Equine Intradermal Test Threshold Concentrations for House Dust Mite and Storage Mite Allergens and Identification of Stable Fauna

THESIS

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By

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Abstract

The presence of house dust mites (HDMs) and storage mites (SMs) in the human environment has been established worldwide and both contribute to atopic disease consisting of atopic dermatitis, asthma and allergic rhinitis in some individuals. The *Dermatophagoides*, *Acarus*, *Tyrophagus* and *Lepidoglyphus* mite genera contribute to the pathogenesis of atopic disease. HDMs and SMs have also been implicated in atopic dermatitis in veterinary medicine. Extensive work has been done for canine mite induced allergic patients, but relatively little information is available for equine allergic patients. Intradermal testing is performed in veterinary patients to identify environmental antigens that cause disease with the intent of formulating allergen-specific immunotherapy.

Equine HDM and SM intradermal test (IDT) threshold concentrations (TCs) for the Midwestern United States are unknown. The mite stable fauna for the Midwestern region of the United States has not been determined. The objectives of this study were to determine IDT TCs for HDM and SM species, to quantify mite-specific IgE concentrations in thirty-eight clinically normal horses over two seasons and to characterize the mite fauna of a stable in this region across three seasons.

Subjective measurements of IDT reactions were used to determine the TCs for *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, *Acarus siro*, *Tyrophagus*
putrescentiae and Lepidoglyphus destructor. The reactions were scored using a scale of 0 to 4+. Allergen testing concentrations ranged from 1:320,000-1:20,000 w/v for HDMs and 1:160,000-1:5,000 w/v for SMs. Threshold concentrations were defined as the highest concentration of a mite allergen where ≤10% of horses had a positive subjective reaction (≥2+) at 15 min. Analysis of equine serum-specific IgE was performed using a commercially available allergen-specific IgE ELISA test. Specialized mite traps and modified flotation methods were used to collect mites in spring, late summer and winter from nine locations on one farm. Selected locations for mite collection represented the three different stabling environments used, bedding types, feed materials or combinations thereof. A single-baited mite trap was placed at each of the locations for a four-day period (96 h), while 200 g of material was gathered from each site on the fourth day for flotation. An acarologist morphologically identified and quantified the species of HDMs and SMs collected.

Subjectively determined TCs were: 1:80,000 w/v for Dermatophagoides farinae in both seasons, 1:80,000 w/v in spring and 1:160,000 w/v in late summer for Dermatophagoides pteronyssinus, 1:40,000 w/v in spring and 1:20,000 w/v in late summer for Acarus siro, 1:20,000 w/v for Lepidoglyphus destructor in both seasons, and 1:20,000 w/v in spring and 1:10,000 w/v in late summer for Tyrophagus putrescentiae. In both seasons, at least one horse had a positive serum IgE result for each HDM or SM evaluated. Negative serum IgE concentrations for all mite species were present in 55% of
horses in spring and 66% in late summer. At least one mite from all four genera specific to this study was identified. *Tyrophagus* mites were the most prevalent with *Dermatophagoides* mites being the least numerous. Collectively, *Oribatida*, *Cheyletus*, *Glycyphagus* and *Tarsonemidae* represented the majority of the genera detected.

The determined TCs from our study differ from published recommendations for equine HDM and SM IDT dilution concentrations, suggesting the need to consider seasonal and regional influences on IDT TCs and reactivity. These results establish that horses stabled in the Midwestern United States are exposed to a diverse Acari population. Provocation and allergy testing of allergic horses with specific mite allergens would be necessary to determine the significance of these mites in relation to disease.
Dedicated to Mom, Amy and Andy
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Chapter 1

Introduction

Allergy, an immune reaction to an antigenic trigger, affects both human and veterinary patients, and has been reported since the early 1800s.1,2 Specifically, atopy is a genetically predisposed syndrome in which allergen-specific IgE is produced to at least a single allergen and one or more of the following diseases are present: allergic rhinitis, asthma or atopic dermatitis.3-5 Many factors contribute to atopic disease including genetic predisposition, environmental exposure, barrier dysfunction and an abnormal immune response. Often individuals affected by atopy have a skewed type 2 helper T cell response in the acute phases of disease, which may be followed a type 1 helper T cell biased immune reaction in more chronic phases.6-8

Atopic dermatitis (AD) is a chronic or relapsing, pruritic, inflammatory skin disease that features erythema, dry skin, papules, plaques, lichenification and exudative lesions.9,10 It can be induced by food proteins or environmental aeroallergens that are either inhaled, ingested or epicutaneously absorbed.3 Pollens such as grasses, trees, weeds, feline dander and house dust mites have been identified as known environmental allergic triggers.9-12 In humans, the worldwide prevalence in children is 10-30% while adults account for 1-10% of affected cases.13,14

While human atopic dermatitis has been extensively studied, little is known about equine atopic dermatitis including prevalence and definitive genetic predisposition. It is
reported in a wide age range of horses with a median age of onset at 6.5 years.\textsuperscript{15} The disease may be seasonal or nonseasonal depending on the allergens involved, and major cutaneous manifestations include pruritus and/or urticaria.\textsuperscript{16} Other common clinical signs are angioedema, alopecia, crusting, papular dermatitis, excoriations, lichenification, hyperpigmentation and eosinophilic granulomas.\textsuperscript{15-17} Symptoms can reduce an affected patient’s performance, lead to unsightly cosmetic defects and can cause significant irritation.

Diagnosis of equine allergic disease is primarily based on history, physical examination and exclusion of differential diagnoses\textsuperscript{15} Once diagnosed, allergy testing is used to identify potential environmental allergens that may be contributing to the allergic disease.\textsuperscript{15} Once identified, they are either avoided, if possible, or placed in allergen-specific immunotherapy (ASIT). The aim of ASIT is to deliver increasing concentrations of selected allergen extracts at a given frequency in order to “desensitize” the immune response or induce tolerance to the allergic stimuli being administered, thus reducing clinical signs.\textsuperscript{18-23}

Intradermal and serologic allergy testing are used to identify potential environmental allergic triggers in veterinary patients for the purpose of ASIT.\textsuperscript{24-27,77-78,28,29} The reliability of the IDT is dependent on many factors. False positive reactions have been reported in healthy horses suggesting possible subclinical hypersensitivity, previous exposure without disease, irritant reactions, contamination of allergens with microorganisms, or allergen cross-reactivity.\textsuperscript{27,30-34} False negative results can be a consequence of using too low allergen concentrations, testing off-season or the administration of medications to the animal that are known to inhibit IgE reactions.\textsuperscript{27}
Limited data is available regarding serologic allergy testing (SAT) in horses. When compared to the gold standard of IDT, the sensitivity and specificity of the SAT are often poor.\textsuperscript{35,36}

Allergen concentrations used for IDTs are determined from threshold concentrations (TCs). The TC, based on reactions produced from injection of serial dilutions of allergens, is determined to distinguish between the concentration of allergen that produces irritant reactions \textit{vs.} the concentration that produces allergic reactions in patients with hypersensitivity. Threshold concentrations should be determined in non-diseased animals that have been raised and maintained in allergen-free environments because these individuals have no level of allergen exposure, thus, making any IDT reaction in this population representative of irritant reactions. Threshold concentrations have been arbitrarily defined as the concentration where <10\% of non-diseased animals have a positive reaction, but this percentage is often considered too high or too low by various clinicians.\textsuperscript{25,32} Allergen concentrations used for equine IDTs are commonly extrapolated from the human, canine or feline literature as well as equine studies, and are limited both in number and in scope.

House dust mites (HDMs) represent one of the most common environmental allergens and are known to be significant contributors to the antigenic burdens in allergic patients.\textsuperscript{24,37-40} HDMs are found in homes with carpeting, upholstered furniture and mattresses and feed primarily on epidermal scale.\textsuperscript{40,41} Storage mites (SMs) are found in grains such as oats, corn, and hay, as well as facilities that manufacture and store grains. Relative humidity limits mite survival and is the key factor that determines where these mites can survive and live. In the temperate seasonal climates of North America, the
prevalence of live mites fluctuates seasonally. Highest live-mite levels occur during the humid summer months when the indoor relative humidity conditions for survival and breeding are most favorable. Live-mite levels drop during the late fall months. The residue of dead mites and the level of mite allergen-produced show similar seasonal trends that parallel the live-mite densities.\textsuperscript{40}

Relatively little information has been reported regarding the potential of HDMs and SMs to act as allergens in equine allergic disease. Based on evidence from other species and limited information in horses, it is probable that HDMs and SMs thrive in the hay, grain, barn dust and bedding of horses and may play an important role in the pathogenesis of equine allergic disease. The aims of this study were to determine the prevalence of cutaneous sensitization and TCs in horses without allergic disease to HDMs and SMs during the spring and late summer, to quantify seasonal serum specific HDM and SM IgE concentrations in healthy horses and finally, to characterize the mite fauna of a stable over three seasons. We postulated that the stable environment would contain HDMs and SMs year-round. Furthermore, we hypothesized that HDM and SM allergen intradermal TCs could be determined and would have seasonal variation. Additionally, we proposed that SAT results would not significantly correlate with the IDT TCs of any mite species.
Chapter 2

Literature Review

2.1 History of Allergy

The current definition of allergy is an immune reaction to specific antigens or allergens, which are often innocuous environmental constituents. John Bostock reported the most primitive concept of allergy in 1819 when he described his own symptoms of *catarrhus aestivus* or hay fever, which he believed to be caused by the summer heat.\(^1\,^2\) In 1873, Charles Blackley recognized that *catarrhus aestivus* was not caused by a change in temperature, but rather by the presence of pollen grains.\(^42\) Clemens P. Piquet was the first to use the word “allergie” in 1906 to describe “adverse reactions to antitoxins that defined the immunopathogenesis of serum sickness.”\(^43\) Years later in 1921, Prausnitz and Kustner discovered a human serum factor that reacted with allergen, which would later be determined to be the antibody, immunoglobulin epsilon (IgE), by Ishizaka et al in 1967.\(^2\,^4\,^44\) In veterinary medicine, allergic disease was first described throughout the 1930s as “eczema” in response to food allergens in the dog.\(^24\,^45\,^46\) Wittich reported a canine patient with rhinitis, conjunctivitis and urticaria secondary to ragweed exposure that responded to allergen-specific immunotherapy in 1941.\(^27\) The first case of equine seasonal dermatitis was reported in 1840 and published in 1937.\(^47\,^48\) Subsequently in 1946, an additional case of equine allergic disease was documented by Reddin and Stever
as a contact dermatitis caused by saddle soap and leather conditioner. Allergy research has evolved from this basic information into an extensive category of knowledge involving the immune system, genetic inheritance and environmental factors that affect human and veterinary patients across the world.

2.2 Hypersensitivity Reactions in Humans

Allergy in the body can produce various types of complex immunologic reactions that result in undesired clinical signs. Normally these immune responses were designed to eradicate the body of the infecting organism or allergen. However because of inadequate control, inappropriate targeting of host cells or stimulation by normally harmless antigens or organisms, these entities can produce disease and adverse effects. The reaction patterns are traditionally classified into four categories or hypersensitivity types: type I immediate IgE-mediated, type II antibody-mediated cytolytic, type III immune complex, and type IV delayed hypersensitivity reactions. Gell and Coombs first published this classification scheme in 1963. Sell et al. suggested a classification system, which takes into account that hypersensitivity reactions have multiple immune system components. The seven categories are as follows: inactivation/activation antibody reactions, cytotoxic or cytolytic antibody reactions, immune-complex reactions, allergic reactions, T-cell cytotoxic reactions, delayed hypersensitivity reactions, and granulomatous reactions. Still, the Gell and Combs classification remains in use today although there is some controversy as to whether it should be completely abandoned, modified or if additional categories need to be added.
2.2.1 Type I Immediate IgE-Mediated Reactions

Immediate hypersensitivity disorders are characterized by the degranulation of inflammatory cells, particularly mast cells, when an antigen crosslinks two IgE antibodies bound to the cell surfaces. In health, individuals exposed to antigens ingested in food, absorbed through the skin or inhaled in the air respond by producing immunoglobulin gamma (IgG) or immunoglobulin alpha (IgA), and have no obvious clinical consequence. Antigens can include pollens (tree, grass, weed), insect venom (bees, wasps), fungi (*Aspergillus, Histoplasmosis*), house dust mites, food proteins (eggs, peanut) or drugs (penicillins, cephalosporins, sulfonamides, local anesthetics). However clinical disease or allergy is observed when certain individuals produce an exaggerated type 2 helper T cell (Th2) response with increased production of IgE antibodies after exposure to the same antigens. This is the immunological basis for atopy. In addition to IgE, some subclasses of IgG such as IgG4 have also been shown to bind mast cell receptors and mediate type I hypersensitivity reactions although their significance in allergy appears much lower than that of IgE. Clinical conditions associated with atopy include asthma, atopic dermatitis and allergic rhinitis. Other type 1 hypersensitivity disorders include acute anaphylaxis, drug reaction, food allergy, lymphocytic-plasmacytic enteritis, urticaria, angioedema and parasitic allergy.

2.2.1.1 Immediate phase reaction

An immediate allergic reaction occurs when the allergen cross-links two IgE molecules attached to the alpha chain of the high-affinity IgE receptor (FcεRI) on mast cells or basophils. After the IgE antibodies are cross-linked, tyrosine kinases are activated.
and through a series of intracellular signaling pathways, the cellular contents fuse with the plasma membrane of the cell releasing preformed mediators, newly formed lipid mediators, chemokines and cytokines within minutes.\textsuperscript{5,56,57} Preformed mediators are contained within cytoplasmic granules and include histamine, serine proteases (tryptase, chymase, carboxypeptidase A) and proteoglycans (heparin, chondroitin sulfates).\textsuperscript{58,59} Once released, histamine induces vascular permeability, edema, erythema, vasodilation, smooth muscle contraction and mucous secretion.\textsuperscript{58,59} In vitro, serine proteases such as tryptase have been shown to digest fibrinogen, fibronectine, pro-matrix metalloproteinase 3, protease-activated receptor 2 and complement component C3.\textsuperscript{58} Tryptase can also trigger fibroblasts and recruit inflammatory cells.\textsuperscript{58} Newly formed lipid mediators include prostaglandins (PGDs) and leukotrienes (LTs).\textsuperscript{58,59} Mast cell mediators can also promote dendritic cell migration and maturation as well as initiating an influx of neutrophils, eosinophils, basophils and effector T cells through up regulating adhesion molecules and secreting proinflammatory cytokines.\textsuperscript{59} The chemokines and cytokines stored and released by mast cells include CXCL8, CCL3, tumor necrosis factor alpha (TNF-\(\alpha\)), interleukin (IL)-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-5, IL-6, IL-10 and IL-13.\textsuperscript{59} Epithelial adhesion molecule upregulation is stimulated by TNF-\(\alpha\), while IL-3, IL-5 and GM-CSF are essential for eosinophil development.\textsuperscript{59}

Like mast cells, basophils also secrete preformed mediators, newly synthesized lipid mediators and cytokines. Histamine is the predominant stored preformed mediator in the granules of basophils, while LTs are the main newly synthesized lipid mediators.\textsuperscript{58} Cytokines released by basophils include IL-4, IL-13 and GM-CSF.\textsuperscript{58} Basophil mediators have similar actions as those in mast cells, which include vascular permeability,
promotion of IgE synthesis and recruitment of inflammatory cells. Immediate-phase reactions typically occur within 20 minutes of exposure of the allergen.

### 2.2.1.2 Late-Phase Reaction

A late-phase reaction peaks 6-12 hours and is macroscopically resolved by 24 hours following allergen exposure. These reactions are marked by the presence of chemokines from degranulated mast cells and infiltration of various inflammatory cells including neutrophils, basophils, lymphocytes and eosinophils. Both eosinophils and basophils release their own biologically active granules such as arylsulfatase, peroxidase and major basic protein that cause further inflammation. Clinical signs include erythema, edema and pruritus depending on the site of exposure.

### 2.2.2. Type II Antibody-Mediated Cytolytic

Antibody-mediated hypersensitivity reactions are caused by IgG or immunoglobulin mu (IgM) antibodies that bind to antigens on specific cells or tissues, or by antigen-antibody complexes that are formed in circulation and deposited in vascular walls. When cells are targeted, they can either be directly opsonized by the antibodies or by complement proteins through activation of the complement system. Neutrophils and/or macrophages that contain receptors for the Fc portion of the antibody or complement protein then phagocytize and destroy the opsonized cells. Antibodies can also bind to normal cellular receptors or other proteins leading to dysfunction and disease without inflammation. When IgM or IgG antibodies are deposited in tissues or vessel walls, activated neutrophils and macrophages are recruited and release lysosomal
enzymes as well as reactive oxygen species causing tissue damage and inflammation. Examples of type II hypersensitivities include drug reactions that typically occur more than seven days after exposure, autoimmune hemolytic anemia, autoimmune thrombocytopenic purpura, pemphigus vulgaris, vasculitis, acute rheumatic fever, Myasthenia gravis and Graves’ disease.  

2.2.3 Type III Immune Complex Hypersensitivity

Immune complexes form when antibodies combine with self or foreign antigens. Type III hypersensitivity occurs when these immune complexes are deposited in tissues or vessels and subsequently produce chemotactic peptides through activation of complement that attract neutrophils, which release enzymes and oxidants into the surrounding tissues. This causes local destruction and inflammation of the tissue. Immune complex-mediated disease often affects multiple tissues and organs with a particular predisposition for the kidneys and joints. Clinical signs include erythema, edema, hemorrhage, thrombosis or vasculitis. Examples of immune complex hypersensitivities include serum sickness, glomerulonephritis, polyarthritis, drug reactions, systemic lupus erythematosus and purpura hemorrhagica.

2.2.4 Type IV Delayed Hypersensitivity

A delayed hypersensitivity reaction occurs through activation of T cells resulting in cytokine-mediated or direct cytotoxic inflammation which develops 24 to 48 hours after antigen exposure. Inflammation caused by CD4+ cells usually involves type 1 helper T cell (Th1) or type 17 helper T cell (Th17) subsets that secrete cytokines, which
recruit neutrophils, macrophages and various other leukocytes. Activation of the leukocytes causes release of lysosomal enzymes, reactive oxygen species, nitric oxide and proinflammatory cytokines. In some instances, CD8+ cytotoxic T cells directly destroy target cells that have class I major histocompatibility complex-associated antigens. T cells may be autoreactive or may be specific for foreign antigens located in cells or tissues. Examples of delayed hypersensitivities include allergic contact dermatitis, acute or chronic rejection of a transplanted solid organ, photoallergic drug reaction (sulfonamides, sulfonylureas, chlorpromazine, furosemide, isoniazid, naproxen, amiodarone) and hypersensitivity pneumonitis.

2.3 Atopic Disease in Humans

The term atopy was first used in 1923 and is currently defined as a genetically predisposed syndrome in which allergen specific IgE is produced to at least a single allergen and one or more of the following diseases are present: allergic rhinitis, asthma or atopic dermatitis. These diseases can be divided into two categories: extrinsic (atopic) and intrinsic (nonatopic). Extrinsic atopic disease occurs with sensitization of the individual to environmental allergens and when an elevation in allergen-specific serum IgE levels is observed. Intrinsic disease is a nonallergic variant where no detectable sensitization is present and low to normal serum IgE levels are detected.

2.3.1 Immunology of Atopic Disease

Although there are many factors to atopic disease including genetic predisposition and environmental exposure, an abnormal immune response is a common feature in
patients suffering from true atopic disease. After the allergen has made contact with a mucosal surface or through epithelial tight junctions, it is taken up by antigen presenting cells (APCs) and carried to the nearest lymph node for presentation to T cells.\textsuperscript{2} Individuals without atopy mount a mild immune response to these antigens by producing the allergen-specific IgG antibodies, IgG1 and IgG4, along with interferon-\(\gamma\) (IFN-\(\gamma\)) manufactured by Th1 cells.\textsuperscript{6,7,68} However, those affected by atopy respond to allergens with a Th2 cell response by producing IL-4, IL-5, and IL-13.\textsuperscript{6,7} Factors that influence this shift to a Th2 response include amount and duration of allergen exposure, low affinity binding of the antigen to the major histocompatibility complex (MHC) class II on APCs and decreased secretion of IL-12 by dendritic cells.\textsuperscript{2,69,70} The Th2 cytokines, IL-4 and IL-13, along with costimulation for CD40 promote isotype switching to IgE through the initiation of transcription for the gene encoding for the epsilon class of the constant region on the immunoglobulin heavy chain.\textsuperscript{5,56} IL-4 and IL-13 also induce expression of vascular adhesion molecules that down regulate Th1 cytokines and simultaneously cause an infiltration of eosinophils.\textsuperscript{9} IL-5 promotes development, activation and survival of eosinophils.\textsuperscript{9}

Acute allergic reactions occur when the allergen cross-links two IgE molecules attached to the alpha chain of Fc\(\varepsilon\)RI receptor on mast cells or basophils eliciting a type 1 immediate hypersensitivity reaction.\textsuperscript{5,57} The Fc\(\varepsilon\)RI receptor is also located on the surfaces of APCs where IgE bound allergen can attach and interact with T cells.\textsuperscript{71} The release of the preformed granules like histamine and lipid mediators such as cysteinyI leukotrienes from mast cells cause vasodilation, vascular permeability, contraction of smooth muscle
and increased mucous production.\textsuperscript{5,72} Chemokines recruit eosinophils and neutrophils causing additional inflammation.\textsuperscript{2}

2.3.2 Atopic Dermatitis

Atopic dermatitis (AD) is a chronic or relapsing, pruritic, inflammatory skin disease that features erythema, dry skin, papules, plaques, lichenification and exudative lesions on the face and extensor surfaces in children as well as flexural surfaces in adults.\textsuperscript{9,10} AD can be induced by food proteins or environmental aeroallergens that are either inhaled, ingested or epicutaneously absorbed.\textsuperscript{3} Pollens such as grasses, trees, weeds, feline dander and house dust mites have been identified as known environmental allergic triggers.\textsuperscript{9-12} Pruritus can be exacerbated with allergen contact, sweating, reduced humidity and increased contact with irritants such as soaps and detergents.\textsuperscript{9,73} The disease often first presents in infancy or childhood with 85\% of cases beginning prior to 5 years of age, but has been less commonly reported to begin in adulthood as well.\textsuperscript{10} The worldwide prevalence in children is 10-30\% while adults account for 1-10\% of affected cases.\textsuperscript{13,14} Approximately 70-80\% of affected patients have extrinsic AD with IgE sensitization while the remaining 20-30\% have intrinsic, non-IgE mediated sensitization.\textsuperscript{9,74}

2.3.3 Pathogenesis of Atopic Dermatitis

The pathogenesis of AD is believed to be derived from combination of genetic predisposition, immunological factors and environment.\textsuperscript{12} Parents with AD have an increased risk of having offspring affected by the disease compared to other atopic
diseases such as asthma and allergic rhinitis. However, approximately 80% of children affected by atopic dermatitis will also develop asthma or allergic rhinitis, which increases the likelihood that cutaneous sensitization may predispose patients to respiratory disease. While the exact pathway of inheritance is still to be determined, chromosome 5q31-33, which contains a cluster of Th2 cytokine genes such as interleukin-2, 4, 5, 13 and GM-CSF is suspected to have relevance to AD. In addition, 16q12, 17q11, 3q21, 1q21 and 17q25 are chromosomes believed to be linked to expression of AD.

