obsoletus complex contigs.

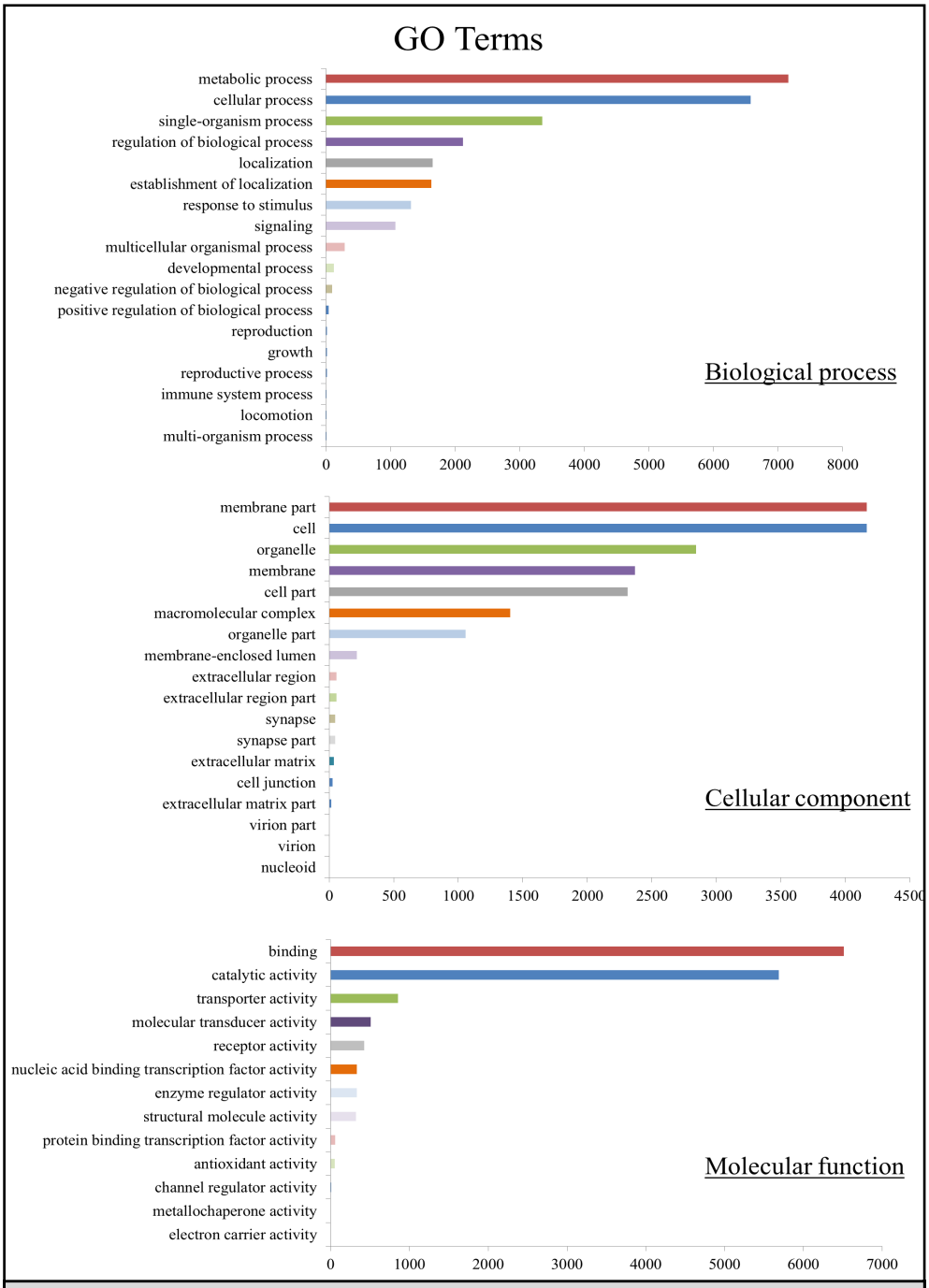


Figure 3: Distribution of Gene Ontology (GO) for the Obsoletus complex transcriptome. Main functional categories in the biological molecular functions, cellular functions and biological process found in the transcriptome of the Obsoletus complex.

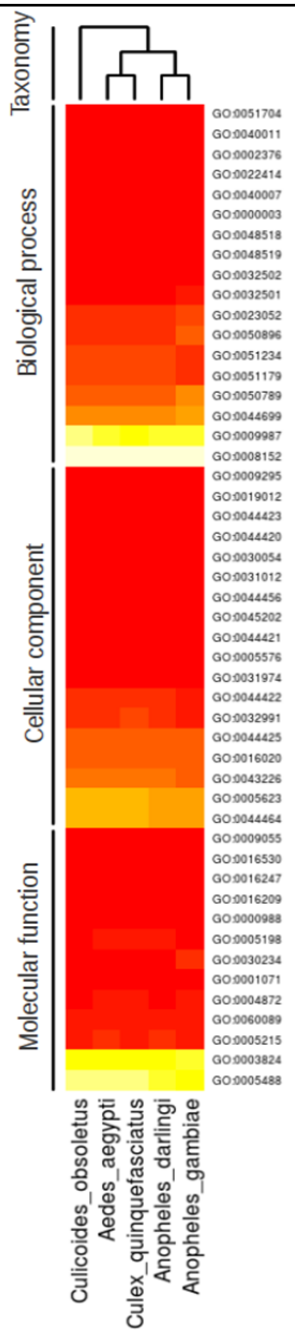


Figure 4: Functional annotation and divergence between the *C. obsoletus complex* genes and genes from 4 other species of *Nematocera*. The transcriptome of the Obsoletus complex and genes from the genome of 4 closely related species underwent functional annotation using Gene Ontology (GO). Colours indicate gene abundance per GO term from white (high abundance) to red (low abundance).

functions are present in the transcriptome.

Sequence comparisons of C. obsoletus with C. nubeculosus and C. sonorensis.

We were interested in differences between sequences of Obsoletus complex insects, *C. nubeculosus* and *C. sonorensis*. Limited sequences are available for *C. sonorensis* (midgut and salivary gland) and *C. nubeculosus* (salivary gland) [20, 21]. These *Culicoides* species have all been reported to be associated with Insect Bite Hypersensitivity (IBH) in horses. IBH is an IgE mediated allergy and symptoms occur when mast cells in the skin degranulate due to the bite of a *Culicoides* insect. For degranulation of mast cells to occur, (salivary) proteins need to have at least two IgE binding epitopes to cause cross-linking of mast cell bound IgE. Horses in Europe are not (or hardly) exposed to *C. nubeculosus* and *C. sonorensis*, in contrast to *C. obsoletus* [32]. However, allergens from *C. nubeculosus* and *C. sonorensis* showing reactivity with horses in Europe have recently been identified, suggesting cross-reactivity between *Culicoides* species [22, 23]. Nevertheless, we previously reported much better *in vitro* reactivity by IBH affected horses with *C. obsoletus* compared to *C. nubeculosus* and *C. sonorensis*, suggesting low cross reactivity between these species [24].

In this study, we were therefore interested in the amount of sequence similarity between these species to see if this might be an explanation for the low cross reactivity between these species. Contigs were compared with sequences from *C. nubeculosus* and *C. sonorensis,* publically available at NCBI using BLAST-x alignments. Proteins which showed more than 40% identity and are larger than 75 Amino acids were considered a match. Results are presented in a Venn diagram created in "R" [33] using the package 'VennDiagram' [34] (Figure 5A).

C. sonorensis has more publicly available sequences, including both whole midgut and salivary gland genes, whereas for *C. nubeculosus* only genes from salivary glands are available. Only about a quarter of the *C. nubeculosus* salivary gland proteins seem to have an identity of at least 40 % with candidate proteins from the Obsoletus complex transcriptome. The percentage of publically available proteins of *C. sonorensis* that match with candidate proteins from Obsoletus complex is higher, more than half of the proteins match.

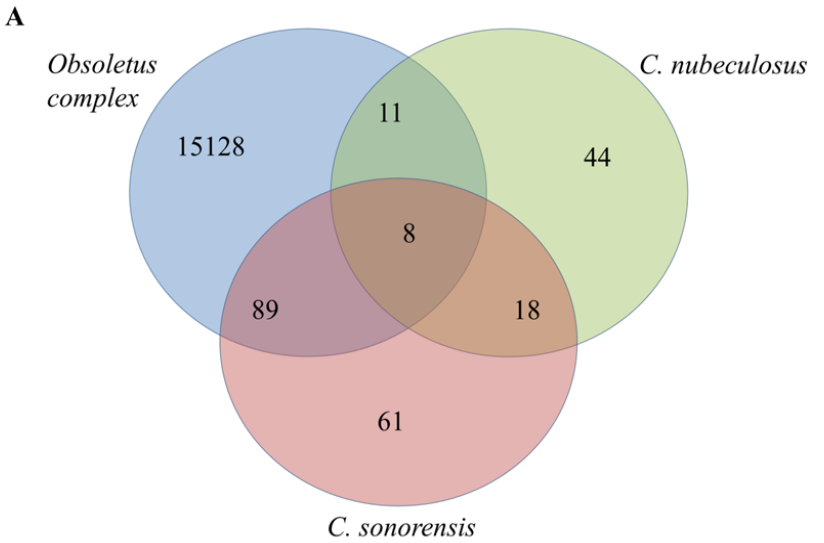
An explanation might be that non-salivary gland proteins, that are available for *C.*

sonorensis, but not for *C. nubeculosus*, contain more conserved proteins than the salivary gland proteins. This explanation is supported by Calvo et al. (2004) [35], who found that salivary proteins of *Anopheles* species are less conserved than the housekeeping proteins within the salivary glands, which suggests that salivary proteins are changing at a faster evolutionary rate. The non-salivary gland part of *C. sonorensis* probably contains more housekeeping genes. To enforce this assumption, we selected the salivary proteins from *C. sonorensis.* Proteins were considered salivary proteins if 'salivary protein' was part of the protein description. This resulted in sixty two proteins which were compared with Obsoletus complex and *C. nubeculosus* sequences (Figure 5B).

Only about a quarter of the salivary proteins of *C. sonorensis* was found to match Obsoletus complex contigs which is similar as with *C. nubeculosus*. Thus, there appears to be a lot of sequence differences between salivary proteins of the different *Culicoides* species, by showing very low similarity. Low similarity was previously reported between genes coding for similar allergens of *C. obsoletus* and *C. nubeculosus* relevant for IBH in horses [36]. The presence of species shared-proteins, and consequently cross-reactivity of antibodies induced by salivary proteins is therefore likely to be low between the different *Culicoides* species.

Conclusions

Prior to this work, no transcriptome has been available for Obsoletus complex insects in public databases. The transcriptome described here provides a first and reliable tool for vector biology studies and identification of allergens relevant for Insect Bite Hypersensitivity in horses.



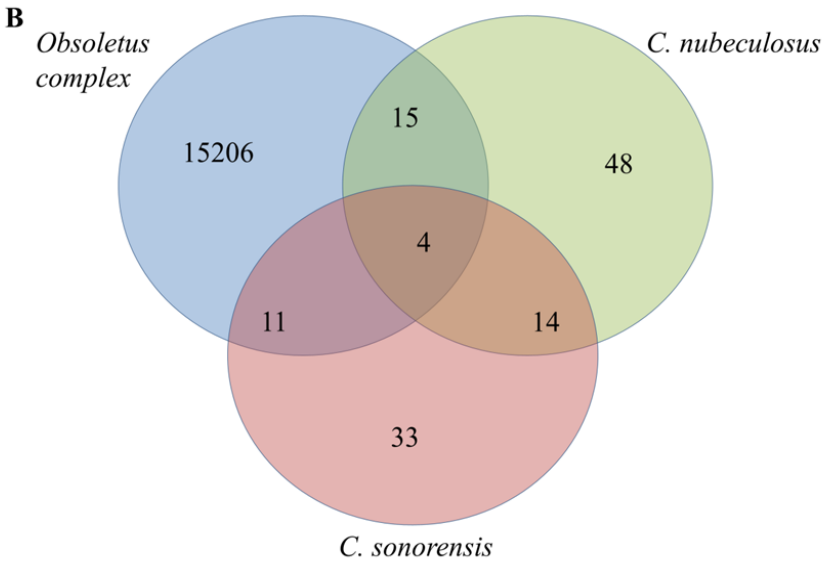


Figure 5: Venn diagram of three *Culicoides* species. The Obsoletus complex transcriptome was compared with sequences from *C. nubeculosus* and *C. sonorensis* , publically available at NCBI , using BLAST-x alignments. Aligned (sequence-based) protein sequences identical for at least 40% and a minimum alignment sequence of at least 75 amino acids are considered a match. **A.** Comparison with *C. sonorensis* salivary gland plus whole midgut. **B.** Comparison with salivary gland sequences of *C. sonorensis*

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CHAPTER

5

Cloning and expression of candidate allergens from *Culicoides obsoletus* for diagnosis of Insect Bite Hypersensitivity in horses

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Abstract

Insect bite hypersensitivity (IBH) is an IgE-mediated (Type I) hypersensitivity reaction induced by allergens from biting midges of the *Culicoides* spp. The aim of the present study was to identify, clone and express recombinant allergens from *C. obsoletus*, the main species found feeding on horses in the Netherlands, by sequence similarity searches on the *C. obsoletus* specific RNA database, with previously described allergens from *C. nubeculosus* and *C. sonorensis*. BLAST searches with these described allergens resulted in similarity hits with 7 genes coding for *C. obsoletus* allergens. These allergens were expressed as hexahistidine tagged recombinant proteins in *E. coli*. Allergens were termed Cul o 1 – Cul o 7. A maltase (Cul o 1) plus Cul s 1 (maltase of *C. sonorensis*) were additionally expressed in insect cells using the baculovirus expression system to compare similar allergens from different species produced with different expression systems in diagnostic *in vitro* and *in vivo* tests. We demonstrate that IBH affected horses in the Netherlands show higher IgE levels to Cul o 1 than to Cul s 1, as determined by an IgE ELISA. Furthermore, we show that Cul o 1 produced in *E. coli* is at least as suitable for *in vitro* diagnosis of IBH affected horses as Cul o 1 produced in the baculovirus/insect cell expression system.

The resulting proteins were evaluated for their ability to discriminate IBH affected and healthy horses by ELISA and intradermal testing. The frequency of positive test results by ELISA within IBH affected horses ranged from 38 % to 67 % for the different allergens. When results of IgE-binding to Cul o 1 – Cul o 7 were combined the test had a sensitivity of 92 % and specificity of 85 %. The capability of the allergens to induce Type I hypersensitivity reaction in IBH affected horses was demonstrated by an intradermal test.

The results show that *E. coli* expressed recombinant allergens from *C. obsoletus* are valuable tools to determine the allergen specific sensitisation profile (component resolved diagnosis) in horses with IBH in countries were *C. obsoletus* is the most abundant species and may facilitate in the development of future immunotherapy.

Introduction

Insect bite hypersensitivity (IBH) is an IgE-mediated (Type I) hypersensitivity reaction found worldwide and is believed to be mainly induced by allergens from biting midges of the *Culicoides* spp., although other insect species, such as *Simulium* and *Stomoxys* spp. were also described to be of importance [1-4]. Although IBH is not life threatening, it causes a lot of discomfort to affected horses, due to the self-mutilation to alleviate the symptoms of pruritus.

Current methods to prevent IBH include insect avoidance by stabling the horses during dusk and dawn when midges are most active, use of blankets to cover the horse, and insect repellents. Skin-related symptoms are sometimes suppressed by the use of topically applied corticosteroids. Some treatment options, like anti-histamines, dietary supplements and ointments are available on the market, but studies on horses using these treatments have shown inconsistent results [5-7].

Immunotherapy could alleviate the symptoms for horses suffering from IBH and some trials have already been carried out with *Culicoides* whole body extracts, but with varying results [8, 9]. Anderson et al. [9], however did see a clear reduction of clinical signs in most and even a complete disappearance in some horses after immunotherapy with *C. variipenis* extract. The quantity of insects required to obtain these results though was very high, being more than 10.000 insects per horse. The use of purified allergens from *Culicoides* insects could simplify and improve future immunotherapeutic approaches.

Several studies have shown that many proteins from *Culicoides* can bind IgE in IBH-affected horses and are therefore potential allergens [10, 11].

Several allergens have now been identified and produced as recombinant proteins. These allergens were derived from the laboratory bred *Culicoides* species, *C. sonorensis* and *C. nubeculosus* [12-14].

The first allergen associated with IBH was identified to be a 66 kDa maltase from *C. sonorensis* and was expressed as recombinant protein (Cul s 1) via the baculovirus expression system [14]. This recombinant protein was found to be highly allergenic in both *in vivo* and *in vitro* tests.

Later, a gene encoding an antigen 5-like allergen was cloned, sequenced and expressed as recombinant protein. This was the first described allergen from *C. nubeculosus* and was therefore designated Cul n 1 [13]. In addition, using phage display technology, a set of 10

recombinant salivary gland allergens from *C. nubeculosus* was produced and named Cul n 2 to Cul n 11. These allergens were recognized by IgE of 13 % to 56.5 % of IBH-affected horses, depending on the allergen [12].

Although many *Culicoides* species have been described to be associated with IBH, apparently *C. obsoletus* is the most important species in the Netherlands in relation to IBH, as this is by far the most abundant *Culicoides* species found feeding on horses in this country [15]. Furthermore, a comparison for *in vitro* diagnosis of IBH using whole body extracts of *C. sonorensis*, *C. nubeculosus* and *C. obsoletus*, showed the importance of using *C. obsoletus* for sensitive and specific diagnostics for horses in the Netherlands. An ELISA with *C. obsoletus* WBE was developed with high sensitivity and specificity that can be used both with sera collected in the *Culicoides* active season as well as with sera collected in the off season [16, 17].

C. obsoletus insects still need to be collected from the wild, because they are not available from laboratory bred colonies and are difficult to rear [18]. Therefore recombinant allergens would be an attractive alternative for whole body extracts for diagnostic assays.

The aim of this study was to develop recombinant allergens from *C. obsoletus* that can be used to improve diagnosis and may be applied in immunotherapy for horses that are exposed to *C. obsoletus.* Furthermore, we compared the reactivity of recombinant maltase from *C. obsoletus* (Cul o 1) with that of *C. sonorensis* (Cul s 1) [14]. These allergens were expressed both in an *E. coli* as well as a baculovirus expression system, to evaluate whether these allergens are more allergenic when expressed in an eukaryotic system, due to possible post translational modifications. Additional *C. obsoletus* genes with sequence similarity to previous described allergens from *C. nubeculosus* were cloned and expressed as recombinant proteins in *E.coli*. These proteins were evaluated for their ability to discriminate IBH and healthy horses *in vitro* by ELISA and their ability to induce immediate and delayed type skin reactions *in vivo*.

Material and methods

Insects

Wild *C. obsoletus* insects were collected in the Netherlands and whole body extracts (WBE) were prepared as previously described [16]. A small fraction (± 5%) of the insects

was checked under a stereo microscope and indeed only contained *C. obsoletus*. Identification of *C. obsoletus* was based on size and wing patterns [19].

Horses

A total of 217 Shetland ponies and Icelandic horses located in different regions of The Netherlands were included in this study. Pairs of clinically confirmed IBH affected and healthy control horses kept at the same location were formed [20]. Seven IBH affected Icelandic horses that showed recurrent symptoms for at least several years and 7 healthy controls from the same pasture were selected for the intradermal test. The remaining 203 horses (76 Icelandic horses and 127 Shetland ponies) were used to evaluate *E. coli* expressed Cul o 1 to Cul o 7 and Cul s 1 in a diagnostic ELISA.

Blood samples were taken from all horses and serum was frozen in aliquots not later than 24 hours after blood sampling and stored at -20 ^{o}C until use. The experimental procedures were approved by the Board on Animal Ethics and Experiments from Wageningen University.

cDNA synthesis

RNA was isolated from ± 200 wild-caught *C. obsoletus* insects by homogenization in TRIzol® (Invitrogen, Paisley, UK) according to the instructions of the manufacturer. The concentration was measured on a spectrophotometer (Nanodrop Technologies) at OD_{260nm}. The integrity of RNA was analysed by electrophoresis on a 1% agarose gel. Total RNA was stored at -80 °C until use.

Prior to cDNA synthesis, a DNase treatment was performed by mixing 2 µg RNA with 1 µl 10x DNAse I Reaction buffer, DNAse I (1 U/µl) and DEPC-treated water up to a final volume of 10 µl. The mixture was incubated for 15 minutes at room temperature (RT), followed by the inactivation of DNAse I by adding 25 mM EDTA (Merck, Darmstadt, Germany) and heating for 10 minutes at 65 °C. Synthesis of cDNA was performed using the Superscript III First Strand Synthesis system (Invitrogen, Paisley, UK), according to manufacturer instructions. Briefly, 300 ng random primers (Invitrogen, Cat. no. 48190-011, 300 ng/µl) and 10 mM dNTPs were added to the DNase I-treated RNA sample. The mixture was heated at 65 °C for 5 minutes and held on ice for a minimum of one minute. 5x First strand buffer (Invitrogen), 0.1 M DTT (Invitrogen), RNase OUT Inhibitor

(Invitrogen, Cat. no. 10777-019, 40 U/µl), and SuperScript III (Invitrogen, 200 U/µl) was added to the mixture and incubated for 10 minutes at RT, followed by incubation for 50-60 minutes at 50 °C. The reaction was terminated by heating at 70 °C for 15 minutes.

Cloning of C. obsoletus cDNA's encoding potential allergens

cDNA of *C. obsoletus* was synthesized using random primers, modified and enriched for attachment to the Illumina flowcell (HiSeq2000, Illumina) at the Genome Analysis Facility of the Universitair Medisch Centrum Groningen (UMCG), The Netherlands. One 100-cycle paired-end lane was sequenced. De novo sequence assembly was done with Velvet (version 1.1.04) [21] using a coverage cut-off of 5 and a minimum contig length of 200 yielding 26936 cDNA contigs (manuscript in preparation). The cDNA contigs were used as an input to create a *C. obsoletus* specific RNA blast database. A protein BLAST with the sequence coding for Cul s 1 was performed on this RNA database to find a similar gene for *C. obsoletus* and specific primers were designed.

Meanwhile, new allergens from *C. nubeculosus* were identified [12, 13] and similarity searches with the sequences coding for these genes were performed as well using BLAST on the *C. obsoletus* RNA database. Six complete sequences coding for genes similar to, respectively, Cul n 1 (antigen 5 like protein), Cul n 2 (hyaluronidase), Cul n 4 (secreted salivary protein), Cul n 7 (unknown salivary protein), Cul n 9 (D7-related salivary protein) and Cul n 11 (trypsin) were found and specific primers for these allergens from *C. obsoletus* were designed. The primers are listed in Table 1, set 1. The 7 allergens were termed according to the recommendation of the allergen nomenclature sub-committee (www.allergen.org) and according to their MW; Cul o 1 (similarity to Cul s 1), Cul o 2 (similarity to Cul n 2), Cul o 3 (similarity to Cul n 1), Cul o 4 (similarity to Cul n 11), Cul o 5 (similarity to Cul n 7), Cul o 6 (similarity to Cul n 9) and Cul o 7 (similarity to Cul n 4). The recombinant proteins with their characteristics are listed in Table 2.

Signal peptides for each allergen were predicted by submitting the sequences to SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP). To clone the *C. obsoletus* allergens in expression vectors, the sequence corresponding to the mature part of the allergens was amplified from *C. obsoletus* cDNA by nested PCR (for primers see Table 1) using KOD hot start DNA polymerase according to manufacturer's instructions (Novagen, Nottingham, UK). The inner primer pairs contained additional restriction sites (Table 1,

Table 1: Primers used to amplify cDNA's coding for allergens Cul o 1 - Cul o 7. Set 2 includes primers to amplify the mature part of the protein including restriction sites (underlined).

Cul o 1	Set 1	Fw	5'-GAAGAATGATAATTTTCAAAACATT-3'
		Rv	5'-TTATTGTTCATATTGTCCGGGA-3'
	Set 2	Fw	5'-CGCGGATCCCCTCAGGGAGCTCGTGAA-3'
		Rv	5'-GCGCTCGAGTTATTGTTCATATTGTCCGGGATC-3'
Cul o 2	Set 1	Fw	5'-TACACTTGTTGTGTGTTTCTG-3'
		Rv	5'-CTCTACATCATAACATAGATAATAATG-3'
	Set 2	Fw	5'-CGCGGATCCCAACATCTGATTCAGGCAGA-3'
		Rv	5'-GCGCTCGAGTACATCATAACATAGATAATAATG-3'
Cul o 3	Set 1	Fw	5'-GAAAATGTTTCGTATTTGTTTATTTAC-3'
		Rv	5'-TATTTGATGGCAAAACTCTGTT-3'
	Set 2	Fw	5'-CGCGGATCCACAGATTTTTGTGACCGAAAGC-3'
		Rv	5'-GCGAAGCTTCCAATTACGGTAAAAATGGCA-3'
Cul o 4	Set 1	Fw	5'-GCAATAATATCGGCTGTTTATGC-3'
		Rv	5'-TTAGTTCATGTTTTGCGTTATCC-3'
	Set 2	Fw	5'-CGCGGATCCGCTGATCATCAATCGC-3'
		Rv	5'-GCGAAGCTTTTAGTTCATGTTTTGCG-3'
Cul o 5	Set 1	Fw	5'-GCGTAATCTTAGGAGGAAT-3'
		Rv	5'-TGAATAGATCATCATTTCT-3'
	Set 2	Fw	5'-CGCGGATCCTTTGATTTAAGTGATG-3'
		Rv	5'-GCGCTCGAGTCATCATTTCTTTATTT-3'
Cul o 6	Set 1	Fw	5'-CCGTTACTTGCCGTTATTGG-3'
		Rv	5'-TCATTATAGACCTTCGTCAAC-3'
	Set 2	Fw	5'-CGCGGATCCTCATTCGTTCCAAAGGAGAG-3'
		Rv	5'-GCGAAGCTTTTATATTGTATTCGGTTTTCCATC-3'
Cul o 7	Set 1	Fw	5'-GTTCTCCGTGTCTAAGCTATC-3'
		Rv	5'-TTAATGAAGAGCTGCGAGTG-3'
	Set 2	Fw	5'-CGCGGATCCTGCTCCTCAATGGCAAATAAG-3'
		Rv	5'-GCGAAGCTTTTAATGAAGAGCTGCG-3'

Table 2: Characteristics of the *C. obsoletus* allergens.