2.3.3.1 Barrier Dysfunction

The relationship between the innate and adaptive immune system contribute significantly to the pathogenesis of AD. Three entities comprise the cutaneous innate immune system, which consist of the physical barrier (stratum corneum and intercellular junctions), cellular components (keratinocytes, APCs, mast cells and polymorphonuclear leukocytes (PMNs)) and the secretory elements (cytokines and antimicrobial peptides). Skin acts as a defense organ to environmental dangers such as allergens, toxins, radiation and microorganisms. The stratum corneum, the upper most layer of the epidermis, contains much of the protective barrier and is composed of layers of corneocytes enveloped in intracellular lipids, which are approximately 50% ceramides, 25% cholesterol and 10-20% fatty acids. Defects in the epidermal barrier such as reduction in ceramides, filaggrin mutations and increased transepidermal water loss lead to irritation and release of proinflammatory cytokines from keratinocytes. These changes also bring about increased permeability and allow allergens to gain direct contact to the immune system. The pruritus threshold is subsequently lowered and substances...
such as histamine, Substance P, prostaglandins and tachykinins further aggravate the pruritic patient.\textsuperscript{81} Elevations in the level of glucosylceramide/esfingomielina deacylase, a ceramide-damaging enzyme, in atopic patients has been reported to decrease levels of ceramides 1 and 3 in the stratum corneum.\textsuperscript{79,81,84,85} Reduction in barrier lipids contributes to cutaneous dryness or xeroderma, and subsequently, the natural antimicrobial agent, sphingosine. This leads to a predisposition of atopic patients to colonization of bacteria, specifically \textit{Staphylococcus aureus} in humans.\textsuperscript{79,86}

\textbf{2.3.3.2 Filaggrin Mutations}

Filaggrin mutations have a multifactorial contribution to inflammation and allergen presentation in AD. A key component in the keratohyalin granules is profilaggrin, which is eventually expressed in terminally differentiated keratinocytes in the stratum corneum.\textsuperscript{87} After being dephosphorylated and cleaved by proteases, profilaggrin becomes filaggrin.\textsuperscript{88} Filaggrin monomers contribute to aggregation and alignment of keratin bundles in the cornified cell envelope, thus contributing to the stratum corneum’s strength and integrity.\textsuperscript{80,89} The filaggrin gene, \textit{FLG}, is located on the epidermal differentiation complex on the human chromosome, 1q21.\textsuperscript{90} Loss-of-function mutations in \textit{FLG}, particularly on exon 3, lead to abnormal lamellar body packing, disorganization of the keratin filaments, reduction in corneodesmosome compactness and increased tight junction gaps.\textsuperscript{90-92} Also, breakdown of filaggrin amino acids causes an increase in stratum corneum pH predisposing the epidermis to proinflammatory cytokines.\textsuperscript{80,93} Approximately 50\% of AD patients with moderate to severe disease have
functional *FLG* mutations, while only 15% of patients with mild to moderate disease carry these mutations.\textsuperscript{60,80,92-94}

### 2.3.3.3 Antimicrobial Peptides

Antimicrobial peptides (AMPs) are key components of the innate immune system that have the direct ability to kill gram-positive and -negative bacteria, fungi, enveloped viruses and cancerous cells.\textsuperscript{78,95} The mechanisms for their antimicrobial actions arise from the ability to insert themselves into and disrupt the cellular membrane of the invading microbe, attract leukocytes and activate pattern recognition receptors (PRRs).\textsuperscript{96} Keratinocytes are responsible for the production of several AMPs including LL-37, human β-defensin (HBD), S100 proteins, ribonuclease 7, dermcidin and sphingosine.\textsuperscript{78,97} Recently, Th17 cells that generate IL-17A and IL-17F cytokines have been implicated as an enhancer for AMP production from keratinocytes.\textsuperscript{98} Although produced by cutaneous cells, only HBD-1 is found in the skin during the noninflamed state, while the remainder is produced following inflammation or injury.\textsuperscript{97,99-101} LL-37 and HBDs are stored in the lamellar bodies of keratinocytes along with cornified envelope proteins and lipids.\textsuperscript{78} Both AMPs act as chemoattractants with LL-37 recruiting PMNs, monocytes and T cells while HBDs bind to the chemokine receptor, CCR6, on immature DCs.\textsuperscript{102-104}

Angiogenesis and epithelial growth are also stimulated by LL-37 in wound healing.\textsuperscript{105} Studies have shown that patients with AD have a significant reduction in AMPs, particularly HBD-2, HBD-3 and LL-37, compared to healthy individuals as well as those affected by psoriasis.\textsuperscript{106,107} This is partially due to the inhibitory properties of the Th2 cytokines, IL-4 and IL-13, and the immunomodulatory cytokine, IL-10, on
keratinocytes.\textsuperscript{106-108} Specifically, Th2 cytokines prevent Th17 development including release of IL-17A, a suggested potent inducer of AMPs.\textsuperscript{98,109} This inhibition has been hypothesized as to being the reason for chronic colonization of \textit{Staphylococcus aureus} in patients with AD.\textsuperscript{98,110} Additionally, sphingosine, a ceramide metabolite that has been found to have antimicrobial actions against \textit{Staphylococcus aureus}, and dermcidin, a broad spectrum AMP, have been discovered to be reduced in AD compared to healthy patients.\textsuperscript{78,111} Reduction in AMPs predisposes these individuals to bacterial, fungal and viral infections.

2.3.3.4 Role of Keratinocytes

Multiple immune or immune-related cells including keratinocytes, dendritic cells, activated T cells, mast cells, eosinophils, basophils and monocytes are abnormal in AD.\textsuperscript{79} Of these cells, the most important to disease pathogenesis include the Th2 biased cells, keratinocytes and dendritic cells.\textsuperscript{79} The Th2 biased immune response has been previously discussed in the immunology of atopy section. Keratinocytes play a vital role in the pathogenesis of AD by releasing or stimulating production of inflammatory cytokines in response to activation of their PRRs. Studies examining mechanical trauma and inflammation of the skin indicate that the cytokines, IL-1α, IL-1β, TNFα and GM-CSF are produced with disruption of the epidermis leading to the production of chemokines that bring leukocytes to the site of damage.\textsuperscript{112-114} Specifically, CCL1, CCL2, CCL3, CCL5, CCL8, CCL11, CCL13, CCL17, CCL18, CCL20, CCL22, CCL26 and CCL27 have been implicated in the recruitment of leukocytes to lesional skin in patients with AD.\textsuperscript{115-124} Additionally, activated keratinocytes release cytokines that attract naïve,
polarized and memory T cells. Chemokine recruitment occurs through CXCL10 for Th1 cells, CCL17 and CCL22 for Th2 cells and CCL27 and CCL18 for memory T cells.\textsuperscript{81} Pivarcsi \textit{et al.} found CCL18 to be the most highly expressed ligand in patients with AD compared to patients with other inflammatory skin diseases such as psoriasis and lupus erythematosus.\textsuperscript{117} Keratinocytes have also been demonstrated to induce the expression of human leukocyte antigen (HLA) class II and intercellular adhesion molecule 1 (ICAM-1) molecules to heighten the presentation of antigens to T cells present in the skin of atopic patients.\textsuperscript{81,125}

\textbf{2.3.3.5 Role of Dendritic Cells}

Cutaneous professional APCs also play a vital role in T cell bias and production of inflammatory cytokines. Resident skin dendritic cells (DCs) act as a link between the innate and adaptive immune system. Once activated by antigens including environmental allergens or microbial agents, they undergo maturation as they migrate to the regional lymph node and signal an adaptive immune response through up regulation of HLA expression and co-stimulatory molecules. Additionally, DCs in the epidermis lose the expression of E-cadherin molecules that anchor them to keratinocytes giving way to migration to the lymph node.\textsuperscript{126} Dendritic cells in the skin can be divided into Langerhans cells (LCs), myeloid derived dermal DCs (mDCs) and plasmacytoid DCs (pDCs).\textsuperscript{81} In noninflamed skin in healthy individuals, LCs are the only epidermal dendritic cell and are thought to maintain tolerance in the skin because they contain surface molecules that hinder T cell response.\textsuperscript{126-128} Loser \textit{et al.} demonstrated in mouse models that the receptor activator of nuclear factor kappa B (NFkB) and its ligand (RANKL) were expressed in
keratinocytes of inflamed skin and regulated T-cell/dendritic cell communication.\textsuperscript{129} Overexpression of RANKL resulted in functional changes of LCs and increase in number of regulatory T cells.\textsuperscript{129} Although their exact mechanism of action is still being investigated, regulatory T cells have been found in abundance in normal skin and are implicated as being important in the preservation of immune homeostasis and inhibition of inflammatory disease.\textsuperscript{130-133}

Allergens penetrating the epidermis first come in contact with LCs given their superficial cutaneous location. Stimulation of LCs occurs when its key receptors FCεRI and thymic stromal lymphopoietin receptor (TSLPR) are bound leading to the activation of T cells and subsequently the release of Th2 favored cytokines in atopic patients.\textsuperscript{134} Activation of DCs through FCεRI binding have specifically induced the expression of IL-4, IL-5, IL-13 and IL-31 from T-cells intensifying IgE production.\textsuperscript{81} Additional cytokines such as IL-8, CCL2 and IL-16 have been produced when LCs are stimulated with FCεRI.\textsuperscript{135} Expression of FCεRI also takes place on mDCs, but unlike LCs, they produce Th1 biased cytokines such as IFN-γ, IL-12 and IL-18 in addition to IL-1, IL-16 and macrophage inhibitory protein 1α.\textsuperscript{135} A subpopulation of mDCs called inflammatory dendritic epidermal cells (IDEC) can be distinguished from LCs because of their lack of Birbeck granules and expression of the mannose receptor (CD206).\textsuperscript{126} They are recruited from the dermis and blood in the acute inflammatory phase of atopic dermatitis and remain during the chronic phase.\textsuperscript{136,137} An \textit{in vitro} study showed that cross-linking of the FCεRI on IDECs causes the release of Th1 biased proinflammatory cytokines such as IL-12 and TNF-α as well as IL-8.\textsuperscript{135} Lesser numbers of pDCs are found within the skin
lesions of atopic patients, but activation of the FCεRI on circulating blood pDCs showed a slight biased towards a Th2 response.\textsuperscript{138}

2.3.3.6 Thymic Stromal Lymphopoietin

Thymic stromal lymphopoietin (TSLP), an IL-7-like cytokine produced by epithelial cells, has been discovered to greatly influence the Th2 differentiation process in allergic disease.\textsuperscript{139} It can also activate natural killer T cells, basophils, eosinophils and mast cells at the start of the inflammatory process.\textsuperscript{140} Allergen proteases from sources such as house dust mites, cockroach and fungi bind to proteinase-activated receptor 2 (PAR-2) on keratinocytes and prompt the release of TSLP.\textsuperscript{141-143} Microbial factors can also induce TSLP production from epithelial cells through the activation of a series of toll-like receptors (TLR) that include TLR-2, TLR-3 and TL-5.\textsuperscript{144} TSLP then acts on dendritic cells, which subsequently produce Th2 attracting chemokines as well as primed T cells that eventually differentiate into Th2 cells.\textsuperscript{134} Previous studies have shown that TSLP is highly expressed in the epidermis in AD and that an elevated TSLP serum level from murine models indicates a barrier defect.\textsuperscript{145} Lee \textit{et al.} reported that higher concentrations of epidermal TSLP were found under filaggrin knockdown circumstances suggesting filaggrin mutations increase TSLP and thus Th2 differentiation.\textsuperscript{146}

Receptors for TSLP are found on natural killer T cells (NKTs), DCs, T cells, B cells, mast cells, basophils and monocytes.\textsuperscript{140,147-149} For NKTs, TSLP binds directly to receptors stimulating the release of IL-13, which then amplifies the Th2 response.\textsuperscript{140,150} Likewise, TSLP acts on basophils causing them to interact in a MHC-II dependent manner with T cells to induce Th2 differentiation. It has also been suggested that TSLP
recruits mast cells and eosinophils in a non-adaptive immune response.\textsuperscript{151} Eosinophil apoptosis is also delayed while cell surface molecules are upregulated by TSLP.\textsuperscript{152}

\textbf{2.3.3.7 JAK-STAT}

The JAK-STAT pathway is made up of a receptor, Janus kinase (JAK) and Signal Transducer and Activator of Transcription (STAT) and is classified as a signal transduction pathway for cytokine and growth factor production.\textsuperscript{153,154} There are multiple members of the JAKs, which includes JAK1, JAK2, JAK3 and TYK2, as well as members of the STAT family, which includes STAT1, STAT2, STAT3, STAT5A/B and STAT6.\textsuperscript{154} Regulators of the JAK-STAT pathway are part of the suppressor of cytokine signaling (SOCS) group that is comprised of cytokine-inducible SH2 domain containing proteins and SOCS1-7.\textsuperscript{154} Binding of specified ligands to the receptor portion activates JAK that then phosphorylates and triggers STAT. STAT moves into the nucleus and regulates targeted genes.\textsuperscript{154} Th2 differentiation is an important part of the AD and can be regulated by JAK-STATs.\textsuperscript{140} Generally, JAK1, JAK3 and STAT6 are considered Th2 pathways, and TYK2, JAK2 and STAT4 are associated with Th1 responses.\textsuperscript{155} The major Th2 cytokine, IL-4, uses the JAK-STAT6 pathway to target nuclear transcription in T cells influencing Th2 responses.\textsuperscript{156} Genes controlled by STAT6 are involved in B cell maturation, IgE class switching and MHC class II production.\textsuperscript{156,157} Studies indicate that TSLP drives a Th2 bias through STAT3 and STAT5 in mice and STAT1, STAT3, STAT5/JAK1 and JAK2 in humans.\textsuperscript{155,158} Loss-of-function mutations in TYK2 and the JAK-STAT suppressor, SOCS2, have led to a predisposition to AD in human and murine models.\textsuperscript{159-162}
2.3.3.8 Cytokines in Atopic Skin

In previous studies evaluating normal skin biopsy specimens from healthy individuals as well as clinically unaffected, acute (<3 days) and chronic (> 2 weeks) skin specimens from patients with atopic dermatitis, it was identified that unaffected atopic skin had more cells expressing IL-4 and IL-13 mRNA than normal skin.\textsuperscript{163,164} Acute atopic skin specimens had significantly more expression of IL-4, IL-5, IL-13 mRNA and IL-16 compared to normal and unaffected skin.\textsuperscript{163-165} T cells in unaffected and acute atopic skin have been shown to express increased levels of IL-4, IL-5 and IL-13, but decreased levels of IFN-γ.\textsuperscript{73} Chronic lesions expressed more IL-4, IL-5, IL-13 and IFN-γ mRNA than normal and unaffected skin.\textsuperscript{163,164} These findings support the biphasic T-cell mediated disease theory where initial inflammation is preferential towards the Th2 phase cytokines, IL-4, IL-5 and IL-13, while chronic stages demonstrate type 0 helper T cell (Th0) pattern which is a mixture of the Th1 and Th2 cytokines, IL-5, IL-12, GM-CSF and IFN-γ.\textsuperscript{8} Additionally, increased IgE levels and eosinophilia has been noted in the peripheral blood atopic individuals.\textsuperscript{73}

2.3.3.8.1 Newer Cytokines

Besides the traditional Th2 and Th1 biased cytokines, studies have indicated that newer cytokines may also influence AD. A member of the IL-2 family, IL-21, is expressed by Th17 cells.\textsuperscript{166-169} Its role in AD is not fully elucidated, but it appears to promote Th17 differentiation and suppression IgE synthesis.\textsuperscript{170-172} Some studies indicate a reduction in IL-21 influences development of allergic disease, but other data suggests a
higher level in atopic lesions. Elevated levels of type 22 helper T cells (Th22), which produce IL-22, have been noted in the peripheral blood and inflamed skin of atopic patients. Secretion of IL-22 from Th22 cells leads to epidermal hyperplasia and downregulates profilaggrin expression in keratinocytes leading to disruption of the protective barrier. Release of IL-25 from epithelial cells after exposure of an allergen causes increased serum IgE levels, eosinophilia and Th2 cytokine production. Like IL-22, IL-25 inhibits filaggrin synthesis and causes barrier defects. High levels of IL-31, a member of the IL-6 cytokine family, have been correlated with the Th2 biased cytokines, IL-4 and IL-13, in patients with atopic dermatitis. Nobbe et al. demonstrated an overexpression of IL-31 in atopic lesions compared to nonlesional skin and healthy controls. Production of IL-31 comes from T cell populations including Th2 committed cells. Their receptors can be found on dorsal root ganglia and cutaneous nerve fibers leading to transmission of pruritus in AD. Previous studies have demonstrated severe pruritus and AD-like lesions in the presence of IL-31, and amelioration of pruritus after the administration of an anti-IL-31 monoclonal antibody. Induction of IL-33 production comes from several sources including presence of antigens, mechanical trauma and microbial agents. Once created, IL-33 provokes expression of IL-5 and IL-13, which elevates serum IgE and eosinophil recruitment. It has been theorized that overproduction of IL-33 may play a key role in the onset of AD.

2.3.4 Pathogenesis of Pruritus in Atopic Dermatitis

Pruritus associated with AD appears to be largely influenced by the crosstalk between keratinocytes and immune cells with cutaneous nerve fibers. Sensory nerve
fibers, primarily C-fibers, that innervate the skin originate in the dorsal root ganglia (DRG) and have peripheral nerve branches in the dermis with free ends reaching into the epidermis.\textsuperscript{198,199} Damage to the stratum corneum through mechanical stimuli, allergens, proteases, toxins or microbial agents triggers a release of neuromediators from skin cells (keratinocytes, fibroblasts) and immune cells (T cells, mast cells, macrophages) that act on pruriceptors on the terminal ending of the sensory nerve.\textsuperscript{197,198,200} Neuromediators include, but are not limited to cytokines (TNF-\(\alpha\), IL-31), leukotrienes, serotonin, histamine, nerve growth factor (NGF), proteases and Endothelin-1.\textsuperscript{197,198,201,202} Pruriceptors are categorized as G-protein coupled receptors, TLRs and cytokine receptors.\textsuperscript{197,202,203} Once pruriceptors are activated, two mechanisms occur: antidromic release of neuropeptides (Substance P, calcitonin gene-related peptide, pituitary adenylate cyclase-activating peptide) and initiation of the phospholipase C\(\beta\) (PLC\(\beta\)) pathway.\textsuperscript{197,198,204,205} Release of antidromic neuropeptides acts as a form of communication from the nerve endings back to the keratinocytes and immune cells triggering further release of neuromediators and an increase in inflammation.\textsuperscript{198,204,205} Activation of the PLC\(\beta\) leads to increased intracellular calcium and transduction of the pruritus signal through the sensory nerve to the dorsal horn of the spinal cord by way of the DRG.\textsuperscript{197,198} Signal transduction crosses the spinal cord where it moves through the spinothalamic tract and into the brain cortex.\textsuperscript{197,206} Studies indicate that the anterior and posterior cingulate cortex and dorsal lateral prefrontal cortex, which are normally reserved for emotions, reward and memory of negative experiences, are significantly more activated in patients with AD compared to healthy individuals.\textsuperscript{206,207} The brain then process the pruritus signal and responds with a resultant scratch reflex.\textsuperscript{203}
2.4 Equine Allergic Disease

2.4.1 Equine Atopic Dermatitis

While human atopic dermatitis has been extensively studied, little is known about equine atopic dermatitis including prevalence and definitive genetic predisposition. Because of a reported familial occurrence and breed disposition, equine AD is believed to have some genetic bias. Other factors include involvement of the immune system, environmental exposure, microbial exacerbation and physiologic reactions.\textsuperscript{15,208-210}

2.4.1.1 Pathogenesis

The pathogenesis of equine allergic disease is similar to that of human allergy. Exposure in sensitized allergic horses occurs through a percutaneous, inhaled or oral route, which then triggers a type-I hypersensitivity by binding to allergen-specific IgE to the FcεRI located on mast cells and basophils.\textsuperscript{211,212} Additionally, allergen-specific IgE binds to dendritic cells allowing for efficient capture of the allergen.\textsuperscript{210} Once the allergen has induced IgE cross-linking on eosinophils and basophils then an elicitation phase occur through release of inflammatory mediators from the cells causing an immediate allergic reaction.\textsuperscript{212} Like humans, it is also hypothesized that horses with equine allergic disease exhibit a Th2-cytokine response consisting of IL-4, IL-5 and IL-13, in addition to increased allergen-specific IgE level.\textsuperscript{210,213}

Recent studies have examined the role of equine TSLP (EqTSLP) in conjunction with the production Th2 biased cytokines.\textsuperscript{214-216} In addition to cloning EqTSLP from the skin of a horse with chronic urticaria to produce specific antibodies, the EqTSLP gene
has also been identified and sequenced. The gene was found on the equine chromosome 14 and encodes a 143 amino acid protein. Equine chromosome 14 also contains the genes for the Th2 cytokines, IL-4, IL-5, and IL-13. Elevated levels of EqTSLP have been found in bronchoalveolar cells from horses with recurrent airway obstruction as well as in equine peripheral blood cells after stimulation with ConA, *Culicoides* extract and IgE cross-linking.

Allergen-specific IgE plays a vital role in equine allergic disease. Foals originally obtain IgE through colostral transfer, which is first detected in serum within 8-12 weeks of life. Self-production does not occur within the foal until 6-9 months. Healthy horse serum IgE concentrations are up to 1000 times higher than humans with a range of 425-82,610 mcg/mL. This range has not been determined as significantly different between allergic and nonallergic horses. Eder *et al.* demonstrated that when measuring serum IgE against crude mold and storage mite extracts, no significant difference was noted between healthy horses and horses with chronic obstructive pulmonary disease (COPD). However when recombinant allergens were used, horses with COPD showed significantly higher levels of IgE than healthy controls. Serum IgG, but not IgA, levels were also significantly higher in COPD horses when tested against a specific *Aspergillus fumigatus* recombinant extract, rAsp f 8. Therefore, the type of testing and allergens used may influence the IgE results in equine patients.

An additional study by Eder *et al.* looked at the association between genetic makeup and environment with mold (*Aspergillus* and *Alternaria*) allergen-specific IgE in 448 Lippizan horses from six stud farms. They found significant links between equine leukocyte antigen (ELA) A1 and elevated serum IgE levels. The gene, ELA A14, was
associated with lower mold-specific IgE, while ELA A8 correlated to undetectable rAsp f7 and rAsp f8 IgE levels. A heritability of 0.33 and 0.21 was calculated for the two mold extract-specific IgE levels and rAsp f8-specific IgE, respectively. In regards to environment, significant differences were observed in sex and age for the two mold extracts between stud farms. Also on a single stud farm, an association was noted between ELA Be27 and lower *Aspergillus*-specific IgE levels. Curik et al. found evidence supporting that the equine MHC region, specifically MHC class II, affected the mold-specific IgE response in the serum from the same 448 Lippizan horses. The strongest associations were noted between the DQA and UM-011 loci and IgE. These studies suggest in addition to a genetic link, various environmental factors at different farm and even barn locations may affect the serum allergen-specific IgE levels in horses.

Skin barrier function plays an important role in human atopic dermatitis, but little is known in regards to equine disease. Marsella et al. performed a pilot study to assess skin barrier dysfunction in allergic disease. Two normal and two atopic horses were selected to assess differences in transepidermal water loss (TEWL) and observations on electron microscopy. No significant difference was noted for TEWL between healthy controls and atopic horses. However, the stratum corneum of normal horses was found to be compacted with continuous lipid lamellae, while abnormal amorphous lipids and irregularities were detected in the allergic animals. This pilot study suggests that barrier dysfunction may be a part of equine AD.
2.4.1.2 Clinical Signs

Clinical signs of equine AD are most commonly reported in a wide age range of horses with a median age of onset at 6.5 years. No sex predilection has been identified, but there is a reported increased prevalence in Thoroughbreds and Arabians. Disease may be seasonal or nonseasonal depending on the allergens involved and major cutaneous manifestations include pruritus and/or urticaria. The pruritus can be mild to severe as the horse demonstrates biting, rubbing of the body on hard surfaces, stomping of the feet, swishing of the tail and sometimes head shaking. Other common clinical signs are angioedema, alopecia, crusting, papular dermatitis, excoriations, lichenification, hyperpigmentation and eosinophilic granulomas. The face, pinnae, ventrum and extremities are the most commonly affected areas for pruritus and skin lesions. Patients with AD can often have secondary bacterial pyoderma, which may exacerbate pruritus and clinical lesions such as papules and crusting. Symptoms of equine allergic disease can reduce an affected patient’s performance, lead to unsightly cosmetic defects and can cause significant irritation. Given the sometimes delayed onset of clinical signs, some horses have already been used for breeding purposed leading to the potential passing of allergy associated genes to the offspring.

2.4.2 Recurrent Urticaria

Urticaria is a clinical sign of underlying disease that can be caused by both immunologic and nonimmunologic factors. Immunological urticarial lesions or wheals result from mast cell or basophil degranulation through type I and III hypersensitivity reactions secondary to aeroallergens, food, insect bites/stings or drugs.
Nonimmunological factors such as light, pressure, heat, cold, stress, drugs, vaccinations and exercise can induce a nonallergic form of urticaria.\textsuperscript{224,225}

\textbf{2.4.2.1 Pathogenesis}

Although the exact mechanism in horses is not completely known, two studies have looked at the histopathologic features of the skin of horses with recurrent urticaria.\textsuperscript{226,227} Rufenact \textit{et al.} found that horses with urticarial lesions had significantly more IgE-bearing cells on immunohistochemistry than the skin of healthy control horses suggesting that IgE may play a pivotal role in urticaria’s pathogenesis.\textsuperscript{226} Subsequently, Hinden \textit{et al.} compared inflammatory cell infiltrate and cytokine expression patterns in lesional and nonlesional skin of eight horses with a history of recurrent urticaria compared to the skin of eight healthy control horses.\textsuperscript{227} They found increased numbers of eosinophils and mast cells in the skin of recurrent urticaria horses when compared with healthy controls as well as more eosinophils in lesional compared to nonlesional skin in affected horses. There was no significant difference in infiltrating cells between nonlesional skin and skin of healthy horses. Expression of IL-4, IL-13 and IL-4 receptor-alpha was increased in lesional skin of affected horses compared with that of control horses, while expression of IL-4 was higher in lesional compared with nonlesional skin of the horses with recurrent urticaria.\textsuperscript{227} These findings suggest that Th2 cytokines, eosinophils and mast cells may also contribute to the pathogenesis of urticaria in horses.
2.4.2.2 Clinical Signs

The primary lesion in urticarial diseases consists of a wheal formed from edema in the dermis with erythema and pruritus.\textsuperscript{224,228} In horses, both erythema and pruritus are variable. The wheal clinically appears as sharply circumscribed, raised, plaques that blanches on diascopy and pits when pressure is applied.\textsuperscript{229,230} Given the variability in size and shape of the urticarial lesions, a classification nomenclature has been established and is as follows: 1. \textit{Conventional urticaria}- papules and wheals that measure 2mm to 5cm in diameter, 2. \textit{Papular urticaria}- small, papules that are 3-6 mm in diameter, 3. \textit{Giant Urticaria}- large wheals that are up to 40 cm in diameter, 4. \textit{Exudative urticaria}- lesions with severe oozing of serum with subsequent matting of the hair and/or alopecia, 5. \textit{Gyrate urticaria}- lesions exhibit arciform, serpiginous or annular shapes, and 6. \textit{Linear urticaria}- parallel bands or bilaterally symmetric urticarial.\textsuperscript{230,231} Any age or breed of horse can be affected although Thoroughbreds and Arabians may be predisposed.\textsuperscript{226,230,231} Angioedema rarely results in some patients secondary to serum leakage or hemorrhage and commonly affects the muzzle, eyelids and ventrum.\textsuperscript{224} The onset of the wheals may be acute and resolve within 24 hours; however, it has also been reported that some lesions will persist for days to months.\textsuperscript{226,227} Chronicity is defined by the human model and is reported as acute (less than 6-8 weeks duration) or chronic (greater than 6-8 weeks duration).\textsuperscript{224,226}

2.4.3 Insect-Bite Hypersensitivity

Insect-bite hypersensitivity (IBH) refers to the most common allergic skin disease of the horse with a prevalence of 2.8-60% of the equine population depending on region
and country. Other names synonymous with IBH include Queensland itch, sweet itch, summer itch, summer sores, summer eczema and summer mange. Culicoides gnats, often referred to as biting midges, sandflies, punkies, and no-see-ums, represent the most important insect implicated in IBH. While over 700 species of Culicoides have been identified, only 130 species have been discovered to be blood feeders. Other insects such as Simulium species (black flies), Stomoxys calcitrans (stable fly) and Haematobia (Lyperosia) have also been anecdotally implicated in the IBH disease process. However, it has been reported that these species may have similar effects of Culicoides given the cross-linking of phylogenetically conserved salivary antigen proteins. While feeding on blood, these species inject the allergic antigens in the surrounding skin and cause intense pruritus secondary to the hypersensitivity reactions.

Seasonal manifestations are the most common with IBH as many insect species, specifically Culicoides, increase in number in the warmer, more humid months such as spring through fall and when the wind is at a minimum. In chronic, severe cases, lesions may persist throughout the winter months as well. The prime feeding times are often noted at dawn and dusk. Meulenbroeks et al. examined the seasonal differences in Culicoides obsoletus-specific serum IgE and cutaneous cytokine expression in IBH-affected and healthy control horses. The IBH peak season samples were taken in August, while the off-season samples were taken in March. Culicoides obsoletus-specific serum IgE was significantly higher in IBH affected horses compared to healthy controls across both seasons. However, the IgE levels in the diseased horses did not differ between March and August. An increase in T cells as well as expression of IL-4, IL-13 and IFN-γ was observed in the skin from both groups of horses in the IBH
peak season.\textsuperscript{243} This study suggested that a Th2-type response was seen secondary to IBH although a Th2/Th1 balance was maintained. Because of the response of the healthy controls, Meulenbroeks et al. inferred that the immune system is activated in both groups of horses during the IBH peak season.\textsuperscript{243}

2.4.3.1 Pathogenesis

Like atopic dermatitis, equine IBH appears to a type-1 immediate hypersensitivity reaction, but may also have a type-IV delayed reaction component.\textsuperscript{210} Wagner et al. confirmed the specific role of IgE by using a modified Prausnitz-Kustner experiment that demonstrated that serum collected from an IBH affected horse and injected into a healthy horse elicited a type-1 hypersensitivity response at the site of injection.\textsuperscript{211} Additional studies have shown that there are significantly more type-1 reactions in IBH affected horses compared to healthy controls.\textsuperscript{244-246} There has also been reported evidence that IBH may elicit a delayed type hypersensitivity, but the presence of this type of reaction as a major factor in IBH has yet to be confirmed on histopathology or cytokine composition.\textsuperscript{234,247,248}

Based on previous studies, IBH appears to have a genetic or familial predisposition with an overrepresentation presented in Icelandic ponies, German shires, Welsh ponies, Shetland ponies, Arabians, Connemaras, Fresians, Swiss Warmbloods and Quarter horses.\textsuperscript{48,237,238,242,249-251} The estimated hereditability of IBH is between 0.08 and 0.3.\textsuperscript{48,252-254} Insect allergen is presented by APCs in the lymph node to naïve T cells via the peptide, MHC class II.\textsuperscript{48} In the horse, these peptides are termed equine leukocyte antigen (ELA) class II.\textsuperscript{255} Chromosome 20q contains the ELA class II region and has
three $DQA$, three $DRB$ and two $DQB$ loci. Variants in the ELA class II region and specific alleles such as Be8, W23 and COR112:274 are associated with a greater risk for IBH. Non-MHC genes associated with IBH and IgE levels have also been identified. Genotypes that had two to five genes encoding for $TSLP$, IFN-$\gamma$, $JAK2$, involucrin ($IVL$) and transforming growth factor beta 1 ($TGF\beta1$) were linked to IBH when single nucleotide polymorphisms were analyzed. Additionally, significant increases were noted in mRNA from the markers, $TGF\beta1$ and IFN-$\gamma$, as well as expression of the gene encoding the CD14 receptor in samples taken from skin lesions IBH-affected horses compared to normal controls. When examining IgE levels, $IGHE$, $IL1RA$, $IL4$, $IL4R$, $IL10$, $IL10/b$, $FCER1A$, $JAK2$ and $ELA$-$DRA$ had significant associations with the IBH trait.

The Icelandic ponies have been well documented in relation to IBH as it has been reported that 26-72% of horses living in Culicoides endemic areas are affected. As the climate does not allow for Culicoides survival, ponies living in Iceland do not exhibit signs of disease. However those born in or shipped to countries with Culicoides, have an increased likelihood of demonstrating the hypersensitivity. Offspring of mares affected by IBH also have an increased risk of developing disease. Sommer-Löcher et al. examined risk factors for development of IBH in affected Icelandic horses as well as other breeds born in Germany, Austria, Switzerland and the Netherlands. They also compared IBH affected and healthy imported Icelandic horses. The group found that duration of exposure during the first 10 months of life and time of birth did not influence development of IBH in the locally bred horses. These horses had their highest chance for developing IBH at between ages three and four. However for the imported
horses, older adults were at a higher risk of developing IBH within the first year following their relocation. Also, prevalence for disease development was similar for imported foals that had their first allergen exposure between 4 and 9 months of age compared to locally bred foals.\textsuperscript{261}

While the route of allergen exposure and source of antigen differ, IBH appears to have overlapping etiologic features with AD. Both involve IgE-mediated, reoccurring, pruritic diseases that are based on multiple factors including genetics and environment. Similar to AD allergens, antigens present in insect saliva elicit immediate type I reaction as well as delayed type IV hypersensitivity in affected patients.\textsuperscript{247} Salivary gland extracts from \textit{Culicoides} species contain as many as 10 IgE-binding proteins bands, which range in size from 12 to 75 kDa and are recognized by 93\% of IBH affected horses.\textsuperscript{262-264} More than 50\% of affected patients commonly reacted to five of the proteins, sized 13, 15, 22, 47 and 48 kDa, and these proteins are now considered potential major allergens.\textsuperscript{262} The lesional skin of IBH horses has been observed to have increased amounts of CD5\(^+\) and CD4\(^+\) T lymphocytes, IgE-bearing cells such as mast cells in the dermis and Langerhans cells in the epidermis, leukotrienes and inflammatory cytokines such as IL-1, IL-3, IL-4 and IL-5.\textsuperscript{265-267} Patients with IBH also have increased numbers of peripheral blood eosinophils, lymphocytes, monocytes and histamine levels.

In addition to elevated numbers of Th2 cells in the acute hypersensitivity response, the suppression of regulatory T cells (Tregs) also appear to impact equine allergic disease. Treg cells are a subpopulation of CD4\(^+\) T cells that also express the IL-2 receptor alpha chain (CD25).\textsuperscript{268} In human medicine, they have been found to uphold self-tolerance and also to have the potential to limit allergic disease, limit chronic
inflammation, impede the immune response to tumors and allow for acceptance in transplantations.\textsuperscript{269-273} The majority of Treg cells found in normal, nondiseased conditions are CD4+ CD25+ population originating in the thymus called natural T regulatory (nTreg) cells.\textsuperscript{269} This population expresses factor forkhead box P3 (FoxP3), which dictates the T cell development in the thymus.\textsuperscript{274} Once in the periphery, Tregs can be induced (iTreg) and produce the inhibitory cytokines, IL-10 and TGF-\(\beta\).\textsuperscript{275,276}

Although the exact mechanism is not completely elucidated yet, Treg cells possess the ability to suppress the other effect T cells including Th1 and Th2 cells through direct cell-to-cell contact, secretion of inhibitory cytokines and/or capability to degrade IL-2.\textsuperscript{277-281}

Limited information regarding Treg cells and their ability to produce immunological tolerance in equine allergic disease is available. Hamza \textit{et al.} was able to isolate a population of FoxP3+ Tregs from peripheral blood mononuclear cells (PBMCs) obtained from healthy horses.\textsuperscript{282} Suppressor functions mediated by IL-10 and TGF-\(\beta\) were produced by the activated FoxP3+ Tregs.\textsuperscript{282} In a separate study, Treg cells were significantly decreased in healthy horses over 15 years of age compared to younger healthy horses.\textsuperscript{283} When examining IBH horses and healthy controls, the number of Treg cells did not differ between the two groups, but the ratio of FoxP3+ Tregs to CD4+ T cells was significantly lower in skin lesions of horses with severe IBH compared to moderate IBH and healthy animals.\textsuperscript{284} Additionally, lesional IBH skin had elevations in IL-13 and reductions in FoxP3+ Treg mRNA and IL-10.\textsuperscript{284} Hamza \textit{et al.} found that PBMCs stimulated with \textit{Culicoides} extracts had reduced suppressor activity, specifically production of IL-10, against CD4+ T cells in IBH patients compared to normal horses.\textsuperscript{285} This reduced suppression was also associated with elevations of IL-4.\textsuperscript{285} These studies
suggest that there is an imbalance between FoxP3+ Treg and Th2/Th1 cells in IBH affected horses.

2.4.3.2 Clinical Signs

Clinical signs of IBH vary with chronicity and severity. Papules, crusting, excoriations, erosions, alopecia and secondary infections follow the self-trauma. With chronic disease, lichenification, fibrosis and hyperpigmentation may be noted. The dorsal aspect of the horse, particularly the base of the mane and tail, and ventral midline are preferred feeding sites for insects, and therefore, represent the most common distribution of IBH associated lesions. In severe cases, patients may exhibit complete loss of hair on the tail and mane as a result of secondary pruritus.

2.5 Diagnosis of Equine Allergic Disease

Diagnosis of equine allergic disease is primarily based on history, physical examination and exclusion of differential diagnoses such as parasitic infestation (Dermanyssus gallinae, pediculosis, Chorioptes equi), allergic contact dermatitis, a drug reaction and cutaneous adverse food reaction. Unfortunately, there is no single test that definitively diagnoses allergic disease in any species of patient. While it has been suggested that allergy testing may be a way to diagnosis an allergy, it is actually used to identify potential environmental immunostimulants that may be contributing to the allergic disease. Once identified, they are either avoided, if possible, or placed in allergen-specific immunotherapy.
2.5.1 Allergy Testing

2.5.1.1 Intradermal Testing

In order to prepare ASIT, allergy testing must be performed to identify the potential environmental allergens inducing disease. While there are multiple types of tests available in human medicine such as patch, skin prick/scratch, intradermal (IDT) or allergen-specific serum IgE testing, only IDT and serum allergen-specific IgE quantification are commonly performed in veterinary patients. IDT is generally thought of as the preferred method for equine allergy testing. It identifies allergens injected directly into the dermis that lead to a dermal type I hypersensitivity reaction via antigenic crosslinking of IgE antibodies on mast cells with subsequent degranulation and wheal formation.212 A benefit of IDT is that the skin represents an example of local and systemic immune reactions.237 The cutaneously-fixed IgE represents the actual IgE mediating atopic dermatitis rather than the circulating IgE in the blood, which has been questioned as to its contribution to skin related allergy.16 As such, the sensitivity of the IDT is higher than that of serum allergy tests making it the preferred test in diagnosing allergic disease.16,19,286,287

Prior to testing, specific drugs such as antihistamines and glucocorticoids need to be withdrawn so not to interfere with the inflammatory components of the hypersensitivity reaction such as mast cell degranulation. The suggested withdrawal time for an antihistamine is 7-14 days although only two drugs, hydroxyzine and cetirizine, and one drug, hydroxyzine, have been studied in dogs and horses, respectively.288-292 Glucocorticoid withdrawal times vary based on route of administration (topical, oral, injectable) and potency of the drug.288 Topical and short-acting oral glucocorticoids are
recommended to be withdrawn 14-30 days prior to the IDT.\textsuperscript{16,25,28,89,293-301} No optimal withdrawal time has been established for long-acting injectable steroids, but the minimal suggested withdrawal times is 28 days with 8+ weeks being preferential.\textsuperscript{25,28,302} Only the effects of injectable dexamethasone given daily over seven days was evaluated in the horse, and the recommended withdrawal time based on the results was found to be 14 days.\textsuperscript{292} Drugs such as ketoconazole, tacrolimus, cyclosporine, essential fatty acids and pentoxyfylline have been shown to not affect IDT results in the dog although studies with these drugs are limited.\textsuperscript{25,28} The effects of other drugs such as tricyclic antidepressants, $\beta_2$-adrenergic agonists, theophylline and bronchodilators are yet to be determined.\textsuperscript{25}

Evaluation of IDT type I IgE immediate hypersensitivity reactions are performed by a trained clinician and assessed subjectively or objectively.\textsuperscript{25} Reactions scored subjectively use a scale of 0 (negative) to 4+ based on wheal size, erythema and turgidity.\textsuperscript{25} Positive controls vary with a 0.001\% histamine phosphate being the most commonly used, however, other solutions such as compound 48/80, codeine phosphate, anti-IgE antibodies and substance P have been utilized. A negative control using a 0.9\% phosphate buffered saline solution is most frequently used.\textsuperscript{25} A 4+ score is comparable to the positive control whereas a 0 score corresponds to the negative control.\textsuperscript{16,25} An objective scoring system can also be implemented by measuring the wheal diameter. Objective measurements $\geq \frac{1}{2}$ diameter of the positive reaction are considered positive.\textsuperscript{16,25}

The subjective scoring method can contain interobserver variation leading to possible false positives or negatives while the objective scoring system fails to take into account erythema and firmness of the wheal which are often significant in IDT.\textsuperscript{303}
Additional IDT drawbacks include reported positive reactions in healthy horses indicating a possible subclinical hypersensitivity, previous exposure without disease, irritant reactions, poor clinical technique, contamination of allergens with bacteria or fungi or ecto-parasitism cross-reaction with dust or storage mite allergens.\textsuperscript{30-33,304} It has also been demonstrated that horses with chronic laminitis have increased number of positive reactions at 15 minutes, 30 minutes and 4 hours compared to nonlaminitic, nonallergic horses.\textsuperscript{305} Of the seven laminitic horses tested, only one had a single previous episode of skin disease manifested as spontaneously resolving urticaria.\textsuperscript{305} False negative reactions can stem from too low of allergen concentration, testing off-season when clinical signs have resolved, stress behavior with induction of endogenous steroids, patients with atopic-like dermatitis or hyporeactivity upon positive control administration.

The IDT is used in horses as a diagnostic tool to determine environmental triggers for allergen-specific immunotherapy and avoidance. Choosing specific allergens for IDT can be challenging. Selection is based on regional distribution of pollens, molds, insects and mites.\textsuperscript{303} Commercially available allergens for use in veterinary medicine are rarely standardized in regards to quality of allergen leading to differences in protein content, manufacturing technique, extract type (crude allergen vs. purified extract), potency, purity and stability.\textsuperscript{24,303}

Use of IDT as a specific screening test for AD is unreliable, as healthy horses can have positive reactions due to one of the causes previously discussed.\textsuperscript{31} However, horses with diseases such AD and recurrent urticaria have more hypersensitivity reactions on IDT compared to clinically healthy horses.\textsuperscript{31} This includes immediate (30 minutes), late
phase (4 to 6 hours) and delayed (24 hours) reactions.\textsuperscript{24,30,62}

2.5.1.1 Threshold Concentrations

Allergen concentrations used for skin tests are determined from threshold concentrations (TCs), which distinguish irritant reactions vs. appropriate potency. Threshold concentrations for the IDT are established by subjectively and objectively scored reactions produced from the injection of serial dilutions of allergens. Threshold concentrations are determined in non-diseased animals and when possible, in animals that have been raised and maintained in sterile allergen-free environments. Therefore, these animals would not have IDT reactions that represent subclinical disease as there has been no previous sensitization. Thus, any IDT reaction in this population will represent irritant reactions. Threshold concentrations have been arbitrarily defined as the concentration where <10% of non-diseased animals have a positive reaction. It is proposed that if a greater percentage of healthy animals react to the allergen concentration used, then the reaction should be considered a false positive result as the allergen was acting as an irritant.\textsuperscript{24,25} The goal for determining TCs is to perform IDT at a concentration below the irritant threshold to avoid influencing the specificity of the test and avoid false positives.

Allergen concentrations used for equine IDTs are commonly extrapolated from the human, canine or feline literature as well as a few equine studies, which are limited both in number and in scope. Five studies have examined IDT TCs in horses.\textsuperscript{32,33,246,306,307} Morris \textit{et al.} examined five dilutions (manufacturer’s recommendation as well as one dilution more potent and three serial dilutions less potent) of 13 commercial insect allergen extracts.\textsuperscript{33} Nine of 13 allergenic insect extracts had TC determined and were
reported as 125 PNU/mL (mayfly); 250 PNU/mL (caddisfly, horsefly, deerfly, fire ant, house fly); 500 PNU/mL (cockroach); 1000 PNU/mL (mosquito); and 1:10,000 w/v (C. nubeculosis). The remaining 4 insect allergens TCs were not determined due to lack of reactivity or excessive reactivity at the least potent dilution. Similarly, Baxter et al. determined the TCs for 27 allergens, and they are as follows for the nine pollens (2000 to > 6000 PNU/mL), four molds (4000 to > 6000 PNU/mL), seven insects (ant, horse fly 125 PNU/mL; house fly, cockroach 250 PNU/mL; moth 60 PNU/mL; mosquito 1000 PNU/mL; Culicoides nebeculosis 1:5000 w/v) and three storage mites (1:10,000 w/v).