Allergen	Genbank Accession nr	MW	Sequence similarity to	% aa identity
Cul o 1	KC339671	66.8	Maltase (Cul s 1) AY 603565	78 %
Cul o 2	KC339672	42.3	Hyaluronidase (Cul n 2) HM 145950	75 %
Cul o 3	KC339673	27.9	Antigen 5 like protein (Cul n 1) EU 978899	70 %
Cul o 4	KC339674	27.1	Trypsin (Cul n 11) HM 145959	51 %
Cul o 5	KC339675	17.9	Unknown salivary protein (Cul n 7) HM 145955	41 %
Cul o 6	KC339676	15.2	D7-related salivary protein (Cul n 9) HM 145957	40 %
Cul o 7	KC339677	15.0	Secreted salivary protein (Cul n 4) HM 145952	33 %

set 2) for cloning of the PCR products into the corresponding sites of the used expression vectors. For expression by *E. coli* the obtained PCR products were ligated into a pET15b (Novagen) derived vector, pET15bNew, restricted by *BamHI* and *XhoI* or *HindIII*, downstream of the sequence coding for a hexahistidine tag. The mature part of Cul s 1 was ordered as a synthetic gene (Mr. Gene, Regensburg, Germany) codon optimized for *E.coli* expression and directly cloned between the *BamHI*- and *HindIII*-sites of pET15bNew.

Searches for sequence similarity within the database were performed using the BLAST method (http://blast.ncbi.nlm.nih.gov/Blast.) Nucleotide sequences were translated using the BCM search launcher translation tool (http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html) and aligned with Clustal W (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The cloned cDNAs encoding for Cul s 1 or Cul o 1 including the N-terminal hexahistidine tag, were sub-cloned into pFB-HBM, a derivative of the pFastBac1vector (Invitrogen) downstream of a honey bee mellitin (HBM) signal sequence [22], kindly provided by Dr. P. Sondermeyer, MSD Animal Health, Boxmeer, NL, for subsequent expression in the baculovirus/insect cell expression system.

E. coli expression and purification

The isolated pET15bNew plasmids encoding the different *Culicoides* antigens were used to transform *E. coli* BL21_CodonPLus (DE3) (Agilent Technologies, Amsterdam, The Netherlands). Proteins were expressed similarly as described by van der Aa et al. (2010) [23].

Baculovirus expression and purification of Cul o 1 and Cul s 1

Recombinant *Autographa californica* nucelopolyedrovirus (AcMNPV) encoding Cul o 1 and Cul s 1 with N-terminal hexahistidine tag were generated using the Bac-to-Bac system (Invitrogen). Briefly, the pFB-HBM recombinant plasmids containing either Cul o 1 or Cul s 1 cDNA were used to transform DH10ß competent cells containing an AcMNPV bacmid with a chitinase and cathepsin deletion [22] followed by isolation of the recombinant bacmid DNA. Screening of transposition-positive constructs was done by PCR with bacmid and gene specific primers.

Preliminary experiments revealed that an optimal production was obtained with a multiplicity of infection (MOI) of 10 $TCID_{50}$ units/cell 72 hours after infection.

The recombinant bacmid DNAs were used to transfect Sf9 cells with CELL FECTIN reagent (Invitrogen) using standard protocols and the virus was harvested 72 h later, followed by amplification of the viral stock on Sf9 cells in suspension cultures with a cell density of 2 x 10^6 viable cells/ml in serum free medium (Sf-900 II SFM, Invitrogen) to produce working viral stocks without serum. The titre of the viral stocks was determined by an End-Point Dilution Assay using the Sf-9 Easy Titre cell line, encoding EGFP under control of the polyhedrin promoter [24]. Immediately after titre determination, Sf9 cells in suspension cultures (5 x 60 ml) were infected at a density of 2 x 10^6 cells/ml with a multiplicity of infection (MOI) of 10 50% Tissue Culture Infective Dose ($TCID_{50}$) units/ cell. At 72 hours post infection, the cell suspensions were centrifuged at 1000g for 10 min at RT and supernatant and cells were collected. The cell-free supernatants were concentrated using Centricon Plus-70 centrifugal Filter Devices (Merck Millipore, Billerica, Massachusetts, US) and subsequently Cul o 1 and Cul s 1 were purified on Ni^{2+}charged chelating sepharose (Fast Flow, GE Healthcare, Uppsala, Sweden) columns. Proteins were eluted with 500 mM imidazole and afterwards dialysed against PBS.

SDS-Page and Western blot analysis of recombinant proteins

To confirm localization and to analyze the purification of the baculovirus expressed recombinant proteins, samples were analysed by SDS-PAGE (12.5 % gel) and immunoblotting. To analyze the purity of the recombinant proteins, dialyzed eluted fractions were loaded on a 12.5 % SDS-PAGE and the gel was stained with Pierce Silver Stain Kit (Pierce, Rockford, USA) for the baculovirus recombinant proteins or stained with Gelcode Blue Stain Reagent (Pierce) for the *E. coli* recombinant proteins.

For immunoblotting, nitrocellulose membranes (Protran, Schleicher & Schuell Bioscience GmbH, Dassel, Germany) were blocked in 5% non-fat cow's milk in Tris buffered saline (TBS)-Tween (10 mM Tris/HCL, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20) for 1 h at RT and then incubated for 1.5 hour with an anti-His6 tag specific monoclonal antibody that was earlier developed in house. Membranes were then incubated for 1 hour with goat anti-mouse IgG horseradish peroxidase (Dako, 1:1000 in milk powder/TBS-Tween). Between each incubation step, membranes were washed three times with TBS-Tween. Signal was detected by development with an enhanced chemiluminescence (ECL) western blotting detection reagent (GE Healthcare) according to the manufacturer's protocol and visualized by the use of Lumni-fil chemiluminescent Detection Film (Roche, Woerden, The Netherlands).

ELISA

Specific IgE serum levels against the recombinant *C. obsoletus* allergens (Cul o 1 to Cul o 7) and Cul s 1 were determined in an indirect ELISA as previously described [16]. Briefly, wells (384 wells plates) were coated with 20 µl/well of 10 µg/ml of recombinant protein overnight at 4°C, followed by incubation with horse serum samples diluted 1:5 in a 1.5% casein buffer (SDT GmbH, Baesweiler, Germany). IgE binding was detected by mouse monoclonal anti-equine IgE-176 [25], followed by goat anti-mouse peroxidase conjugate (multispecies adsorbed, AbD Serotec, Oxford, UK). High sensitivity tetramethylbenzidine (SDT GmbH) was used as a substrate. The reaction was stopped with 1% HCL. Absorbance was measured with a multi-mode microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, US) at a wave length of 450 nm

corrected for 650 nm. For each allergen, sera of all the IBH affected and healthy horses were tested on the same 384-wells plate to avoid "between plate" OD value differences. Wells without horse serum, but all the other components showed background OD450nm values < 0.07.

The cut-off for positive IgE-binding was set at the mean + 3 times the standard deviation of the OD_{450nm} reading of the healthy horses. The OD_{450nm} values of the healthy horses that were evidently positive (> 1.0, arbitrarily decided) were left out to determine this cut-off, but were taken into consideration to determine false positives.

Intradermal test

An intradermal test was performed on seven clinically confirmed IBH affected and seven healthy control Icelandic horses to test the capability of the *Culicoides* recombinant allergens to induce Type I (IgE mediated) hypersensitivity reactions. All horses were living under the same circumstances (bedding, feeding, pasture) for at least one year. The middle region of the neck of the horses was clipped. The injection sites were indicated with a permanent marker and were placed about 5 cm apart. One concentration (10 µg/ml) per recombinant allergen or *C. obsoletus* WBE were injected intradermal in a volume of 0.1 ml [26]. Histamine phosphate was used as a positive control (10 mg/ml) for a positive wheal reaction and a phosphate buffer as a negative control (Artuvetrin, Artu Biologicals, Almere, The Netherlands). The wheal diameter was measured in millimeters at 30 min and 1 h (immediate phase reaction) and 4 h and 24 h (late phase cell-mediated reaction).

Skin reactions were considered positive when the absolute wheal diameter was larger than the highest wheal diameter provoked by the negative control at the same time point.

Statistical analysis

IBM SPSS statistics, version 19, software program was used for statistical analysis. The non-parametric Mann–Whitney *U* test was used to compare OD_{450nm} values from IBH-affected horses with those from healthy controls against Cul o 1 and Cul s 1 and to compare wheal diameters from IBH affected horses and healthy horses after the intradermal test. Results were considered significant at $p \leq 0.05$.

Results

Cloning of Cul o 1 cDNA

A *C. obsoletus* specific RNA blast database was used to identify the gene encoding for maltase with the sequence encoding for the maltase of *C. sonorensis* (Cul s 1). Specific primers were designed and used to amplify the gene encoding for the maltase (Cul o 1).

To detect a putative polymorphism in the gene encoding for Cul o 1 in a *C. obsoletus* population, ten independent PCRs were performed on the cDNA from a pool of *Culicoides obsoletus* insects. Three isoforms were found with only minor differences on the amino acid level. Alignment of the most detected Cul o 1 isoform with the published Cul s 1 maltase revealed an identity of 78 % on amino acid level (Supplementary figure 1).

E. coli expression and purification of Cul o 1 and Cul s 1

Cul o 1 and Cul s 1, expressed in *E. coli*, were found to be in the insoluble fractions (inclusion bodies) and their molecular masses were in agreement with the expected size (69 kDa). After purification and refolding, a yield of 10.6 mg/L bacterial culture was obtained for Cul o 1 and 11.2 mg/L bacterial culture for Cul s 1. The proteins were found to be >95 % (Figure 1).

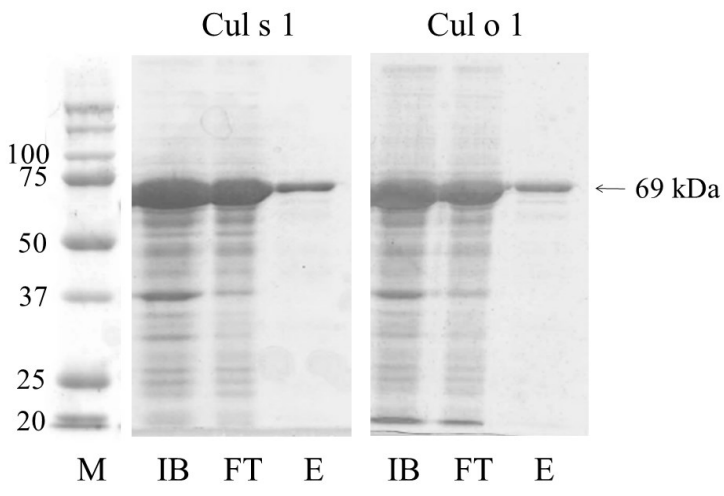


Figure 1: Purification of *E. coli* expressed Cul s 1 and Cul o 1. M = marker, IB = solved inclusion bodies, FT = flow through (unbound proteins), E = refolded and dialysed eluted fraction. Proteins were separated by SDS-PAGE (12,5 % gel) and stained with Gelcode coomassie blue-stain.

Baculovirus expression and purification of Cul o 1 and Cul s 1

The Bac-to-Bac system was used for the production of recombinant Cul o 1 and Cul s 1 in a baculovirus expression system. Immunoblot analysis with an anti-6X His tag specific antibody showed that the recombinant Cul o 1 and Cul s 1 were mainly found in the cell-free supernatant (Figure 2A). After purification, a substantial reduction of recombinant protein was observed in the flow through and bound proteins were recovered by elution with 500 mM imidazole (Figure 2B). Eluted Cul o 1 and Cul s 1 were found to be > 95% pure (Figure 2C). A yield of approximately 100 μg/10^9 cells was obtained for both Cul s 1 and Cul o 1.

Determination of IgE antibodies against E. coli expressed Cul o 1 and Cul s 1

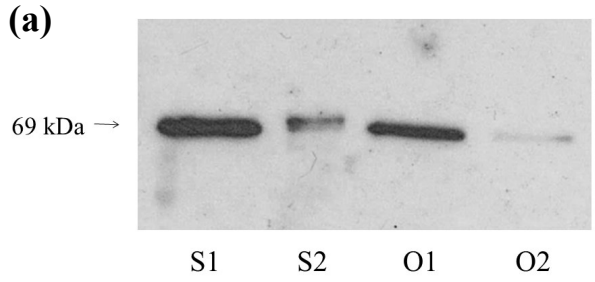
An initial IgE and IgG ELISA with 10 IBH affected and 10 healthy control horses with all three Cul o 1 isoforms did not show any clear differences in OD_{450nm} values (data not shown). and therefore the isoform that was most abundant (isoform 3) was chosen as the antigen in further experiments.

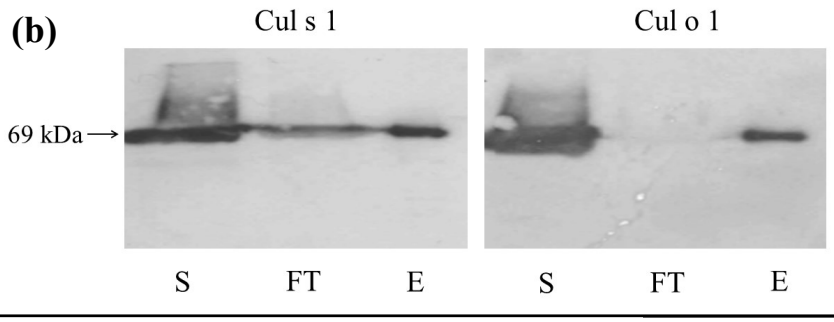
Sera of 103 IBH affected and 100 healthy horses were evaluated for IgE levels (OD_{450nm} values) against Cul o 1 and Cul s 1 in an indirect ELISA. Test results were considered positive when above the cut-off level (mean + 3 times the standard deviation of IgE levels of healthy horses).

Almost double the percentage of positive tested IBH affected horses was obtained with Cul o 1 (43 %) compared to Cul s 1 (22 %). A significant difference between the OD_{450nm} values of IBH affected and healthy horses was observed for both Cul o 1 and Cul s 1 ($p < 0.001$), however OD_{450nm} values obtained with Cul o 1 for IBH affected horses were significantly higher compared to OD_{450nm} values obtained with Cul s 1 ($p < 0.001$, Figure 3).

Determination of IgE antibodies against baculovirus expressed Cul o 1 and Cul s 1 versus E. coli expressed Cul o 1 and Cul s 1

To compare the IgE levels (OD_{450nm} values) against baculovirus expressed Cul o 1 and Cul s 1 with IgE levels against *E. coli* expressed Cul o 1 and Cul s 1, an additional IgE ELISA was performed. Because of the low yield of baculovirus expressed Cul o 1 and Cul s 1,





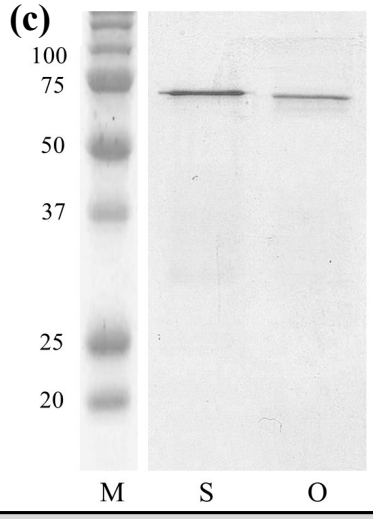


Figure 2 a) Western blot analysis of cell-free supernatants (1) and cell lysates (2) of 72h post infection with recombinant baculovirus expressing Cul o1 or Cul s1. The presence of Cul o 1 (O) and Cul s 1 (S) was detected by an anti-[His]6 antibody, followed by horseradish peroxidase-labelled detection antibody. **b)** Western blot analysis of purified baculovirus expressed Cul o 1 and Cul s 1 from the cell-free supernatants. S: Concentrated supernatant prior to purification; FT: flow-through (unbound proteins); E: Eluted fraction after dialysis**. c)** Silverstaining of purified baculovirus expressed Cul s 1 (S) and Cul o 1 (O)

only a small number of horses could be tested by ELISA. These horses were selected to

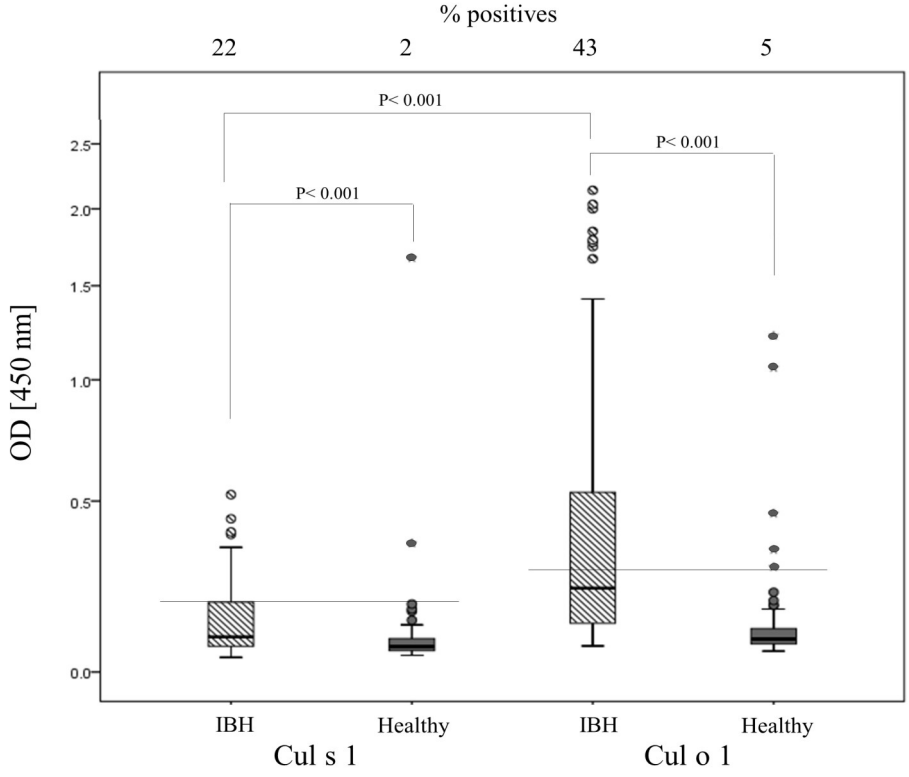


Figure 3: IgE levels presented as OD_{450} values against *E. coli* expressed Cul s 1 and Cul o 1 in sera diluted 1:5 of 103 IBH affected horses and 100 healthy control horses. Results are presented in box plots. The horizontal lines inside of the box is the median of the measurements. The horizontal lines crossing the box plots are the cut-off values determined by the mean OD_{450} values of the healthy horses + 3 x Standard deviation. The bottom and top of the box are the 25th and 75th percentile, respectively. The end of the whiskers represent the minimum and maximum value. The dots represent the outliers. Lines connecting two box plots denote a statistically significant difference with the *p* value on top. The percentage of positive tested horses for each allergen is indicated on the top.

belong to one of the following groups: Group A) Seven horses that were clinically IBH affected and previously tested positive in ELISA with WBE as well as with *E. coli* Cul o 1; Group B) Seven horses that were clinically IBH affected and previously tested positive in ELISA with WBE, but negative with *E. coli* Cul o 1; Group C) Ten horses that were clinically healthy and previously tested negative ELISA with WBE as well as with *E. coli* Cul o 1 (Figure 4).

Horses from group A on average showed similar IgE levels (OD_{450nm} values) against *E. coli* expressed Cul o 1 as against baculovirus expressed Cul o 1. However, IgE levels against baculovirus expressed Cul s 1 were higher compared to *E. coli* expressed Cul s 1.

All horses in group A, except for 1 horse with Cul s 1, had a positive test result with both recombinant proteins regardless of the used expression system.
Horses from group B that were previously tested positive on WBE but negative on *E. coli* expressed Cul o 1, on average had higher IgE levels against the baculovirus expressed Cul o 1 and Cul s 1 and the OD values of all these horses were above the cut-off level. However, clinically healthy horses (group C) showed similar IgE levels against these baculovirus expressed proteins. In contrast, IgE levels against the *E.coli* expressed proteins were not above the cut-off level, suggesting non-specific reaction with baculovirus expressed recombinant Cul o 1 and Cul s 1 (Figure 4).

Amplification of Cul o 2 – Cul o 7 cDNA

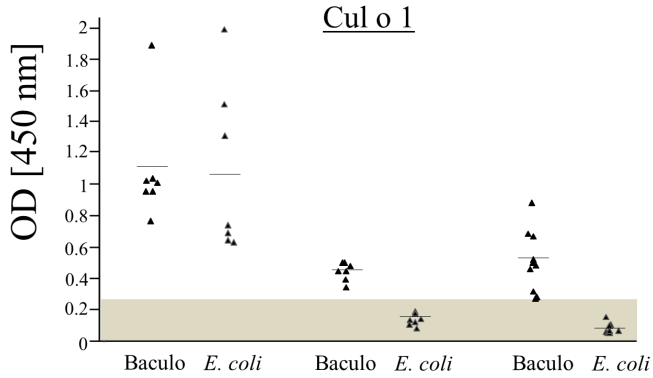
Six complete genes coding for *C. obsoletus* allergens with sequence similarity to Cul n 1, Cul n 2, Cul n 4, Cul n 7, Cul n 9 and Cul n 11 [12, 13] were found in a *C. obsoletus* RNA database. Specific primers were designed and used to amplify these genes from *C. obsoletus* cDNA.
Alignments of the allergens Cul o 2 – Cul o 7 with their similar *C. nubeculosus* allergens showed global similarity with amino acid identities ranging from 33 – 75 %, depending on the alignment (Supplementary figure 2-7).

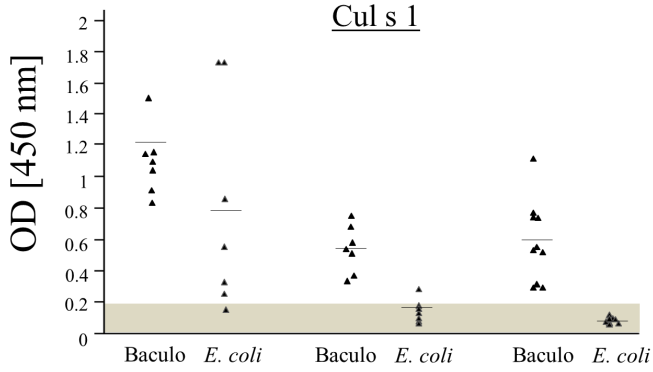
E. coli expression and purification of Cul o 2 – Cul o 7

Cul o 2 – Cul o 7 were successfully expressed in *E. coli*. The recombinant proteins were found to be in the insoluble fractions and were dissolved, purified, refolded and dialysed (Figure 5A). All *E. coli* expressed proteins were found to be > 95 % pure (Figure 5B). The sizes of the proteins that were purified were in good agreement with the expected sizes based on the calculation from the amino acid sequences including the hexahistidine tag.

Determination of IgE antibodies against E. coli expressed Cul o 2 – Cul o 7

Sera of 103 IBH affected and 100 healthy horses were evaluated for IgE levels (OD_{450nm} values) against *E. coli* expressed Cul o 2- Cul o 7. Test results were considered positive when above the cut-off level (i.e. mean + 3 times the standard deviation of IgE levels of healthy horses). The frequency of positive test results varied between the allergens, with Cul o 6 resulting in the lowest percentage of positive tested IBH affected horses (38 %)





Group	A	B	C
Clinical signs	+	+	-
ELISA WBE *C. obsoletus*	+	+	-
ELISA *E. Coli* Cul o 1	+	-	-

Figure 4: IgE levels presented as OD_{450} values against Cul o 1 (upper graph) and Cul s 1 (lower graph) expressed in baculovirus or *E. coli* of **A**. Seven clinically IBH affected horses, tested positive with *C. obsoletus* WBE as well as for *E. coli* Cul o 1; **B**: Seven clinically IBH affected horses, tested positive with *C. obsoletus* WBE, but negative for E. coli Cul o 1 **C**. Ten clinically healthy horses, tested negative with *C. obsoletus* WBE as well as for *E. coli* Cul o 1. The cut-off level is highlighted in light gray and determined by the mean OD_{450} values of the healthy horses + 3 x standard deviation. The triangles represent the individual values. The horizontal lines are the means of the IgE levels.

and Cul o 5 the highest percentage of positive tested IBH affected horses (67 %). Percentages of positive tested clinically healthy horses were between 2 – 6%, depending on the allergen (Figure 6).

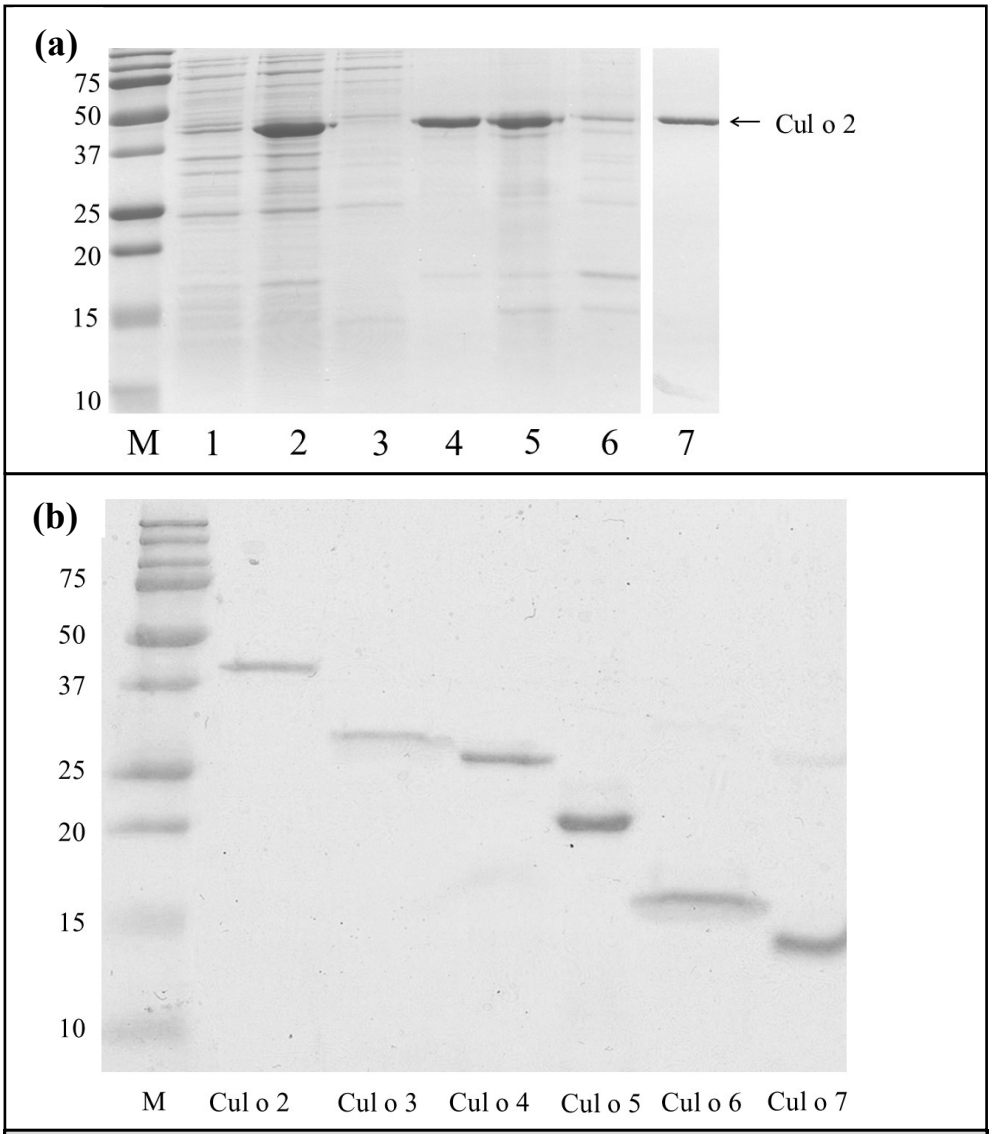


Figure 5: a) Typical example of induction and purification of *E. coli* expressed protein (Cul o 2). 1: Uninduced protein; 2: Induced with IPTG; 3: soluble fraction; 4: insoluble fraction (inclusion bodies); 5: solved inclusion bodies; 6: Flow through (unbound proteins); 7: refolded and dialysed eluted fraction. Proteins were separated by SDS-PAGE (12,5 % gel) and stained with Gelcode coomassie blue-stain. **b)** SDS-PAGE (12,5 % gel) and Gelcode coomassie blue-stain of the *E. coli* expressed proteins Cul o 2 t/m Cul o 7.