The TC was not determined due to excessive reactivity at the lowest concentrations tested for dust mites (Dermatophagoides farinae < 1:12,000 w/v, Dermatophagoides pteronyssinus < 1:30,000 w/v), and the storage mite, Acarus siro (< 1:10,000 w/v). In an Austrian study, 81 Icelandic horses, 43 of which were affected with recurrent seasonal dermatitis, were intradermally tested with 22 various allergens relevant to the region. Only the Culicoides variipennis antigens were tested at multiple concentrations. It was reported that the recommended testing concentrations of 1:50,000 w/v and 1:25,000 w/v did not evoke significant reactions in either the healthy control horses or seasonal dermatitis horses, but that positive reactions were noted at 1:10,000 w/v. The final two studies were assessed by groups from the Netherlands and recommended the following IDT concentrations: 1:1000 w/v for Culicoides extracts and 1000 Noon units/mL for Tyrophagus putrescentiae and Acarus siro.
2.5.1.2 Allergen-Specific Serum IgE Testing

*In vitro* serologic allergy tests (SAT) detect the presence of elevated allergen-specific IgE. Some of the most common techniques to quantitate IgE include radioallergosorbent testing (RAST), the ImmunoCap System™ and enzyme-linked immunosorbent assays (ELISA). RAST was the first test developed to detect allergen-specific IgE in serum. In RAST, the allergen is adhered to a solid medium, often a paper disk, and incubated with the patient’s serum. A buffer wash is added to remove any unbound proteins, then radiolabeled anti-IgE detects bound IgE and results are reported in IgE per milliliter. The ImmunoCap System™ expands upon RAST by using a cellulose sponge instead of a paper disk to increase the binding of allergens as well as using fluorescent anti-IgE so that a fluorescent enzyme assay can be more accurately quantitate allergen-specific IgE. ImmunoCap results are given <0.10 to >100 kU/L.

In veterinary medicine, allergen-specific ELISA testing is the most common test employed. ELISA testing involves coating the wells of an assay plate with allergens, then adding the patient’s serum. The plates and serum are incubated allowing allergen-specific IgE in the serum to bind to the allergens. Subsequently, polyclonal or monoclonal anti-IgE is added, which binds to the IgE. Specialized enzymes and substrates are then added that cause a color change. A popular veterinary ELISA test detects allergen-specific IgE by using a biotinylated recombinant form of the extracellular part of the alpha chain of the human high-affinity IgE receptor (FcεRIα). This assay uses diluted serum and streptavidin-alkaline phosphatase reagents for detection of the FcεRIα. Test results are expressed in ELISA antibody (EA) units based on optical density values with results
at or above 150 EA units considered positive.

Pitfalls of these include variable sensitivities and specificities especially between manufacturers, test type and reagents used.\textsuperscript{19,286,287} False positives have been found in patients with high total serum IgE secondary to nonspecific binding of the allergens.\textsuperscript{19} \textit{In vitro} testing reagents may also cross react with IgG antibodies, which may also lead to false positives.\textsuperscript{287} The level of allergen-specific IgE measured by these assays has also been questioned as to whether it accurately depicts the actual hypersensitivity causing mast cell-fixed IgE antibodies.\textsuperscript{19} Because SAT appears less sensitive, epicutaneous testing including IDT is the preferred allergy testing method in both veterinary and human medicine.\textsuperscript{19,286,287}

Unfortunately, limited data is available regarding serologic allergy testing horses. In the previous studies that compared SAT in healthy horses and clinically diseased horses, no significant difference was found for the majority of specific allergens between IgE levels in the two groups.\textsuperscript{35,220,309,310} Additionally when compared to the preferred method of IDT, the sensitivity and specificity of the SAT were often low or unpredictable.\textsuperscript{35,309} Given the unpredictable results with low sensitivity and specificity of SAT in horses, IDT is the recommended test for identification of environmental allergens and creation of ASIT.\textsuperscript{15,16,212,311}

2.6 Treatment

2.6.1 Allergen-Specific Immunotherapy

Allergen-specific immunotherapy (ASIT) has been defined by the World Health Organization as the administration of progressively increasing amounts of an allergen to
an allergic patient with the goal of diminishing symptoms upon allergen re-exposure. Allergen tolerance is the “adaptation of the immune system characterized by a specific noninflammatory reactivity to a given allergen that in other circumstances would likely induce cell-mediated or humoral immunity leading to tissue inflammation and/or IgE production.” Although the exact mechanism of action is still yet to be determined, ASIT appears to work in atopic disease by inducing allergen tolerance through alteration of antigen presentation in APCs, generation of Treg cells with allergen-specific suppressing abilities, decreased mast cell, eosinophil and basophil provoked inflammation, and antibody isotype switching from IgE to IgG4. Immunotherapy was first used in 1911 by Leonard Noon and John Freeman when they administered increasing doses of pollen extract every 7-14 days to human patients with histories of conjunctival hypersensitivities to grass pollens. The patients treated with pollen specific immunotherapy exhibited a decrease in symptoms during the grass pollen season as well as an increased tolerance to conjunctival challenge when retested. Today, ASIT is successfully used to significantly reduce or control symptoms of people with allergic rhinitis and asthma. Although not traditionally used in human patients with AD, some studies have found significant improvement in clinical signs as well as reduction in other medications required for subjects receiving ASIT.

2.6.1.1 ASIT Mechanism of Action in Humans

Allergen-specific immunotherapy has been reported to decrease serum levels of IL-4, decrease release of IL-4 and IL-13 from peripheral lymphocytes, increase numbers of Th1 T cells secreting IFN-γ, and decrease the numbers of eosinophils and basophils
present at the affected organ.\textsuperscript{289,291} Other studies have discovered that allergen-specific IgE levels initially increase with ASIT administration, but then decrease following after several months of therapy.\textsuperscript{288,317} With ASIT, immature APCs, particularly DCs, pick up the antigen in the tissue and travel to a local lymph node where it is presented to T cells.\textsuperscript{318} Since pro-inflammatory signals are absent in ASIT, the APCs remain in a partially mature state, which causes production of co-stimulatory molecules that lead to the induction of Tregs.\textsuperscript{318,319} Multiple studies have shown that Tregs can be enhanced with ASIT, and that they regulate the inflammatory effects of Th2 cells through direct suppression.\textsuperscript{280,320-324} Because of the decrease in Th2 cells, an increase in the Th1 to Th2 response is often noted in patients using ASIT therapy.\textsuperscript{314,324} Once inhibited, Th2 no longer release IL-4, IL-5 and IL-13, which are required mast cell, basophil and eosinophil differentiation and survival.\textsuperscript{313,325} Tregs produce IL-10 and TGF-β, which further suppress the inflammatory activity of mast cells, basophils and eosinophils as well as Th0, Th1 and Th17 effector cells.\textsuperscript{280,320,326-331} Both cytokines also quell IgE production while stimulating that of IgG4 and IgA.\textsuperscript{313,332} ASIT-induced IgG blocks IgE by binding to allergens, reduces allergic inflammation by decreasing IgE-mediated mast cell degranulation, lessens the volume of allergen-specific memory cells that have undertaken isotype switching to IgE, and decreases late phase reactions by inhibiting IgE antigen presentation to T cells.\textsuperscript{333-337} Specifically, IgG4 has been implicated has having increased blocking activity against IgE in allergic patients.\textsuperscript{313,327} It is considered anti-inflammatory as IgG4 does not fix complement and prevents immune-complex formation from other IgG isotypes.\textsuperscript{313,314,338}
2.6.1.2 ASIT Mechanism of Action in Veterinary Patients

The effects of ASIT have also been studied in veterinary medicine. After exposure with house dust mite allergen, a shift to a Th1 was seen with an increase in IFN-γ compared to IL-4 in atopic dogs. Kepple et al. compared serum of atopic dogs receiving ASIT with that of untreated, healthy dogs and found increased levels of IL-10 and Treg cells. Like dogs, cats with asthma that were treated with immunotherapy had a decrease in IL-5 and IL-4 and an increase in IFN-γ as well as IL-10 in bronchoalveolar lavage fluid. Studies evaluating IgG production after ASIT in veterinary medicine have varied. A few reports have described elevations in serum IgG in atopic dogs and asthmatic cats after immunotherapy administration. However, Hou et al. did not detect a significant increase in total IgG or IgG subclasses in dogs receiving ASIT. Likewise, no change was detected in IgA levels in asthmatic cats receiving therapy. Only Willemse et al. examined intradermal test reactions before and after implementing ASIT, and reported that six of 16 dogs had a decrease in intradermal test reactivity as well as improved clinical signs. However, four of five dogs receiving a placebo also had diminished intradermal test reaction sites.

2.6.1.3 Patient Selection

Veterinary patients are selected for immunotherapy based on multiple factors and criteria. Generally, veterinary patients diagnosed with atopic disease based on identification of positive offending allergens on IDT or SAT for which avoidance of the allergen is not possible are considered for ASIT. Furthermore, animals who do not significantly improve with symptomatic therapy, even seasonally affected animals, are
candidates. While a few studies have indicated that older patients or animals with a longer duration of disease may have a decreased response to ASIT, the majority of reports indicate that disease age of onset, age at the start of immunotherapy and chronicity of disease do not effect efficacy of ASIT. In fact, it has been suggested that older animals may be better candidates as they are unlikely to develop new hypersensitivities to environmental allergens that may influence the result of ASIT. Conflicting studies exist regarding whether seasonality, breed or gender affect the response in canine patients.

2.6.1.4 Formulation

Specific guidelines have been established in humans for formulation and administration of immunotherapy for allergic disease. Unfortunately in veterinary medicine, there is not a standardized protocol for dose or frequency of administration, potency or number of allergens to be placed in an ASIT vaccine, type of allergens used (aqueous, alum precipitated) or route of administration (subcutaneous, sublingual, intradermal). ASIT is therefore formulated based on a veterinarian’s preference, results of allergen-specific testing and patient’s history. Therefore, ASIT will vary from patient to patient. Regionally specific immunotherapy (RESPIT) is the formulation of immunotherapy consisting of a standard set of allergic triggers for the area and eliminates the need for allergy testing as all patients receive the same allergens in immunotherapy. One double-blinded veterinary study compared dogs treated with a standard set of allergen immunotherapy compared to ASIT and found a median improvement in their clinical scores to be only 18% compared to the 70% with patient
specific immunotherapy based on IDT. Several studies have also suggested that the efficacy of ASIT formulated based on SAT results is comparable to that of IDT devised immunotherapy, but these results have not been consistent. Although limited studies are available, the effectiveness may also be dependent on species as dogs and cats may respond to in vitro test based ASIT, while horses do not.

2.6.1.5 Allergen Types

Over 500 allergenic extracts exist from mites, insects, fungi, pollens and animal danders. Extracts are composed of proteins, carbohydrates, lipids, enzymes and glycoproteins obtained from the natural allergenic source. They are used in allergy testing and to promote desensitization as in ASIT. The optimal dose for ASIT is that of which produces clinical improvement in the majority of patients without causing undesirable adverse effects. Approximately 5-20μg of the major allergen is reported as the optimal dose for mites, cat dander, ragweed and hymenoptera venom in people. In human medicine, they are classified as standardized or non-standardized.

Currently, 19 standardized allergen extracts exist, and they are labeled based on the contents and type of potency testing performed. In the United States, the Federal Drug Administration regulates allergen extract standardization and testing is based off of in vivo IDT in allergic individuals and/or in vitro serum IgE methods. The potency is measured as bioequivalent allergy unit (BAU) and reported as BAU/mL. There is no validated method for potency testing in non-standardized extracts, and they are often labeled according to their weight per volume (w/v) ratio or amount of Protein Nitrogen
Units per milliliter (PNU/mL).\(^{367}\) Consistency of nonstandardized allergen extracts may vary by lot and manufacturer depending on the source of collection, filtration process, manufacturing and storage.\(^{367}\) Allergen extracts are further categorized by if they are naturally occurring or modified/recombinant as well as if they are aqueous or contain an adjuvant or preservative (alum precipitated, propylene glycol suspended or glycerinated).\(^{383,384}\) The modified or recombinant forms are allergenic proteins synthesized in a purified form so that only relevant molecules are present opposed to mixture of allergenic and non-allergenic proteins, carbohydrates and lipids present in traditional, natural extracts.\(^{383}\) Adjuvants are non-immunogenic compounds that enhance antigenicity though a non-pathogenic immune response when combined with an allergenic extract.\(^{383}\) By increasing the immune response with an adjuvant, the amount of allergen can be decreased or the frequency of administration can be lengthened, thus reducing possible side effects.\(^{383}\) Types of adjuvants include, but are not limited to oil emulsions, natural or synthetic microbial derivatives (monophosphoryl lipid A, immunostimulatory sequences oligo-deoxynucleotide) or aluminum salts (aluminum hydroxide, aluminum phosphate).\(^{383,385-387}\)

The most common form of allergen used in the United States for veterinary patients is the aqueous based form obtained from a commercial laboratory, while alum precipitated allergens are preferred in Europe.\(^{346,359}\) Aqueous extracts are quickly absorbed and for this reason frequent injections must be given.\(^{362}\) Preservation is often achieved by adding phenol or glycerin.\(^{362}\) Phenol-preserved extracts lose more potency compared to glycerin-preserved, but the glycerin can also cause an increase in local reactions.\(^{362}\) Alum-precipitated extracts delay absorption leading to longer time between
injections. However, cutaneous nodules and sterile abscesses have been reported with use. There is one report of better patient response with aqueous vs. alum precipitated allergens, but further studies would be required to validate this. Allergens are selected based on allergy test results and the seasonal nature of disease for each patient.

2.6.1.6 ASIT Administration

Frequency and amount of immunotherapy administered also vary based on patient and clinician. Typically, a loading phase is implemented by giving increasing amounts and concentrations of ASIT every two to seven days. Once a given concentration and amount is reached, a maintenance phase is implemented and immunotherapy is administered at regular intervals ranging from every five days to once yearly. The generally recommended period for maintenance is every five to 20 days. However, this can vary based on the patient’s response, as concentrations and frequency of ASIT may need to be increased or decreased if the patient clinical signs worsen. Clinical improvement is not immediate and often takes 2 to 12 months before a response is noted. Time to response may also be species dependent as this time in dogs, cats and horses is reported as 2-9, 1-4 and 8-12 months, respectively. The long-term efficacy has only been evaluated in dogs via uncontrolled studies. Continued improvement after discontinuation of ASIT has been reported in 4-23% over variable amounts of time. This may suggest that long-term administration is necessary for lifelong effects in veterinary patients.
2.6.1.7 Types of Immunotherapy

Various types of ASIT are used in veterinary medicine although more forms such as intrabronchial, intralymphatic and epicutaneous immunotherapy are available in human medicine. Subcutaneous immunotherapy (SCIT) is administered via injection and is the most common form of ASIT in veterinary medicine. More recently, oral, specifically sublingual (SLIT), immunotherapy has become commercially available for veterinary patients. Despite this, only one study to date has evaluated the efficacy of oral immunotherapy in dogs, which showed no significant clinical improvement. While an additional study did not assess clinical response, Deplazes et al. detected the development of tolerance in two ovalbumin-sensitized dogs administered oral immunotherapy, but not in two house dust mite-sensitized dogs. Another variation on ASIT is called rush immunotherapy (RIT), and it consists of rapidly completely the loading phase with a short period of time, often less than 24 hours, in order to hurriedly reach the maintenance phase. It is unclear whether RIT in dogs may lead to an increased incidence of adverse reactions and/or premature discontinuation of ASIT. However, Mueller et al. found a higher success rate as well improvement within 6 months of therapy in 11 dogs using RIT compared to another 11 dogs using conventional ASIT.

2.6.1.8 Adverse Effects

While ASIT has been reported to be of benefit to allergic patients, adverse reactions have been described in dogs, cats and horses. Increased generalized pruritus is the most common side effect reported in the canine patient. Although less common,
local injection site reactions manifested as edema, pruritus and/or pain have also been observed.\textsuperscript{314,390,395} Anaphylaxis, weakness, lethargy, diarrhea, vomiting, anxiety, urticaria and edema are rare and occur in $\leq 1\%$ of veterinary patients.\textsuperscript{314,390,403}

### 2.6.1.9 Use in Horses

Like humans, dogs and cats, ASIT is used in equine patients as a form of therapy to treat allergic disease. It is recommended when the disease causes clinical signs for a prolonged period of time, and the offending allergen cannot be avoided such as in the case of environmental pollens. ASIT has been reported to have a good to excellent success rate for treatment in approximately 60-84\% of the equine allergic dermatitis cases where it was used.\textsuperscript{15,374,404-407} However, other studies have documented inconsistent or no response to ASIT in horses with allergic disease.\textsuperscript{249,317,408,409} No studies have been published documenting the specific immunologic effects of equine ASIT, but it is possible that it may be similar to that in humans, canines and felines where allergen-specific IgE levels decrease in time, numbers of basophils and eosinophils decrease, and the Th2 mediated reaction is shifted to a Th1 or regulatory T cell response.

### 2.6.2 Alternative Therapy in Equine Allergic Disease

In addition to ASIT, horses suffering from allergic disease can alternatively be treated with systemic and topical medications. Topical medications include shampoos, rinses, lotions and ointments. Active in ingredients in the topical medications can include antipruritics (glucocorticoids, colloid oatmeal, anesthetics), antibacterials (chlorhexidine, benzoyl peroxide, ethyl lactate) or antifungals (elotrimazole, ketoconazole). Systemic
medications have also been used to control allergic disease. Oral (prednisolone, dexamethasone) or injectable (dexamethasone) glucocorticoids have been administered in short and long term courses.\textsuperscript{15} Adverse side effects of glucocorticoids in equine patients include hepatopathy, laminitis and iatrogenic hyperadrenocorticism.\textsuperscript{15} Antihistamines (hydroxyzine, cetirizine, diphenhydramine), phosphodiesterase inhibitors, tricyclic antidepressants with antihistaminic properties (doxepin) and fatty acid supplementation have also been used with success.\textsuperscript{15,410} Additionally systemic antibiotics and/or antifungals may be warranted to treat secondary bacterial and fungal infections.

2.7 Mites

Phylogenetically, mites belong to the phylum Arthropoda in the subphylum Chelicerata, the class Arachnida and the subclass Acari.\textsuperscript{411,412} In the 1880s, a former United States Department of Agriculture employee, Nathan Banks, began researching the impact of mites in environment and in 1915, he published the first English manual of mites including the earliest mite categorization.\textsuperscript{413} Today, there are approximately 40,000 mite species, but a single universal classification system does not exist for the Acari.\textsuperscript{411} Acarologists usually recognize three superorders Opilioacarida, Parasitiformes and Acariformes.\textsuperscript{412} Opilioacarida is a small group of large mites that resemble daddy long-leg spiders (Opiliones).\textsuperscript{412} Parasitiformes are further divided into three suborders including the Mesostigmata, which includes parasitic mite species of birds, mammals, and plants as well as free-living and predatory mites.\textsuperscript{412} Acariformes suborders include Prostigmata, Oribatida and Astigmata.\textsuperscript{412} Unlike other mites, Astigmata lack a distinct respiratory organ or trachea. Instead exchange of gasses and water occurs directly
through the cuticle, thus temperature and humidity levels play an important role in Astigmata survival.\textsuperscript{26} Half of the Astigmata mites such as \textit{Sarcoptes}, \textit{Demodex} and \textit{Otodectes} are known parasites of birds, mammals and insects.\textsuperscript{412} The remaining half of the Astigmata mites species are considered nidicoles, which live in nests of birds and mammals.\textsuperscript{412} Many of the nidicoles mites have adapted to living in houses and storage facilities.\textsuperscript{412} Broadly, the nidicoles can be divided into the Pyroglyphid family of mites, referred to as house dust mites (HDMs), and the nonpyroglyphid mites, referred to as storage mites (SMs).\textsuperscript{411,412} Species from both divisions of mites have been found to cause significant allergic disease in both humans and veterinary patients.\textsuperscript{40,412}

### 2.7.1 House Dust Mites

The term house dust mite describes the family of Pyroglyphidae, which includes 49 species, and are found in homes.\textsuperscript{28} Mite species in this group include \textit{Dermatophagoides farinae}, \textit{Dermatophagoides pteronyssinus}, \textit{Blomia tropicalis} and \textit{Euroglyphus maynei}.\textsuperscript{40,412} HDMs are generally characterized by an ovoid body with eight legs that is white to yellow in appearance.\textsuperscript{40} Dust mites are estimated to live up to 120 days at the average temperature of 28° C with relative humidity of 80%.\textsuperscript{29} A single female can produce 50 to 80 eggs in her lifetime.\textsuperscript{40,414} The average lifecycle spans 30 days and consists of an egg, larva, three nymphal stages and adult.\textsuperscript{40} Dust mites are found commonly in homes with carpeting, upholstered furniture, fabrics and mattresses. They feed on epidermal scales, fungi, pollens and bacteria.\textsuperscript{40}

Dust mite species are dioecious and successful reproduction requires the direct deposit of a male’s sperm to the female’s bursa copulatrix where it is then transferred to
the seminal receptacle. Over a prolonged period of time, the sperm is released from the seminal receptacle into the connecting oviducts and then fertilizes eggs as they move through the ducts from the ovaries. The egg is the first step in the life cycle, which is followed by a larva, protonymph, tritonymph and finally the adult. The larva resemble adult house dust mites, but contain only three pairs of legs; whereas the nymphal and adult stages contain 4 pairs of legs. Immature stages also lack reproductive organs and therefore are void of sexual dimorphism. The larval and nymphal stages each take an active feeding before entering an inactive phase. Metamorphosis takes place in the old exoskeleton before it is shed giving rise to the next life stage. House dust mite life cycle length is dependent on the environmental conditions, in particularly relative humidity and temperature. Low relative humidity can cause a prolonged larval phase, while lower than preferred temperatures can trigger the development of extended quiescent nymphal stages.

2.7.1.1 Dermatophagoides

Dermatophagoides is of Greek origin and translates to “skin eating.” This genus is divided into two species, *pteronyssinus* which means “feather loving”, and *farinae* which means “flour.” Both names reflect the natural habitat of each species prior to adapting to the human environment. *Dermatophagoides* mites feed on the shed epidermal scales of humans and other mammals, fungi, bacteria and pollen. *Dermatophagoides farinae* (*D. farinae*) has previously been detected in baking mixes, whereas *Dermatophagoides pteronyssinus* (*D. pteronyssinus*) has not. *D. farinae* appears more predominantly in drier climates and has been coined the American house
Dust mite due to its prevalence in the United States. *D. pteronyssinus* appears to thrive in more humid climates and is termed the European house dust mite. However, both species have been found to cohabitate in various parts of the world including all regions of the United States with a higher prevalence observed in the Southeast and Midwest as well as the Atlantic, Gulf and West coasts. The largest mite densities in the United States were observed in the carpeted floors rather than the upholstered furniture or mattresses.

*D. farinae* range in size from 260 to 400 μm with rounded idiosoma and a variably shaped propodosomal shield. All of the legs end with prominent pretarsi and small claws. The anus is surrounded by an oval perianal ring, which also contains prominent anal suckers and a pair of preanal setae. *D. pteronyssinus* is similar in size and features, but varies slightly as the hysterosomal shield is larger and more extensive.

*Dermatophagoides* bodies are composed of 70 to 75% water, and their survival depends on replacement of any lost water through excretion, evaporation, reproduction of defecation. Water consumption through food or oxidation of organic materials is not sufficient for the needs of the mites, so water must be obtained through absorption through unsaturated ambient air. The critical relative humidity ranges from 55 to 73% with resultant death of mites secondary to dehydration if the humidity level drops below 51% for prolonged periods of time. *Dermatophagoides* mites are considered eurythermic and can survive in temperatures ranging 15°C to 35°C.