From the 103 IBH affected horses, 95 reacted with at least one of the *E. coli* recombinant allergens (Cul o 1 – Cul o 7) while 15 out of 100 healthy horses showed IgE reactivity, resulting in a test sensitivity of 92 % and specificity of 85 %. The eight horses that were

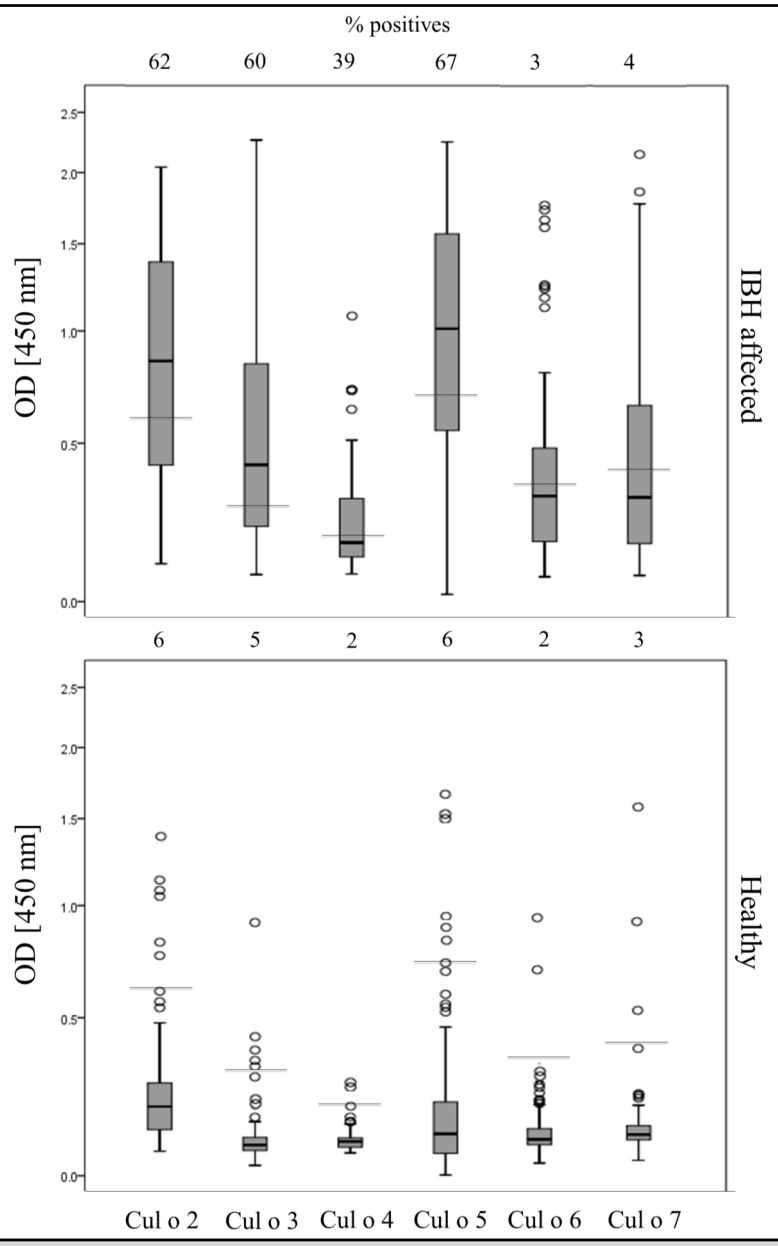


Figure 6: IgE levels presented as OD_{450} values against Cul o 2 – Cul o 7 in sera diluted 1:5 of 103 IBH affected horses and 100 healthy control horses. Results are presented in box plots. The horizontal lines inside of the box are the median of the measurements. The horizontal lines crossing the box plots are the cut-off values determined by the mean OD_{450} values of the healthy horses + three times the standard deviation (excluding OD values above 1.0). The bottom and top of the box are the 25th and 75th percentile, respectively. The ends of the whiskers represent the minimum and maximum value. The dots represent the outliers. The percentage of positive tested horses for each allergen is indicated on the top.

tested negative with the allergens were also tested negative using WBE, except for one horse (results not shown) [16]).

Intradermal test

An intradermal test was performed on 7 IBH affected horses and 7 healthy horses to test whether recombinant allergens and *C. obsoletus* WBE were able to induce type I hypersensitivity (IgE mediated) immediate (0.5 h and 1 h) and late phase (4 h and 24 h) skin reactions *in vivo*.

At 30 min post injection all horses showed a clearly visible wheal at the histamine injection site, which remained visible until 4 hours after injection (Table 3). After 24 hours these wheals had disappeared. The wheal diameters induced by the histamine injection and the negative control (saline) did not differ significantly between IBH affected and healthy horses for any of the time points. The most noticeable skin reactions for WBE and allergens were observed after 1 h and 4 h (Table 3, Figure 7). All IBH affected horses showed positive skin reactions for WBE from time point 1 h onwards, while only 1 healthy horse reacted positive at 0.5 and 24 h and 2 healthy horses at 1 h and 4 h. There was a significant difference between IBH affected and healthy horses for WBE at each time point ($p < 0.01$).

For all allergens at least one IBH affected horse showed a positive type I hypersensitivity

Table 3: Results intradermal skin test of seven IBH affected horses and seven healthy control horses to *C. obsoletus* WBE and recombinant allergens.

Injection	IBH affected horses Number of positives*				Healthy horses Number of positives*			
	0.5 hr	**1 hr**	**4 hr**	**24 hr**	**0.5 hr**	**1 hr**	**4 hr**	**24 hr**
Saline	0	0	0	0	0	0	0	0
Histamine	7	7	7	0	7	7	7	0
WBE	5	7	7	7	1	2	2	1
Cul s 1 E.coli	1	3	2	1	0	0	0	0
Cul o 1 E. coli	0	5	1	0	0	0	0	0
Cul s 1 Baculo	2	4	4	1	0	0	2	1
Cul o 1 Baculo	2	3	4	0	0	0	1	0
Cul o 2	0	0	0	1	0	0	0	0
Cul o 3	0	2	2	0	0	0	0	0
Cul o 4	2	5	4	4	1	0	1	1
Cul o 5	2	3	6	5	0	1	2	1
Cul o 6	1	1	1	1	0	0	0	0
Cul o 7	2	3	3	3	0	0	0	0

* Cut-off = highest wheal diameter provoked by saline

skin reaction, but for Cul o 2 this was only the case in the late phase (at 24 h) of the reaction (Table 3). The highest number of IBH affected horses with positive skin reactions was observed for the baculovirus expressed allergens and Cul o 4 and Cul o 5. For these allergens, positive skin reactions were also observed for 1 or 2 healthy horses at different time points.

A significant difference between IBH affected and healthy horses was only observed with the wheal diameters provoked by Cul o 4 after 1 h. However, the means of the wheal diameters of the IBH affected horses were always larger than the ones of the healthy horses and for each allergen and WBE.

There was no significant difference between the wheal diameters provoked by Cul o 1 versus Cul s 1 for either the baculovirus or *E. coli* expressed variants. Although more horses were tested positive with the baculovirus expressed Cul o 1 or Cul s1 compared to *E. coli* expressed Cul o 1 or Cul s 1, there was no significant difference between the wheal diameters provoked by baculovirus versus *E. coli* produced proteins.

Discussion

In this study we report the identification and production of 7 *C. obsoletus* recombinant allergens that can be used to diagnose IBH with a sensitivity of 92 % and specificity of 85 % when combining the results of each single allergen IgE ELISA. A maltase from *C. sonorensis* (Cul s 1) was the first allergen described to be associated with IBH [14], but *C. sonorensis* is not native in the Netherlands. To evaluate if the maltase from *C. obsoletus* (Cul o 1) is better for IBH diagnosis in the Netherlands than Cul s 1, both were expressed in *E.coli* as well as in a baculovirus/insect cell expression system. Horses with IBH showed a higher average IgE-binding to *E.coli* produced Cul o 1 than to Cul s 1 and more IBH affected horses scored positive with Cul o 1 (43 %) than with Cul s 1 (22 %). These findings are in agreement with previously reported data, where ELISA with WBE of *C. obsoletus* resulted in better diagnosis than such an ELISA with WBE of *C. sonorensis* [16]. Some horses did show IgE-binding with Cul s 1, which is most likely due to cross-reactivity between shared epitopes between Cul o 1 and Cul s 1, since the horses that were used in this study are unlikely to be exposed to the North-American *C. sonorensis*. Reactivity to Cul s 1 was also observed previously in a histamine release test and intradermal skin test using horses from Germany that most likely have not been exposed

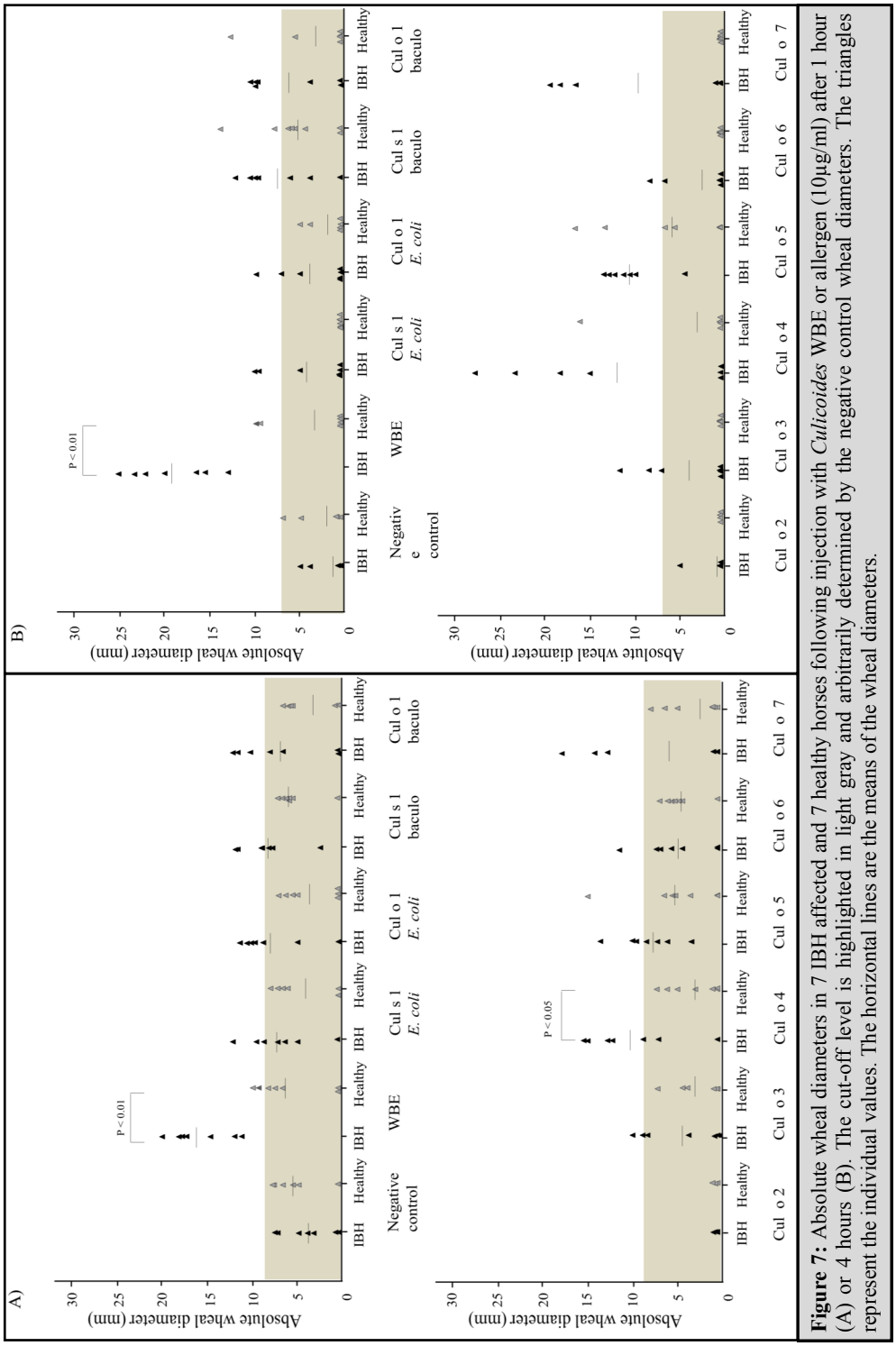


Figure 7: Absolute wheal diameters in 7 IBH affected and 7 healthy horses following injection with *Culicoides* WBE or allergen (10µg/ml) after 1 hour (A) or 4 hours (B). The cut-off level is highlighted in light gray and arbitrarily determined by the negative control wheal diameters. The triangles represent the individual values. The horizontal lines are the means of the wheal diameters.

to *C. sonorensis* [14]. The maltase from *C. nubeculosus* (Cul n 8) was used in a similar ELISA as described here by Schaffartzik et al. [12] and resulted in a positive test result for 21.7 % of the IBH-affected horses, comparable to the reactivity that we found for Cul s 1, whereas we found over 40 % of our horses to be tested positive with Cul o 1. Although we tested different horses, it is likely that for these horses in Switzerland better results would be obtained with the maltase from *C. obsoletus* as it is also one of the most abundant species found in this country [27].

To compare potential differences between the immunoreactivity between the maltase expressed in a prokaryotic system and the same maltase expressed in an eukaryotic system, we produced this allergen in both *E. coli* as well as a baculovirus expression system. The *E. coli* system has the advantage that it is easy to handle and often results in high expression levels of the recombinant protein. However proteins expressed in *E. coli* often accumulate in insoluble inclusion bodies, and therefore require chemical refolding procedures to obtain the protein in a native, biological active form [28, 29]. The baculovirus expression system is characterized by post-translational processing and therefore often results in the production of (glycosylated) biologically active soluble proteins [29].

Baculovirus expression can display better IgE-binding to certain allergens compared to *E. coli* expressed allergens [30, 31]. However, some studies report no difference between *E. coli* and baculovirus expressed allergens in relation to their IgE-binding properties *in vitro* or skin prick reactivity *in vivo* in allergic individuals [32, 33] and an *E. coli* expressed allergen has shown to have similar immunological properties as its natural form [28]. This suggests that for some allergens glycosylation and correct folding obtained by an eukaryotic system might be important for biological activity, but for other allergens a prokaryotic system might be sufficient. Here, we report Cul o 1 and Cul s 1 expressed in *E. coli* to be at least as suitable in IgE-binding compared to the same allergens expressed in the baculovirus expression system in horses with IBH.

The high background values within the healthy horses tested in ELISA with baculovirus expressed Cul o 1 and Cul s 1, compared to E. coli expressed proteins, might be due to antibodies to N-glycans present on the baculovirus expressed allergens. Insect cells produce structural different N-glycans than mammalian cells [34]. Horses are constantly exposed to different types of insects and therefore the presence of pre-existing antibodies

against insect produced N-glycans is not unlikely. This kind of antibody will not bind to the unglycosilated *E. coli* expressed allergens. Also non-specific binding to minor contaminations in the purified protein fraction can not be excluded, but the allergens seemed pure as shown by SDS-PAGE and silverstaining

Because of the easy-handling of *E. coli* cells and the high expression of Cul o 1 and Cul s 1 compared to the baculovirus expression system, 6 additional allergens from *C. obsoletus* were expressed solely in *E. coli*. These allergens were found based on similarity searches within the *C. obsoletus* RNA database using previously described allergens from *C. nubeculosus* [12, 13]. Similar genes of four described allergens from *C. nubeculosus* were not found in the RNA database. Either similar genes for these proteins are not present in the *C. obsoletus* specific RNA database or the database is not yet complete.

Sequence analysis revealed different percentages of amino acid similarities between the individual cloned allergens and the allergens from *C. nubeculosus* or *C. sonorensis* [12-14]. The highest similarity was found for maltase and lowest similarity with a secreted salivary protein (Cul n 4, 33 %). Even though Cul o 1 and Cul s 1 have a high amino acid identity (78 %), Cul o 1 clearly has a higher IgE reactivity with IBH affected horses compared to Cul s 1. We found lower sequence similarities for the allergens Cul o 2 – Cul o 7 with their similar *C. nubeculosus* allergen (33 – 75 %) and therefore the difference between reactivity with IgE for these allergens might even be higher when compared with their similar allergen derived from *C. nubeculosus*. We already previously reported *C. obsoletus* to have more IgE-binding proteins compared to *C. nubeculosus* and *C. sonorensis* as detected by western blot analysis in all tested IBH affected horses [16].

The IgE-binding capacity of each allergen was determined by ELISA for both IBH affected as wells as healthy horses. IBH-affected horses that were tested positive ranged from 38 – 67 %, depending on the allergen. Although we tested different horses, we report higher percentages of positive tested horses with our *C. obsoletus* allergens, than was reported for the corresponding allergen from *C. nubeculosus* by [12, 13]. However, to be more conclusive about the cross-reactivity between these allergens, they should be tested together in the same ELISA for the same horses.

When IgE-binding ELISA results to Cul o 1 to Cul o 7 are combined, a sensitivity of 92 % and a specificity of 85 % is obtained, which is close to the sensitivity and specificity obtained with the same ELISA using *C. obsoletus* WBE [16]. Horses that did not show

positive test results with any of the seven recombinant allergens, also did not show positive test results with WBE (data not shown). A possible explanation for the lack of reactivity, might be that these horses are sensitised to other specific allergens e.g. from other insects that were able to cause IBH but were not included in this study. Schaffartzik et al. [12] reported 11 allergens from *C. nubeculosus*, while we were able to express 7 allergens based on similarity searches with these allergens. Cul n 3 and Cul n 5 were both reported to show IgE reactivity with a large percentage of horses [12] and therefore similar *C. obsoletus* allergens might exist that are crucial for diagnosis of some horses by ELISA. However we were not yet able to find complete genes coding for the proteins similar to Cul n 3 and Cul n 5 in the *C. obsoletus* RNA database and were therefore not able to clone and express these allergens.

Thus, our findings show that serum IgE of IBH affected horses recognize the *E. coli* produced *C. obsoletus* allergens and can therefore be used as a diagnostic tool for IBH in horses where *C. obsoletus* is the most abundant species. However, to provide information about the capability of the recombinant allergens to induce a Type I hypersensitivity reaction (immediate and late phase) *in vivo,* an intradermal test was performed. Type I skin reactions were observed for all allergens within IBH affected horses.

Some healthy horse showed type I skin reactions with WBE, Cul o 4, Cul o 5 and the baculovirus expressed allergens. These horses might be asymptomatic cases of IBH. Indeed, type I hypersensitivity reactions after intradermal injection with *Culicoides* allergens are more often reported in healthy horses [12, 13, 26, 35]. Thus, horses might be sensitised to *Culicoides* allergens without showing clinical signs of IBH. On the whole, greater skin reaction in response to the recombinant allergens were observed for IBH affected horses compared to healthy horses. Interestingly, Type I skin reactions in IBH affected horses, provoked by WBE or individual allergens were stronger after 1 and 4 hours than after 30 min. Other studies also reported greater wheals after 1 and 4 hours compared to 30 min after intradermal injections with *Culicoides* WBE [26, 36]. This suggests that the immediate reaction induced by degranulation of mast cells in horses provoked by allergens of *Culicoides* spp probably peaks after 30 min. Wheals measured at 4 hour are more likely to be caused by the influx of other inflammatory cells, which contribute to the late phase of the response.

In conclusion, the 7 recombinant allergens Cul o 1 – Cul o 7, expressed in *E. coli,* provide

essential tools to determine IgE-binding of horses to individual components of *C. obsoletus* and are capable to induce immediate and delayed skin reactions in horses *in vivo*. These allergens can be useful to determine the sensitisation profile (component resolved diagnosis) of horses and may contribute to the development of allergen-specific immunotherapy for individual horses suffering from IBH.

Acknowledgements

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Supplementary data

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Culo1           MIIFKTLTILISSACLVLTVPQGAREKDWWETGNFYQVYPRSFMDSDGDGIGDLKGVQQK 60
Culs1           MIPFKKLTILLSIACSVLAAPEGAREKDWWEIGNFYQVYPRSFMDSDGDGVGDLKGISEK 60
                ** **.****:* ** **:.*:********* ******************:*****:.:*

Culo1           VAYLKEIGMDGVWLSPIFDSPMADFGYDISNFTKVYPKFGDMQSIQELIDECNKNDIKLI 120
Culs1           VGYLKEIGMDGVWLSPIFDSPMADFGYDISNFTKVFPQFGDLSSIDELVAEFNKKDMKLI 120
                *.**********************************:*:***:.**:**: * **:*:***

Culo1           LDFVPNHSSDECEWFKKSVKRDPYYSDFYIWHPGKPNPNGGRNLPPTNWVSAFRSSAWEW 180
Culs1           LDFVPNHTSDQCEWFKKSIQRDPEYNDYYIWHPGKPNPDGGRNLPPTNWVSAFRSSAWEW 180
                *******:**:*******::*** *.*:**********:********************

Culo1           NDERQEYYLHQFLAAQPDLNYRNPEVVKTMKNVLKFWLNKGINGFRIDAVPYLFEVGPDA 240
Culs1           NEERGEYYLHQFLAQQPDLNYRNPKVVETMKNVLRFWLSKGINGFRIDAVPYLFEVGPDA 240
                *:** ********* *********:**:******:***.********************

Culo1           SGNYPDEPLTYKCADPLSQCYYDHIYTQMRSETVNMVTQWRTVMDDWQKENGGPTRVLLV 300
Culs1           NGNYPDEIETHACSDPLSQCYLYHDYTQNRPETFEMVTEWRATLEEFKQKNGGPTRVLMV 300
                .******  *: *:*******  * *** *.**.:***:**:.::::::::********:*

Culo1           EAYAPLAKVVEIYGENGKLNGAEFPFNFEFLNYLGPKSDAADFKAIIDNYLATIPQGSTP 360
Culs1           EAYAPLTKVIQIYGQNGQLNGAQIPFNFEFLNNLGAVSNARDFKDVIDNYLSTIPEGATP 360
                ******:**::***:**:****::******** **. *:* *** :*****:***:*:**

Culo1           NWVQGNHDQHRSASRLGPQKADAVNMLLQVLPGAAITYYGEELAMEDVFIPWYRTVDPQA 420
Culs1           NWVQGNHDQHRSASRLGPQKADAVNMLLQVLPGAAVTYYGEELAMEDVFVPWSRTVDPQA 420
                ************************************:*************:** *******

Culo1           CTTDPVTFHKYSRDPARTPMIWDDQRNAGFSNANSTWLPVGPDFRSNNVELQRSERSSHL 480
Culs1           CTTDPNIFHAKSRDPARTPMIWTSQKNAGFSSSNYTWLPTGPDYRKNNVEVQRSQRGSHL 480
                *****  **  *********** .*:*****.:* ****.***:*.****:***:*.***

Culo1           NIFKKLMQLRKQDILMYGDYDSVLLNDDVLAVKRELSNNRTLIAVLNLGFTEQMVKLSTI 540
Culs1           NIFKKLTQLRKQDILMYGTYDSYLANDDVLVIKREIENNRTLIAVLNLGFTEQVVNLNLN 540
                ****** *********** *** * *****.:***:.***************:*:*.

Culo1           S-QWQIPQVMEVYTASVGSGMFERQPVYTDEVYVAAGIGVVLDYQVGKRLPQPRGDDPGQ 599
Culs1           DRDWKVPERMEVATASVNAGMFERQPVVTSEVYVSAGVGVVLDYQVGRQIPEPRGDDPGL 600
                . :*::*: *** ****.:******** *.****:**:**********:::*:*******

Culo1           YEQ 602
Culs1           YE- 602
                **
```

Supplementary figure 1: Alignment of Cul o 1 and Cul s 1 with an identity of 78 % on amino acid level. The amino acids highlighted in grey represent the predicted signal peptide. The underlined amino acids within the boxes represent the places of the polymorphisms of Cul o 1 of isoforms 1. Amino acids underlined: isoform 1 = A/S/E, isoform 2 = S/T/E, isoform 3 (A/T/D)

```
Culo2           MWSSVVNISHIMAVVTTAHLISAQHLIQAELPDSPLNIVKEFDDDG-------------- 46
Culn2           MWLNVVNVSQFMTAWATFNLINAQQLIQVEPENVPYEIVDAKDDASESRGIFFNNFITSK 60
                ** .***:*::*:. :* :**.**:***.*  : * :**.  ** .

Culo2           RNNNNQNDFNFYWNIPSFMCAQHNITFTDMTSSYNIVQNKDDKWRGDKIVILYDPGKFPA 106
Culn2           NNDNKRHDFTFYWNIPSFMCSKYNVTFTDMTSSYNIVQNKDDKWRGDQIIILYDPGKFPA 120
                .*:*:::**.**********:::*:**********************:*:**********

Culo2           LLEHQGQLYRRNGGVPQEGNLQEHIDILAEHINKLIPDTQFSGIGVIDFESWRPIFRQNS 166
Culn2           LLEHQGKLYRRNGGVPQGGNLQEHIDYFAESVNTLIPDQNFSGIGVIDFESWRPIYRQNS 180
                ******:********** ******** :** :*.**** :***************:****

Culo2           GVLQPYKDLSYKLVHRDHKLWNRKRVEIEAARLFEAAGRTFVEETINVAKILRPKAKWGY 226
Culn2           GVLQPYKDLSYKLVQRKHRLWSKKLIEEEAAREFETAGRSFVEETVKVAKYLRPNAKWGY 240
                **************:*.*:**.:* :* **** **:***:*****::*** ***:*****

Culo2           YGFPYCFNMNGGANMNEDCPANVKEENDQIKWLWDIVDVVLPSVYLNNKITSAQRVQFVR 286
Culn2           YGFPYCFNMNGGANMKEDCPSNVKEENNRIKGLWDIVDVVLPSVYLNNKITASQRVQFVR 300
                ***************:****:******::** ********************::*******

Culo2           GRMREGYRVAKMSKKSPKPPVLAYLRYVYTDTLKFLSNEDLKQAIKVSKEQKSKGMIFWG 346
Culn2           GRMREGYRVSQLSKQPVKPPVYSYLRYVYTDNLKYISNDDLKQSIKVSKEQKGSGLIFWG 360
                *********:::**:. **** :********.**::**:****:********..*:****

Culo2           SSYDVKTKEQCIDFRKYVDNNLGPIVLLANNKSPKVLTPNLA 388
Culn2           SSYDVKTKDQCFDFRNYVDNNLGPIVLSANDNTPKILTPNLS 402
                ********:**:***:*********** **:::**:*****:
```

Supplementary figure 2: Alignment of Cul o 2 and Cul n 2 with an identity of 75 % on amino acid level. The amino acids highlighted in grey represent the predicted signal peptide.