In locations with temperate seasonal climates, the population of living mites fluctuates with seasonal changes. The number of *Dermatophagoides* mites is highest in the summer months when it is believed that indoor relative humidity is most favorable for mite survival and breeding. Mite levels drop during the fall and are lowest during
the winter months when relative humidity is low due to indoor heating.\textsuperscript{34,412,418} Additionally, the amount of rainfall and cleaning practices also impact the amount of live mites as well as allergen.\textsuperscript{412,418}

\subsection*{2.7.1.2 Other House Dust Mites}

\textit{Blomia tropicalis} and \textit{Euroglyphus maynei} are additional house dust mite species. In the United States, they are primarily found in highly humid climates such as in Florida and Gulf Coast homes, but \textit{Euroglyphus maynei} has also been found in Ohio homes.\textsuperscript{423-425} The clinical importance of these species in veterinary and human medicine is not completely clear as there are limited studies investigating their role in allergic disease.\textsuperscript{40,414,426,427}

\subsection*{2.7.2 Storage Mites}

Traditionally, SMs were primarily found in agricultural environments where they cause occupational allergy in farmers and grain manufacturers; however, there has been a more recent increase SMs in the house dust of urban dwellings.\textsuperscript{411,428,429} SMs are found in grains such as oats, corn, wheat and hay as well as facilities that manufacture and store such grains.\textsuperscript{411} Mites may be carried over from these raw ingredients to contaminate processed foods especially when the products become moist or are stored in humid environments.\textsuperscript{412} A relative humidity of greater than 70\% is preferable for SM survival.\textsuperscript{412} SMs feed on protein-rich substances plants and microorganisms including bacteria and molds.
2.7.2.1 *Acarus*

*Acarus siro* (*A. siro*) is known as the flour mite and ranges in size from 320-650 μm. While the body is colorless, the legs and gnathosoma vary in color from pale yellow to reddish brown. The hind margin is smoothly rounded, and the chelicerae have distinct teeth. The legs end with well-developed pretarsus and stalked claws.

It is an economically important pest of stored food products that had been reported to cause lesions and digestive ulcers in livestock; however it is considered primarily an indoor species occurring in storage areas. *A. siro* is more frequently found on processed cereals such as flour rather than whole grain or hay. It has also been detected in cheese, beehives and broiler house litter. This mite species is mycophagous and preferentially feeds on species of *Eurotium*, but can also be maintained on *Aspergillus* and *Penicillium*. *Eurotium* species thrive in grains that have a moisture content of 13-15%, and *A. siro* mites are predominantly found in grains with a moisture content of 14% or greater. The optimal relative humidity for *A. siro* was determined to be 85%. Likewise, the preferred temperature for *A. siro* is approximately 28.5°C although the mites are considered eurythermic and can adapt to survive in temperatures ranging from 13-31°C.

While *A. siro* has traditionally been the most studied, two other *Acarus* species, *A. farris* and *A. immobilis*, are also considered storage pests. *A. farris* is smaller at approximately 350 μm with more lightly colored appendages than *A. siro*. It is a field species of mite that has been detected in barley, hay, cheese, poultry feed, oats, birds’ nest and in litter of poultry houses. It is most predominant during August and
September, and is often associated with Tyrophagus longior.\textsuperscript{34} A. farris has also been found to inhabit grasslands, haystacks, soil and on the leaves of plants. A. immobilis closely resembles A. farris except for distinct egg-shaped terminal head on the first and second tarsi of adult mites.\textsuperscript{34} This species is predominantly found outdoors in birds’ nests and in ground vegetation, but can also be detected in unprocessed cereals, cheese and storage facilities in temperate climates.\textsuperscript{34}

\textbf{2.7.2.2 Lepidoglyphus}

\textit{Lepidoglyphus destructor (L. destructor)} is a major source of mite allergy in European rural environments, but it also causes allergy in urban populations around the world. The adult mite ranges in size from 350 to 560 μm with a pear-shaped idiosoma and dull, white cuticle.\textsuperscript{34} The legs are long and thin, and each one terminates with a pretarsus and small claw.\textsuperscript{34} Each tarsus is surrounded by a claw-like subtarsal scale, which gives it a ciliated appearance.\textsuperscript{34}

\textit{L. destructor} was determined to be the most important allergen causing symptoms from both upper and lower airways among farmers in one Swedish study.\textsuperscript{432} It has been found in soil samples in various countries including Scotland, England, Canada, Japan, the United States and Russia, and is frequently associated with \textit{A. siro, Cheyletus eruditus} and \textit{Cyperus malaccensis}.\textsuperscript{34} In a single study, the most abundant aeroallergens found in air of commercial dairy barns, near Cooperstown NY and Rochester MN, USA, was \textit{Aspergillus fumigatus} followed by \textit{L. destructor}.\textsuperscript{433} In the midland counties of England, \textit{L. destructor} was discovered to always be present in stacks of grain, straw and hay in open fields; however, none were detected in grasslands.\textsuperscript{34} In addition to grain, this mites
species has also been found in oats, rye, wheat, barley, linseed, rice, dried fruit, sugar beet seed, in dead insects, on dried mammal skin, in rodent nests, within mattress stuffing and in poultry feces. L. destructor mites are strictly mycophagous and occur in a great range of conditions with periodic outbreaks affected by the abundance of field fungi, thus this species has been used as a biomarker for grain spoilage. The optimal relative humidity for L. destructor was determined to be 85% with an ideal temperature of approximately 29.5°C. Because of these optimal conditions, higher mite densities are noted in the early summer and sharply decline during the fall and winter months. Like D. farinae and A. siro, L. destructor is also considered eurythermic and can survive in temperatures ranging from 21-33°C. Adults, nymphs and larvae are more susceptible to lower temperatures, but the hypopal or diapause stage can tolerate temperatures as low as -18°C.

2.7.2.3 Tyrophagus

Tyrophagus putrescentiae (T. putrescentiae), a mold mite, is a major pest in stored products worldwide as well as a known cause of allergic asthma and rhinitis. It is approximately 280-450 μm in size and has a smooth, shiny cuticle with varying degrees of tanning on the appendages. The body appears more slender than other Tyrophagus species and each leg terminates with a stalked claw and well-developed pretarsus. The chelicera have teeth as well as a spur-like outgrowth and mandibular spine.

These mites are pests of fungi and preferentially feed on readily digestible genera such as Eurotiun and Penicillium. Stored products such as wheat, flour, cheese, dried
eggs, ham, sunflower seeds, dried bananas, tobacco, barley, nuts and cereal have been found to contain high numbers of *T. putrescentiae* mites as well as dry dog food. The species is stenothermic with an ideal temperature range at 29.0-34.8°C and approximately 90% or greater for relative humidity. Allergies induced by *T. putrescentiae* are most commonly reported in farmers and food industry workers, which correlates to the presence of antigen for the mite species found in cattle farms and farmers’ living quarters in Germany.

Another *Tyrophagus* mite of importance is *T. neiswanderi*, which is similar to *T. putrescentiae* in size, but has distinctly pigmented corneae on the anterolateral corner of the dorsal propodsomal shield. *T. neiswanderi* mites are pests of foliage and have been reported in greenhouse cucumber plants in Northern Ohio. These mites appear to be mycophagous and fungal hyphae have been detected within the gut.

### 2.7.2.4 Glycyphagus

*Glycyphagus domesticus* (*G. domesticus*) is broad bodied storage mite that has been found to induce dermatitis in humans known as Grocer’s itch. Their bodies are 400 to 750 μm in length with a round idiosoma and dull cuticle covered by tiny papillae. Each leg is long and slender terminating in a pretarsus and small claw. Optimal living conditions occur at a relative humidity of 80-90% with a temperature of 23-25°C. These mites are found in flour, wheat, hay, linseed, tobacco, cheese, ham and on dried plant and animal remains. They have also been detected in moldy wallpaper and in barn dust. *G. domesticus* feed on fungi and are the most common pest of stockfish in
Ireland. Additionally, the species has been implicated in causing a pruritic dermatitis in workers handling cheese as well as asthma in some individuals.  

2.7.3 Other Mite Genera and Species

2.7.3.1 Oribatida

Oribatida is an order of Acariforme mites that are often referred to as moss or beetle mites. The bodies of these mites typically range in size from 300 to 750 μm and can be tan to black in color depending on the level of sclerotization of the cuticle. Oribatid mites have wing-like extensions at the anterolateral aspect of their bodies with distinctly shaped sense organs. They are primarily terrestrial and can be found in decaying vegetation, moss, algae or the soil. Food sources include bacteria, fungi, carrions, mammalian feces and in some cases other mites.

Oribatida mites are of agricultural importance as they have been discovered to be an intermediate host for tapeworms. This discovery was first made in 1937, and it has since been found that 127 Oribatida species serve as intermediate hosts for 27 species of Anoplocephalidae that affect many herbivores including horses, sheep and cattle. Transmission occurs when mites consume tapeworm eggs shed in the feces of the definitive host. The infective cysticercoid larvae develops within the mite’s body and is eventually consumed by a herbivore grazing on mite infested grass or feed.

2.7.3.2 Aeroglyphus robustus

*Aeroglyphus robustus* is a species of mite belonging to the Acariformes suborder. Often referred to as the warty grain mite, they have been reported in stored wheat, oats
and barley in Ohio, Belgium, Canada and Russia.\textsuperscript{446,447} The cuticle of these mites is ornamented with small microtrichae and sometimes irregular protuberances. Their preferred temperature is approximately 28°C although they were discovered to be capable of overwintering in immature stages at temperatures as low as -39°C.\textsuperscript{446,447} A relative humidity of 80-95\% is considered ideal for their peak reproduction, which occurs between July and August.\textsuperscript{446,447}

2.7.3.4 Cheyletus

*Cheyletus* is a genus of mites belonging to the Cheyletidae family that belongs to the Trombidiformes, which are part of the Acariformes superorder. These mites are primarily free-living predatory mites that feed on storage or grain mites.\textsuperscript{34} *Cheyletus eruditus* (*C. eruditus*) is one species that is approximately 450 to 620 \(\mu\)m in length with a colorless, diamond-shaped body.\textsuperscript{34} The tibial claw is prominent and terminates with two teeth at the base. Optimal temperatures for *C. eruditus* range from 26.4-29.4°C.\textsuperscript{34} *C. eruditus* is a known predator of the storage mites, *L. destructor* and *A. siro*, but will also feed on the young life stages of moths, beetles and psocids. Preferentially, *C. eruditus* will feed on *A. siro* compared to *L. destructor*. When food sources are scarce, *C. eruditus* mites have been observed to exhibit cannibal-feeding activities.\textsuperscript{34} A trend has been observed that these mites are able to reduce the population of *A. siro* in the summer months, but fail to reduce them in the winter due to the fact that *A. siro* reproduces at higher rate than *C. eruditus* during that time. *C. eruditus* can also be found in mammal and birds nests as well as in house dust.\textsuperscript{34}
2.7.3.5 Tarsonemidae

Tarsonemidae is a family of mites known as the thread footed, broad or white mites. The 31 genera in this family include phytophagus, fungivorous and insectivorous mites. Species of Tarsonemidae mites are relatively small, ranging in size from 100-400 μm in length with an oval shape and a colorless to yellow cuticle. The mouthparts are distinct and adapted for piercing and sucking. Each leg contains five segments that terminate in a pretarsus with one or two claws. Members of the Tarsonemidae family primarily reside in tropic and subtropic climates. Large populations have been found in El Salvador, Costa Rica and Panama. They are considered agricultural pests and have lead to strawberry, bell pepper and tomato crop losses.

2.7.4 Mite Collection and Identification Techniques

Mite sampling involves removal of mites from their environment by vacuuming, brushing or trapping. Vacuuming is often a preferred method because of the potential to standardize the type of vacuum apparatus, size of space to be vacuumed and surface of collection. However some authors contend that while standardization may be attempted, only 0.5-10% of the mite population in a given environment may be removed via this method. Increasing the time spent vacuuming has led to increased dust gathered, but not necessarily increased mite collection. Brushing consists of using a bristle instrument to remove material from upholstered materials or carpets. While it is difficult to directly compare vacuuming to various brushing techniques, one study found that in
assessing a single brushing with vacuuming of adjacent areas, the mean number of mites per squared meter was 95 times higher in vacuumed mattresses and 24 times higher in vacuumed carpet.\textsuperscript{453} However, it has been suggested that brushing more readily acquires species of Pyroglyphidae, Acaridae, Glycyphagidae and Cheyletidae while vacuuming only collects \textit{D. pteronyssinus}, \textit{D. farinae} and \textit{Euroglyphus maynei} from the pyroglyphid species.\textsuperscript{454,455}

Trapping is another method of mite collection, which can use heat, adhesive materials or bait to lure mites to/away from specified traps. Like brushing, directly comparing trapping to vacuuming is often difficult as vacuuming collects eggs, dead mites, fecal pellets and immobile immature mite life stages while trapping requires live mites to move into the trap apparatus. This may skew the results of the overall mite population present. Recently a commercially available baited-device, the BT Mite Trap\textsuperscript{TM}, was developed to trap and monitor storage mites in stored food products. Experimentally, the BT Mite Trap\textsuperscript{TM} had a mean minimal mite capture rate of 62\% using laboratory and field strains of \textit{T. putrescentiae} and \textit{A. siro} mites in 10 replicate experiments over four day periods with controlled humidity and temperature.\textsuperscript{456} An additional study by Wakefield and Dunn found that the trap was effective at detecting \textit{L. destructor}, \textit{Tyrophagus longior} and \textit{A. siro} over a 10-day period in a laboratory setting at a temperature of 20 \textdegree C and a relative humidity of 65\%.\textsuperscript{457} The trap was also tested in four different storage facilities that were known to manufacture and/or store pet food, cheese, specialty animal feed and cereal products. In comparison to two traditional, non-commerically available trapping devices, the BT Mite Trap\textsuperscript{TM} collected significantly more mites including 17 different genera of storage and predatory mites.\textsuperscript{456} These studies
indicate that the BT Mite Trap™ may be an important commercially available tool in the 
standardization and collection of mites in stored products.

Once mites have been collected, the next step in identifying the population 
involves removal from the sample through flotation or suspension with subsequent 
morphologic classification or through molecular techniques such as measurement of 
guanine content, enzyme-linked immunoassay or polymerase chain reaction. Traditional 
flotation and suspension methods use specific gravity gradients, lactic acid and dynamic 
flotation concentrations to separate mites by their densities from the aqueous solution 
where the mites are then removed.\textsuperscript{451,458-460} These methods are often considered costly, 
time consuming or limited in the materials that can be tested.\textsuperscript{458-460} In 2000, Thind 
developed a modified flotation technique that when tested with dust specimens, which 
sometimes contained fragmented insects and rodent hair, from various domestic living 
areas (bedroom, living room) and occupational spaces (office, warehouse, factory) found 
mites in 78\textendash88\% of samples.\textsuperscript{461,462} Of the mites obtained, 48 different taxa were identified 
with 26\% of domestic samples and 15\% of office samples containing more than 100 
mites per gram of dust, which is considered to be a sensitization factor in allergic 
disease.\textsuperscript{461-463} This modified flotation technique also improved upon the efficiency of the 
older collection tests as it takes two hours to collect mites from dust samples compared to 
the 12 to 208 hours of other sampling methods.\textsuperscript{462}

Once mites are collected and filtered, they are then mounted to microscope slides 
and morphologically examined for classification purposes. Identification requires a 
certain degree of acarology expertise, as it is based anatomic features including 
placement of shields, mouthparts and orientation of hairs. Several published keys are
available to aid in this process, but mounted mite samples are often fragmented or in immature stages, which makes identification even more difficult. Because of the difficulty in morphologically recognizing distorted, legless or fragments of mites, molecular techniques are being developed to provide better tools for identification.

Enzyme-linked immunoassays (ELISA) exist for species of house dust mites as well as for several species of storage mites. Early ELISA testing for *L. destructor* lacked sensitivity and demonstrated cross-reactivity in the presence of grain. A more recent ELISA using polyclonal antibodies for *A. siro* has yet to been validated. In 2008, Dunn *et al.* developed rapid species-specific ELISAs using monoclonal antibodies for *A. siro, L. destructor, Glycyphagus domesticus, Tyrophagus longior* as well as for other mites of the *Tyrophagus* and *Glycyphagus* genera. These immunoassays were validated with a high specificity in the presence of predatory mites, insects, fungi and grain. Further work demonstrated the ELISAs of *A. siro* and the *Tyrophagus* genera were comparable to the flotation method in detecting mites in laboratory and field samples.

Wong *et al.* used polymerase chain reaction-restriction fragment length polymorphism of the internal transcribed spacer 2 region to distinguish between six specific house dust mite and storage mite species. Based on the digestion patterns with the restriction enzymes, it was discovered that Hinf I and Ple I enzymes could be used to distinguish between *D. pteronyssinus* and *D. farinae*. Additionally, Bfa I and Alu I differentiated between *Blomia tropicalis* and *Glycometus malaysiensis*, and the Ple I enzyme was used for *T. putrescentiae* and *Aleuroglyphus ovatus*.​
Another method of mite detection does not specifically identify house dust mites, but rather their specific allergens. Immunochemical assays such as RAST inhibition, radioimmunoassays and ELISA have been developed to detect allergens such as Der p 1, Der f 1 and Der p 2. An ELISA using monoclonal antibodies is among the most commonly used methods to detect mite allergens because of its availability and ease of use. Results are quantified as a amount of allergen per gram of dust. Approximately 2 μg of allergen per gram of dust equates to 100 mites/g while 10 μg are approximately equivalent to 500 mites/g. Based on guidelines published by the International Workshop on House-Dust Mite Allergy, exposure to 2 μg of Dermatophagoides group 1 allergen per gram in the home is considered a risk factor for sensitization and development of asthma, while exposure of 10 μg of the same allergen is regarded as a major risk factor.

An indirect measurement of house dust mites by measuring their nitrogenous waste product, guanine, was originally developed in 1984. However, this test was not specific as it also detects guanine produced from any mite species and birds as well as it cross-reacts with xanthine found in human tissues. After using pure mites samples with house dust, Hallas et al. found that 63% of the guanine detected was produced by non-house dust mite sources giving rise to the possibility of a high false positive rate. However, given its enzymatically rich nature, mite fecal excrement was still suspected as being an antigenic source although its use as a detection of actual mite numbers may be misleading. Further work validated this suspicion in three mite species. In addition to fecal excrement, the outer cuticle, eggs and internal structures related to the digestive and reproductive tracts in three mite species are highly immunogenic sites and may also be
responsible for allergic sensitization. Tang et al. successfully produced polyclonal antibodies from sensitized rodents to identify the most antigenic parts of the house dust mite, Blomia tropicalis, and two storage mites, Aleuroglyphus ovatus and Glycycometrus malaysiensis. These capturing antibodies may have future use detecting antigenic sites in more mite species, which may also aid in the recognition of mites in the environment.

2.7.5 Cross-Reactivity and Co-variation of Sensitization

Allergic cross-reactivity can be defined as when a hypersensitivity reaction occurs against an allergen to which the individual has no prior sensitization to, but this allergen shares similar antigenic structures with another allergen to which the individual has been sensitized. Covariation of sensitization expands on the concept of cross-reactivity and is defined as a “higher observed frequency of sensitization to two or more allergens than the expected occurrence.” Cross-reactivity among various species of house dust mites and storage mites has been previously reported in humans. In 2012, Zhang et al. found a higher prevalence of positive skin prick and allergen-specific IgE test results for the house dust mite species, D. farinae and D. pteronyssinus, compared to five species of storage mites. Through a series of IgE inhibition measurements, the study found that none of the storage mites could inhibit IgE against D. pteronyssinus, but all storage mites could inhibit IgE against the storage mite species. The group concluded that positive reactions to storage mites could be explained by reactivity to other storage mites as well as cross-reactive to D. pteronyssinus. This could rationalize why patients in one geographic area that are not exposed to a specific allergen such as a storage mite could have a positive allergen specific test result to that mite. Additionally, Shafique et al.
discovered through sequencing amino acids that covariation of sensitization might occur between house dust mites and some species of insects and mollusks.\textsuperscript{477} Therefore, other invertebrates may also influence allergy test results.

Limited studies have examined the cross-reactivity of house dust mites and storage mites as well as other allergens in veterinary medicine.\textsuperscript{482-485} A recent in vitro study demonstrated the possibility of cross-reactivity between \textit{D. farinae}, \textit{D. pteronyssinus}, \textit{A. siro}, \textit{L. destructor} and \textit{T. putrescetiae} based on the results on intradermal, serum allergen-specific IgE and IgE ELISA cross-inhibition testing.\textsuperscript{484} Naturally occurring atopic dogs in this study had a co-sensitization of 45% or higher for all possible pairs of the five mite species.\textsuperscript{484} In 2013, Buckley \textit{et al.} also demonstrated cross-reactivity or co-sensitization between the five previously mentioned mite species, but not between the mites and epidermals, molds, grasses, trees and weed pollens.\textsuperscript{485} They concluded that phylogenetically related allergens were statistically more likely to react with one or more allergens within the given group; whereas reactions between unrelated groups were no more likely to occur than that expected with chance.\textsuperscript{485}

\textbf{2.7.6 House Dust Mites and Storage Mites in Allergic Disease}

Worldwide, dust mites represent one of the most commonly reported environmental allergens. A reported 40-80\% of human patients with atopy were found to be sensitized to house dust mite allergens with positive skin allergy tests and allergen specific IgE titers.\textsuperscript{486-489} Their usefulness was found in that they recycle human waste products by feeding on shed keratinocytes in the environment. The concept of mite induced allergic disease was first considered over 70 years ago and several early studies
implicated *Dermatophagoides* species as the main source of allergenicity in urban homes. All three atopic disorders, asthma, allergenic rhinitis and atopic dermatitis, have been clinically induced with provocation of HDM antigens. There has been much debate regarding if species such as *D. farinae* or *D. pteronyssinus* have cross-allergenicity or if people affected are co-sensitized to both mites. Recent investigation has determined that there is typically a 15-20% difference in amino acid sequence between the two species, and although they have different epitopes, they appear immunologically cross-reactive.