```
Culo3           MFRICLFTVL--CVNFVVATDFCDRKLCRRQIEPNVYQNIPHIGCNHDGRNSPACPSDAK 58
Culn1           MIKKLSIVILFSCISFVLSTNFCNKDLCKRQNGPQSFTYLKHIGCRHTGKNANTCPRDAK 60
                *::   :.:*  *:.**::*:**::.**:**  *: :  : ****.* *:*: :** ***

Culo3           ILPMSTKRKNLILRVHNRLRNKVALGQLPGYPKAVRMPILRWDDELAYLAELNVKQCEMK 118
Culn1           ILPMSTKRKNLILKVHNRLRNKVALGKLPGFPKAARMPILRWDDELAYLAELNVKQCKME 120
                *************:************:***:***.**********************:*:

Culo3           HDQCRNTDKFRYAGQNLAYIGGGKEPNAVRIKTLVRAWFDEYKDANSSFIDKYRSHPNGK 178
Culn1           HDQCRNTDKFKYAGQNLAYTMGTPQKNAVRIKKLIRAWFKEHENATASFIGKYRDHPQGR 180
                **********:********  *  : ******.*:****.*:::*.:***.***.**:*:

Culo3           AIGHFTAMVQDRTDTVGCAILRHTKNTYFFLACNYSFTNMVKDKVYTRGAKSCSKCRTGC 238
Culn1           VIGHFTAMIQDRTDTVGCAILRHSGNKYFFLACNYGFTNMMNQKVYTKSNKSCSKCRTGC 240
                .*******:***************: *.********.****:::****:. **********

Culo3           SPVYKGLCKPHEYVNPDPDEDLD 261
Culn1           SKVYKGLCAAHEYVNPDPYEDLD 263
                * ****** .******** ****
```

Supplementary figure 3: Alignment of Cul o 3 and Cul n 1 with an identity of 70 % on amino acid level. The amino acids highlighted in grey represent the predicted signal peptide.

```
Culo4           MNIKSTIIVIAIISAVYAADHQSQKAVAVPSYTYRRP--STKIIGGAPAFSHQFPWQASI 58
Culn11          MKSLKFIFIFIAFSVLEVYCNEVQEVIAVPPRNYTKPRYSIKIVGGSPAREHQFPWQAS- 59
                *:  . *:::  :*.: .  :: *:.:***. .* :*  * **:**:** .******** 

Culo4           TVTACSGDWCSLCGGSLISRKHVLTAAHCTKGLSSFTIGLGSNTRNRPAVTVVAKSKTEH 118
Culn11          -VTSCSEKSCLICGGSLISRRYVLTAAHCTAGLSEFQIGVGSTDRNRPAFSVFSYGKIEH 118
                 **:** . * :*********::******** ***.* **:**. *****.:*.: .* ** 

Culo4           PKYNPESLANDVSIITLSLNVNLNNNIKVISLANSGIGTLVNRNAFVSGYGKTSSSSEGS 178
Culn11          PQYNPEKLFNDIAILKLPRPIRFIKGVETIKLARNNV-SYTNQNATLSGYGKTSSSGPVS 177
                *:****.* **::*:.*.  :.: :.::.*.**...: : .*:** :**********.  * 

Culo4           NTLNYLSMRLISNSDCYKVFGPQIYSTTLCAVARSSVHKNVCSGDSGGPLVIKRNGNYVQ 238
Culn11          DKLNFVDMRIISNQECANEYS-IVRNTTLCALGRNRSVENACQGDSGGPLVVKERSSFVQ 236
                :.**::.**:***.:* : :.  : .*****:.*.   :*.*.********:*....:** 

Culo4           VGIVSFVAKVGCDAGFPSGYARVSSFRNWITQNMN-- 273
Culn11          VGIVSFVAKVGCEYGYPSGYARVSSFYKWIANNTDLN 273
                ************: *:********** :**::* :
```

Supplementary figure 4: Alignment of Cul o 4 and Cul n 11 with an identity of 51 % on amino acid level. The amino acids highlighted in grey represent the predicted signal peptide.

```
Culo5           MKRVILGGIILFLTSKFVLSFDLSDALPGHITDDMTTTEKPTNVAAATADFSDDDLLAVI 60
Culn7           -MQAITVAIILILSVVFVGCFDLRDALPGHVTDDQTTTVKPG-VVQRTADFSDDDLWAVL 58
                  :.*  .***:*:  ** .*** ******:*** *** **  *.  ********* **: 

Culo5           NESKKKVKSS---DVKPTNKLLTNVKNKLESVDFKKIDQKVASLLIPIYKKASEAILNWI 117
Culn7           KEDNKKSKSSSGSQAKDKQTILKTMQAKLKSIKFDQVDTKLASRISSPYVQSANSVMQWI 118
                :*.:** ***   :.* .:.:*..:: **:*:.*.::* *:** : . * ::::::::** 

Culo5           SMTGAAIETSPCFEKIDRIFTKILQDTSKYFTPARAN--IAQKSYVEKLTKALSTIRQCI 175
Culn7           SSITNIKKVVNCFDNIEIYFQSIINEASNNYTKKKVQLQVVEKQFKEKLYATLSSTRQCL 178
                *      :.  **::*:  * .*::::*: :*  :.:  :.:*.: ***  :**: ***: 

Culo5           ISKIKK- 181
Culn7           ISRLKAK 185

                **::*
```

Supplementary figure 5: Alignment of Cul o 5 and Cul n 7 with an identity of 41 % on amino acid level. The amino acids highlighted in grey represent the predicted signal peptide.

```
Culo6           MKFLIPLLAVIGLISCS--FVPKESDYDTNNLKKVQKCELKHQVNDSEKAIWWAWKIPAK 58
Culn9           MKLIILIFTIIGLALCAPDEVPSGDQYDTDNLLIVRDCEEEKDLPAAEKAEWWDWKVPAN 60
                **::* ::::***  *:   **. .:***:**  *:.** ::::  :*** ** **:**: 

Culo6           PARCYLACVMSAFKWIDKKGNPKLD-AIKKSYKAVGHEDSAPNQQFIKKNCGRKLPKNPC 117
Culn9           PTECYIDCIFQKYGWLSGEGGSIVNSAVEASYAAVGHSNPS------LASCN--PSKSGC 112
                *:.**: *::. : *:. :*.. :: *:: ** ****.:.:        .*.   .*. * 

Culo6           DKAILLYACFLVRSENLQHFKDAFDGKPNTI 148
Culn9           SKADELYACLLN--ADGQKFKDAFDGKRDAK 141
                .**  ****:*    : *:******** ::
```

Supplementary figure 6: Alignment of Cul o 6 and Cul n 9 with an identity of 40 % on amino acid level. The amino acids highlighted in grey represent the predicted signal peptide.

```
Culo7           MKFSVSKLSLLLCITILCICFATAAPQWQISELSEQSNVIKCSNENNFGIYKELCQFLKK 60
Culn4           MKFPT----FLILAFFLSLYISSTASRRKHFRHLKRIEAANDCPAKNSGTYQKVCKQLQK 56
                ***..    :*:   :*.: ::::*.: :  .  :: :. : .  :* * *:::*: *:*

Culo7           IYIKAPDEDLGSYLRGGLQSAANRLLDPTVTLPKNTLKNVEDCMKNFQAVINEYNVVALK 120
Culn4           YYVLTPDDKLGSYLKGGLQEAANRVLTPVSKSDKITFDIVQNCLKNFQVMVNKHNKEALR 116
                 *: :**:.*****:****.****:* *. .  * *:. *::*:****.::*::*  **:

Culo7           KYQECDDQCAKQAGQLFENDVSKTAGRMGDCIVSLAALH 159
Culn4           KYRECKKECFTEVGKEFSSALDKTGVQIAECLNESL--- 152

                **:**..:* .:.*: *.. :.**. ::.:*: .
```

Supplementary figure 7: Alignment of Cul o 7 and Cul n 4 with an identity of 33 % on amino acid level. The amino acids highlighted in grey represent the predicted signal peptide.

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CHAPTER

6

Evaluation of a diagnostic ELISA for insect bite hypersensitivity in horses using recombinant Obsoletus complex allergens

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Abstract

Culicoides species of the Obsoletus complex belong to the most important species of midge involved in causing Insect Bite Hypersensitivity (IBH) in horses in The Netherlands. The aim of the current study was to evaluate seven different Obsoletus complex derived recombinant allergens (Cul o 1 – Cul o 7) and to compare these with Obsoletus complex whole body extract (WBE) in an IgE ELISA using sera of 194 clinically-confirmed cases of IBH and 175 unaffected horses were assessed. The highest test accuracy was obtained with WBE, followed by Cul o 2, 3 and 5. Two ELISAs with a combination of recombinant allergens, combi-1 (Cul o 3, 5 and 7) and combi-2 (Cul o 1, 2, 5 and 7) were additionally performed and both resulted in high test accuracies close to that obtained with WBE. Combi-1 resulted in the best sensitivity and specificity, both 89%. Both combi-1 and combi-2 performed less well with samples collected in winter, but over 70% of the IBH affected horses could still be identified. In conclusion, a combination of three Obsoletus complex recombinant allergens (Cul o 3, 5 and 7) could potentially replace Obsoletus complex WBE in an IgE ELISA for diagnosis of IBH in horses.

Introduction

Culicoides midges are the primary cause of a worldwide, seasonal recurrent skin allergy in horses, called Insect Bite Hypersensitivity [1]. The welfare of affected horses is compromised, as a result of severe itch which leads to rubbing, scratching and localized hair loss, with excoriations and sometimes secondary infection [2, 3]. Affected horses generally develop IgE antibodies against allergens from *Culicoides* species [4-6].

Culicoides obsoletus is often reported as the most abundant midge species attracted to horses in different countries [7-10]. Whole body extracts (WBE) of this particular species have been shown to be more immunoreactive in different *in vitro* diagnostic tests for IBH compared to WBE of other common non-native *Culicoides* species [11]. Based on this species WBE a diagnostic IgE ELISA with high sensitivity and specificity has been developed.

C. obsoletus is often identified purely based on wing characteristics. *C. obsoletus* is part of the Obsoletus complex, which in central and northern Europe also includes *C. scoticus* and *C. chiopterus* [12]. *C. dewulfi* has also been considered a part of this group for a long time due to its morphologically similarity, but was recently described to belong to a different taxonomic group [13]. However, because female midges of all these species are morphologically very difficult to distinguish, these species are often referred as Obsoletus complex, when identification is based on morphology.

Insect whole body extracts are complex mixtures containing many proteins and other molecules. Like other natural allergenic extract preparations, they can be contaminated with allergens from other sources and other unwanted components, like proteases which can interfere with allergenicity testing [14, 15]. Moreover, they are difficult to standardize and do not provide information about disease-eliciting allergen content. Additionally, *Obsoletus complex* insects still need to be collected from the wild, because so far they have not been successfully maintained in laboratory bred colonies [16]. For these reasons, defined recombinant allergens are an attractive alternative. Moreover, an individual's IgE reactivity profile can be more precisely determined with recombinant allergens, thus allowing component resolved diagnostics (CRD). Subsequently, those allergens to which the patient carries specific IgE can be selected for tailor made specific immunotherapy [17].

We previously identified and produced seven recombinant allergens from Obsoletus

complex that were cloned based on similarities to previously described allergens for *C. nubeculosus* and *C. sonorensis* [18-21]. We found a maltase of Obsoletus complex (Cul o 1) to result in better *in vitro* diagnosis compared to its homolog from *C. sonorensis,* a species that horses were not exposed to, thus showing the importance to use the dominant *Culicoides* species that feeds on horses for diagnosis on allergen level as well.

In this study we evaluated seven individual Obsoletus complex derived recombinant allergens with Obsoletus complex WBE in an IgE ELISA using sera of a large number of IBH affected and unaffected horses. Two combinations of several of these recombinant allergens were used to design an IgE ELISA with sensitivity and specificity comparable to the previously developed Obsoletus complex WBE ELISA.

Material and methods

Preparation of Obsoletus complex WBE and allergens

Culicoides insects from the Obsoletus complex were collected from the wild and WBE was prepared as previously described [11]. Copy DNA encoding 7 Obsoletus complex allergens (Cul o 1, 2, 3, 4, 5, 6 and 7, Genbank accession numbers KC339671-KC339677) were cloned and expressed in *E. coli* and the recombinant proteins were purified as previously described [18].

Horses and blood sampling

A total of 194 IBH affected (105 Shetland ponies and 89 Icelandic horses) and 175 healthy control (97 Shetland ponies and 78 Icelandic horses) horses were included in this study. Horses were selected by a strict protocol and according to a case-control design described in detail by [22, 23]. In a previous study 103 of the clinically confirmed IBH affected horses and 100 healthy control horses were already used to evaluate a diagnostic ELISA [11]. Fourteen clinically confirmed IBH affected Shetland ponies with mild IBH symptoms and 11 healthy control ponies were used to compare IgE ELISA results from serum collected in IBH season (August 2011) versus serum collected off season (March 2011). The remaining 180 IBH affected horses that were used in this study to evaluate the IgE ELISA showed clinical symptoms (mild to severe), while the 164 healthy controls did not show any symptoms despite similar exposure to *Culicoides* spp. IBH affected horses

and healthy controls were matched on various factors as described by [22]. Most of the Icelandic horses were not imported, but born in The Netherlands (> 85%). Blood samples of these horses were collected in The Netherlands during autumn of 2009 and 2010. All serum samples were frozen in aliquots not later than 24 hours after blood sampling and stored at -20 ^{o}C until use. Blood sampling was approved by the Board on Animal Ethics and Experiments from Wageningen University.

ELISA

Specific IgE serum levels against WBE or recombinant Cul o 1 to Cul o 7 were determined in an indirect ELISA as previously described [18]. In short, Costar 384-wells plates were coated with 20 µl/well of 10 µg/ml Obsoletus complex WBE or recombinant allergen, except for Cul o 5, which was coated at 5 µg/ml. Two ELISAs with a combination of only a few allergens were also performed. Combi-1 containing allergens Cul o 3, Cul o 5 and Cul o 7, coated as a mix at concentrations of 2.5 µg/ml, 1.25 µg/ml and 2.5 µg/ml respectively for each of the allergens and combi-2, containing allergens Cul o 1, Cul o 2, Cul o 5 and Cul o 7, coated as a mix at concentrations of 2.5 µg/ml, 2.5 µg/ml, 1.25 µg/ml and 2.5 µg/ml respectively for each of the allergens. After coating, wells were incubated with horse serum samples diluted 1:5 in a 1.5% casein buffer (SDT GmbH, Baesweiler, Germany). IgE binding was detected by mouse monoclonal anti-equine IgE-176 [24], followed by goat anti-mouse peroxidase conjugate (multispecies adsorbed, AbD Serotec, Oxford, UK). High sensitivity tetramethylbenzidine (SDT GmbH) was used as a substrate. The reaction was stopped with 1% HCL. Absorbance was measured with a multi-mode microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, US) at a wave length of 450 nm corrected for 650 nm. For each ELISA, sera of all the IBH affected and healthy horses were tested on the same 384-wells plate to avoid “between plate” OD value differences. Optimal coating concentrations were determined prior to the experiment by titration.

SDS-PAGE and Western blotting

SDS-PAGE and western blotting were performed as previously described [11]. For WBE 20 µg/protein/lane was loaded and recombinant allergens were pooled and 100 ng/individual allergen/lane was loaded on a 15% SDS-PAGE gel. Separated proteins were

transferred to a nitrocellulose membrane (Protrans, Schleicher & Schuell, Bioscience GmbH) by means of electrophoresis. IgE binding was detected with a monoclonal antibody against horse IgE (αIgE-176) [24], followed by goat anti-mouse IgG horseradish peroxidase (Dako, 1:1000 in milk powder/TBS-Tween).

Statistical analysis

The Mann–Whitney *U* test was used to compare OD_{450nm} values or log_2 dilutions of the standard from IBH affected horses with those from healthy horses against WBE or allergens using IBM SPSS statistics, version 19. Results were considered significant at $p \leq 0.05$.

The overall diagnostic performance of each ELISA (allergens and WBE) was determined from receiver-operating characteristic (ROC) curves [25], computed in IBM SPSS statistics, version 19. The cut-off value of each ELISA was set as the value that corresponds to a balanced optimal sensitivity and specificity.

The predicted probability of IBH-status (IBH or healthy) for a certain OD_{450nm} value expressed as log_2 IgE titres was determined by logistic regression. The analysis was performed with the PROC LOGISTIC of SAS (SAS Inc, V9.2).

Results

IgE ELISAs with Obsoletus complex allergens

ROC analysis was performed on the IgE ELISA data of all individual recombinant allergens and WBE of Obsoletus complex tested on 194 IBH affected and 175 healthy control Shetland ponies and Icelandic horses. The test accuracies (area under the curve (AUC), sensitivity, specificity) were obtained from the analysis and the cut-off values determined by these ROC curves (Table 1, Figure 1). The highest test accuracy based on the AUC was obtained for WBE, followed by Cul o 2 and Cul o 3. For all allergens and WBE, IBH affected horses had significantly higher OD_{450nm} values than healthy horses (Figure 2, $p < 0.001$). The highest percentage of positive tested IBH affected horses based on the cut-off determined by the ROC curve, was obtained for WBE (86 %). Within individual allergens, Cul o 3 resulted in the highest percentage of positive tested IBH affected horses (85%) and with Cul o 1 the lowest percentage of IBH affected horses was tested positive (76%). The proportion of false positive tested horses within the healthy

Table 1: Test accuracy parameters for indirect IgE ELISAs on sera of IBH affected and healthy control horses using *Obsoletus complex* whole body extract (WBE) or *Obsoletus complex* or recombinant allergens (Cul o 1 – Cul o 7).

IBH affected vs. Healthy horses

	WBE	*Cul o 1*	*Cul o 2*	*Cul o 3*	*Cul o 4*	*Cul o 5*	*Cul o 6*	*Cul o 7*	*Combi-1*	*Combi-2*
AUC	0.95	0.85	0.90	0.90	0.86	0.88	0.83	0.86	0.94	0.92
Cut-off OD*	0.32	0.15	0.41	0.37	0.15	0.40	0.17	0.12	0.57	0.54
Cut-off std c**	-	-	-	-	-	-	-	-	2.5	2.7
Sensitivity (%)	86	76	83	85	78	82	77	80	89	86
Specificity (%)	86	76	84	85	79	81	77	80	89	86
PPV (%)	87	78	85	86	80	83	79	81	90	87
NPV (%)	85	74	82	84	77	80	75	78	88	85

*Cut-off: $OD_{[450nm]}$ cut-off that corresponds to balanced optimal sensitivity and specificity.
**Cut-off std c: The cut-off value that corresponds to a balanced optimal sensitivity and specificity, expressed as $\log_2$ IgE titer of the standard that can be used as a reference in future ELISA's when using the serum pool of 17 IBH affected horses to prepare a standard curve of 2x dilution steps.

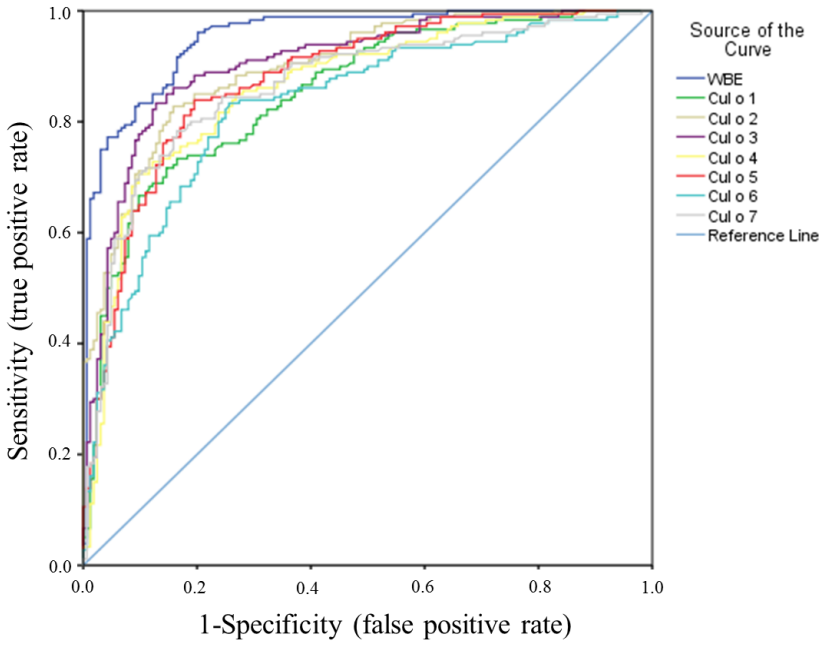

Figure 1: ROC curves of IgE ELISA against *Obsoletus complex* whole body extract (WBE) and recombinant allergens (Cul o 1 – Cul o 7) on sera samples of 180 insect bite hypersensitivity (IBH) affected and 164 healthy horses

control group ranged from 15-24% among the recombinant allergens.

Nearly half (46%) of the IBH affected horses had positive test results with all seven allergens, followed by 21 % of these horses reacting to 6 allergens and 16 % to five allergens (Figure 3). Only 1% of IBH affected had no positive test results with any of the allergens. In contrast, most healthy horses (61%) had a negative test result with all of the allergens, followed by 21% of these horses reacting to 1 allergen and 11% to 2 allergens. Only 1 % of the healthy horses had positive test results with all 7 allergens.

Western blot analysis

Four IBH affected horses that were tested in the ELISAs showed high OD_{450nm} values with WBE, but did not have IgE reactions with any of the 7 allergens or a relatively low OD_{450nm} value for just one allergen, suggesting that other allergens present in the WBE are responsible for the high signal in this ELISA. Western blot analysis using Obsoletus complex WBE and a pool of all 7 recombinant allergens was performed on these horses to evaluate the presence of other allergens in the extract. Sera of all four horses showed IgE-

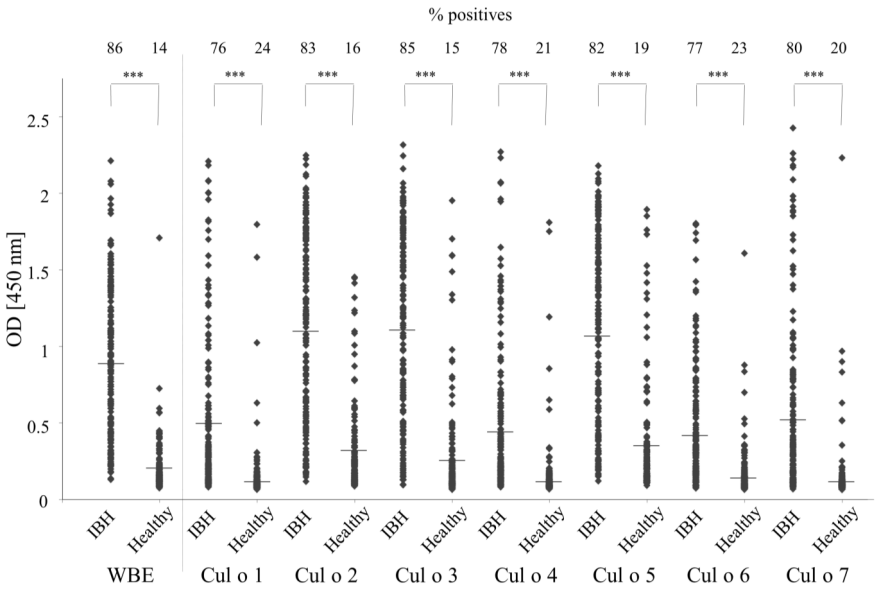

Figure 2: IgE levels presented as OD_{450nm} values against Obsoletus complex whole body extract (WBE) and 7 recombinant allergens (Cul o 1 – Cul o 7) from sera of 180 insect bite hypersensitivity (IBH) affected and 164 healthy horses. Individual values are presented as squares. The horizontal lines crossing each group of squares represent the median of the measurements. The dotted horizontal lines crossing the two groups (IBH and healthy) within each ELISA represent the cut-off determined by the ROC curves. Lines connecting two groups denote a statistically significant difference with the *p* value indicated on top. The percentage of positive tested horses for each allergen or WBE is indicated on top of the figure.

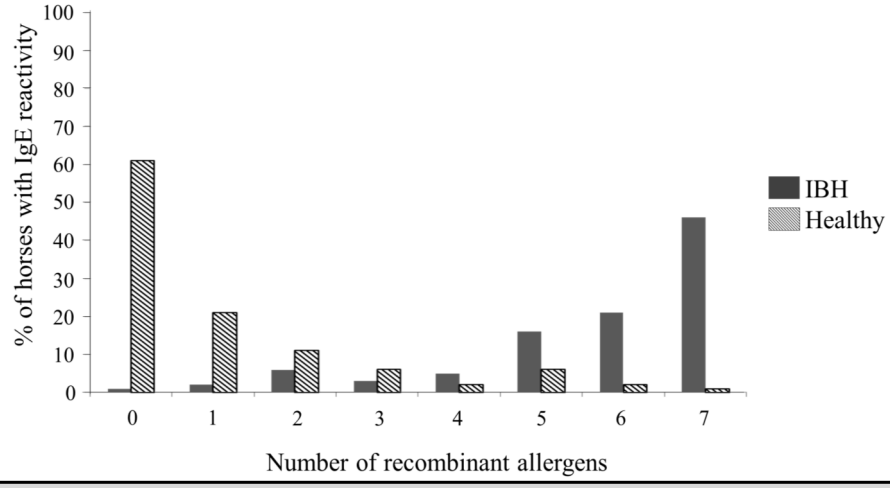

Figure 3: Percentages (y-axis) of insect bite hypersensitivity (IBH) affected and healthy horses and the number of different recombinant allergens to which they react (x-axis). IgE reactivity is defined as an OD_{450nm} values in the IgE ELISAs above the cut-off levels as determined by the ROC curves (Fig. 1).

binding with a protein band of around 15 kDa in WBE and three of the horses also to a band of 20 kDa (Fig. 4). Two horses also showed IgE reactivity with some higher MW proteins present in the WBE and with Cul o 4 in the pool of recombinant allergens, based on its MW.

IgEs ELISAs with combination of allergens

The highest number of IBH affected horses could be discriminated from healthy horses with the allergens Cul o 2, Cul o 3 and Cul o 5. However, Cul o 2 and Cul o 3 showed an extensive overlap in positive tested horses. Therefore two additional ELISA's were performed with a combination of either Cul o 2 with Cul o 5 and Cul o 7 (Combi-1) or Cul o 3 with Cul o 5, Cul o 7 and additionally Cul o 1 (Combi-2). Cul o 1 was included, because this allergen was found to result in increased number of positively tested IBH affected horses.

A pool of equal volumes of sera of 17 IBH affected horses with previously determined high *Culicoides* allergen-specific IgE levels was used as a reference serum to generate a standard curve with 2x dilution steps, using a start dilution of 1:2.5 (serum:sample buffer).

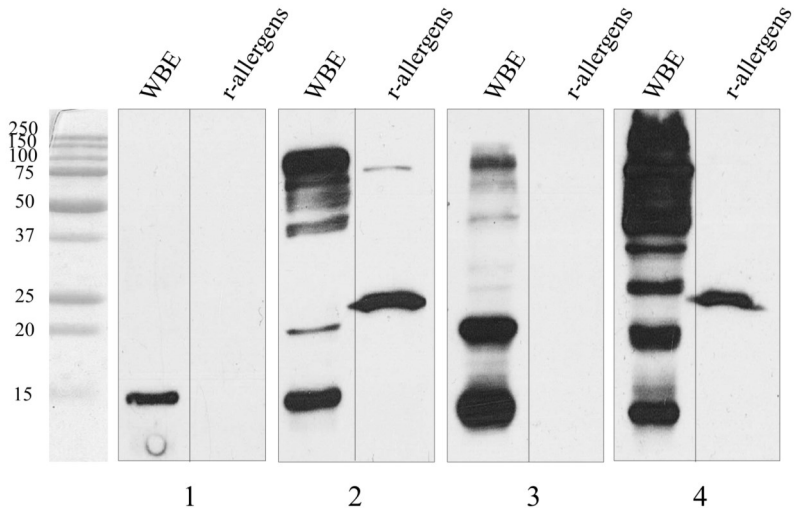

Figure 4: Western blot analysis of 4 IBH affected horses (1-4) using *Obsoletus complex* whole body extract (WBE, 20 µg) or a pool of all recombinant allergens (r allergens = Cul o 1 – Cul o 7, 100 ng each) Proteins were separated on 15 % SDS-PAGE gels and transferred to nitrocellulose membranes. Binding of IgE from horse sera was detected with an anti-equine IgE mouse mAb and HRP goat anti-mouse IgG. The molecular weight marker (M) is indicated on the left in kDa.

For the combination ELISA's the OD_{450nm} values of tested samples were converted to the titer of the corresponding dilution of the reference serum, run on the sample ELISA plate, that resulted in the same OD_{450nm} value. The highest reference serum dilution that still had an $OD_{450\,nm}$ above the cut-off value ($OD_{450\,nm}$ = 0.1, average of wells without serum incubation + 3 SD) was assigned a titer of 1. This was obtained at a 1280x dilution of the reference serum, which sets the titer of the undiluted reference serum at 1280. This reference serum can be used in future ELISAs to convert OD_{450nm} values to IgE titers of the standard dilutions to avoid test result differences caused by environmental or other factors influencing ELISA results.

A high test accuracy from the ROC curves was observed for both combi-1 (Cul o 3, 5, 7) and combi-2 (Cul o 1, 2, 5, 7), which came close to WBE, with slightly better results for combi-1 (Figure 5, Table 1). OD_{450nm} values were expressed as $\log_2$ IgE titres obtained from the standard reference serum. IgE levels of IBH affected horses were significantly higher (i.e. corresponded to a lower dilution of the reference sera pool) than those of

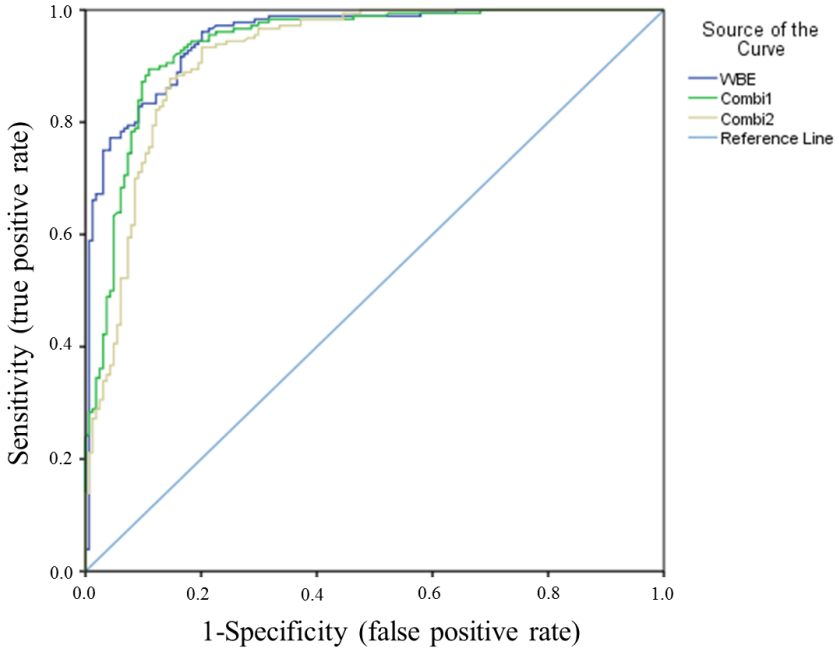

Figure 5: ROC curves of IgE ELISA against *Obsoletus complex* whole body extract (WBE) and a combination of recombinant allergens: combi-1 (Cul o 3, Cul o 5, Cul o 7) and combi-2 (Cul o 1, Cul o 2, Cul o5, Cul o 7) on sera samples of 180 insect bite hypersensitivity (IBH) affected and 164 healthy horses.

healthy horses (Figure 6). The highest sensitivity and specificity (89 % and 89 %) was obtained with combi-1.

IgE levels in summer versus winter samples

For 14 IBH affected horses and 11 healthy horses sera samples of both IBH-season (August) and off season (March) were available. To test the applicability of the combi ELISAs to detect IBH in samples obtained in the off season, an additional ELISA was performed on sera of these horses. WBE was included as a positive control, because we previously reported no significant difference between OD_{450nm} values in serum obtained in off season compared to IBH-season [26]. A significant decrease in the IgE titer was

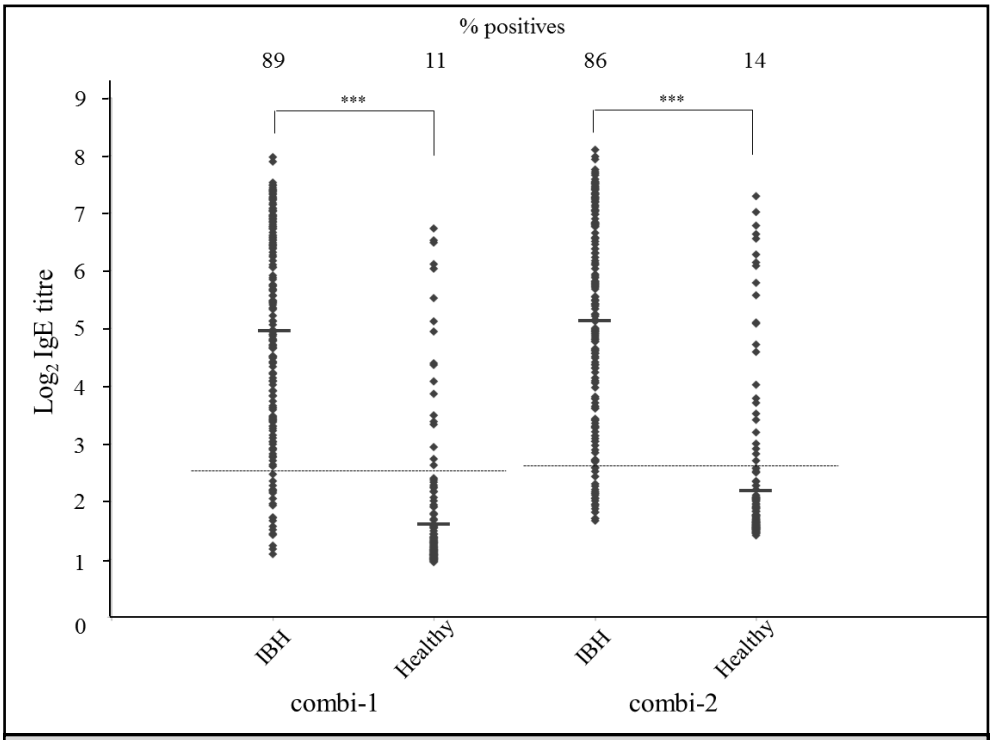

Figure 6: IgE levels presented as log_2 IgE titers the standard (a pool of 17 insect bite hypersensitivity (IBH) affected horses), against a combination of recombinant allergens: combi-1 (Cul o 3, Cul o 5 and Cul o 7) and combi-2 (Cul o 1, Cul o 2, Cul o 5 and Cul o 7) from sera of 180 IBH affected and 164 healthy horses. Individual values are presented as squares. The horizontal lines crossing each group of squares represent the median of the measurements. The dotted horizontal lines crossing the two groups (IBH and healthy) within each ELISA represent the cut-off determined by the ROC curves. Lines connecting two groups denote a statistically significant difference with the *p* value indicated on top. The percentage of positive tested horses for each ELISA is indicated on top of the figure.

observed with off season sera from IBH affected horses for both combi-1 and combi-2 ELISA, but not for WBE (Figure 7). Although a significant decrease was observed for the whole IBH affected group, for some horses there was only a small decrease in the IgE titer observed during off season. Horses showing a clear decrease in IgE titer for combi-1, also showed this for combi-2. For WBE, some horses showed an increase in the IgE titer during the off season. Although not significant, an overall small decrease in the average of the off season samples was observed for healthy horses as well.

Predicted probability of IBH-status related to IgE titers of combi-1 ELISA

Because best results were obtained with combi-1 ELISA, logistic regression was performed to predict the probability of the IBH-status (i.e. IBH affected or healthy) in

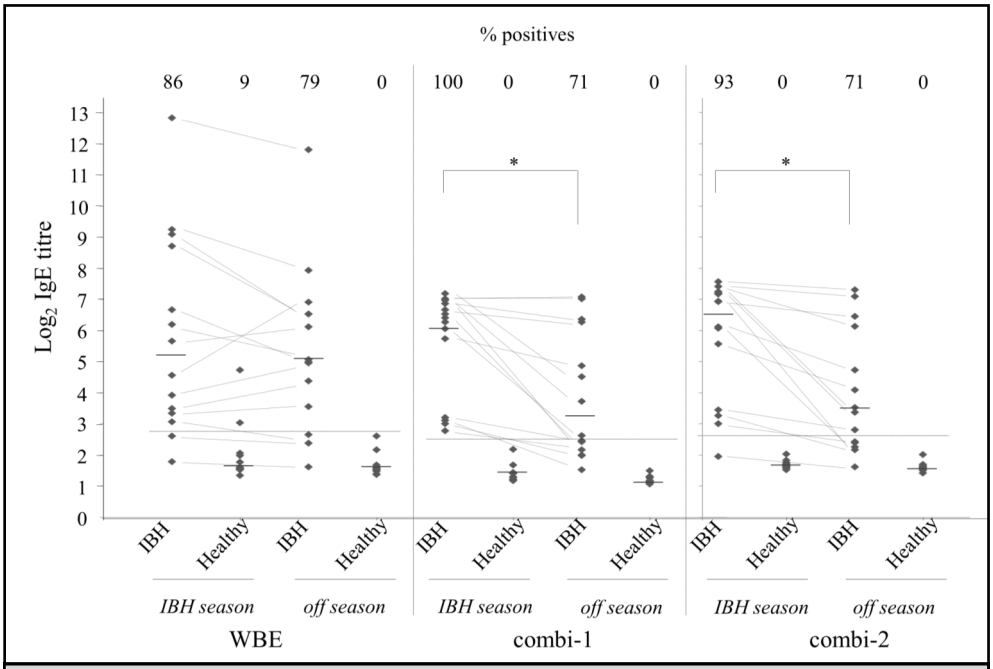

Figure 7: IgE levels presented as $\log_2$ IgE titers of the standard (a pool of 17 insect bite hypersensitivity (IBH) affected horses) against *Obsoletus complex* whole body extracts (WBE) and combinations of recombinant allergens: combi-1 (Cul o 3, Cul o 5 and Cul o 7); combi-2 (Cul o 1, Cul o 2, Cul o 5 and Cul o 7) from sera collected during IBH season and off season of 14 IBH affected and 11 healthy horses. The horizontal lines crossing each group of squares represent the median of the measurements. The dotted horizontal lines crossing the four groups (IBH and healthy for both IBH season and off season) within each ELISA represent the cut-offs determined by ROC curves. Lines connecting two squares denote the measurements of the same horse. Lines connecting two groups denote a statistically significant difference with the *p* value indicated on top. The percentage of positive tested horses for each allergen or WBE is indicated on top of the figure.

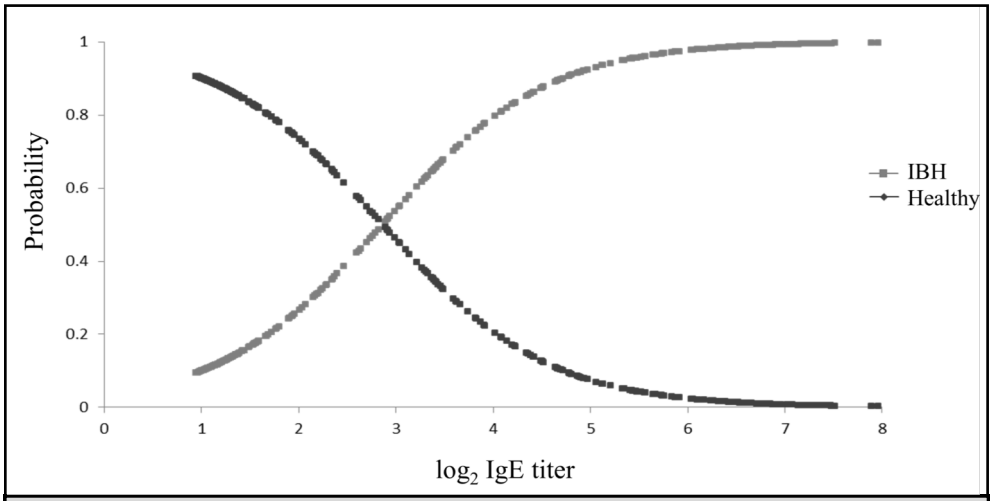

Figure 8: Estimated probability to be IBH affected or healthy in relation to the log_2 IgE titer against combi-1 (Cul o 3, Cul o 5, Cul o 7). Determined by logistic regression analysis of the obtained ELISA data.

relation to IgE titres of combi-1. Figure 8 shows the predicted probabilities of both IBH-outcomes related to log_2 IgE titres. With values corresponding to a log_2 IgE titre of approximately 5.0 or higher the probability of being IBH affected is 90-100 %, whereas values corresponding to a log_2 IgE titre of approximately 1.8 or lower the probability of being healthy is 80 – 90 %. At the inflection point at a log_2 IgE titre of approximately 3 the probability of being IBH affected or healthy is equal.

Discussion

Insect Bite Hypersensitvity is an IgE mediated allergic skin disease in horses caused by the bites of *Culicoides* species [27]. We previously reported a *Culicoides* species from the Obsoletus complex to be a particular important species to be used for reliable diagnosis of IBH in countries where this is the main species found feeding on horses [11].

Although we previously described an IgE ELISA with high sensitivity and specificity using Obsoletus complex WBE, to date there is no such test available using recombinant allergens. The use of recombinant allergens would provide an attractive alternative for WBE, because with these recombinant allergens insects do not need to be collected from the wild. Moreover, they are easy to standardize and testing on the individual recombinant allergens will enable component-resolved diagnosis of IBH in horses and tailor-made

immunotherapy. In this study we evaluated the IgE reactivity to WBE, 7 individual Obsoletus complex recombinant allergens and a combination of few allergens in an IgE ELISA for the diagnosis of IBH in horses.

We found the sensitivity and specificity of the WBE IgE ELISA to be somewhat lower than previously reported [11]. The same horses were included in this study, but additional horses, selected by the same criteria, were included as well. More healthy horses of this additional group were found to be sensitised against allergens. Furthermore, more clinically confirmed IBH affected horses of the additional group showed lower IgE levels, below the cut-off value, against WBE. However, most of these IBH affected horses did show IgE reactivity with one or more of the single allergens, which suggest that clinical symptoms of these horses are caused by IBH and not some other skin condition. The positive tested healthy horses used in this study did not show clinical symptoms, but some of these horses did have clear IgE reactivity against several recombinant allergens as well as against WBE. The IgE ELISA described in this study only detects the binding capacity to IgE and does not determine whether the allergens can cause cross-linking of FceR1 bound IgE antibodies on basophils and mast cells, which is necessary for symptoms to occur. Furthermore, horses have been reported before to be sensitised to *Culicoides* allergens without showing clinical symptoms [28]. Why these animals do not develop clinical allergy despite their sensitisation is still unknown, but regulatory immune mechanisms might play a role in suppressing the immune reactions, as regulatory T cells have shown to suppress mast cell activation in mice [29] and there is evidence that suggests that healthy horses have a higher number of regulatory T cells compared to IBH affected horses [30, 31].

A combination of only three allergens, combi-1 (Cul o 3, Cul o 5 and Cul o 7), resulted in a sensitivity and specificity (89% and 89%) slightly higher than that obtained with WBE. The corresponding probability curve can be used for individual tested horses to determine for a certain titer the probability that the tested horse has IBH.

Interestingly, nearly 90% of IBH affected horses showed a positive test result with four or more recombinant allergens, whereas only 11% of healthy horses did, suggesting that a larger number of IgE reactive allergens implies a higher chance of truly having IBH. IBH affected horses that did not have IgE reactivity with one of the allergens, but did have IgE reactivity with WBE, specifically reacted with proteins of 15 and 20 kDa. A protein of 20

kDa was already previously indicated to be an important IgE-binding protein in IBH affected horses [11]. The identity of these additional putative allergens is currently under investigation.

We compared IgE levels of sera obtained in the IBH season with those of sera from the same horses during the off season. As was previously observed with the WBE IgE ELISA [26], again there was no significant difference between these "in-season" and "off season" sera. Individuals with hay-fever have also been reported to have high titers of pollen-specific IgE in the serum even out of season [32]. However when we performed the ELISA with a combination of three or four allergens, we did find a significant decrease of IgE reactivity during off season, although this decrease was not consistent for all horses. A decrease in the test sensitivity during off season was also observed in a histamine release test performed by [33].

In comparison to for instance humans, horses have high levels of IgE [34]. The discrepancy between the WBE and the recombinant IgE ELISAs may be due to different amounts of coated allergens. The total protein concentrations used for the WBE and combi-1 ELISAs were 10 ug/ml and 6.25 ug/ml, respectively. However, in the case of the combi-1 ELISA this consisted all of (recombinant) allergens. In contrast, only a small fraction of all the proteins in the WBE will actually consists of allergens, which have to compete with all the other proteins in the WBE for binding to the well. Therefore, in comparison to the recombinant allergens ELISAs, only a small amount of each individual allergen will become coated in the WBE ELISA. A drop in IgE titer in the off season may therefore not be directly reflected in a drop the OD450 value in the WBE ELISA, because the specific IgE might still be in excess of the amount of coated allergen. Coating with recombinant allergens can result in a drop of the OD450 value, because allergen-specific IgE in serum is much less likely to be present in excess of much higher amounts of coated allergens. Nevertheless, we were still able to detect high IgE reactivity off season in most IBH affected horses, even with recombinant allergens and therefore it seems that the production of IgE is maintained even in the absence of *Culicoides* insects or that IgE in horses has a long half-life. Serum levels of healthy horses also seemed to decrease during off season. Therefore, a lower cut-off level for the IgE ELISA may be more appropriate for testing samples collected in the winter season.

Conclusions

A combination of 3 recombinant Obsoletus complex allergens (Cul o 3, 5 and 7) in a single IgE ELISA is able to diagnose IBH with a high sensitivity and specificity. The individual allergens Cul o 1 – Cul o 7 can be used to develop tailor-made immunotherapy as most of the IBH affected horses show sensitivity with at least one of these allergens.

Acknowledgements

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CHAPTER

7

Factors associated with *Culicoides obsoletus* specific IgE levels in two horse breeds in The Netherlands

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*Equal contribution

Submitted paper

Abstract

Insect bite hypersensitivity (IBH) is a common allergic disease in horses caused by bites of *Culicoides* spp. In The Netherlands, *Culicoides obsoletus* is the most important species involved in IBH. The aim of this study was to identify and quantify the association between several factors and IgE levels (OD_{450nm} values) against *C. obsoletus* whole body extract and 7 recombinant allergens. Data from 143 Icelandic horses and 177 Shetland ponies were analyzed using multivariable models. Also, the relation between IgE levels and severity of symptoms of IBH affected horses was examined. Positive correlations (0.06 – 0.44) were found between *C. obsoletus* specific IgE levels and severity of symptoms. IBH status (case or control), breed and the interaction between status and breed were significantly associated with log transformed IgE levels against several *C. obsoletus* allergens. Cases had significantly higher IgE levels compared to controls. Differences in IgE levels between cases and controls were largest in Icelandic horses. Shetland pony controls had significantly higher IgE levels compared to Icelandic horse controls, while differences in IgE levels between Shetland pony cases and Icelandic horse cases were not significant. Other factors associated with IgE levels were age, month of scoring, interaction between IBH status and month of scoring and degree of itchiness. In conclusion, severity of symptoms and IgE levels in IBH affected horses against several *C. obsoletus* allergens appeared to be related. Incorporation of the factors associated with *C. obsoletus* specific IgE levels in horses may further improve the interpretation and accuracy of IgE ELISA test results.