Storage mite allergies have also been found to be common in atopic humans with or without house dust mite allergies. Allergies to storage mites have been associated with damp housing conditions as well as occupational exposure. Storage mites have also been implicated as a cause of asthma in farmers based on previous studies using bronchial provocation. One study followed 1577 Swedish farmers over 12 years and reported that 41.7% had some degree of asthma or allergic rhinoconjunctivitis by the end of the time period. Of these farmers, 500 were tested with radioallergosorbent tests and a prevalence of 6.5% was found that had allergic hypersensitivities to storage mites. Similarly, another study examined Wisconsin farmers for signs of allergic respiratory disease and reported that 11.6% of affected individuals had a positive reaction on skin or RAST testing to storage mites. Of those positive to storage mites, 88% were reactive to *L. destructor* while there was a 75% and 25% prevalence to *T. putrescentiae* and *A. siro*, respectively. While the majority of the literature focuses on the occupational exposure of farmers, there has been increasingly
more information showing that storage mites may also induce allergy in a more urban environment.\textsuperscript{519-521}

\subsection*{2.7.6.1 House Dust Allergen}

Although house dust is often referred to as an aeroallergen, it is more likely to be the components of dust, which include house dust mites, storage mites, animal danders, molds, bacteria, plant materials and insect fragments, that is the true antigenic source of allergic disease. Partially digested food and enzymes are excreted from live mites as fecal particles. These particles are surrounded by a chitinous peritrophic membrane which helps to keep the digested materials intact.\textsuperscript{522} Mite fecal pellets are believed to be a major form of allergen and can rapidly release proteins that lead to disease.\textsuperscript{522} In 1980, investigators were able to purify the first major mite allergen, which was a 24 kD glycoprotein and called it \textit{Dermatophagoides pteronyssinus} allergen I (Der p 1).\textsuperscript{523} Subsequently other major allergens were identified from \textit{D. pteronyssinus}, Der p 2, as well as \textit{D. farinae}, which were termed Der f 1 and Der f 2.\textsuperscript{504,524}

Further studies found that allergens produced by \textit{D. farinae} and \textit{D. microceras} contained homologous cross-reacting antigenic components which led to the creation of a house allergen group classification system that consists of group 1 (Der p 1 and Der f 1) and group 2 (Der p 2 and Der f 2).\textsuperscript{504,525} Both groups represent proteolytic enzymes that are present in mite feces.\textsuperscript{24} Group 1 allergens (25 kD) are larger than group 2 allergens (15 kD) and are 20-33 times more likely to be found in the feces.\textsuperscript{24} Group 2 allergens are the most similar in structure and strongly cross-reactive with >90\% sequence
homology. Both groups are carried by particles greater than 10 μm, which are only aerosolized when disturbed.

More recently, over 24 groups of Dermatophagoides allergens exist as well as multiple allergens for the storage mites, A. siro, L. destructor and T. putrescentiae (Figure 1). Allergen nomenclature has been structured to reflect the first three letters of the genus name as well as the first letter of the species name and the number in which the allergen was discovered. Studies using solid phase immunoassays and immunoblotting, studies have indicated that the Dermatophagoides group 1 and 2 allergens account for approximately 50% of IgE binding in atopic patients. Group 4, 5 and 7 allergens made up 30% of the IgE binding. Currently, only Der p 1, Der f 1, Der p 2, Der f 2 and Der p 10 are available for commercial use.

As opposed to the stored food products, more storage mite species are being detected in house and barn dust in both rural and urban environments. Because of this, it has been proposed to change from calling them “storage mites” to “domestic mites.” Like the cross-reactivity of house dust mites, there is still much debate if storage mites and house dust mites share antigenic properties. For Dermatophagoides and Tyrophagus species, there appears to be some similar antigenic components in both the mites and feces, but the majority of their allergens appear to be mite specific. In a single English study, 196 individuals from an urban environment with no history of occupational exposure were found to have a prevalence of 24% and 14% via radioallergoabsorbent testing (RAST) to D. pteronyssinus and at least one storage mite (A. siro, T. longior or L. destructor), respectively. Considerable cross-reactivity between D. pteronyssinus and the storage mites A. siro and T. longior was
detected, but only a limited amount cross-reactivity between *D. pteronyssinus* and *L. destructor* was noted.\textsuperscript{519} This may suggest that some of the response to storage mites observed by direct RAST is a consequence of cross-reactivity with the more abundant *Dermatophagoides* mites.\textsuperscript{519} However this theory has is still yet to be definitively proven.

### 2.7.6.2 Role of Innate Immune System

Although atopic dermatitis is considered a Th2 biased allergic response, the innate immune system also plays a vital role in its manifestation. The activation of the innate immune system occurs through the mite allergen itself as well as through danger signals in the allergen and/or the environment.\textsuperscript{530} Stimulation is mediated via germline encoded receptors, PRRs, which recognize conserved structures known pathogen-associated molecular patterns (PAMPs).\textsuperscript{442,531} PRRs are expressed in macrophages, dendritic cells and nonprofessional immune cells such as epithelial cells, endothelial cells and fibroblasts. Proinflammatory cytokines and chemokines are upregulated by the activation of PRRs through the downstream signaling pathways such as nuclear factor kappa B, mitogen activated protein kinase and type I interferon pathways.\textsuperscript{531} Four classes of PRRs exist that contribute to these proinflammatory pathways and include TLRs, NOD-like receptors (NLR), RIG-I-like receptors and C type lectin receptors. Examples of PAMPS include lipopolysaccharides (LPS), β-glucans and damage-associated molecular patterns (DAMPs).\textsuperscript{442,531} Due to its natural environment, HDMs, in addition to their endogenous allergen, contain LPS, β-glucans and fungi, which may act as DAMPs.\textsuperscript{531}

The role of TLR4 has been discovered to be an important part of house dust mite allergy. Recent studies have shown that mice deficient in TLR4 were absent of the
features necessary to produce allergic asthma. Dendritic cells activated by TLR4 in the draining lymph nodes were decreased in these mice leading investigators to suspect that TLR4 is a vital component in the HDM sensitization process. Hammad et al. demonstrated that activation of TLR4 in the airway epithelium through its ligand, LPS, from HDMs led to the production of innate pro-Th2 cytokine, TSLP, GM-CSF, IL-25 and IL-33. This led to the belief that epithelial cells are important in the creation of allergic inflammation through TLR4 and LPS containing HDM interaction. Additional studies have proven that group 2 mite allergens are LPS-binding proteins.

Another pathway activated by HDM exposure is the secretion of the chemokine, CCL-20, which is believed to be mediated by the binding of HDM-derived β-glucans to C type lectin receptor Dectins. Dectin-2 is important for the invasion of eosinophils and neutrophils in the lungs during an allergic response as well as the production of Th2 cytokines. House dust mite exoskeletons also contain chitin, which acts as a size dependent PAMP that stimulates Dectin-1, TLR-2 and the mannose receptor, leading to production of both pro- and anti-inflammatory cytokines. In regards to AD, keratinocytes appear to express TLR 1-6, and 9 as well as Dectin-1. This suggests that LPS and β-glucans carried by HDM may enable the innate response in the skin as well as the lung. NLR pathways are also activated by HDM derived keratinocyte stimulation through the initiation of caspase-1 and release of IL-1β and IL-18.

Additional innate, non-PRR, mechanisms can also lead to allergic disease. Proteinase-activated receptors (PARs) sense extracellular microbial proteinases that lead to inflammation. PAR-2 activation through Der p 1, Der p 3 and Der p 9 has been hypothesized to cause the secretion of GM-CSF, eotaxin, IL-6 and IL-8 in airway
epithelium and keratinocytes.\textsuperscript{540-543} Another mechanism of innate immune activation occurs when proteolytically active HDM allergens anti-protease lung defenses in the mucosa.\textsuperscript{442,531} Both Der p 1 and Der f 1 can enhance tissue local tissue damage by dysregulating human neutrophil chemotaxis and inactivating lung surfactant proteins.\textsuperscript{544,545} HDM protease allergens also cleave the tight junction proteins occludin and zonula occludens-1 leading to the destruction of the epithelial barrier integrity and increased permeability for allergen presentation.\textsuperscript{546,547}

### 2.7.6.3 Antibody Levels to Mites

Controversy surrounds serum antibody levels, specifically IgE, in relationship to the diseased state of an individual. Elevations in allergen-specific serum IgE have been thought to indicate sensitization, but this does not always appear to be the case.\textsuperscript{10,548} In regards to house dust mites, IgE responsiveness is impacted both by environmental exposure and is also frequently associated with the human leukocyte antigen gene complex.\textsuperscript{76,549-551} Recent studies have shown that genetic makeup may contribute up to 40\% of the variability in the IgE responsive phenotype in humans.\textsuperscript{552,553}

Similarly in dogs, a genetic predisposition may influence the presence of high IgE responsiveness in healthy canine patients.\textsuperscript{548,554} Roque \textit{et al.} determined that a population of nonatopic West Highland White Terriers (WHWTs) had significantly higher positive serum allergen-specific IgE results for 44 out 48 environmental allergens tested compared to a group of atopic WHWTs.\textsuperscript{555} The most commonly reported positive allergens in the nonatopic WHWTs included \textit{D. farinae}, \textit{D. pteronyssinus} and \textit{T. putrescentiae}.\textsuperscript{555} Later work by the same group discovered a genetic predisposition in the
healthy WHWTs to high IgE with a significant allelic association between *D. farinae*-specific IgE and 2.3-Mb area on CFA-35. Thus elevated serum IgE may be caused by many factors in the canine patient including environment and genetic phenotype.

Like IgE, the role of IgG in HDM allergy in both humans and dogs is debatable. Some argue that the subclass, IgG4, blocks the body of allergen to IgE while others feel the IgG4 immune response is closely correlated with that of IgE. In 1985, Willemse *et al.* detected a non-IgE antibody in the serum of atopic dogs and coined it IgGd. Further investigation found that allergen-specific IgG was found in both healthy and atopic dogs, but that levels were higher in the atopic patients and that serum IgE and IgG were significantly correlated in these patients. Using specially developed monoclonal antibodies to the four canine IgG subclasses, Mazza *et al.* discovered that IgG4 was the predominant responder to *D. farinae* and *D. pteronyssinus*. In 1998, an additional study found that while there was a significant correlation between allergen-specific canine IgE and IgGd for *D. farinae* and *D. pteronyssinus*, IgGd levels were higher in healthy dogs compared to atopic dogs. Atopic dogs also had higher IgGd to unrelated allergens. In horses, Eder *et al.* demonstrated that horses with COPD had higher levels of serum allergen-specific IgG to a recombinant mold allergen than healthy controls. Further work is needed in both human and veterinary medicine to define the exact role of IgG in mite induced allergic disease.
2.7.6.4 Mite Induced Allergic Disease in Animals

2.7.6.4.1 Canine

Similar to humans, house dust and storage mites have been described as a common cause of canine nonseasonal atopic dermatitis.\cite{38} House dust mites of the *Dermatophagoides* species have been identified on the skin and hair of dogs as well as in house dust.\cite{563,564} There is much evidence based intradermal tests, IgE serum testing, epicutaneous patch tests, peripheral blood mononuclear cell proliferation, exacerbation of clinical signs following exposure, improvement in pruritus after avoidance and responses to allergen-specific immunotherapy that house dust mite allergens play a significant role in canine allergic dermatitis.\cite{565-572} Storage mites such as *A. siro*, *L. destructor*, and *T. putrescentiae* have also been implicated in canine atopic dermatitis. Sensitization to these mites are also commonly observed in atopic dogs.\cite{38,240,573} Some commercial dog food, both unopened and opened stored bags, have been reported to contain some species of storage mites although further investigation is warranted if the bags were contaminated in their stored environment vs. where the food was manufactured.\cite{440,574}

Based on numerous studies investigating intradermal test results in atopic dogs across the world, the percentage of patients with a positive result varies from location and extract used. However anywhere from 2-100\% of dogs examined had a positive intradermal test to *D. farinae* and/or *D. pteronyssinus*.\cite{484,575-581} For storage mites that included *A. siro* and/or *T. putrescentiae*, 18-52\% of dogs had a positive result.\cite{484,578-581} Similarly serum IgE levels were measured for HDM and SM, and found that 32-90\% and 37-80\%, respectively, had an elevated allergen-specific IgE concentration.\cite{577,580-582}

Multiple studies have also begun to investigate the specific antigen present in house dust.
mites that cause canine allergic disease. The results show that unlike humans, who react more to group 1 and group 2 house dust mite allergens, dogs appear to be more reactive to a high molecular weight antigen (90-110 kD) which is currently being referred to as Der f 15.\textsuperscript{449,566,583,584}

2.7.6.4.2 Mite Allergen in the Canine Microenvironment

Detection of HDMs in the environment of dogs with and without canine atopic dermatitis has successfully been performed.\textsuperscript{563,571,585-589} Based on these studies, dust mites and/or their allergens were detected in 60-100\% of homes with atopic dogs, 35-65\% of homes with healthy dogs, and 48-84\% of homes without dogs.\textsuperscript{563,571,585,587-589} Additionally, \textit{D. farinae} was found to be statistically more prevalent in homes housing canines in the United States and Greece, while \textit{D. pteronyssinus} is statistically more prevalent in homes in England.\textsuperscript{563,571,585,587-589} Higher concentrations of mite allergen and/or mite densities were detected in dog bedding, basements, and homes without central air conditioning.\textsuperscript{585,588} Raffan \textit{et al.} reported that frequent washing of dog beds and environmental flea control in living rooms significantly decreased the levels of Der p 1.\textsuperscript{571} Seasonal fluctuations and humidity also affected mite densities with statistically more HDMs being detected in the summer and fall or when the relative humidity was $\geq$75\%.\textsuperscript{585,588}

Only Farmaki \textit{et al.} reported on the presence of SMs in the canine microenvironment.\textsuperscript{588,589} Limited numbers were found in both studies with a prevalence of 0-10\% for \textit{A. siro}, 0-5\% for \textit{T. putrescentiae} and 0-5\% \textit{L. destructor}. Total numbers of mites were also more limited when compared to \textit{D. farinae} and \textit{D. pteronyssinus}.\textsuperscript{588,589}
2.7.6.4.3 Equine

Unlike in canines and humans, relatively little information has been reported regarding the potential of dust and storage mites as allergenic sources in equine allergic disease. The first known report of storage mites in association with equine recurrent airway obstruction (RAO) examined IgE antibodies detected in serum by ELISA testing in both normal horses and horses diagnosed with RAO. Although it was found that IgE antibodies could be detected in sera using ELISA testing, no significant difference was found between the serum IgE antibody levels to crude storage mite extracts in normal horses and that in affected horses.²²⁰

In 2008, an Australian study was published which investigated threshold concentrations for multiple allergenic extracts in a group of forty-one normal horses over three seasons.³² Currently, the concentrations of injected mite allergens in equine intradermal testing are non-standardized and based off of the concentrations used in canine intradermal testing.²⁵ Of the multiple allergenic extracts used in this 2008 study, two dust mites (D. farinae, D. pteronyssinus) and four storage mites (A. siro, L. destructor, T. putrescentiae, Blomia tropicalis) were investigated.³² Threshold concentration was defined as the highest concentration of allergenic extract intradermally injected where less than 10% of the normal horses had a “positive” reaction. Intradermal tests were subjectively scored based on a scale of 0 (negative) to 4 based on wheal size, erythema and turgidity by comparison to the positive (histamine) and negative (saline) controls. Positive reactions were defined as scores of 2 or greater.³² Threshold concentrations for three out of the four storage mites (L. destructor, T. putrescentiae, B.
tropicalis were found to be 1:10 000 w/v. Threshold concentrations could not be determined for the dust mites, *D. farinae* and *D. pteronyssinus*, and the storage mite, *A. siro*, due to greater than 10% of normal horses having a positive reaction at the weakest concentration tested. 32 Further studies are warranted to investigate threshold concentrations of house dust and storage mites in non-allergic horses especially those residing outside of Australia.

### 2.7.6.4.4 Mites in the Equine Environment

A study by Wallace et al in 2010 evaluated the presence of house dust mites in horse rugs and saddle blankets. *Dermatophagoides* mites were found via a modified floatation technique in eight of the sixteen rugs and blankets sampled. 590 This appears to be the first study to confirm the presence of dust mites in the equine environment. No literature has been published specifically investigating the presence of storage mites in the equine environment. However in previous work investigating mites as potential allergens in the environment of Dutch, it was found that hay had ten times as many mites as grain and three times as many mites as straw. 429 It was also discovered that *L. destructor* was the dominant mite in grain and straw, while *A. siro* was the dominant mite in hay. 429 Based on this data and what is historically known about mites, it is suspected that both dust and storage mites live in the hay, grain, barn dust and bedding of horses and that they play a significant role in equine allergic disease.
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Chapter 3

Equine Intradermal Test Threshold Concentrations for House Dust Mite and Storage Mite Allergens and Identification of Stable Acari Fauna

3.1 Abstract

Background - House dust mite (HDM) and storage mite (SM) stable fauna and their associated equine intradermal test (IDT) threshold concentrations (TCs) for the Midwestern region of the U.S. are unknown.

Objectives - To determine IDT TCs and serum IgE concentrations for two HDM and three SM species in clinically-normal horses over two seasons, and to identify the mite taxa and habitats in a stable.

Animals - Thirty-eight clinically normal horses.

Methods - Threshold concentrations for HDMs and SMs were determined using IDT subjective measurements and a statistical model. An ELISA was used to quantify serum IgE concentrations for the same mite species. A modified-flotation method and BT mite traps™ were used to identify morphologically HDMs and SMs.

Results - Subjective IDT TCs were: 1:80,000 w/v for Dermatophagoides farinae in both seasons, 1:80,000 w/v in spring and 1:160,000 w/v in late summer for Dermatophagoides pteronyssinus, 1:40,000 w/v in spring and 1:20,000 w/v in late summer for Acarus siro,
1:20,000 w/v for *Lepidoglyphus destructor* in both seasons, and 1:20,000 w/v in spring and 1:10,000 w/v in late summer for *Tyrophagus putrescentiae*. Statistically significant associations for increased serum IgE and a positive IDT reaction were evident for *Dermatophagoides farinae* in the spring and *Dermatophagoides pteronyssinus* in both seasons. One mite from all four genera specific to this study was identified; however, two HDM and *Acarus siro* species were not detected.

**Conclusions** - This study established HDM and SM IDT dilution concentrations for the horses in this region. The modified flotation technique was superior to the BT mite traps™ in mite collection in the far environment. Exposure to diverse acaridae fauna may contribute to the pathogenesis of equine allergic disease.

### 3.2 Introduction

Intradermal and serologic allergy testing are used to identify potential environmental allergic triggers in atopic patients.\textsuperscript{17,25,403,592-594} The results of these tests help form the rationale for the therapeutic plan. Intradermal tests (IDT) and skin prick tests provoke localized, small, controlled allergic responses, which are given subjective and/or objective scores. Subjective evaluation of IDT reactions are performed by a trained clinician and assessed by scoring wheal size, erythema or turgidity of the injected allergen site, whereas objective evaluations are performed by measuring the wheal diameter.\textsuperscript{25} The subjective scoring method has interobserver variation leading to possible false positives or negatives. Objective scoring of wheal size fails to take into account erythema and firmness, characteristics thought to be of importance in interpreting the severity of disease.\textsuperscript{303,593}
The reliability of the IDT and the resultant reactions are dependent on many factors. False positive reactions have been reported in healthy horses suggesting possible subclinical hypersensitivity, previous exposure without disease, irritant reactions, contamination of allergens with microorganisms, or may be the result of ectoparasitism known to produce IgE that can cross-react with dust or storage mite allergens.\textsuperscript{30-33,304,593} False negative reactions can be a consequence of using too low allergen concentrations, testing off-season or the administration of medications to the animal that are known to inhibit IgE reactions.\textsuperscript{593} Limited data is available regarding serologic allergy testing (SAT) in horses. In previous studies that compared SAT in healthy horses and clinically diseased horses affected by atopy, insect hypersensitivity or recurrent airway obstruction, no significant difference was found for the majority of specific allergens between IgE levels in the two groups.\textsuperscript{35,220,309,310} When compared to the gold standard of IDT, the sensitivity and specificity of the SAT were often low or unpredictable.\textsuperscript{35,309}

Choosing specific allergens for inclusion in the IDT is of utmost importance in detecting the correct environmental sensitizers. Selection is based on regional distribution of pollens, molds, insects and mites.\textsuperscript{303} By convention, allergen concentrations used for IDTs are determined from threshold concentrations (TCs). The TC is determined to distinguish between the concentration of allergen that produces irritant reactions vs. the concentration that produces allergic reactions in patients with hypersensitivity. Threshold concentrations for the IDT are established by subjectively and objectively scored reactions produced from the injection of serial dilutions of allergens. Ideally, TCs should be determined in animals that are non-diseased and have been raised and maintained in sterile allergen-free environments. These animals would not have IDT reactions that
represent subclinical disease as there has been no previous sensitization. Any IDT reaction in this population would represent irritant reactions. Although raising horses in specific pathogen-free conditions has been done, this is costly and beyond the scope of many research budgets. Threshold concentrations have been arbitrarily defined as the concentration where <10% of non-diseased animals have a positive reaction.\textsuperscript{25,32} It is proposed that if a greater percentage of healthy animals react to the allergen concentration used, then the reaction should be considered a false positive result as the allergen was acting as an irritant.\textsuperscript{25,403} Allergen concentrations used for equine IDTs are commonly extrapolated from the human, canine or feline literature. Equine studies for TCs are limited both in number and in scope. Five studies have examined IDT TCs in horses.\textsuperscript{32,33,246,306,307} Three studies examined TCs of insect extracts\textsuperscript{33,246,307} and the other two reported the seasonal variation of TCs for pollen, insect, mold and mite extracts.\textsuperscript{32,306} In one study, the TCs of two house dust mite (HDM) and one storage mite (SM) extract could not be determined due to excessive reactivity at the lowest TC used.\textsuperscript{32}

Worldwide, HDMs represent one of the most common environmental allergens and are known to be significant contributors to the antigenic burdens in atopic dermatitis, allergic rhinitis and asthma.\textsuperscript{37-40,403} HDMs are found in homes with carpeting, upholstered furniture and mattresses and feed primarily on epidermal scale.\textsuperscript{40,595} Experimentally, HDMs have also survived by feeding on equine keratinocytes \textit{in vitro}.\textsuperscript{596} The ambient relative humidity limits their survival and is the key factor that determines where these mites can survive and live. The cold hardiness of the HDM, \textit{Dermatophagoides farinae}, (\textit{D. farinae}) is impressive as it can survive for 7 days at -18°C thus; it is likely to survive in barns during the Midwestern U.S. winter months. Three species of HDM have been
previously found in Midwestern U.S. homes.\textsuperscript{425} *D. farinae* was the most prevalent species followed by *Dermatophagoides pteronyssinus* (*D. pteronyssinus*) and *Euroglyphus maynei*. Storage mites are found in grains such as oats, corn, and hay, as well as facilities that manufacture and store grains. *Acarus siro* (*A. siro*), *Tyrophagus putrescentiae* (*T. putrescentiae*), *Lepidoglyphus destructor* (*L. destructor*) and *Glycyphagus domesticus* are the most common species found in cereals, flour and baking mixes in homes in the United Kingdom.\textsuperscript{597} In the temperate seasonal climates of North America, the prevalence of live mites fluctuates seasonally. Highest live-mite levels occur during the humid summer months when the indoor relative humidity conditions for mite survival and breeding are most favorable. Live-mite levels drop during the late fall months. The residue of dead mites and the level of mite allergen-produced show similar seasonal trends that parallel the live-mite densities.\textsuperscript{40}

Relatively little information has been reported regarding the potential of HDMs and SMs to act as allergens in equine allergic disease. The first report of SMs in association with equine chronic bronchitis examined serum IgE antibodies detected by ELISA in both normal horses and horses diagnosed with chronic bronchitis.\textsuperscript{220} In 2010, Wallace \textit{et al.} evaluated horse rugs and saddle blankets for the presence of HDMs. *Dermatophagoides* mites were found via a modified flotation technique in eight of the 16 rugs and blankets sampled.\textsuperscript{590} A European study evaluated mites in the equine environment but no species identification was performed.\textsuperscript{598} In the Netherlands, hay from dairy farms had 10 times as many SMs as grain and three times as many SMs as straw.\textsuperscript{429} *L. destructor* was the dominant mite in grain and straw, while *A. siro* was the dominant mite in hay.\textsuperscript{429} Based on evidence from other species and limited information in horses, it
is probable that HDMs and SMs thrive in the hay, grain, barn dust and bedding of horses and may play an important role in the pathogenesis of equine allergic disease. As such, a significant need exists to identify and determine the prevalence of the HDM and SM fauna of the stable. Furthermore, TCs for selected HDM and SM allergen extracts need to be established in non-allergic horses so that IDT results from a patient with allergic disease can be interpreted. We propose that the stable environment provides a nourishing habitat to maintain a viable HDM and SM population year-round. Furthermore, we hypothesize that HDM and SM allergen intradermal TCs can be determined and will vary with the season and that SAT results will not significantly correlate with the IDT TCs of any mite species. The aims of this study were to determine the IDT TCs of horses without allergic disease to HDMs and SMs during the spring and late summer, to quantify seasonal serum specific HDM and SM IgE concentrations in healthy horses and finally, to characterize the mite fauna of a stable over three seasons.