Introduction

Insect bite hypersensitivity (IBH) is a common allergic disease observed in many horse breeds worldwide and is mainly caused by bites of *Culicoides* spp. Prevalence varied greatly, from 7.5% in Shetland ponies to 18.2% in Friesian horses in The Netherlands [1]. Insect bite hypersensitivity is accompanied with a severe itch, which results in self-inflicted trauma. Common clinical symptoms are hair loss, bald spots, thickening of the skin, crusting, scaling, and sometimes open wounds and secondary infections [2]. At present, fully effective measures to prevent or cure IBH are unavailable [3]. Welfare and economic value of affected horses is reduced due to discomfort and disfiguration and severely affected horses sometimes need to be euthanized [4, 5].

So far, diagnosis of IBH is based on observation of clinical symptoms combined with the typical seasonality and recurrence of symptoms in subsequent years. Reduced itch after elimination of exposure to *Culicoides* spp. (e.g. stabling, anti-insect blanket) confirms the diagnosis, while other conditions causing itch need to be ruled out [6]. *In vivo* and *in vitro* test results like release of specific allergic mediators [7, 8], wheal size in intradermal testing [9, 10] *Culicoides* spp. specific IgE or IgG determined by ELISA [9, 11] can contribute to the accuracy of IBH diagnosis. We recently reported the characteristics of an IgE ELISA to discriminate healthy horses from IBH affected horses using *C. obsoletus* whole body extract (WBE) with a sensitivity of 93.2% and specificity of 90.0% [11]. Additionally, 7 recombinant allergens of *C. obsoletus* were identified and produced as recombinant proteins and tested for their usefulness to diagnose IBH by ELISA as a replacement for WBE [12, 13].

Various factors might affect *in vivo* and *in vitro* test results. For instance, Baselgia et al. [7] found a significantly higher sulphidoleukotriene release in IBH affected geldings compared to affected mares. In the same study it was reported that during time of exposure the proportion of positive test results in IBH affected horses (97%) was significantly higher compared to half a year after exposure (74%). However, effects of factors on diagnostic test results often are not determined and accounted for. Knowledge of these factors might further improve the interpretation of the IgE ELISA test results and thereby the differentiation of cases from controls. The aim of our research was to identify and quantify factors associated with serum IgE levels against *C. obsoletus* WBE and

recombinant allergens determined by ELISA, in Shetland ponies and Icelandic horses in The Netherlands.

Materials and methods

Study design and data collection

Both Shetland ponies and Icelandic horses in The Netherlands were selected according to a case-control design described in detail by Schurink et al. [14, 15]. In short, cases and controls were matched on various factors and selected following a strict protocol to increase phenotype reliability (i.e. true healthy or IBH affected).

Cases were defined as horses with IBH clinical symptoms, whereas controls were free of symptoms (despite exposure to *Culicoides* spp.). An inquiry (for details see [15]) was conducted to gain knowledge about history and management of IBH. Clinical symptoms were scored by an experienced veterinarian using a score sheet. Severity of hair loss, hyperkeratosis, scaling, crusting, excoriation, open wounds and secondary infections on the crest, base of the tail, hindquarters, abdomen, shoulder, neck and head was separately graded in four categories from mild (category 1) to profound (category 4). The most severe symptom on any location was defined as the severity score for IBH clinical symptoms of a horse. Blood was collected by jugular venipuncture and allowed to cloth. Serum was frozen not later than 24 h after collection and was stored in eppendorf tubes at -20 °C until use. Blood sample collection was approved by the Board on Animal Ethics and Experiments from Wageningen University (experiments 2009055 and 2010109).

Data included observations on 143 Icelandic horses and 177 Shetland ponies (Table 1). Cases and controls were successfully matched on various factors (Table 1).

Preparation of C. obsoletus whole body extract and recombinant allergens

Culicoides spp. were aspirated from a horse wearing an anti-insect blanket using a pooter. Random samples of the aspirated *Culicoides* spp. were identified to be *C. obsoletus* based on wing pattern. WBE was prepared as previously described by van der Meide et al. (2012) [11]. Seven *C. obsoletus* allergens (Cul o 1 – Cul o 7, GenBank accession numbers KC339671 – KC339677) were recently identified by similarity searches with sequences coding for *C. nubeculosus* and *C. sonorensis* allergens [16-18] on a *C. obsoletus* specific

transcriptome database and produced as recombinant proteins [12].

C. obsoletus specific IgE levels

IgE levels – expressed as OD_{450nm} values – against WBE and recombinant allergens Cul o 1 through Cul o 7 (single allergens or a combination of allergens; Combi-1 contained Cul o 3, 5 and 7, and Combi-2 contained Cul o 1, 2, 5 and 7) were determined by ELISA as previously described by van der Meide et al. [11, 13].

Statistical analysis

Log transformation. IgE levels against *C. obsoletus* allergens were log transformed (referred to as e.g. ln_WBE), as preliminary analysis showed presence of heterogeneous variance and deviations from a normal distribution.

Table 1: Data description.

	Icelandic horses			Shetland ponies		
Variable	Case	Control	Total	Case	Control	Total
Number	72	71	143	91	86	177
Sex						
Female	50	42	92	91	86	177
Male	22	29	51	-	-	-
Mean age, yr. (SD)	13.2 (6.0)	12.4 (5.8)	12.8 (5.9)	7.2 (4.5)	8.0 (4.3)	7.6 (4.4)
Coat colour						
Black	16	11	27	48	45	93
Bay	13	9	22	3	5	8
Chestnut	8	14	22	19	22	41
Other	35	37	72	21	14	35
Pedigree						
Number of sires	57	60	95	76	77	118
Year of collection						
2009	-	-	-	71	67	138
2010	72	71	143	20	19	39
Month of collection						
September	21	29	50	45	46	91
October[a]	51	42	93	46	40	86

[a] In total 7 Shetland ponies were scored in the first week of November, but grouped with the ponies

Breed differences in severity of symptoms. Ordinal logistic regression (LOGISTIC procedure SAS® 9.2; SAS Institute Inc. Cary, NC) was used to test whether severity score differed between breeds.

*Correlation between IgE levels and severity of symptoms***.** To test whether severity of IBH clinical symptoms of cases was associated with their IgE levels, Spearman correlation coefficients were calculated (within breed) between severity score and log transformed IgE levels (CORR procedure SAS® 9.2; SAS Institute Inc. Cary, NC). Also, correlations among IgE levels against various *C. obsoletus* specific allergens were calculated.

Quantification of factors associated with IgE levels. A general linear model (GLM procedure SAS® 9.2; SAS Institute Inc., Cary, NC) was used for the analysis of factors associated with log transformed IgE levels. Horses from different breeds might respond differently to specific *C. obsoletus* allergens. To investigate this, the factors breed, IBH status (case or control) and their interaction were tested for an association with all *C. obsoletus* allergens using data from both Shetland ponies and Icelandic horses simultaneously. Identification and quantification of other factors associated with IgE levels in a multivariable breed-specific model was performed only on the most promising allergens: WBE, Combi-1 and Cul o 2. WBE was included because it contains a mixture of all proteins and is therefore likely to contain most or even all relevant allergens associated with IBH. Combi-1 resulted in the best sensitivity and specificity obtained with a mixture of recombinant allergens (van der Meide et al., unpublished data) and was therefore chosen as an alternative for WBE to be included in this study. Finally, Cul o 2 was one of the best single recombinant allergens to discriminate a large number of cases from controls [13]. The factors that were tested for an association with ln_WBE, ln_Combi-1 and ln_Culo2 were IBH status, month and year of scoring, age (both linear and 2nd order polynomial), coat color, withers height category (mini, small, middle, tall) and inbreeding coefficient for Shetland ponies. For Icelandic horses, IBH status, month of scoring, age, coat color, import from Iceland and sex were used as independent variables. Factors with P <0.25 in a univariable model were further analyzed in a multivariable model. For this, a backward elimination procedure was followed by deleting the least significant factor. Confounding was considered present in case of a change in β-coefficient estimate of another factor greater than 25% or a change greater than 0.1 when values of estimated β-coefficients were between -0.4 and 0.4. Two-way interactions

between all factors were tested.

Not all owners answered all inquiry questions. The available information per case varied, while the number of cases with complete information was limited (n = 23 Icelandic horses and 65 Shetland ponies). Analysis of inquiry information using a multivariable model would decrease power. Answers to inquiry questions were therefore tested separately as independent variables in breed-specific univariable models using IBH cases only. Factors that were tested for an association with *C. obsoletus* specific IgE levels were degree of itchiness, application of anti-insect blanket, age at start of IBH, number of seasons affected, month in which symptoms appear and month in which symptoms regress.

Results

Descriptive statistics C. obsoletus specific IgE levels and severity of symptoms

Mean and standard deviation of untransformed IgE levels against *C. obsoletus* allergens are presented in Table 2. Mean IgE level against *C. obsoletus* allergens showed large variations between breeds and also between cases and controls (Table 2).

In Shetland ponies, more severe symptoms were observed compared to Icelandic horses (Table 2). Ordinal logistic regression showed that Shetland ponies had a 1.9 times higher probability (95% confidence interval: 1.1 – 3.4 with $P = 0.03$) to have more severe clinical symptoms compared to Icelandic horses (data not shown).

Correlation between IgE levels and severity of symptoms

In Shetland ponies, correlations between severity of IBH clinical symptoms and log transformed *C. obsoletus* specific IgE levels ranged from 0.16 to 0.44; in Icelandic horses correlations ranged from 0.06 to 0.39 (Table 3). In Shetland ponies, correlations between severity of symptoms and IgE levels were significantly larger than 0 (more severe symptoms, more IgE), except for Cul o 1. However, in Icelandic horses, correlations were only significantly positive for Cul o 1, Cul o 7, Combi-1 and Combi-2.

Correlations among IgE levels against various *C. obsoletus* specific allergens were moderate to high and highly significant ($P < 0.0001$), ranging from 0.61 to 0.97 in Icelandic horses and from 0.42 to 0.98 in Shetland ponies (data not shown). However, individual differences in response to the various *C. obsoletus* specific allergens were

Table 2: Distribution of severity of IBH clinical symptoms in cases and mean IgE levels, expressed as OD_{450nm} values, against various allergens in Icelandic horses and Shetland ponies.

	Icelandic horses			Shetland ponies		
Variable[a]	Case	Control	Total	Case	Control	Total
Severity of symptoms, n (%)						
Mild (1)	9 (12.5%)	-	-	11 (12.1%)	-	-
Moderate (2)	14 (19.5%)	-	-	10 (11.0%)	-	-
Severe (3)	34 (47.2%)	-	-	34 (37.4%)	-	-
Profound (4)	15 (20.8%)	-	-	15 (39.5%)	-	-
OD_{450nm} values, mean (SD)						
WBE	0.98 (0.53)	0.18 (0.21)	0.58 (0.57)	0.81 (0.44)	0.22 (0.12)	0.52 (0.44)
Cul o 1	0.54 (0.56)	0.14 (0.27)	0.34 (0.48)	0.45 (0.46)	0.16 (0.12)	0.31 (0.37)
Cul o 2	1.09 (0.62)	0.22 (0.24)	0.66 (0.64)	1.18 (0.62)	0.35 (0.30)	0.78 (0.65)
Cul o 3	1.20 (0.63)	0.25 (0.31)	0.73 (0.69)	1.09 (0.62)	0.29 (0.39)	0.70 (0.65)
Cul o 4	0.46 (0.50)	0.15 (0.28)	0.30 (0.44)	0.48 (0.46)	0.17 (0.15)	0.33 (0.38)
Cul o 5	1.06 (0.60)	0.26 (0.28)	0.66 (0.62)	1.21 (0.56)	0.43 (0.44)	0.83 (0.64)
Cul o 6	0.42 (0.42)	0.14 (0.20)	0.28 (0.35)	0.47 (0.34)	0.19 (0.15)	0.33 (0.30)
Cul o 7	0.60 (0.59)	0.18 (0.31)	0.39 (0.51)	0.49 (0.55)	0.11 (0.05)	0.30 (0.43)
Combi-1	1.42 (0.59)	0.28 (0.37)	0.85 (0.75)	1.47 (0.62)	0.35 (0.44)	0.92 (0.77)
Combi-2	1.34 (0.66)	0.30 (0.43)	0.82 (0.76)	1.44 (0.63)	0.41 (0.48)	0.94 (0.77)

[a] Severity score of IBH clinical symptoms was defined as the most severe symptom on any location, graded 1 – 4. Mean (standard deviation) of untransformed IgE levels against *C. obsoletus* whole body extract (WBE), Combi-1 (a combination of recombinant allergens Cul o 3, 5 and 7) and Combi-2 (a combination of recombinant allergens Cul o 1, 2, 5 and 7). Combi-1 and Combi-2 was missing for 6 controls (all Shetland ponies) and 9 cases (7 Shetland ponies and 2 Icelandic horses).

Table 3: Spearman correlation coefficients between severity of IBH clinical symptoms and log transformed IgE levels against several allergens within breed.

	Severity of symptoms	
Trait[a]	Icelandic horses	Shetland ponies
ln_WBE	0.06^{ns}	0.31*
ln_Culo1	0.30*	0.16^{ns}
ln_Culo2	0.15^{ns}	0.44**
ln_Culo3	0.18^{ns}	0.41**
ln_Culo4	0.19^{ns}	0.30*
ln_Culo5	0.19^{ns}	0.41**
ln_Culo6	0.14^{ns}	0.30*
ln_Culo7	0.39*	0.24*
ln_Combi-1	0.33*	0.30*
ln_Combi-2	0.39*	0.34*

[a] Severity of IBH clinical symptoms was defined as the most severe symptom on any location, graded 1 – 4. Log transformed IgE levels (expressed as OD_{450nm} values) against *C. obsoletus* whole body extract (WBE), Cul o 1 – Cul o 7, a combination of recombinant allergens (Combi-1) containing Cul o 3, 5 and 7 and a combination of recombinant allergens (Combi-2) containing Cul o 1, 2, 5 and 7.
^{ns} $P > 0.05$
* $0.0001 \leq P < 0.05$
$^{**}P < 0.0001$

observed (data not shown), which reflect differences between horses in sensitization against allergens.

Factors associated with C. obsoletus specific IgE levels

IgE levels (mean of both cases and controls) were higher in Shetland ponies compared to Icelandic horses for all investigated allergens except Cul o 7 (Table 4). The difference in IgE levels between Shetland ponies and Icelandic horses was significant for Cul o 2, Cul o 4 to 7 and Combi-2 (Table 4). Differences in IgE levels between cases and controls against all allergens were highly significant across and within breeds (Table 4). The interaction between breed and IBH status was significant for WBE, Cul o 1, 2 and 5. These interactions were due to Shetland pony controls having significantly higher IgE levels compared to Icelandic horse controls (Table 4), while differences in IgE levels between cases of both breeds were not significant.

Table 4: LS-means and significance (*P*-value, italicized) for several *C. obsoletus* specific IgE levels in Icelandic horses and Shetland ponies of the factors breed and IBH status[a].

	IgE levels, expressed as OD_{450nm} values									
Factor	WBE	Cul o 1	Cul o 2	Cul o 3	Cul o 4	Cul o 5	Cul o 6	Cul o 7	Combi-1	Combi-2
Breed	*0.55*	*0.18*	*0.0005*	*0.94*	*0.02*	*<0.0001*	*0.0001*	*0.08*	*0.11*	*0.006*
Icelandic horses	0.34	0.19	0.38	0.41	0.18	0.40	0.18	0.21	0.47	0.46
Shetland ponies	0.36	0.21	0.51	0.41	0.21	0.57	0.24	0.18	0.54	0.58
IBH status	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*
Case	0.74	0.32	0.90	0.90	0.32	0.93	0.32	0.33	1.27	1.19
Control	0.17	0.12	0.21	0.18	0.12	0.24	0.13	0.11	0.20	0.23
Breed × IBH status	*0.006*	*0.005*	*0.03*	*0.12*	*0.21*	*0.04*	*0.27*	*0.46*	*0.07*	*0.06*
Icelandic horse cases	0.81^{b}	0.34^{b}	0.85^{b}	0.97	0.31	0.84^{b}	0.29^{b}	0.37	1.27	1.14^{b}
Icelandic horse controls	0.14^{c}	0.10^{c}	0.17^{c}	0.17	0.10	0.19^{c}	0.11^{c}	0.12	0.18	0.19^{c}
Shetland pony cases	0.67^{b}	0.30^{b}	0.84^{b}	0.84	0.33	1.03^{b}	0.36^{b}	0.30	1.26	1.23^{b}
Shetland pony controls	0.19^{d}	0.14^{d}	0.19^{d}	0.19	0.14	0.32^{d}	0.16^{d}	0.11	0.23	0.28^{d}
R^2, coefficient of determination	0.62	0.33	0.51	0.52	0.33	0.50	0.32	0.33	0.62	0.57

[a] Analyses are based on log transformed IgE levels, but LS-means were transformed back to OD_{450nm} values for interpretation purposes. LS-mean IgE levels against *C. obsoletus* whole body extract (WBE), a combination of recombinant allergens (Combi-1) containing Cul o 3, 5 and 7 and a combination of recombinant allergens (Combi-2) containing Cul o 1, 2, 5 and 7.

Several other factors were tested in breed-specific models for a potential association with IgE levels against WBE, Cul o 2 and Combi-1 only (Table 5). In Shetland ponies, significantly decreased IgE levels against Cul o 2 were found with age until 15 years-of-age, as indicated by a positive coefficient for the quadratic term (Table 5). The difference between cases and controls regarding IgE levels against Combi-1 was larger in September (1.17) compared to October (0.89) (Table 5). In Icelandic horses, IgE levels against WBE significantly increased with age and IgE levels against Cul o 2 were significantly higher in October compared to September (Table 5). In cases, IgE levels against WBE and Cul o 2 were significantly higher in October compared to September, whereas differences in IgE

Table 5: LS-means and significance (*P*-value, italicized) of several factors for IgE levels against WBE, Cul o 2 and Combi-1 in Icelandic horses and Shetland ponies[a]

	Shetland ponies			Icelandic horses		
Factor	WBE	Cul o 2	Combi-1	WBE	Cul o 2	Combi-1
IBH status	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*
Case	0.67	0.93	1.26	0.75	0.74	1.20
Control	0.19	0.28	0.23	0.15	0.17	0.18
Month of scoring	ns	ns	*0.597*	*0.241*	*0.017*	ns
September			0.53	0.31	0.31	
October			0.56	0.35	0.42	
IBH status × month of scoring	ns	ns	*0.041*	*0.031*	*0.004*	ns
Case September			1.37	0.62[b]	0.53[b]	
Case October			1.16	0.89[c]	1.04[c]	
Control September			0.20	0.15[d]	0.18[d]	
Control October			0.27	0.14[d]	0.17[d]	
Age	ns	*0.007, 0.041*	ns	*0.016*	ns	ns
Linear term		-0.0397		0.0029		
Quadratic term		0.0014				
R^2	0.55	0.48	0.58	0.70	0.60	0.68

[a] Analyses are based on log transformed IgE levels, but LS-means were transformed back to OD_{450nm} values for interpretation purposes. LS-mean IgE levels against *C. obsoletus* whole body extract (WBE), Cul o 2 and a combination of recombinant allergens (Combi-1) containing Cul o 3, 5 and 7.
[b, c, d] Values with different superscripts differ significantly (P <0.10).
ns = not significant, several non-significant factors (month of scoring) are presented, as the interaction between IBH status and month of scoring proved to be significant.

levels between months in controls were small and not significant (Table 5). Confounding of factors was not observed. Coefficients of determination were higher in Icelandic horses compared to Shetland ponies (Table 5).

Answers to inquiry questions were tested for their association with IgE levels against WBE, Cul o 2 and Combi-1 in breed-specific univariable models using IBH cases only. In Shetland ponies, degree of itchiness was positively associated with IgE levels against Cul o 2 ($P = 0.018$). No such association was present with IgE levels against Combi-1, while the association with IgE levels against WBE showed a *P*-value of 0.082. In Icelandic horses, degree of itchiness was not associated with IgE levels. Shetland pony cases with anti-insect blanket (n = 23) tended to have higher IgE levels compared to Shetland pony cases without anti-insect blanket ($P = 0.13$ for WBE and $P = 0.11$ for Combi-1). In Icelandic horses, no significant effect of anti-insect blanket was present, 52 out of 60 cases did wear a blanket. Number of seasons affected, month during which symptoms normally appear, month during which symptoms normally regress and age at start of IBH did not have a significant effect on any of the tested *C. obsoletus* specific IgE levels in neither Icelandic horses nor Shetland ponies.

Discussion

The aim of our research was to identify and quantify factors associated with serum IgE levels against *C. obsoletus* WBE and *C. obsoletus* recombinant allergens determined by ELISA. Various factors were identified to be associated with IgE levels in Shetland ponies and Icelandic horses in The Netherlands. Severity of IBH clinical symptoms was associated with IgE levels against *C. obsoletus* allergens. Most of the correlations between severity score and IgE levels were significantly larger than 0. In agreement with our results, Peeters [19] found that the performance and accuracy of their allergen specific IgE ELISA in Belgian warmblood horses improved when IBH clinical symptoms at time of sampling were more pronounced. In human insect allergic patients, no such a significant correlation was found between venom-specific IgE levels and severity of clinical reactions [20, 21]. In addition, human studies of atopic asthma, allergic rhinitis and food allergies reported no correlation between specific IgE levels and severity of disease [22, 23]. An explanation for the presence of a correlation between IgE levels and severity of symptoms in horses might be because of the general high serum IgE concentrations in horses

compared to humans [24]. In humans, unbound IgE concentrations are very low and IgE has a short half-life due to binding to the high-affinity IgE receptor (FceRI) on mast cells and basophils (for review: [25]). Due to the much higher concentrations of unbound total IgE in horses many mast cells and basophils might already be saturated with IgE, increasing the half-life of IgE. Therefore, levels of unbound IgE in horse sera are relatively high and give a better representation about the sensitization than in humans.

Correlations between severity of IBH clinical symptoms and IgE levels against *C. obsoletus* specific allergens were higher and more significant in Shetland ponies compared to Icelandic horses. Severity of symptoms was significantly higher in Shetland ponies compared to Icelandic horses. In Shetland ponies less preventive and curative measures were applied, whereas the vast majority of IBH affected Icelandic horses wore anti-insect blankets. Severity of symptoms in Shetland ponies therefore might better represent the true exposure to *Culicoides* spp. and thereby result in higher correlations between severity of symptoms and IgE levels against WBE and recombinant allergens.

The mean IgE level (case and control) against several allergens was significantly higher in Shetland ponies compared to Icelandic horses. However, when looking within IBH status, significant differences in *C. obsoletus* IgE levels between Icelandic horses and Shetland ponies were only found for control horses. Lower levels were found for the Icelandic horse controls. Interestingly, total IgE levels were also reported to be lower in Icelandic control horses compared to control horses of other breeds [26]. Breed differences in antigen-specific serum antibody levels (including IgE) was also reported for sheep [27]. Genetic factors might be of influence, as significant genetic variance against specific IgE levels was found in Lipizzan horses [28]. An alternative explanation for higher IgE levels in Shetland pony controls might be related to a difference in age: mean age of Shetland ponies was 5 years lower than mean age of Icelandic horses (Table 1). An age effect was present for IgE levels against Cul o 2 in Shetland ponies and for IgE levels against WBE in Icelandic horses (Table 5). In a human allergy study specific IgE levels were reported to decrease with age in human patients with insect allergy [29]. In non-allergic individuals a similar decrease in IgE levels with increasing age might well exist. As mean IgE levels against several *C. obsoletus* allergens differed significantly between Shetland pony controls and Icelandic horse controls, breed specific thresholds can be set to further improve the sensitivity and specificity of the IgE ELISA used to diagnose IBH.

Differences in IgE levels between cases and controls were highly significant across breeds, but were larger in Icelandic horses compared to Shetland ponies. Wilson et al. [26] reported significantly higher total IgE levels in Icelandic horses with IBH compared to healthy Icelandic horses regardless of where they were born, which was not observed in other breeds (including Shetland ponies). Furthermore, total IgE levels of IBH affected Icelandic horses were higher than total IgE levels of IBH affected of other breeds. They suggested that insect specific IgE may constitute the major component of total serum IgE in IBH affected Icelandic horses. However, here we did not observe significantly higher *C. obsoletus* specific IgE levels in IBH affected Icelandic horses compared to IBH affected Shetland ponies.

In our data no significant differences in IgE levels between females and males were present. In contrast to Baselgia et al. [7], who reported a significantly higher sulphidoleukotriene release in IBH affected geldings compared to affected mares. They also reported month of sampling to affect diagnostic outcomes. In agreement, we found a significant effect of month of scoring on *C. obsoletus* specific IgE levels, which is likely to be a result of *Culicoides* spp. activity depending on weather conditions. This is supported by our previously observed decrease in IgE levels against recombinant allergens in off season sera compared to IgE levels in IBH season sera [13].

Conclusions and implications

Severity of IBH clinical symptoms and IgE levels against several *C. obsoletus* allergens appeared to be correlated. Several other factors were found to be associated with IgE levels against *C. obsoletus* (recombinant) allergens. Incorporation of these factors might further improve the interpretation and accuracy of the test results and will thereby better differentiate cases from controls.

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CHAPTER

8

General discussion

Introduction

Insect bite hypersensitivity (IBH), the most common seasonal recurrent skin allergy in horses, is clinically characterized by intense itch and irritation caused by hypersensitivity reactions mainly to bites of midges of the genus *Culicoides* [1-3]. Currently there is no fully curative treatment available for IBH, while the need for such a treatment is high **(Chapter 1**). Welfare of affected horses is seriously reduced and owners of such horses have to encounter increased costs due to attempts to control the itch and because of reduced commercial value of the horses. Severely affected horses sometimes even have to be euthanized [2].

In human studies, specific immunotherapies applied in individuals with type I allergies have showed promising results. However, for horse allergies, specific immunotherapy has not yet been well investigated, most likely due to the lack of (recombinant) allergens of the causative agents of these allergies.

The research described in this thesis therefore aimed to characterize, identify and produce allergens from the causative agent of IBH. Furthermore, this thesis aimed to develop a suitable and reliable diagnostic test for IBH. An accurate, reliable diagnostic test with allergens is an important part of treatment, because other skin conditions can be ruled out and such a test can be applied to determine for which exact components of *C. obsoletus* the IBH horses are allergic. This will enable a tailor-made composition of (recombinant) allergens for use in immunotherapy. Finally, increasing our knowledge about the immunopathology of IBH was another aim of this thesis. A schematic overview of the aim, most important findings and prospects of this thesis is presented in Figure 1. In this chapter our most important findings will be summarized and discussed.

Diagnosis of IBH using *C. obsoletus*

Observation of clinical signs, combined with well documented medical history and ruling out other conditions that may lead to similar symptoms, is assumed to be the gold standard to diagnose IBH in horses [4, 5]. In addition, intradermal testing, histamine release tests (HRT) and ELISA systems using *Culicoides* or *Simulium* whole body extracts (WBE) have been evaluated for discrimination of IBH affected horses and healthy controls [4-9]. Serological tests are preferred, because it is easier to take a blood sample, it permits

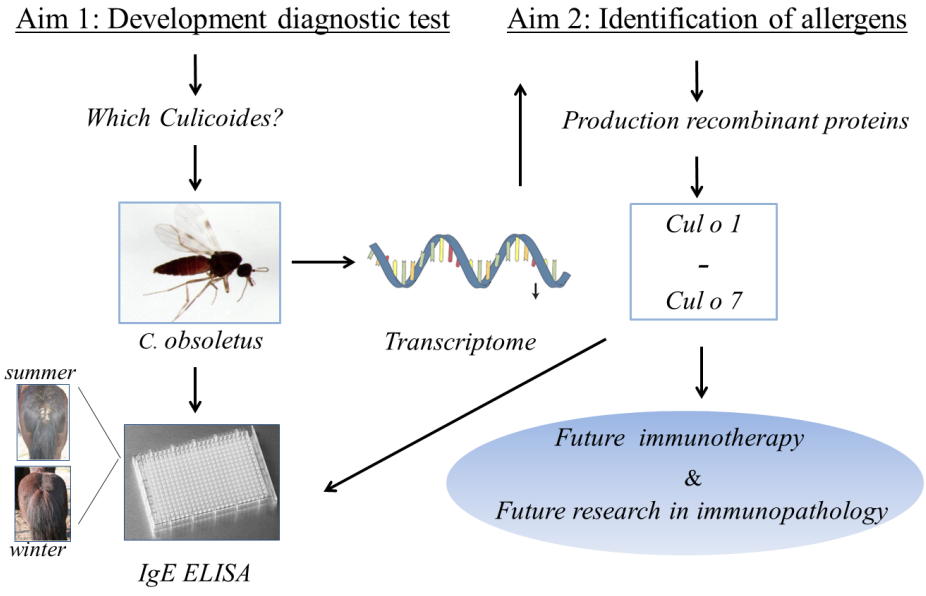

Figure 1: Summarizing overview of the main aims and findings of this thesis. *C. obsoletus* was found to be the most important species responsible for IBH in the Netherlands, a diagnostic ELISA was developed that we can use in summer as well as winter when clinical symptoms are absent. The transcriptome of *C. obsoletus* was sequenced and assembled which allowed allergen identification. *C. obsoletus* allergens (Cul o 1- Cul o 7) were produced as recombinant proteins that can be used for future immunotherapy and research into the mechanisms of immunopathology.

storage of samples until testing, and is more convenient for the veterinarian than performing a labor-intensive *in vivo* intradermal test. Furthermore intradermal tests can be painful and cause false positive reactions when injected to deeply [10].

For these reasons we aimed in our studies described in **chapter 2, 5** and **6** to develop an easy to perform *in vitro* test. Previously, WBE for *in vitro* diagnosis of IBH has been reported to show several drawbacks, for instance low sensitivity and/or specificity in serological tests and HRT [5, 6, 11]. Additionally, HRT requires fresh blood samples and test results have been reported to differ between months of testing [4, 8].

In **chapter 2** we report higher sensitivity and specificity with *C. obsoletus* WBE compared to *C. sonorensis* and *C. nubeculosus* WBE in a HRT and ELISA tested on a small number of horses. The fact that an ELISA can be performed on samples that have been frozen and on a large number of samples at the same time was the decisive factor to further evaluate the ELISA with WBE on a large number of horses. This demonstrated a

high sensitivity and specificity, higher then reported before, and applicability of the diagnostic test also for sera samples collected off season (**chapter 3).** Recombinant allergens are an attractive alternative for natural extracts. The assembly and sequencing of the *C. obsoletus* transcriptome described in **chapter 4**, allowed the identification of allergens from *C. obsoletus.* These allergens were produced as recombinant proteins and evaluated for diagnosis in **chapters 5** and **6.** Factors that can affect the ELISA test results were evaluated in **chapter 7**.

Choice of Culicoides species important for sensitive diagnosis

The main difference with other studies that evaluated *in vitro* diagnostic tests with WBE, was our choice to use *C. obsoletus* WBE. Previous studies performed in the Netherlands found *C. obsoletus* to be most often feeding on horses [12, 13]. These results are confirmed by our findings in chapter 2 were we collected insects from a horse using a pooter as well as with a 'Onderstepoort' light trap. Both capture methods resulted in the large majority of collected *Culicoides* spp to be *C. obsoletus*, which was even nearly 100% when collected directly from the horse with a pooter.

We choose to compare this endogenous species with 2 other *Culicoides* species, *C. nubeculosus* (Europe) and *C. sonorensis* (North America), commonly used in IBH studies. Our findings, reported in **chapter 2**, showed that horses had much better IgE reactivity with *C. obsoletus* WBE compared to *C. nubeculosus* and *C. sonorensis* WBE, suggesting low allergen cross-reactivity between these species. Cross-reactivity between *Culicoides* species has been studied before, but results differ and some report no difference between *Culicoides* species [5, 14], while others do find strong differences [7, 15]. Interestingly, Langner et al. [5] compared a native species (*C. nubeculosus*) with an exotic species (*C. sonorensis*) for that country and found no differences in reactivity. This is in agreement with our findings in chapter 2, where we report similar IgE reactivity with *C. nubeculosus* as with *C. sonorensis*. However, we report much higher IgE reactivity with *C. obsoletus*. Although *C. nubeculosus* might be found in Europe, in contrast to *C. sonorensis*, it is not commonly reported feeding on horses or only in very small numbers [16]. Furthermore, we did not collect any *C. nubeculosus* when aspirating *Culicoides* insect from a horse (chapter 2). Therefore we conclude that horses are not exposed to all *Culicoides* species present in the country were they are located. This is further enforced by our findings in

chapter 2, in which we do demonstrate much stronger IgE reactivity to *C. obsoletus* than to *C. nubeculosis* and *C. sonorensis*, and by Wilson et al. [15] and Sloet van Oldruitenborgh-Oosterbaan et al. [7], who both found better reactivity with *C. obsoletus* compared to *C. nubeculosus* as detected by western blot and an intradermal test. Moreover, in **chapter 5** we compared an allergen (Cul o 1, maltase) of *C. obsoletus* with its homolog from *C. sonorensis* (Cul s 1) and found more IgE reactivity with Cul o 1 than with Cul s 1, suggesting that on single allergen level diagnosis can be improved as well when using allergens from *C. obsoletus*. Furthermore, when we compared salivary proteins from *C. obsoletus* with those from *C. nubeculosus* and *C. sonorensis*, large amino acid differences were revealed between (sequence-based) similar proteins of the different species, as can be concluded from the Venn diagram described in **chapter 4** and by alignments of similar allergens described **in chapter 5**. Approximately a quarter of the *C. nubeculosus* salivary gland proteins have an identity of at least 40 % with candidate proteins from *C. obsoletus* (chapter 4). The remaining of the salivary gland proteins thus has a lower identity. Although more matching proteins were found between C. sonorensis and C. obsoletus, the similarity or cross-reactivity between these two species does not seem to be larger than between *C. nubeculosus* and *C. obsoletus* when looking at the results in **chapter 2**. We assume that the higher number of matching proteins with *C. sonorensis* is caused by the non salivary proteins that are available for *C. sonorensis* in the public database. Salivary proteins between *Anopheles* species were reported to be less conserved than housekeeping proteins [17]. It is likely that the non-salivary gland part of *C. sonorensis* contains more housekeeping proteins and therefore results in more matches with the *C. obsoletus* transcriptome. The low similarity between salivary proteins of different *Culicoides* species decreases the probability of the presence of cross-reactive IgE -binding epitopes in the different allergen preparations and necessitates the use of allergens from relevant species for the development of diagnostic tools.

Thus, the use of *C. obsoletus* allergens so far seems to be crucial for reliable *in vitro* diagnosis of IBH at least in The Netherlands, but probably in many other European countries as well. However, this does not exclude the possibility that the use of other *Culicoides* species might also contribute to the improvement of diagnosis. *C. pulicaris* is also reported feeding on horses in relatively high percentages compared to other species,

although less frequently found than *C. obsoletus* [12, 13, 16]. It would be interesting for further research to compare *C. pulicaris* with *C. obsoletus* in *in vitro* diagnostic tests.

Choice of Culicoides species mainly important for in vitro diagnosis

The 'lack of' cross reactivity between *C. obsoletus* and other *Culicoides* species so far seems mainly to be observed with *in vitro* tests. Although Sloet van Olruitenborgh-Oosterbaan et al. [7] reports an intradermal skin test to be better with *C. obsoletus* compared to *C. nubeculosus*, it is noteworthy to mention that they compared a commercial extract from Greer laboratories with a non-commercial extract prepared and stored in a different manner. In **chapter 2** we report that insect collection method and preparation can have great influence on quality of the extract. Furthermore, we found that immediate freezing by liquid nitrogen is important to avoid precipitation in high concentration protein extracts and that degradation already starts after only a few freeze thaw cycles (data not shown). Although we are not aware of the preparation methods of the Greer extract, we know it is stored at 4°C while it is generally known that many proteins are not stable at 4°C for longer periods. This is especially a problem in natural allergen extracts, like the extract of Greer, because they can contain proteases which affect the stability [18]. The findings observed by intradermal tests by Sloet van Olruitenborgh-Oosterbaan et al. [7] might therefore not necessary be related to lack of cross-reactivity between species, but more related to difference in quality of the used extracts. In an intradermal pilot test performed on 1 IBH affected and 1 healthy horse we observed clear immediate wheals provoked by *C. nubeculosis* and *C. sonorensis* WBE, although *C. obsoletus* WBE resulted in larger wheals (Table 1). The extracts employed were prepared and stored in the same manner. Anderson et al. [14] found no differences at all in skin reactivity provoked by *C. obsoletus* compared to reactivity provoked by 5 other native and non-native *Culicoides* spp. for that area [14]. Horses were injected with 0.1 ml of a 1% *C. obsoletus* extract corresponding to approximately 170 insects.

In our pilot experiment, it seems that at higher doses the intradermal skin test might also be successfully performed with non-native species. However, at lower antigen doses the intradermal skin test performs better with the local feeding *C. obsoletus*. Therefore, Anderson et al. [14] might have observed differences in skin reactivity if they would have used more diluted samples. Several speculations could explain these findings.

Table 1: The wheal diameter of one IBH affected horse and one healthy control horse at 5 different time points after injection with *C. obsoletus*, *C. nubeculosus* and *C. sonorensis* extract.

Injection (0.1 ml)	Wheal diameter (mm)			
	IBH affected horse		**Healthy control**	
	0.5 hr	**1 hr**	**0.5 hr**	**1 hr**
Saline	10		10	
Histamine	21		20	
C. obsoletus (µg/ml)				
10	22	25	9	8
1	18	20	10	8
0.1	16	19	10	7
C. nubeculosus (µg/ml)				
10	19	20	11	11
1	19	21	11	10
0.1	13	15	10	8
C. sonorensis (µg/ml)				
10	17	19	11	11
1	16	17	10	10
0.1	10	10	9	10

It is possible that the amount of histamine released by basophils or mast cells might not be proportional to the severity of the skin reactions. It could well be that above a certain histamine release a maximum wheal size is developed, which does not further increase with increasing histamine release. In contrast, the HRT test measures the actual histamine release and differences obtained with different *Culicoides* species as reported in chapter 2 can therefore be detected. Intradermal tests and *in vitro* test might therefore not be comparable.

Interestingly, in **chapter 7** we report a correlation between *C. obsoletus* IgE levels and severity of the clinical symptoms, suggesting that for *C. obsoletus* diagnosis by ELISA is a good alternative for intradermal test. Such correlation remains to be established for other *Culicoides* species.

Langner et al. [5] performed an HRT using *C. nubeculosus* and *C. sonorensis* saliva and WBE on blood of Icelandic horses located in Germany. Although at 5 µg/ml they were able to diagnose all IBH affected horses to be positive, also some control horses were tested positive. At a concentration of 0.5 µg/ml WBE they found about half of the IBH affected horses to have a positive test result and at a lower concentration only few or no horses were tested positive. In agreement, we found only few IBH affected horses to test

positive in the HRT with WBE of *C. nubeculosus* and *C. sonorensis* at a concentration of 0.5 µg/ml, while 100 % (n = 10) of these horses were tested positive with *C. obsoletus* extract. At the same concentration no control horses were tested positive (chapter 2). It is very likely that the diagnosis of these horses located in Germany would have been improved with the use of *C. obsoletus* WBE, because this is also the most abundant *Culicoides* species found in Germany [19]. Langner et al. [5] however did report an improvement of the HRT using saliva of *C. nubeculosus* and *C. sonorensis* and therefore saliva of these species might be as good for *in vitro* diagnosis of IBH as *C. obsoletus* WBE. However, collecting saliva from *Culicoides* insects requires the laboratory breeding of these insects and special membrane feeding devices to collect the saliva [20], and therefore does not seem to be an attractive alternative for *C. obsoletus* WBE. Furthermore, it is very likely that WBE contains all the relevant saliva allergens as IgE reactivity with WBE was observed to be very high (chapter 2) and genes coding for salivary allergens were present in the *C. obsoletus* transcriptome (chapter 4 and 5).

Off season diagnosis

The half-life of IgE in serum in humans is short, only 2-3 days, while IgE bound to Fc receptors on basophils and mast cells can survive for long periods up to months [21]. IBH is a seasonal allergy; it occurs when *Culicoides* insects are active, which is during the warmer months of the year (April-September in The Netherlands). Due to the short half-life of IgE it is expected that the developed ELISA, described in chapter 2, is less sensitive during the off season. However, as described in **chapter 3** and **5**, we did not find a decrease in *C. obsoletus WBE* specific IgE levels during the off season, compared to the levels during the *Culicoides* season. However we did find a decrease of IgE levels against a combination of three or four recombinant allergens for most of the tested horses in the off season, although IgE still remained clearly detectable in most IBH affected horses (chapter 5). IgE production might be maintained (at least for some horses) even in the absence of allergen contact. This was observed also before by Smurthwaite et al. [22], as individuals with hay-fever were found to have high titres of pollen-specific IgE in the serum even off season. Not long ago, this would raise questions, because in general plasma cells were considered as short-lived end products of B cell differentiation, which is actually still true for many plasma cells [23]. However, for some time already there is

evidence that at least some IgE-secreting plasma cells in allergic individuals are long-lived and that long-lived plasma cells can survive in the bone marrow, lymph nodes or spleen up to years [24-27]. Moreover, these plasma cells are not affected by antigenic challenge or immunosuppression [28, 29]. Thus it might be that horses have long-lived plasma cells specific for *Culicoides* allergens.

An alternative explanation for the absence of a decrease in IgE ELISA signal in winter-samples with ELISA plates coated with WBE could be because the molecules of true allergens actually bound to the plastic is lower than the number of allergen-specific IgE molecules present in a serum dilution of 1:5, even in winter samples, meaning there is an excess of IgE in proportion to coated allergens. This phenomenon will occur only after coating with WBE and not after coating with purified (recombinant) allergens due to competition of the allergens with the many other proteins in the extract for binding to the plastic of the well. The fact that horses have relatively high IgE levels in their serum, compared to for instance humans [30], will make this phenomenon more likely. Therefore, to be more conclusive, the ELISA with WBE should be performed with titrated summer and winter sera.

Baselgia et al. [4] found a decrease in positive test results by HRT using *C. nubeculosus* WBE several months after *Culicoides* exposure, suggesting that IgE levels decrease, or at least the *Culicoides* specific IgE antibodies bound to basophils in the absence of allergen exposure. This study was performed in Switzerland, were horses are likely to be more exposed to *C. obsoletus* than to *C. nubeculosus,* because it is again the main species found in this country [31]. Maybe the number of *C. nubeculosus* allergen-specific plasma cells that are produced is too low to result in long-lived plasma cells or plasma cells specific for only few *C. nubeculosus* allergens to survive, in contrast to *C. obsoletus* allergen specific plasma cells, who are probably relatively more abundant. Another speculation might be the choice of test. HRT is based on degranulation of mast cells by cross-linking of bound IgE antibodies. For cross-linking to occur the allergen needs to have at least two epitopes. A decrease in IgE antibodies will probably have a bigger effect on degranulation induced by *C. nubeculosus* WBE compared to *C. obsoletus,* because they are likely to bind less epitopes of *C. nubeculosus* allergens than of *C. obsoletus*. However, to be more conclusive, a HRT should be performed in summer as well as winter using *C. obsoletus* WBE or recombinant allergens.

Effect of horse breed on IgE ELISA test results

Our ELISA was tested in two different breeds, Shetland ponies and Icelandic horses. Symptoms of these horses varied from mild to profound, but most horses showed severe or profound symptoms of IBH. We found breed differences in IgE levels as reported in **chapter 7**, but only within control horses. Differences in serum antigen-specific IgE levels can also be observed in different sheep breeds [32]. However, our test seems to be applicable for multiple breeds, but the cut-off could be set breed-specifically to obtain the highest test sensitivity and specificity.

Recently a similar serological IgE ELISA test was also described by Peeters [33] who reported a sensitivity of 70 % and a specificity of 97 % for horses with severe symptoms of IBH during several years. The ELISA was performed with Belgian Warmblood horses and a combination of three recombinant allergens plus an Obsoletus group WBE was required to reach this sensitivity and specificity. To explain the relatively poor sensitivity of their ELISA compared to our ELISA, they suggested that our serological test described with WBE in **chapter 2** might only be a valuable diagnostic test for IBH for certain breeds. Indeed, it has been shown that Icelandic horses imported from Iceland display stronger T helper 2 (Th2) type and IgE responses to *Culicoides* allergens than other horse breeds [34, 35]. However, only a small percentage of the Icelandic horses tested in chapter 7 were imported from Iceland and Icelandic horses born in *Culicoides* infected countries do not seem to be more susceptible for IBH than other breeds [36]. Furthermore, Peeters (2013) suggested that our results might differ, because Shetland ponies and Icelandic horses are often kept more in the field compared to Warmblood horses [32]. However, most of our IBH affected Icelandic horses wore anti-insect blankets and are therefore less exposed to insects than Shetland ponies, but their IgE levels were not found to be lower than those of Shetland ponies. Therefore we consider our IgE ELISA using *C. obsoletus* WBE or recombinant allergens applicable for different horse breeds. However to be more conclusive about the sensitivity and specificity of the ELISA for different breeds, the ELISA should also be evaluated on horses of other breeds.

***C. obsoletus* recombinant allergens**

Diagnosis and treatment of allergic diseases have relied for a long time on natural allergenic extracts, which contain many non-allergenic proteins and sometimes allergens

from other sources. Furthermore, they are difficult to standardize [37].

A good alternative is therefore the use of recombinant allergens. Another advantage of the use of recombinant allergens is that a patient's sensitization profile can be determined by component resolved diagnosis, enabling possible adaptation of the composition of the antigen preparation used for specific immunotherapy to the requirement of the individual horse. In **chapter 5** we describe the identification and characterization of 7 allergens (Cul o 1 – Cul o 7) found by homology searches on a recently sequenced *C. obsoletus* transcriptome (**chapter 4**) using sequences of previously reported allergens from *C. nubeculosus* and *C. sonorensis.*

Cul o 1- Cul o 7 are a good alternative for diagnosis of IBH with WBE and are interesting tools for future immunotherapy.

Diagnosis with recombinant C. obsoletus allergens

In **chapter 5**, Cul o 1 – Cul o 7 were reported to be valuable allergens for diagnosis of IBH after testing them by IgE ELISA and an intradermal test. Furthermore, our IgE ELISA with a combination of only three of these allergens (Combi-1) resulted in a sensitivity and specificity of both 89 % close to that obtained with WBE (**chapter 6**). These allergens, for diagnosis therefore provide an attractive alternative for WBE.

Some clinically healthy horses also showed high IgE levels against one or more allergens. Clinically healthy horses are more often reported to be sensitized [12, 38]. These sensitized horses might never develop clinical signs of allergy, but in human studies asymptomatic sensitization is reported be a risk factor for later allergy development [39]. A diagnostic test performed as a decisive factor for treatment can therefore also be important for clinically healthy horses. Interestingly, in our study described in chapter 5, we report most sensitized clinically healthy horses to have specific IgE only for one or a few allergens. On the contrary, most IBH affected horses showed to have specific IgE for a large number of allergens. Sensitization to a minimal number of allergens might therefore be an important factor for clinical symptoms to occur. To be more conclusive about the chances a horse shows (or will show) symptoms of IBH, a positive ELISA result with combi-1 should preferentially be followed by testing on the individual allergens. This will also allow to determine for which exact components of *C.obsoletus* the horses are allergic, which is important to know for treatment.

In **chapter 6** we report that additional *C. obsoletus* allergens seem to be responsible for IBH that can be crucial for diagnosis and treatment of some horses. With combi-1 we reached a sensitivity of 89 % and this sensitivity might possibly be increased with these 'missing' allergens. Hellberg et al. [34] already reported at least 10 IgE binding proteins from *C. nubeculosus* and a lot IgE binding variety between horses [34], whereas in this thesis only 7 allergens were identified and produced as recombinant proteins. Furthermore, 11 allergens were described for *C. nubeculosus* [40, 41]. Since *C. obsoletus* was found to be a more important species for *in vitro* diagnosis than *C. nubeculosus* in chapter 2, it is likely that at least 11 IgE binding proteins should exist for *C. obsoletus* as well.

Additional candidate allergens

In **chapters 2** and **6** we describe two proteins of 15 and 20 kDa to be present as most dominant IgE reactive bands detected by western blot analysis and these are therefore interesting candidates for further characterization. Western blot analysis described in **chapter 6** shows that these proteins are not one of the seven allergens (Cul o 1 – Cul o 7) that were identified by homology searches.

During this research project, we have tried immunoprecipitation and *two dimensional* (2D) gel electrophoresis to isolate these proteins from WBE for characterization by mass spectrometry. Most effort was put into 2D gel electrophoresis, because immunoprecipitation using total IgE of an IBH affected horse with strong reactivity to the 15 and 20 kDa protein covalently coupled to sepharose beads was unsuccessful after several attempts.

Preliminary 2D electrophoresis experiments showed that only a spot of 15 kDa (pH > 8) could be detected by western blot analysis and no spots of 20 kDa. Strips ranging from pH 3-10 were used, because these resulted in the best separation of *C. obsoletus* WBE proteins in the area where our spot of interest was found by western blot analysis. The western blot and a 2D gel stained with Oriole Fluorescent Gel stain (BioRad) were overlaid using a photo manipulator program (Pixlr.com/editor). A protein spot with a MW of 15 kDa on the gel matched the spot visualized by western blotting (indicated by a circle in Figure 2A and 2B). Additional gels were stained with Oriole and these showed a similar spot pattern as shown in Fig. 2B. The same spot on these gels matched with the

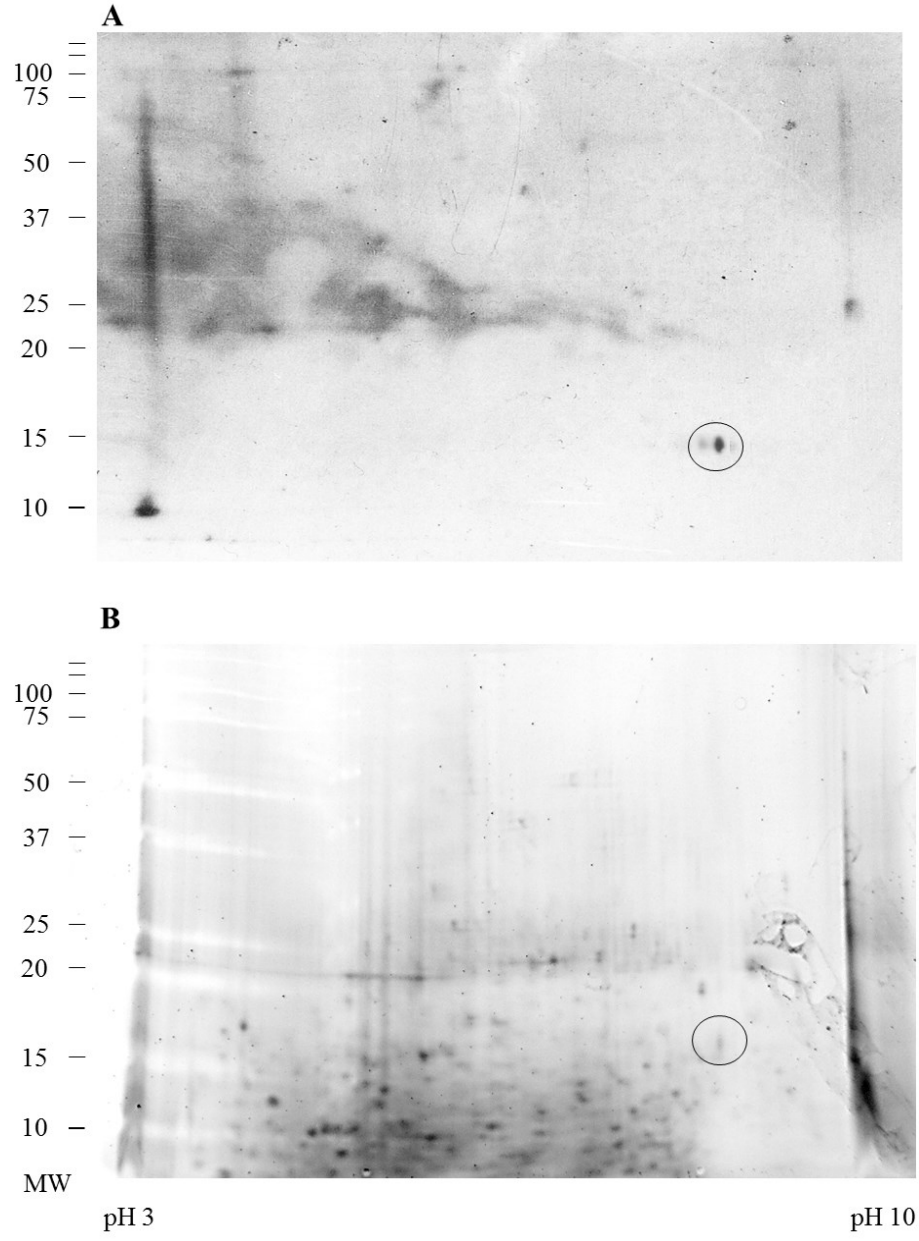

Figure 2: (A) Western blot analysis of two dimensional (2D) separated proteins of *C. obsoletus* WBE incubated with sera of 2 IBH affected horses. Binding of IgE from horse sera was detected with an anti-equine IgE mouse mAb and HRP goat anti-mouse IgG. The molecular weight marker [43] is indicated on the left in kDa. **(B)** 2D gel with proteins of *C. obsoletus* WBE, stained with Oriole Fluorescent Gel Stain.

protein spot detected by the western blot analysis. We used a pool of sera of two horses showing both IgE reactivity with a 15 and 20 kDa band using western blot analysis of a 1D gel, although the 20 kDa band was much fainter than the 15 kDa band (Figure 3). A possible explanation for the lack of a 20 kDa spot on the 2D gel might be that the isoelectric point of this protein was outside the limits of the strip or, more likely, because the protein was not present in sufficient amounts to be detected by 2D gel western blot analysis.

A protein spot of 15 kDa (pI >8) that matched with one of the spots visualised by western blot analysis for IgE binding was excised from the 2D gel stained with Oriole fluorescent Gel Stain (BioRad, Munchen, Germany), proteolytically digested and identified by Mass Spectrometry (MS) analysis.

MSMS spectra from the MS measurements were searched with Bioworks 3.3.1. against the *C. obsoletus* transcriptome database. A set of common contaminants was added and from this a decoy reverse database was constructed using the program SequenceReverser from the MaxQuant package [42] and added as well. Bioworks results were filtered to show only those contigs for which at least 2 peptides were found. Nearly 70 contigs were found to have at least 2 peptides. Candidate proteins within these contigs with an isoelectric point close to 8 and/or MW close to 15 kDa were selected for *E. coli*

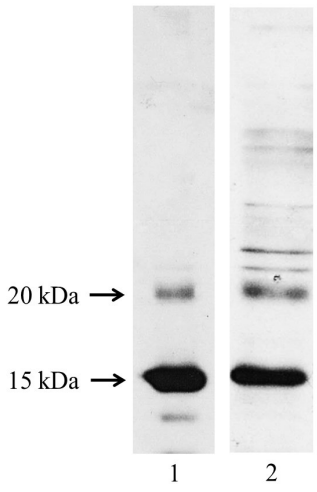

Figure 3: Western blot analysis of a 1 D gel with *C. obsoletus* WBE incubated with sera of 2 IBH affected horses. Binding of IgE from horse sera was detected with an anti-equine IgE mouse mAb and HRP goat anti-mouse IgG. Two proteins with a MW of 15 and 20 kDa are visualized.

expression. However, none of these proteins were found to have a predicted signal peptide.

Two candidate proteins similar to NADH ubiquinone dehydrogenase and a protein similar to hypothetical protein from *Aedes aegypti* (XM_001648736.1) were the most interesting candidates because of their MW (close to 15 kDa) and pI (> 8). After purification, all 9 recombinant proteins, including the NADH ubiquinone dehydrogenase and a hypothetical protein from *Aedes aegypti,* showed no or very low IgE-binding as detected by western blot analysis and ELISA (data not shown). These findings suggest that we did not characterize the protein of interest of 15 kDa as detected by western blot analysis by 2D gelectrophoresis. A possible explanation might be that we did not excise the correct spot from the gel, the protein was not present in sufficient amounts or it could not be found in one of the contigs of the *C. obsoletus* RNA database.

Methods for improvement of separation by 2D gel electrophoresis.

As can be seen in the oriole stained gel (Figure 2B) only spots in the lower MW region can be detected, while coomassie stained proteins of *C. obsoletus* WBE seperated on a 1D gel shows many higher MW proteins (chapter 2). It seems that most proteins are not well resolved or smeared on the gel, as this is a common limitation in 2D gel electrophoresis [44]. This might also explain the absence of detection of the 20 kDa band.

Isolation and characterization of the proteins might be achieved by preparative separation of protein by chromatography methods, like ion exchange chromatography or hydrophobic interaction chromatography. Proteins fractions can afterwards be analysed by western blot analysis and the protein fraction which contains our protein of interest can be further separated by 1D or 2D gelelectrophoresis. Decreasing the complexity of the extract is likely to increase the efficiency of resolving and separation of proteins and therefore to simplify identification by mass spectrometry.

Identification of a candidate allergen by 1D gelectrophoresis and mass spectometry

During this research project we anticipated separation of our 15 kDa and 20 kDa protein from WBE by 1D gel electrophoresis would be unsuccessful because of the complexity of natural allergen extracts. However, we assume that allergens are often highly abundant proteins in an extract. For instance, it is suggested that 5 – 20 % of mosquito salivary

protein consists of D7 proteins, which are major allergens in most insect species [45]. We therefore recently performed a pilot experiment by loading *C. obsoletus* WBE on a 1D gel for separation of proteins based on their MW. A protein band of 15 kDa was excised from the gel that was stained with Oriole fluorescent Gel Stain (BioRad), proteolytically digested and identified by MS analysis, similar as described in the previous paragraph for 2D gel electrophoresis.

As expected, peptide hits were found for many proteins (>100) present in the *C. obsoletus* transcriptome. Nevertheless, one protein was found to be an interesting candidate, because this protein showed most peptide hits (13 peptides) was found to have a MW close to 15 kDa and to have a signal peptide. This protein shows highest sequence similarity with odorant binding proteins from *Anopheles funestus* (34 % identities) and other biting insects. There was no similarity hit with genbank accessible *Culicoides* salivary proteins. Sol I 2, a major allergen from the venom of red imported fire ant, shows strucutal (but not sequence) homology with odorant binding protein of *Dropsohila* [46], suggesting that odorant binding like proteins from biting insects might serve as allergens. Furthermore, an odorant binding protein has recently also be reported to be an allergen present in salivary gland, hair and urine of the Siberian hamster [47] and a dog dander allergen has shown sequence similarity and cross reactivity with a odorant binding protein present in cow dander [48]. These data suggest that the recently identified *C. obsoletus* odorant binding protein might well serve as an allergen for IBH.