3.3 Materials and Methods

3.3.1 Study Design

All phases of the experimental design of the study were approved by the Institutional Animal Care and Use Committee. The study was a prospective study.

3.3.2 Horses

The horses used in this study were donated to the veterinary hospital and were housed at the same farm. All horses had lived at the farm at least 12 months, if not longer. Horses were kept primarily on pasture with access to a shelter. The shelters were
cleaned weekly by removal of all bedding and replacement with fresh shavings and/or straw. Biannual disinfection was performed after removal of bedding by spraying hard surfaces with a solution that has antimicrobial, fungicidal and virucidal properties (0.39% Quat-Stat SC™, Betco Corporation, Toledo, OH, USA). Free choice mixed grass hay, pasture, salt and mineral blocks and water *ad libitum* were provided. Each pasture contained three to 15 horses that had access to either a three-sided run-in shed or to an open metal barn. One run-in shed contained an attached wooden barn with three box stalls. Each enclosure’s bedding contained mixed hardwood sawdust, straw or a combination of the two. Community feeders within each pasture and/or barn contained mixed grass round baled hay.

Thirty-eight clinically normal horses were used for this study. Inclusion criteria required no previous history of seasonal or perennial pruritus, recurrent urticaria or respiratory disease. The horses had not received any medications that could influence the IgE serology or intradermal testing for at least 60 days prior to study commencement or during the duration of the study. These included topical or oral glucocorticoids, non-steroidal anti-inflammatories, antihistamines or supplements in which the exact ingredients could not be determined. Additionally, these horses had never presented with clinical signs of dermatological disease nor had an IDT. Horses received regular rotation anthelminthic treatment.

**3.3.3 Allergens for Intradermal Testing**

A total of five mite allergen extracts were used for testing and included two species of HDMs (*D. farinae, D. pteronyssinus*) and three species of SMs (*A. siro, L. destructor*
and *T. putrescentiae*). Extracts were made from semi-purified whole body mite preparations that were purchased as 10 ml glass vials of a 1:100 w/v aqueous solution with 0.4% phenol added as an antimicrobial preservative (Greer Laboratories, Lenoir, NC, USA). Allergens and dilutions were stored in glass vials at 4°C and removed 10 min before the IDT was performed. One vial of each mite extract was used for all allergen dilutions and all IDTs. Allergen concentrations were derived from a pilot study that commenced two weeks prior to the start of the reported study. The pilot study tested 10 serial dilutions for each mite species in five normal horses. The results from the pilot study are not included in the presented data. For the reported study, five to six serial dilutions of each mite allergen extract were made using 0.4% phenolated saline (Greer Laboratories, Lenoir, NC, USA) as sterile diluents. Histamine phosphate (Histatrol®, Alk Abelló, Port Washington, NY, USA) 0.275 mg/ml at a concentration of 1:100,000 w/v and 0.4% phenolated saline were used as positive and negative controls, respectively. Mite allergen extract IDT concentrations used were as follows: for *D. pteronyssinus* and *D. farinae* 1:20,000 w/v (25 PNU), 1:40,000 w/v (12.5 PNU), 1:80,000 w/v (6.25 PNU), 1:160,000 w/v (3.125 PNU) and 1:320,000 w/v (1.563 PNU). IDT concentrations used for *A. siro, L. destructor* and *T. putrescentiae* were as follows: 1:5,000 w/v (100 PNU), 1:10,000 w/v (50 PNU), 1:20,000 w/v (25 PNU), 1:40,000 w/v (12.5 PNU), 1:80,000 w/v (6.25 PNU) and 1:160,000 w/v (3.125 PNU). The dilutions were made the day before the testing period. A total of thirty intradermal injections were administered for each test per horse, twenty-eight injections were the allergens.
3.3.4 Intradermal Testing

The intradermal tests were conducted during two consecutive seasons, early April (spring) and August (late summer). Seven to eight horses were tested per day over a successive five-day period in the spring and again in the late summer. The horses were kept in the same environment for the duration of the study. Fractious horses were sedated with 0.01 to 0.02 mg/kg detomidine hydrochloride (Dormosedan®, Pfizer, New York, NY, USA) intravenously via jugular venipuncture 5 min prior to testing. A square test site area measuring approximately 20 cm × 20 cm was made on the center of the left or right lateral aspect of the neck by gently clipping the hair with a number 40 blade (Oster Power Pro Ultra Cordless Clipper Kit, McMinnville, TN, USA). A waterproof permanent marker was used to create a testing template, which consisted of two horizontal rows of ten dots. Intradermal injections of controls and allergens were made above and below the dots. Syringes were preloaded with 0.1 ml of the each individual allergen to avoid under or over injection. One investigator performed all intradermal injections for all horses. A volume of 0.1 ml of each allergen extract was injected intradermally to form a visible bleb using a 27-gauge (0.5 mm) needle and 1 ml syringe. Needles on testing syringes were changed between horses to prevent the transfer of any infectious agent. Each skin test site was evaluated subjectively by a single investigator at 15 min for both seasons. The reactions were subjectively scored using a scale of 0 (negative) to 4+ based on wheal size, erythema and turgidity. A 4+ score was comparable to the positive histamine control whereas a 0 score corresponded to the negative saline control. Subjective scores of ≥ 2+ were considered positive for the purpose of TC determination. The subjective TC was determined based on the highest concentration of a mite allergen used in the IDT where
≤10% of horses had a subjective reaction of ≥2+ at 15 min. Concentrations of allergens used that caused >10% of horses to have positive subjective 15 min reactions were considered probable irritant reactions. Following IDT approximately 3 ml of a 1% hydrocortisone leave-on lotion (Resicort®; Virbac Animal Health, Fort Worth, TX, USA) was applied to the test site.

### 3.3.5 Allergen-Specific IgE Serum Testing

Serum from each horse was evaluated for IgE concentrations to *D. farinae*, *D. pteronyssinus*, *A. siro*, *L. destructor* and *T. putrescentiae* mites. A 10 ml aliquot of blood was obtained for serum IgE evaluation prior to the IDT. Blood was collected via jugular venipuncture using a BD Vacutainer® (BD, Franklin Lakes, NJ, USA) 20-gauge needle and holder into a 10 ml no additive, silicone-coated interior serum tube (BD, Franklin Lakes, NJ, USA). Serum was separated by centrifugation for 10 min at 2500 rpm and stored at -80 °C. Samples were shipped overnight on dry ice to the diagnostic laboratory (Heska, Loveland, CO, USA) at the end of each testing season. Analysis of equine serum-specific IgE was performed using a commercially available allergen-specific IgE ELISA test (Heska ALLERCEPT® ELISA, Loveland, CO, USA) performed in CMG-HESKA® Laboratories. Briefly, allergen-specific IgE is detected using a biotinylated recombinant form of the extracellular part of the alpha chain of the human high-affinity IgE receptor (FcεRIα). The assay used diluted horse serum and streptavidin-alkaline phosphatase reagents for detection of the FcεRIα. The allergens used to coat the wells of the assay plate were obtained from HDM and SM extracts manufactured by CMG-HESKA® Laboratories. Positive and negative controls used for each assay are proprietary. The test
results were expressed in ELISA antibody (EA) units based on optical density values with results at or above 150 EA units considered positive. Assays were performed simultaneously on one plate to decrease interassay variability for each season. The ELISA was performed blinded to the IDT results.

3.3.6 Mite Sampling

A 133-acre farm located in the Midwestern United States was selected for mite analysis. This farm serves as a teaching and research farm for large farm animals mostly horses but also cattle. The horses used in this study reside at the farm. Passive mite sampling was conducted over three consecutive seasons (spring, late summer and winter). Sampling was conducted from the same locations at the farm each season by the same individual. Mite numbers and identification analysis were evaluated from contents obtained from nine different locations on the farm. These sites were as follows: 1) wooden community feeder measuring approximately 6 x 0.45 x 0.25 m at palpation stalls which always contained a small amount of corn, grain and mixed grass hay where mares could go and come at free will, 2) community run-in shed attached to a pasture used to house mares for reproductive labs with bedding that consisted of straw, mixed grass hay, dirt, and mixed organic debris, 3) individual stall which housed one horse 12-18 h a day with bedding that contained mixed organic debris, mixed grass hay, shavings and dirt, 4) a bucket that sat in the barn and had continual supply of contained cracked corn, 5) a large round grass hay bale stored in a 3-sided outdoor shed with no horses, 6) a large square bale of mixed grass hay stored in a 3-sided outdoor shed with no horses, 7) a pine wood sawdust pile stored in a barn, 8) a hay feeder that contained mixed grass round
baled hay that had sawdust and mixed organic debris and housed a large group of geldings that could freely go out to pasture and, 9) an uncovered hay feeder in pasture which contained mixed grass round baled hay, mixed organic debris and dirt and two geldings. Approximately 300 g of mixed materials from each testing site was collected, labeled and secured within two polyethylene sealed bags. Additionally, a single BT mite trap™ was unscrewed and placed on horizontal surface at each site for four consecutive days. On the fourth day, the BT mite traps™ were screwed closed and placed within two polyethylene sealed bags. Samples and traps were stored at -20 °C prior to being shipped to The Food and Environment Research Agency (Sand Hutton, York, UK). Relative humidity and temperature were recorded at each location daily over four consecutive days including just prior to sample collection. A handheld RH300 Dual Input Hygro-Thermometer Psychrometer® (Extech Instruments, Nashua, NH, USA) was used to take the measurements.

3.3.7 Mite Counts and Identification

Mites were extracted from the 300 g samples by using a modified flotation technique. Briefly, the test sample was combined with 100 ml of deionized water in a beaker. Subsequently, 20 ml of ethanol, 20 ml of 35% hydrochloric acid and 20 ml of saturated sodium chloride solution were added to the beaker and then transferred to a 750 ml flotation flask. The combined solution was mechanically mixed while 10 ml of ethylene blue stain was added to dye the aqueous phase and any vegetative material. From a bottom inlet, kerosene was added to the flotation flask and allowed to permeate through the aqueous portion. Because of the mite’s lipophilic properties, they adhered to
the kerosene material and were carried to an interface portion near the top of the flask. After decanting the kerosene layer with mites over filter paper, methylene blue was added to the filter paper and examined under a low-powered stereomicroscope for morphologic identification by licensed acarologists.

For the BT mite traps™, the apparatus was disassembled and mites were filtered from the bait as previously described. Briefly, the lid was removed from the base of the trap and the bait used to lure mites into the trap was extracted using forceps. The lure was then placed above a three-piece Hartley funnel that contained filter paper maintained by suction pressure. Approximately 500 mls of deionised water were used to wash the bait and dismantled trap pieces, and the washings were directed into the funnel to be filtered. A methylene blue solution was added, and the filter paper was dried with suction pressure. The filter paper was then placed in a Petri-dish and examined under a low-powered stereomicroscope for morphologic identification by licensed acarologists.

3.3.8 Statistical Analysis

Threshold concentrations were determined for each of the five mite species (D. farinae, D. pteronyssinus, A. siro, T. putrescentiae, and L. destructor) for two seasons (spring and late summer). McNemar’s test was used to compare subjective reaction of ≥ 2+ at 15 min between the two seasons. McNemar’s test was also used to compare allergen-specific IgE serum dichotomized at ≥ 150 to the subjective reaction of ≥ 2+ at 15 min. A random-effects logistic regression model was generated to define the exact TCs for each of the five mite species in both seasons. The model used the seasonal IDT data
from the 38 horses and the same parameters used for the determination of the subjective TCs. The subjective reaction ≥ 2+ (yes vs. no) was regressed on mite allergen concentration, season, and the interaction of concentration and season. If the interaction term was not significant, it was dropped from the model. After fitting each regression model, non-linear contrast statements were used to estimate the 10% TC for each mite species and season and for the difference in the 10% TC across seasons. The resulting p-values from McNemar’s test and the regression analysis were adjusted using the Holm’s procedure to conserve the overall type I error at 0.05 due to the multiple testing. All analyses were run using Stata 12.1 (Stata Corporation, College Station, Texas). A P-value of ≤0.05 was used to determine statistical significance.

3.4 Results

3.4.1 Horses

The study population included 13 mares and 25 geldings ranging from four to 26 years old with a median age of 12 years. The age and breed of one gelding was unknown. Breeds represented were 13 Thoroughbreds, 11 Quarter Horses, six Warmbloods, five Standardbreds, one Appaloosa, and one American Saddlebred. The same population of horses was used in each season.

3.4.2 Intradermal testing

During both seasons, all horses exhibited an adequate subjective reaction graded as a 4+ to the positive histamine control and had subjective reactions graded as a 0 to the negative saline control. In this population of horses, positive wheals (≥2+) at 15-min
were found at more dilute allergen concentrations despite having a negative subjective
(<2+) reaction at the higher concentration for the mite tested. For the spring, this
occurred at 17 mite concentration sites on 14 different horses, while it occurred at eight
mite concentration sites on eight horses for the late summer. Of the horses affected, three
had similar results in both seasons. Two of these three horses were affected for the same
mite species in both seasons although the concentrations that caused these discordant
results were different between the two seasons. These untoward reactions could not be
consistently linked to a specific mite extract or dilution. However, positive reactions to *A.
siro* at lower (i.e., weaker) concentrations with negative subjective reactions at higher
concentrations occurred in seven horses in the spring, which was the greatest number of
these results for any species of mites in any season.

3.4.3 Subjective 15-Minute Intradermal Threshold Concentrations

The spring subjective TCs for both HDMs (*D. farinae* and *D. pteronyssinus*) were
1:80,000 w/v. The spring subjective TCs for SMs were 1:40,000 w/v for *A. siro* and
1:20,000 w/v for both *L. destructor* and *T. putrescentiae*. The late summer subjective TCs
were unchanged for *D. farinae* and *L. destructor*; however, the TC for *D. pteronyssinus*
changed to a more dilute concentration. The TCs dilutions for *A. siro* and *T.
putrescentiae* increased in potency. The changes in the mite subjective TCs between the
two seasons were not statistically significant (Table 2).
3.4.4 Exact 15-Minute Intradermal Threshold Concentrations

In addition to the determination of the subjective TCs, a random-effects logistic regression model was used to estimate the exact 10% TCs for each of the five mite species in both seasons. A significant difference was found for the *D. pteronyssinus* allergen TCs needed for ≤10% of normal horses to react in spring vs. late summer (Table 3). The mite with the most variance between seasons was *D. pteronyssinus* whereas *T. putrescentiae* had the least amount of variance (Figure 1). When considering how the exact model TCs fit within the scope of the determined subjective TCs, all subjective TCs were within 95% confidence interval (95% CI) of the exact TCs with the exception of *T. putrescentiae* and *D. pteronyssinus* in the spring (Table 3). The exact probabilities from the logistic regression model can be used to extrapolate the mite allergen concentration needed to obtain a TC percentage different than 10%. For example, if the desired percentage of normal horses that had a positive (≥2+) reactions was ≤1% for *D. farinae* in the late summer, then the TC would be approximately 0.25 [x 100,000^{-1}] which is then converted to a ratio and a 1:400,000 w/v concentration of allergen should be used for the IDT.\textsuperscript{600}

3.4.5 Allergen-Specific IgE Serum Testing

Positive IgE EA units to HDM and SM ranged from 150 to 1170. In both seasons, at least one horse had a positive serum IgE result for each HDM or SM evaluated. Negative IgE results for all species of mites occurred in 55% (21/38) and 66% (25/38) of the horses in the spring and late summer, respectively. When considering the percentage of horses in the spring that had multiple positive serum IgE results, reactions to two mite
species were the most common occurring in 18% (7/38) of the horses, whereas 5% (2/38) of the horses had positive IgE results to three mite species and 5% (2/38) were considered to have significant IgE levels for four species of mites. In the late summer, the percentage of horses with positive serum IgE results to two mite species was the most common occurring in 13% (5/38) of the horses, whereas 5% (2/38) of the horses had positive IgE results to three mite species with only 3% (1/38) considered to have significant IgE results for four species of mites. There was no significant difference in the percentage of horses with positive serum IgE results in late summer vs. those reported in the spring. Across the two seasons, only eight horses had positive measurable IgE concentrations in the spring for the same mite species in the late summer. All other positive IgE concentrations for a specific mite species were found in horses with a negative serum IgE level in the opposite season. In both seasons, D. farinae was the species with the most positive horses with a mean IgE of 260 EA (range 28-323 EA) for spring and 275 EA (range 2-671 EA) for late summer. The overall results for mite allergen-specific serum IgE concentrations in spring and late summer are listed in Table 4. When considering the subjectively determined IDT TCs, significant associations between positive serum IgE results with a positive IDT reaction were found for D. farinae in the spring and for D. pteronyssinus in both seasons (Table 5). Alternatively when using horses with positive reactions (≥2+) at concentrations above the exact IDT TCs, a significant positive association between a positive IgE result and a (≥2+) positive IDT reaction was only found for A. siro in the late summer (Table 6).
3.4.6 Mite Identification, Counts, Locations, Temperature and Relative Humidity

The morphologic analysis for mites in the environment on this farm revealed a rich Astigmata and Prostigmata fauna. For the modified flotation technique, mites of the suborder Astigmata belonging to 30 different genera were identified. Pyroglyphida species were rare whereas Chortoglyphidae (nonpyroglyphidae) were common. A total of 3332 mites were collected over the three seasons. Of these, 20% (660/3332) were of the four genera relevant to this study. Spring was the only season in which two out of five species and all four genera of mites were identified (Table 7). When considering the seasonal numbers of mites in four genera of this study, the winter mite collection was the greatest at 281, followed by the spring collection with 232 mites and finally, the summer analysis had 78 mites.

Throughout all seasons, the SM fauna was dominated by the abundance of *Tyrophagus*. This SM genera was the most abundant in the winter and least numerous in the late summer. A total of 211 *T. putrescentiae* mites were identified and represented 6% (211/3332) of all mites collected and 32% (211/660) of the four genera SM and HDM used for IDT TC determination. *T. sylvester* was the most plentiful *Tyrophagus* species identified. This species accounted for 192 mites obtained in the winter from the stall that housed a stallion for at least 16 hours a day for the last two years. The greatest number of *T. putrescentiae* were found in the spring in the straw bedding which was the location with the second greatest population of mites collected from one area. The sites sampled revealed that the *Lepidoglyphus* fauna was the only other mite genera present during all three seasons. *Lepidoglyphus* had the greatest number of mites found in the spring. *L. destructor* represented 1.9% (63/3332) of all the mites identified and 9.5% (63/660) of
the four SMs and HDMs evaluated for TCs. *Tyrophagus* and *Lepidoglyphus* were the only genera present in the late summer.

The *Acarus* fauna of *A. farris*, *A. immobilis* and *A. nidicolous* were found in the spring and winter only. Both of these species were identified in different seasons in the stall where the horse was present for most of the day (location 3). *A. siro* was not found. Additionally, *Dermatophagoides* genera was nearly absent in samples collected from this rural environment. *D. farinae* or *D. pteronyssinus* species were not collected in any of the three seasons; however, one *D. microceras* was identified in the long communal wooden feeding trough that had sparse remnants of cracked corn, fine grain dust, oat hulls and hay particles.

The highest infestation of mites was the individual stall, which housed one horse 12-18 hours a day (location #3). The stall had a total of 43 mites in the spring, 60 mites in the late summer and 233 mites in the winter. The only other sites in which mites were found in all three seasons was the sawdust pile stored in the barn. The bucket which had a continual supply of cracked corn was the only site sampled that did not contain any of the five mite species over the three seasons. All other locations contained at least one mite species in at least one season (Table 7).

For the BT mite traps™, 22 genera of Astigmata mites were detected. The majority was in the nonpyroglyphidae family. A total of 1200 mites were collected over the three seasons. Only 2% (12/1200) of the mites belonged to one of the four genera relevant to this study. Of the five pertinent species, only one, *T. putrescentiae*, was collected and only in two seasons, spring and late summer, in two locations (Table 8).
The late summer traps contained 14 *T. putrescentiae* mites, while the spring traps detected eight mites of the same species.

The average relative humidity and temperatures for each site over each season varied in range (Table 9).

### 3.5 Discussion

To optimally define allergy test performance, a method should be reproducible and validated. Evaluation of physiologic responses during direct allergen challenge in healthy animals without allergic disease remains an appropriate method to help define the significance of intradermal reactivity when known concentrations of allergens are used. This study determined the ideal equine IDT cutoff threshold values for two HDM and three SM allergen dilution concentrations for an immediate type-1 reaction. Two evaluation methods (subjective and statistical model) were used in order to ensure that the recommended IDT mite allergen concentrations would be reliable for distinguishing intradermal reactions that represent true sensitization in clinically affected horses vs. irritant or false positive reactions.

Current recommendations for equine IDT HDM testing concentrations are 60 to 250 PNU/mL which represents dilutions of 1:2000 to 1:8333 w/v, respectively.\(^{303,593}\) References for equine IDT SM testing concentrations as determined by subjective TCs are limited and reported as 1000 Noon units/mL for *T. putrescentiae* and *A. siro* for horses in the Netherlands, and 1:10,000 w/v for *L. destructor* and *T. putrescentiae* for horses in Australia.\(^{32,306}\) In the study by Baxter *et al.*, the TCs of *D. farinae*, *D. pteronyssinus* and *A. siro* were not determined due to excessive reactivity at the least
potent dilutions used which were 1:30,000 w/v for *D. pteronyssinus*, 1:12,000 w/v for *D. farinae* and 1:10,000 w/v for *A. siro*. In the present study, the subjective TCs for *T. putrescentiae* of 1:10,000 w/v for late summer did corroborate the recommended TCs for this mite as reported by Baxter *et al.* The subjectively determined TC for *L. destructor* of 1:20,000 w/v in this study was just one serial dilution less than what had been previously reported. In our study the subjective TC for *A. siro* was the most dilute for the SMs ranging from 1:40,000-1:20,000 w/v, therefore it was not surprising that when 1:10,000 w/v was used, the TC could not be determined.