The odorant binding protein of *C. obsoletus* is currently being cloned and expressed in recombinant form to assess this protein as allergen relevant for IBH.

Implications for immunotherapy

The main disadvantage of current treatment options, like horse blankets, insect repellents, corticosteroids and anti-histamines is that they fail to target the mechanisms underlying the allergy, often only have short activity and may have several side-effects.

Allergen-specific immunotherapy is the only causative treatment of allergy at the moment and is based on the repeated administration of the disease-eliciting allergens until the sensitivity to the administered allergens is reduced through various immunological mechanisms [49]. Immunotherapy used in humans with insect allergies has shown to be able to shift the cytokine production to Th1 cytokines [50, 51], to increase the production

of IL-10 [52], to reduce the histamine release by basophils [53] and to reduce allergen specific IgE levels [54].

For insect bite hypersensitivity, only a few immunotherapy trials have been described to date using WBE of *Culicoides* spp. but they reported controversial results [55, 56]. One of the studies however did report a clear reduction of clinical signs after weekly subcutaneous injections with *C. variipenis* extract and even a complete disappearance of symptoms in some horses after two years [55]. Furthermore, a retrospective study on 51 horses reported on the clinical presentation of equine atopic skin diseases and evaluated the response to treatment with allergen-specific immunotherapy based on intradermal testing combined with serum testing. Allergen-specific immunotherapy was found to be successfully used in the management of different types of atopic skin disease in horses [57].

In **chapter 7** we observed for Shetland ponies a decrease in *C. obsoletus* specific IgE levels with increase in age. This was not observed for Icelandic horses, which might be because they were more often protected by insect blankets, therefore being less exposed to bites. In contrast, the Shetland ponies were not protected by blankets and therefore the decrease in IgE levels with increasing age might well be due to a natural and gradual desensitization.

The use of *C. obsoletus* WBE as described in **chapter 2** could be useful for immunotherapy in horses suffering from IBH in The Netherlands. However, the use of natural allergen extract has some major bottlenecks. E.g. Injection of these unmodified extracts can result in local and severe systemic side effects, due to the presence of allergenic materials [58-60]. Because of these side-effects the clinically effective maintenance dose can sometimes not be reached. Furthermore, relevant allergens might not be present in sufficient amount or exhibit poor immunogenicity [61]. Finally, new sensitizations can be induced against allergens present in the extract, which were not recognized by the patient before treatment [62, 63].

The use of recombinant proteins can improve on the use of natural allergen extracts for allergen specific immunotherapy.

Recombinant allergens have the advantages that they can be produced whenever needed under defined conditions, reaching milligram or grams quantities by different high level expression systems, which makes quality control and standardization possible [37].

Many recombinant allergens have showed comparable IgE reactivity *in vitro* and *in vivo* as its natural allergens [64-66]. Although we did not compare our recombinant allergens with their natural form *in vivo*, as described in **chapter 5 and 6** all of our 7 expressed recombinant allergens on individual level showed IgE reactivity with a large number of IBH affected horses and *E. coli* expressed maltase showed comparable IgE reactivity to baculovirus expressed maltase, which is expected to closely resemble the natural form of the protein. Furthermore, in **chapter 6** the IgE reactivity of horses to a combination of 3 recombinant allergens was comparable to the IgE reactivity to *C. obsoletus* WBE as detected by IgE ELISA. For human allergies, cocktails of few recombinant allergens were also reported to successfully replace natural allergen extracts for *in vitro* diagnosis [66]. The advantage of such a cocktail of allergens is that each allergen is present in a defined concentration and no other irrelevant proteins or proteases are present.

Another advantage of the use of recombinant allergens is that they allow for a more precise diagnosis and by identifying which allergens are responsible for the allergic symptoms they form a basis for better prescription of immunotherapy [67]. Many successful immunotherapy studies using recombinant allergens have lately been reported for human allergies [68]. Recombinant Cul o 1 – Cul o 7 are thus interesting tools for future immunotherapy in horses with IBH., because this panel of recombinant allergens will determine for which exact components of *C.obsoletus* the IBH horses are 'allergic' and this will enable a tailor-made composition of (recombinant) allergens for use in immunotherapy.

Concluding remarks

In conclusion, *C. obsoletus* seems to be the most important causative agent of IBH in The Netherlands and its allergens currently provide the most reliably available *in vitro* diagnosis of Insect Bite Hypersensitivity in horses. The transcriptome of *C. obsoletus* has been sequenced and assembled that will allow gene expression analysis and further gene discovery. Seven *C. obsoletus* allergens (Cul o 1 – Cul o 7) were identified that can be produced by *E. coli* as recombinant proteins and used for diagnosis of IBH affected horses by ELISA. With a combination of 3 of these allergens a reliable serological diagnostic test for IBH was developed that can replace *C. obsoletus WBE* without losing sensitivity or specificity and is applicable in different horse breeds.

The panel of available 7 recombinant allergens can be used to determine for which exact components of *C.obsoletus* the IBH horses are allergic and this will enable a tailor-made composition of (recombinant) allergens for use in future immunotherapy.

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SUMMARY

SAMENVATTING

Summary

Insect Bite Hypersensitivity (IBH) is the most common skin allergy in horses and involves a Type I (IgE mediated) hypersensitivity reaction. IBH is clinically characterized by intense itch and irritation caused by hypersensitivity reactions to bites of insects, mainly of the *Culicoides* species. Welfare of affected horses is seriously reduced and they can become unsuitable for riding or showing purposes, because of the discomfort and disfigurement. No fully curative treatment is yet available for IBH. Furthermore, current diagnostic tests are unreliable because of their low sensitivity and specificity. The goal of the research project described in this thesis was to increase our understanding of immunological aspects of IBH, with special attention to improving diagnosis by the characterization and production of recombinant allergens.

Chapter 1 firstly gives an overview of the cells of the immune system that are involved in this allergy, after which the basic mechanism of a Type I allergy and additionally the production of allergens and their use for component resolved diagnosis is discussed. Secondly a literature review about research on IBH, with special attention to the currently available different diagnostic tests is presented. The chapter is finalized with the research aims of this project and a thesis outline.

Most studies performed on IBH are performed with whole body extracts or allergens from laboratory available *C. nubeculosus* (found in Europe, but rarely found in The Netherlands) or *C. sonorensis* (typical for North America). However, *C. obsoletus* is regularly found to be the *Culicoides* species mostly found feeding on horses in European countries, including the Netherlands, but so far cannot be successfully maintained in laboratory bred colonies.

In the first study, described in **chapter 2**, we describe the collection of female *C. obsoletus* directly from a horse using a pooter. Collecting these insects alive, not in alcohol, is important for the preparation of high quality extracts. Whole body extracts (WBE) of the three *Culicoides* species: *C. obsoletus C. nubeculosus* and *C. sonorensis* were evaluated for their applicability for diagnosis of IBH in horses in The Netherlands. They were tested for IgE binding by ELISA and Western blotting and for their capacity to degranulate basophils in a histamine release test. For all tests, best results were obtained with *C. obsoletus*. The ELISA was further evaluated using *C. obsoletus* extract on

approximately 200 IBH affected and healthy horses, which demonstrated high test sensitivity and specificity.

Chapter 3 describes a study in which we compared lesional and non-lesional skin of ponies affected by IBH in the IBH season with those of the same animals off season and those in skin of healthy ponies in both seasons for their Th1/Th2 immune response pattern. *C. obsoletus*-specific IgE serum levels, skin histopathology and cytokine and transcription factor mRNA expression (IL4, IL10, IL13, IFNγ, FoxP3 and CD3ζ) were evaluated. *C. obsoletus*-specific IgE serum levels within each group were the same in the IBH season and off season. A significantly higher histopathology score in lesional skin of affected ponies than in non-lesional skin and skin of healthy ponies in the IBH season was observed. Finally, there was a general up-regulation of the cytokines IL4, IL13 and IFNγ in the skin of all horses in the IBH season compared to off season.

Chapter 4 describes the sequencing and assembly of the transcriptome of *C. obsoletus*. RNA of female *C. obsoletus* insects collected from the wild was sequenced using Illumina paired-end RNA sequencing technology. *De novo* assembly resulted into 41,223 contigs, and the estimated open reading frames (ORFs) were extracted from the contigs and resulted in 28,000 candidate genes. Only a quarter of the salivary gland proteins of *C. nubeculosus* was found to match with contigs of *C. obsoletus*. The remaining salivary gland proteins did not match. For *C. sonorensis* the number of proteins that matched was higher, but most likely because of the presence of non-salivary gland proteins which might be more conserved. Thus, there appears to be a lot of sequence differences between salivary proteins of *C. obsoletus*, *C. nubeculosus* and *C. sonorensis* species, which might explains the low cross-reactivity between allergens from *C. obsoletus* and allergens from the other two species.

In **chapter 5** we describe the identification of 7 allergens from *C. obsoletus* by sequence similarity searches on the *C. obsoletus* transcriptome, with in literature described allergens from *C. nubeculosus* and *C. sonorensis.* These allergens were cloned and expressed as recombinant proteins in *E. coli* and named Cul o 1 – Cul o 7. A maltase (Cul o 1) and Cul s 1 (maltase of *C. sonorensis*) were additionally expressed in insect cells using the baculovirus expression system to compare similar allergens from different species produced with different expression systems in diagnostic *in vitro* and *in vivo* tests. IBH affected horses showed higher IgE levels to Cul o 1 than to Cul s 1. Furthermore,

production of these proteins in *E. coli* was at least as suitable for *in vitro* diagnosis of IBH affected horses as these same proteins produced in the baculovirus expression system. For *E. coli* expressed Cul o 1 – Cul o 7, the frequency of positive test results by ELISA within IBH affected horses ranged from 38 % to 67 %. The capability of the allergens to induce Type I hypersensitivity reaction in IBH affected horses was demonstrated by an intradermal test.

Additionally, in **chapter 6** the applicability of the 7 *C. obsoletus* derived recombinant allergens was further evaluated and compared with *C. obsoletus* WBE in an IgE ELISA using almost 350 IBH affected and healthy horses. The highest test accuracy was obtained with WBE, followed by Cul o 2, 3 and 5. Two ELISA's with a combination of recombinant allergens, combi-1 (Cul o 3, 5 and 7) and combi-2 (Cul o 1, 2, 5 and 7) were additionally performed and both resulted in high test accuracies close to that obtained with WBE. Both combi-1 and combi-2 resulted in a lower test sensitivity with samples collected in winter compared to samples collected in IBH season, but most IBH affected horses could still also be correctly diagnosed in winter.

Chapter 7 describes the identification and quantification of the association between several factors and IgE levels against *C. obsoletus* whole body extract and 7 recombinant allergens. Furthermore, the relation between IgE levels and severity of symptoms was examined. Severity of symptoms and IgE levels against several *C. obsoletus* allergens were found to be related. Breed was found to be associated with IgE levels. Differences in IgE levels between cases and controls were found to be largest in Icelandic horses. Shetland pony controls had significantly higher IgE levels compared to Icelandic horse controls, but differences in IgE levels between Shetland pony cases and Icelandic horse cases were not significant. Other factors associated with IgE levels were age, month of scoring, interaction between IBH status and month of scoring, degree of itchiness and number of seasons horses were affected with IBH.

In the general discussion (**chapter 8**) the most important findings of this thesis are discussed and presented in a summarizing overview. A 2D gel electrophoresis approach to identify additional allergens is discussed and methods for improvement are suggested. An odorant binding protein that was recently identified as a new candidate allergen is discussed. Finally, we discuss the prospects to use the produced recombinant allergens for immunotherapy treatment of IBH affected horses.

In conclusion, *C. obsoletus* allergens currently provide the most reliable diagnosis of insect bite hypersensitivity in horses located in countries where this species is the most dominant *Culicoides* species feeding on horses. With a combination of three recombinant allergens derived from *C. obsoletus* we developed the first reliable serological diagnostic test for IBH. The panel of all 7 recombinant allergens allows to determine for which exact components of *C. obsoletus* the IBH horses are allergic ("component resolved diagnosis"). This will enable a tailor made composition of (recombinant) allergens for use in immunotherapy.

Samenvatting

Staart- en maneneczeem (SME) is de meest voorkomende huidallergie bij paarden en betreft een type 1 (IgE gemedieerde) allergische reactie. SME veroorzaakt jeuk en huidirritatie door een allergische reactie op insectenbeten, voornamelijk van *Culicoides* insectensoorten. Als reactie op de jeuk kunnen de paarden tot bloedens toe gaan schuren. Het welzijn van paarden kan door deze aandoening ernstig worden aangetast en ze kunnen ongeschikt worden om te berijden of voor paardenshows. Momenteel is er geen genezende behandeling beschikbaar voor SME. Daarnaast zijn de huidige beschikbare diagnostische testen onbetrouwbaar door een lage sensitiviteit en specificiteit van de test. Het doel van het onderzoeksproject beschreven in dit proefschrift was om meer inzicht te verkrijgen in de immunologische aspecten van SME, en om een betrouwbare diagnostische test voor SME te ontwikkelen.

Hoofdstuk 1 geeft een overzicht van de cellen van het immuunsysteem die een rol spelen in SME, en beschrijft het mechanisme van een type I overgevoeligheidsreactie. Vervolgens wordt de productie van recombinant allergenen beschreven en hoe deze gebruikt kunnen worden om te diagnosticeren voor welke allergenen een patiënt precies allergisch is (component-resolved diagnostics, CRD). Daarna wordt een literatuuroverzicht van eerdere onderzoeken naar SME gepresenteerd, met extra aandacht voor de verschillende beschikbare diagnostische testen en de overeenkomst met de klinische diagnose als gouden standaard. Het hoofdstuk wordt afgesloten met de onderzoeksdoelen van dit project en een inhoudelijk overzicht van dit proefschrift.

De meeste onderzoeken naar SME worden uitgevoerd met extracten van complete insect of recombinant allergenen van in het laboratorium te kweken *Culicoides* stammen, zoals *C. nubeculosus* (komt voor in Europa, maar wordt zelden gevonden in Nederland) of *C. sonorensis* (voorkomend in Noord Amerika). *C. obsoletus* is echter de *Culicoides* soort die het meest gevonden wordt op paarden in Europese landen, inclusief Nederland, maar deze soort kan tot nu toe nog niet gekweekt worden in het laboratorium.

In **hoofdstuk 2**, beschrijven we het vangen van vrouwelijke *C. obsoletus* direct van een paard met behulp van een zuigbuis (‘pooter’). Deze studie bevestigt dat het overgrote deel van de *Culicoides* insecten die zich op paarden voeden tot C. obsoletus behoren en laat verder zien dat op deze wijze gevangen insecten een veel hogere kwaliteit extract leveren

dan *Culicoides* gevangen met een veelgebruikte val, waarbij de insecten in alcohol terecht komen. Complete extracten van 3 *Culicoides* soorten: *C. obsoletus*, *C. nubeculosus* en *C. sonorensis* werden vergeleken voor hun toepasbaarheid voor SME diagnostiek. De extracten werden getest op IgE binding aan de hand van ELISA en Western blot en er werd getest of ze in staat waren tot het degranuleren van perifere bloed basofiele granulocyten door middel van een basofiele degranulatie test. Het *C. obsoletus* extract gaf de beste resultaten in zowel IgE binding in ELISA en Western blot als ook een degranulatietest voor basofiele granulocyten. De ELISA gebaseerd op *C. obsoletus* extract werd verder geëvalueerd op een groter aantal paarden (ca. 200) allergische en gezonde paarden en liet een hoge test sensitiviteit en specificiteit zien.

Hoofdstuk 3 beschrijft een onderzoek waarin het Th1/Th2 patroon in lesionale en niet-lesionale huid van Shetlanders met SME in het SME seizoen wordt vergeleken met het Th1/Th2 patroon in de huid van dezelfde dieren buiten het seizoen en met huid van gezonde Shetlanders in beide seizoenen. *C. obsoletus*-specifieke IgE serum niveaus, huid histopathologie en cytokinen en transcriptiefactor mRNA expressie (IL4, IL10, IL13, IFNγ, FoxP3 en $CD3_ζ$) werden bestudeerd. *C. obsoletus*-specifieke IgE serum niveaus binnen elke groep waren net zo hoog in het SME seizoen als buiten het seizoen. De lesionale huid van paarden met SME had een significant hogere histopathologie score dan de niet-lesionale huid en huid van gezonde paarden. In de huid van alle paarden werd een algemene verhoogde mRNA expressie van de cytokines IL4, IL13 en IFNγ gevonden in het SME seizoen in vergelijking met buiten het seizoen.

Hoofdstuk 4 beschrijft het bepalen van de nucleotidenvolgorde (sequencen) en het samenstellen (assembleren) van het transcriptoom van *C. obsoletus*. RNA van vrouwelijke *C. obsoletus* wild-gevangen insecten werd gesequenced met de Illumina paired-end RNA sequencing methode. De novo assemblage resulteerde in 41,223 contigs, waarna de open reading frames (ORF's) werden bepaald wat resulteerde in 28,000 kandidaat genen. Slechts een kwart van de eiwitten aanwezig in speekselklierweefsel van *C. nubeculosus* kwam wat betreft sequentie overeen met contigs van *C. obsoletus* (tenminste 40% identiek en tenminste 75 aminozuren in lengte). De overige speekselklier eiwitten kwamen niet met elkaar overeen. Het aantal eiwitten van *C. sonorensis* dat overeenkwam tussen contigs en de bepaalde sequentie was hoger, maar dit komt waarschijnlijk door de aanwezigheid van andere dan speekselklier eiwitten die meer geconserveerd zijn. Er lijkt

dus een groot verschil in de sequenties van de speekseleiwitten van *C. obsoletus*, *C. nubeculosus* en *C. sonorensis* te zitten, wat kan verklaren dat IgE tegen speekseleiwitten opgewekt door beten van *C. obsoletus* slecht kruisreageert met extracten van de andere 2 *Culicoides* soorten.

Hoofdstuk 5 beschrijft de identificatie van 7 allergenen van *C. obsoletus* door middel van zoeken naar sequentie overeenkomst in het *C. obsoletus* transcriptoom met bekende allergenen van *C. nubeculosus* en *C. sonorensis* beschreven in de literatuur. Deze allergenen (Cul o 1 – Cul o 7 genoemd) werden als recombinante eiwitten in *E. coli* tot expressie gebracht. Een van deze allergenen (Cul o 1) en het overeenkomende allergeen van *C. sonorensis* (Cul s 1) werden daarnaast ook met een baculovirus expressiesysteem in insecten cellen geproduceerd. Daarmee kunnen dezelfde eiwitten afkomstig van verschillende *Culicoides* soorten en geproduceerd door verschillende expressiesystemen met elkaar worden vergeleken op hun bruikbaarheid in verschillende diagnostische in vitro en in vivo testen. Paarden met SME hadden hogere IgE niveaus gericht tegen Cul o 1 dan tegen Cul s 1. Daarnaast was de productie van deze eiwitten in het E. coli systeem minstens net zo goed voor in vitro diagnose van paarden met SME als dezelfde eiwitten geproduceerd in het baculovirus expressiesysteem. Met de in *E. coli* tot expressie gebrachte Cul o 1 – Cul o 7, varieerde de frequentie van positieve testresultaten van paarden met SME van 38 % tot 67 %. De capaciteit van deze allergenen om een type I reactie te veroorzaken in de huid werd aangetoond door middel van een intradermale huidtest.

In **hoofdstuk 6** werd de toepasbaarheid van de 7 *C. obsoletus* allergenen verder onderzocht en vergeleken met *C. obsoletus* compleet extract in een IgE ELISA aan de hand van ongeveer 350 SME en gezonde paarden. De beste resultaten werden behaald met compleet extract, gevolgd door Cul o 2, 3 en 5. Twee ELISA's met een combinatie van recombinante allergenen, combi-1 (Cul o 3, 5 en 7) en combi-2 (Cul o 1, 2, 5 en 7) werden daarnaast ook uitgevoerd en beide ELISA's resulteerden in een hoge betrouwbaarheid die gelijk was aan de ELISA met compleet extract. Zowel combi-1 als combi-2 resulteerde in een lagere test sensitiviteit met serum afgenomen in de winter in vergelijking met serum afgenomen in het SME seizoen, maar de meeste paarden met SME konden in de winter nog steeds juist (in overeenstemming met de klinische verschijnselen in de zomer) gediagnostiseerd worden.

Hoofdstuk 7 beschrijft de identificatie en kwantificering van de relatie tussen verschillende SME risico factoren en de IgE serum niveaus tegen *C. obsoletus* compleet extract en de 7 recombinante allergenen. Daarnaast is voor de SME paarden de relatie tussen IgE niveaus en ernst van klinische symptomen onderzocht, waarbij de ernst van symptomen en de hoogte van de IgE niveaus tegen verschillende *C. obsoletus* allergenen significant positief met elkaar correleerden. Het ras van het paard was geassocieerd met de hoogte van de IgE serum niveaus. Het verschil in de IgE niveaus tussen SME paarden en gezonde dieren was het hoogst bij de IJslanders. Gezonde Shetlanders hadden significant hogere IgE niveaus dan gezonde IJslanders, maar het verschil in IgE niveaus tussen Shetlanders met SME en IJlanders met SME was niet-significant. Andere factoren die geassocieerd waren met de IgE niveaus waren leeftijd, maand van scoren, interactie tussen SME status en maand van scoren, mate van jeuk en het aantal seizoenen dat paarden last hadden van SME.

In de algemene discussie (**hoofdstuk 8**) worden de belangrijkste bevindingen van dit proefschrift bediscussieerd en gepresenteerd in een samenvattend overzicht. Een 2D gel elektroforese methode die was toegepast om nieuwe en aanvullende allergenen te identificeren wordt bediscussieerd en suggesties ter verbetering worden voorgesteld. Een odorant-bindend eiwit dat recent is geïdentificeerd als een kandidaat allergeen wordt besproken. Tot slot bediscussiëren we de vooruitzichten om de geproduceerde recombinante allergenen te gebruiken voor immunotherapie ter behandeling van paarden met klinisch verschijnselen van SME.

Uit dit proefschrift kan worden geconcludeerd dat *C. obsoletus* allergenen nu voor de meest betrouwbare diagnose van staart- en maneneczeem zorgen bij paarden die gelokaliseerd zijn in landen waar deze soort de meest dominante Culicoides soort is die zich op paarden voedt. Tevens kunnen daarmee zowel binnen als buiten het insectenseizoen SME lijders van niet-lijders worden onderscheiden. Met een combinatie van drie recombinant allergenen afkomstig van *C. obsoletus* hebben we de eerste betrouwbare serologische diagnostische test ontwikkeld voor SME. De 7 recombinante allergenen kunnen gebruikt worden om te bepalen voor welke componenten van C. obsoletus paarden met SME precies allergisch zijn. Dit biedt mogelijkheden om elk paard een op maat gemaakte immuuntherapie te geven met de juiste (recombinante) allergenen.

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ABOUT THE AUTHOR

Curriculum vitae

Nathalie Maria Adriana van der Meide was born on the 24th of February 1982 in Leiderdorp, The Netherlands. After graduating from the Sint Maartens College in Voorburg, she studied Agriculture at the INHOLLAND University in Delft. After graduation, she choose to continue with the MSc programme Animal Sciences at Wageningen University. During this MSc programme she performed an eight months research project about the effect of early administration of probiotics on the immune system at later age of laying hens. Afterwards, she continued with a minor thesis in which she studied the role of A-Kinase Anchoring Protein 121 (AKAP121) in Steroidogenesis. For this project she stayed for 4 months in Texas, USA, at the Texas Tech University Health Sciences Centre. After graduation, she shortly worked as a research technician at the Molecular Immunology department of Utrecht University Medical Centre. In 2008, she started on a STW funded PhD project at the Cell Biology and Immunology Group of Wageningen University, of which the outcomes are described in this thesis. The research was carried out under the supervision of prof. dr. ir. Huub Savelkoul and dr. ir. Edwin Tijhaar. During this project she attended several courses and international conferences, presented her work by oral and poster presentations and supervised 5 students that performed their theses on her PhD project.

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* equal contribution

Training and Supervision Plan

The basic package (3 credits)

WIAS Introduction Course, Wageningen,	2008
Ethics and philosophy of animal science, Wageningen	2009

Scientific exposure (13 credits)

International conferences

3rd European Veterinary Immunology Workshop, Berlin, Germany	2009
9th international veterinary immunology symposium, Tokyo, Japan	2010
7th World Congress of Veterinary Dermatology, Vancouver, Canada	2012
4th European Veterinary Immunology Workshop, Edinburgh, Scotland	2012

Seminars and workshops

Annual meetings Dutch Society for immunology (NVVI), Noordwijkerhout	2008, 2011
NVVI, Guiding the action of the immune system, Lunteren	2010
WIAS Science day, Wageningen	2009- 10, 12
WIAS seminar, Regulation of the immune response in Teleost Fish, Wageningen	2008
WIAS seminar, Immunomodulation and Allergy, Wageningen	2008
Symposium emerging vector born viral diseases, Lelystad,	2008
Seminar: Tour on Protein Purification and Analysis, Wageningen	2010
Minisymposium: Genetics and immunology of Insect Bite Hypersensitivity ,Wageningen	2009

Presentations

Food4You festival Wageningen:	
'Allergisch voor voedsel' (poster)	2008
Wageningen University:	
‘Ontwikkeling van interventie strategieën voor staart en maneneczeem bij paarden’	2009
Minisymposium STW, Wageningen:	
‘Allergen identification of *Culicoides obsoletus* relevant for IBH’ (oral)	2009
WIAS Science Day, Wageningen:	
‘Allergen identification of *Culicoides obsoletus* relevant for IBH’ (poster)	2010
7th World congress of Veterinary Dermatology, Vancouver, Canada:	
‘Improved diagnosis of Insect Bite Hypersensitivity in horses with allergens from indigenous *Culicoides obsoletus’* (oral)	2012
4th European Veterinary Immunology workshop, Edinburgh, Scotland:’	
‘Improved diagnosis of Insect Bite Hypersensitivity in horses with allergens from local midges *(Culicoides obsoletus)’*	2012

In-Depth studies (7 credits)

Disciplinary and interdisciplinary courses

11th Fish immunology workshop, Wageningen	2010
Introduction Course Immunology, Utrecht	2010

ELISA Course Huub Savelkoul, Wageningen	2010
Comperative Proteomics, Wageningen	2010

Advanced statistics courses

WIAS advanced statistics course: Design of animal experiments, Wageningen	2009

MSc level courses

Bioinformation Technology, Wageningen	2010

Skills (15.9 credits)

Professional Skill support courses

Phadia, ImmunoCAP training, Wageningen	2008
Information literacy, including introduction EndNote, Wageningen	2009
PhD competence assessment, Wageningen	2009
Project & time management, Wageningen	2009
Techniques for writing and presenting a scientifc paper, Wageningen	2011

Didactic skills

Supervising practicals Cell Biology I	2008-2009
Supervising 4 theses (2 Bsc, 4 Msc)	2009-2012

Management skills

Organising WIAS Science day, Wageningen	2009
Organising minisymposium on insect bite hypersensitivity, Wageningen	2009

Total number of credits: 38.9

It always seems impossible until it's done

-Nelson Mandela-

Colophon

The study is part of a larger project entitled: “Development of intervention strategies for insect bite hypersensitivity (IBH) in horses”, and was financially supported by Technology Foundation STW, the Dutch Federation of horse breeding ('s-Hertogenbosch, The Netherlands) and ALK-Abelló / Artu Biologicals (Almere, The Netherlands).

Cover : photograph of an IBH affected horse, made by Nathalie van der Meide
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