The subjective TCs for both HDMs were very dilute at 1:80,000 w/v for *D. farinae* and 1:80,000-1:160,000 w/v for *D. pteronyssinus*. Although it was expected that more dilute TCs for HDMs compared to TCs for SMs would be found based on our pilot study and previous studies, what was surprising was the apparent lack of exposure of this population to HDMs in this agricultural setting. The reactivity to HDM likely reflects cross-reactivity among mite allergens that are taxonomically related. Cross-reactivity between allergens may cause “covariation of sensitization,” i.e., a higher observed frequency of sensitization to two or more allergens than the expected frequency.

Sensitization to several HDM and SM species exist in mite-allergic humans and dogs. The presence of multiple mite hypersensitivities often reflects the likeness of mite allergens, which share similar IgE binding epitopes and therefore, may be a cause of clinically reported cross-reactivity. Humans allergic to *Dermatophagoides* spp. may experience allergic symptoms when exposed to mollusk, crustaceans and multiple other insects. This cross-reactivity is reported to be due to the group 10 muscle allergen, tropomyosin, present in mites and various insects (chironomids, mosquito and
cockroach), snails and shrimp as well as a 25-kDa allergen present in several arthropod groups. The Chironomidae (commonly known as midges) is a group of diverse flies with over 10,000 species worldwide. This group of horses would have had continual exposure to chironomids. Collectively the evidence of the exposure of the horse to the enormous range of insects and mites in its environment, the identified low TCs to *D. farinae* and *D. pteronyssinus*, the statistically significant associations between positive serum IgE results with a positive IDT reaction (≥2+) to *D. farinae* in the spring and *D. pteronyssinus* in both seasons and finally, the lack of *Dermatophagoides* in the environment would all support subclinical sensitization to allergens that cross-react with this genera. Recently, Liao *et al* reported that 97% of humans with rhinitis that were sensitized to *T. putrescentiae* were also sensitive to *D. pteronyssinus* and using the IgE-binding inhibition analysis found complete absorption of IgE binding activity by *D. pteronyssinus* which indicated that *T. putrescentiae* hypersensitivity was due to cross-reactivity not dual-sensitization.

Although cross-reactivity is a possible mechanism for the reactivity to very dilute HDM concentrations, HDM extracts have microbial contaminants. The microbial compounds lipopoly saccharide and β-glucan can be routinely detected in HDM extracts obtained from whole-mite cultures or mite bodies. In addition, chitin and fungi cell wall are also in mite extracts. Whether or not these microbial compounds are derived from endosymbionts and/or stable contaminants in mite culture remains to be fully addressed. Data have suggested that *Dermatophagoides* carry endosymbiotic bacteria that produce bacterial lipopeptides as well as have ecological relationships between xerophilic fungi like *Aspergillus penicillodies* conidia that have been identified in their digestive
system and fecal pellets. Moreover, yeast enters the composition of the mite growth medium. With such a diverse composition of inflammatory mediators and sensitizers in these extracts, it is impossible to discount the potential contribution of these to the low \textit{D. farinae} and \textit{D. pteronyssinus} TCs detected in the horses.

Positive IDT reactions were observed at more dilute concentrations for some mite species while negative results were found at higher concentrations. To the author’s knowledge, this has not been previously reported. While the exact cause of this occurrence is unknown, potential explanations may include subjective observer error or traumatic injection at the individual intradermal site, which may have caused false positive reactions. In the future having multiple investigators read any reaction site and averaging the results may decrease intraobserver error, but may also lead to interobserver error. Another possible explanation would have been contamination with irritants precipitates or proteases of the vial(s) containing the more dilute concentrations. Only three horses had these discordant results for both seasons and of the three, only two had them for similar mite concentrations across spring and late summer. There may have been some unidentified variation in the cutaneous sensitization of these animals such as a focal increase in mast cells or epidermal Langerhans cell in the area of skin tested that may have led to these results.

Investigation of seasonal TC determination was done to determine if a concentration-response relationship between the level of exposure to HDM and SMs and cutaneous sensitization was present in cohort of normal horses. The prevalence of allergic signs during annually recurring periods remains the standard best indicator of potential importance and reflects the dose response relationship where the degree of clinical
sensitivity varies with the environmental allergen concentration. Allergen load may also influence the development of serum specific-IgE. Seasonal variation in the subjective TCs for the mites did exist with changes representing one serial dilution for *D. pteronyssinus, T. putrescentiae* and *A. siro*, however the differences were not statistically significant. It must be recognized that magnitude of mite allergen concentration does not alone denote clinical relevance as low levels of some mite genera may be more sensitizing than at higher levels of others. The determined TCs and the very low prevalence of HDMs in this study would seem to suggest that horses are sensitized to one or more of these mites at very low levels that do not provoke clinical signs.

There is no universally accepted or defined percentage of normal animals that should have negative intradermal reactions in order to prevent interpreting reactions as false positives. Therefore, we developed a random-effects logistic regression model that can be used by the clinician to prevent testing with mite allergen concentrations above the threshold for a type-I mediated reaction. The overall outcome of using extrapolated allergen concentrations from this model will be to prevent “irritant” or false positive reactions and allow detection of true sensitization in affected horses. When comparing the exact TCs from the model to those found with the subjective reading of the IDT, the determined subjective TCs were predominately within the 95% CI for the model, however, two TCs were not included within the CI. This is due to the limited number of serial titrations used. For example, the subjective TC for *D. farinae* in the spring was determined to be 1:80,000 w/v while the exact statistical model TC was 1:42,195 w/v with a 95% CI of 1:31,250-1:64,516 w/v. Our study found that > 10% of the normal horses reacted at 1:40,000 w/v, but not at the next weaker serial dilution of 1:80,000 w/v.
Therefore, the model predicts that the TC lies somewhere between 1:40,000-1:80,000. If the testing panel would have included concentrations of 1:50,000 w/v or 1:60,000 w/v then our subjective TC may have been within the exact CI. As this is a statistical model, complete agreement between it and the subjective findings could not be achieved. A potential reason for the exceptions of *T. putrescentiae* and *D. pteronyssinus* in the spring, could be due to the presence of outliers on the statistical model which may have skewed the logistic regression line and caused a stronger statistically exact TC.

Concordance between the IDT and *in vitro* specific IgE assay in this study was dependent on the mite species evaluated. Positive allergen-specific serum IgE for mites was present in 45% of horses in spring and 34% of the horses in late summer. Positive allergen-specific IgE would indicate the presence of subclinical sensitization, which would make any corresponding positive IDT reaction likely true positives and not irritant reactions as these should not be IgE-mediated. However, the presence of a positive IDT reaction below the TC with a negative serum allergen-specific IgE result would likely indicate a false positive for that patient on the IDT. The greatest percentage of positive serum IgE results were for the HDMs, supporting the likelihood of subclinical sensitization due to cross-reactivity in this population. To determine the IgE cross reactivity between *A. siro*, *L. destructor*, *T. putrescentiae*, *D. farinae* and *D. pteronyssinus*, a sensitized population to each mite would need to be identified, serum collected and then IgE immunoassay and immunoblotting inhibition experiments performed.

An aim of this study was to identify the mite fauna from various areas of a typical farm. The intent was twofold. First, as North American inhalant and cutaneous allergens
are demographically, botanically and ecologically diverse, it is important to ensure that key allergens used for the IDT reflect the range of potential mite antigens that could act as environmental sensitizers. Inclusion of regional specific allergens maximizes the patient’s chances to benefit from allergen-specific immunotherapy. Second, knowledge of the local indigenous mite fauna, can be used to help provide recommendations that would limit environmental exposure as well as pinpoint potential sources of infestation before allowing the infestation to spread. Currently, there are no studies that have identified and quantitated the mite fauna in the rural horse farm environment of the U.S. In this study, the modified flotation technique proved superior to the BT mite trap™ in both number and various species/genera of mites detected. The morphologic identification of mites over the three seasons was very successful not only due to the flotation method of collection used but also because the identification was performed by an experienced acarologist who can reliably identify partial and intact mites. No commercially available detection method developed to date has been able to consistently detect mixed mites in samples. Our study identified all four genera of mites used for determination of TCs; however the different species of *Acarus* and paucity of *Dermatophagoides* detected by the flotation method suggests a possible re-consideration of relevant allergen extracts for use in the horse.

The *A. siro* mite was not part of this stable’s microenvironment. *A. siro* is known as the flour mite and is an economically important pest of stored food products that had been reported to cause lesions and digestive ulcers in livestock, however it is considered primarily an indoor species occurring in storage areas. The *Acarus* fauna of *A. farris, A. immobilis* and *A. nidicolous* were identified. *A. farris* and *A. immobilis* occur
primarily in outside environments, such as the nest of birds and mammals, bat roosts, grass, and on farm products such as vegetables.\textsuperscript{431} \textit{Acarus farris} and \textit{L. destructor} dominate the fauna of one-year-old hay in New Zealand.\textsuperscript{603} They occur together in hay samples in such a way that \textit{A. farris} is the most numerous in the most humid hay samples; whereas \textit{L. destructor} takes advantage of the drier part of the hay. This relationship was found in one of our square hay bale samples in the winter although the numbers were low. This may be due to the presence of the predator mite \textit{Cheyletus eruditus} that is a stylet feeder that gradually replaces these two species in hay. This sample did have several \textit{Cheyletus eruditus} identified and over 200+ Tarsonemidae, which are known predatory mites that feed on other mites, mycelia of fungi, algal bodies and higher plants (data not shown). Interestingly, fresh cut hay in the field was not found to contain any recognizable numbers of SMs; however upon arrival in the barn hay becomes infested from old hay remaining there.\textsuperscript{603} The maximum concentration of mites found in one-year old hay is 50,000 live mites/kg. Thereafter, the density slowly decreases but the mites never die out. Twenty-nine year old hay still holds live mites.

Mites in the \textit{Tyrophagus} genera were found in multiple locations across all three seasons in this study.\textsuperscript{441} \textit{T. putrescentiae}, a mold mite, is a major pest in stored products worldwide as well as a known cause of allergic asthma and rhinitis.\textsuperscript{529,604,605} Products such as wheat, flour, cheese and cereal have been found to contain \textit{T. putrescentiae} mites as well as stored dry dog food.\textsuperscript{440,529} The ideal survival conditions have been found to be 10.0-34.8 °C for temperature and approximately 90% for relative humidity.\textsuperscript{29,436,441} Allergies induced by \textit{T. putrescentiae} are most reported in farmers and food industry workers, which correlates to the presence of antigen for the mite species found in cattle.
farms and farmers’ living quarters in Germany. For the present study, *T. putrescentiae* mites were found in multiple horse feeders, hay, straw bedding and sawdust in spring, late summer and winter. In addition to the aforementioned species, two other *Tyrophagus* mites, *T. neiswanderi* in the spring and *T. sylvester* in the winter, were also detected. Although little information is available on these two species, *T. neiswanderi* has been reported to have a similar optimal temperature as *T. putrescentiae* (7.39–31.7 °C), and *T. sylvester* mites have been detected in the outdoor urban environment in Poland. For the time periods sampled in this study, the relative humidity was not ideal for the *T. putrescentiae* mites, but the average temperatures in spring and late summer were within the optimum range for both *T. putrescentiae* and *T. neiswanderi*. The lower then preferred temperatures in the winter may explain the decrease in mite numbers of both species during this study period.

The SM, *L. destructor*, is a major source of mite allergy in European rural environments, but it also causes allergy in urban populations around the world. On the island of Gotland, Sweden, five genera of SMs were detected on 16 farms but the different mite species were not represented on all farms. *L. destructor* was the dominating species on 13 of 16 farms. The authors earlier studies showed that *L. destructor* was the most important allergen causing symptoms from both upper and lower airways among farmers. The most abundant aeroallergens found in air of commercial dairy barns, near Cooperstown NY and Rochester MN, USA, was *Aspergillus fumigatus* followed by *L. destructor*. *L. destructor* occurs in a greater range of conditions with periodic outbreaks affected by the abundance of field fungi, thus this species has been used as a biomarker for grain spoilage. The described pattern of *L. destructor* fluctuation of
large numbers of this mite developing in the late spring and early summer was verified in our study.\textsuperscript{610}

The bucket which had continual supply of contained cracked corn was the only site sampled that never contained any of the four genera of mites that were evaluated for IDT in this study over the three seasons. However, the corn bucket was not mite free as during the spring collection two different SM genera were identified and included 71 \textit{Aeroglyphus robustus}, a SM that commonly occurs after grain has been stored for two years as well as 15 \textit{Glycyphagus domesticus}, which is phylogenetically closely related to \textit{L. destructor} (data not shown). \textit{Glycyphagus domesticus} was reported to be the second most common SM found in German farms infesting 108/121 farms.\textsuperscript{611}

This was an exploratory study to identify mite genera and species and their habitat. Sinha and Wallace\textsuperscript{612} have determined that three factors regulate acarine numbers (i) basic determinants (moisture, temperature, food and intrinsic rate of increase in species) (ii) influencers (seed cracks, microflora, predator mites, dockage); and (iii) seasonal and cyclic regulators (seasonality in temperature and several density-dependent factors). To develop a better understanding of determinants and main species of the equine mite fauna for this region, samples from additional locations and different types of farms over all four seasons are be needed. Additionally detection of the presence of a mite’s specific allergen, if known and diagnostic reagents are available, in the environment would be of use to distinguish the level of allergen present \textit{vs.} the number of mites present.

There are recognized limitations of this study. The first is that the horses were not specific-pathogen free and therefore likely had developed cutaneous sensitization from
continuous exposure to insects and mites. This exposure may make the determined TCs less potent than if they had been assessed in horses that had always lived in a sterile environment. The second is that the same population was used for the determination of TCs in both seasons. Iatrogenic exposure to mite allergen extracts in spring could have the potential to sensitize them to the mites influencing the late summer TCs. As the TCs did not change significantly over the two seasons, it is unlikely that this low level of allergen concentration prejudiced the late summer TCs results. Finally, the population of horses and farm used in this study were located in a single region. Geographical differences may yield different mite populations or various TCs.

In conclusion, our findings would suggest that the equine IDT panel of allergens may not need to include *D. farinae*, *D. pteronyssinus* and *A. siro* but rather the mites more commonly found in the stable microenvironment. Additionally, the apparent mite cross-reactivity in this population would advocate for the use of purified mite allergens to increase the specificity of the equine IDT. Exact dilution concentrations for the SM allergens relevant to this population would be in the range of 1:12,550-1:13,100 for *L. destructor*, with a dilution of 1:6,600 for *T. putrescentiae*. Future investigations should be conducted not only across different regions of the country to determine the regional differences in TCs for HDMs and SMs, but also during all four seasons. Finally, it is recognized that there is an inherent risk of basing TCs purely on results in clinically normal horses as the ‘irritant’ TC may significantly deviate from the concentration identified in hypersensitive patients. As such, additional studies are needed in horses with recognized allergic disease to SMs to identify the allergen detection TCs.
Table 2. Comparison of Spring & Late Summer Subjective Intradermal Test Threshold Concentrations

<table>
<thead>
<tr>
<th>Mite species</th>
<th>Spring concentration (w/v)</th>
<th>Late summer concentration (w/v)</th>
<th>p-value&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>D.f.</td>
<td>1:80,000</td>
<td>1:80,000</td>
<td>0.655</td>
</tr>
<tr>
<td>D.p.</td>
<td>1:80,000</td>
<td>1:160,000</td>
<td>---&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>A.s.</td>
<td>1:40,000</td>
<td>1:20,000</td>
<td>0.157</td>
</tr>
<tr>
<td>L.d.</td>
<td>1:20,000</td>
<td>1:20,000</td>
<td>0.564</td>
</tr>
<tr>
<td>T.p.</td>
<td>1:20,000</td>
<td>1:10,000</td>
<td>0.654</td>
</tr>
</tbody>
</table>

<sup>1</sup> p-values were not adjusted using the Holm’s procedure since there were not statistically significant comparisons

<sup>2</sup> Could not be estimated since all observations were “no” for both seasons

Table 3. Comparison of Spring & Late Summer Statistically Derived Exact Intradermal Threshold Concentrations

<table>
<thead>
<tr>
<th>Mite species</th>
<th>Concentration (95% CI) [x100,000⁻¹]</th>
<th>Concentration ratio (95% CI) (w/v)</th>
<th>Concentration (95% CI) [x100,000⁻¹]</th>
<th>Concentration ratio (95% CI) (w/v)</th>
<th>Estimate (95% CI) [x100,000⁻¹]</th>
<th>p-value¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D.f.</em></td>
<td>2.37 (1.55-3.20)</td>
<td>1:42,195 (1:31,250-1:64,516)</td>
<td>1.67 (1.14-2.19)</td>
<td>1:59,880 (1:45,662-1:87,719)</td>
<td>0.71 (1:31,250-1:64,516)</td>
<td>0.223</td>
</tr>
<tr>
<td><em>D.p.</em></td>
<td>4.73 (3.42-6.04)</td>
<td>1:21,140 (1:16,556-1:29,239)</td>
<td>2.10 (1.02-3.19)</td>
<td>1:47,620 (1:31,347-1:98,039)</td>
<td>2.63 (1:31,347-1:98,039)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><em>A.s.</em></td>
<td>6.68 (4.69-8.68)</td>
<td>1:14,970 (1:11,520-1:21,000)</td>
<td>10.24 (6.11-14.4)</td>
<td>1:9,765 (1:6,944-1:16,366)</td>
<td>-3.56 (1:6,944-1:16,366)</td>
<td>0.255</td>
</tr>
<tr>
<td><em>L.d.</em></td>
<td>7.97 (5.77-10.17)</td>
<td>1:12,550 (1:9,832-1:17,331)</td>
<td>7.63 (5.56-9.71)</td>
<td>1:13,105 (1:10,298-1:17,985)</td>
<td>-0.34 (1:10,298-1:17,985)</td>
<td>0.714</td>
</tr>
<tr>
<td><em>T.p.</em></td>
<td>15.1 (10.6-19.6)</td>
<td>1:6,620 (1:5,102-1:9,433)</td>
<td>15.0 (11.2-18.7)</td>
<td>1:6,665 (1:5,347-1:8,928)</td>
<td>0.16 (1:5,347-1:8,928)</td>
<td>0.942</td>
</tr>
</tbody>
</table>

¹ p-values are adjusted using the Holm’s procedure to conserve the overall type 1 error rate at 0.05

Table 4. Percentage of Horses with Positive Seasonal Allergen-Specific Serum IgE

<table>
<thead>
<tr>
<th>Mite Species</th>
<th>Horses with IgE ≥150 EA</th>
<th>Horses with IgE ≥150 EA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spring</td>
<td>Late Summer</td>
</tr>
<tr>
<td>D.f.</td>
<td>37% (14/38)</td>
<td>24% (9/38)</td>
</tr>
<tr>
<td>D.p.</td>
<td>18% (7/38)</td>
<td>18% (7/38)</td>
</tr>
<tr>
<td>A.s.</td>
<td>11% (4/38)</td>
<td>8% (3/38)</td>
</tr>
<tr>
<td>L.d.</td>
<td>3% (1/38)</td>
<td>3% (1/38)</td>
</tr>
<tr>
<td>T.p.</td>
<td>16% (6/38)</td>
<td>18% (7/38)</td>
</tr>
</tbody>
</table>

Serum IgE levels expressed as Elisa Antibody Units (EA). Positive allergen-specific serum IgE are considered positive at ≥150 EA. D.f.=Dermatophagoides farinae; D.p.=Dermatophagoides pteronyssinus; A.s.=Acarus siro; T.p.=Tyrophagus putrescentiae; L.d.=Lepidoglyphus destructor.
Concentrations expressed as weight/volume (w/v). Serum IgE levels expressed as Elisa Antibody Units (EA). Positive allergen-specific serum IgE are considered positive at ≥150 EA. *D.f.* = *Dermatophagoides farinae*; *D.p.* = *Dermatophagoides pteronyssinus*; *A.s.* = *Acarus siro*; *T.p.* = *Tyrophagus putrescentiae*; *L.d.* = *Lepidoglyphus destructor*.}

### Table 5. Comparison of Subjective Intradermal Threshold Concentrations to Positive Allergen-Specific Serum IgE (≥150 EA)

<table>
<thead>
<tr>
<th>Mite species</th>
<th>Spring concentration (w/v)</th>
<th>Spring $p$-value$^1$</th>
<th>Late summer concentration (w/v)</th>
<th>Late summer $p$-value$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D.f.</em></td>
<td>1:80,000</td>
<td><strong>0.012</strong></td>
<td>1:80,000</td>
<td>0.139</td>
</tr>
<tr>
<td><em>D.p.</em></td>
<td>1:80,000</td>
<td><strong>0.033</strong></td>
<td>1:160,000</td>
<td>0.041</td>
</tr>
<tr>
<td><em>A.s.</em></td>
<td>1:40,000</td>
<td>0.414</td>
<td>1:20,000</td>
<td>0.472</td>
</tr>
<tr>
<td><em>L.d.</em></td>
<td>1:20,000</td>
<td>0.635</td>
<td>1:20,000</td>
<td>0.564</td>
</tr>
<tr>
<td><em>T.p.</em></td>
<td>1:20,000</td>
<td>0.472</td>
<td>1:10,000</td>
<td>0.412</td>
</tr>
</tbody>
</table>

### Table 6. Comparison of Exact Intradermal Threshold Concentrations to Positive Allergen-Specific Serum IgE (≥150 EA)

<table>
<thead>
<tr>
<th>Mite species</th>
<th>Spring concentration (w/v)</th>
<th>Spring $p$-value$^1$</th>
<th>Late summer concentration (w/v)</th>
<th>Late summer $p$-value$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D.f.</em></td>
<td>1:42,195</td>
<td>0.063</td>
<td>1:58,880</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td><em>D.p.</em></td>
<td>1:21,140</td>
<td>0.063</td>
<td>1:47,620</td>
<td>0.130</td>
</tr>
<tr>
<td><em>A.s.</em></td>
<td>1:14,970</td>
<td>0.081</td>
<td>1:9,765</td>
<td>0.013</td>
</tr>
<tr>
<td><em>L.d.</em></td>
<td>1:12,550</td>
<td>0.225</td>
<td>1:13,105</td>
<td>0.739</td>
</tr>
<tr>
<td><em>T.p.</em></td>
<td>1:6,620</td>
<td>0.331</td>
<td>1:6,665</td>
<td>0.617</td>
</tr>
</tbody>
</table>

$^1 p$-values are adjusted using the Holm’s procedure to conserve the overall type 1 error rate at 0.05
Table 7. Mite Genera and Number in Nine Stable Locations Collected via Modified Flotation Technique

<table>
<thead>
<tr>
<th>Location</th>
<th>Spring</th>
<th>Late Summer</th>
<th>Winter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Feeder bin in indoor mare run-in shed</td>
<td>10 Tyrophagus putrescentiae</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>1 Dermatophagoides microceras</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Straw bedding in indoor mare run-in</td>
<td>88 Tyrophagus putrescentiae</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>4 Lepidoglyphus destructor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Wood shavings inside stall housing</td>
<td>43 Acarus farris</td>
<td>60 Tyrophagus putrescentiae</td>
<td>192 Tyrophagus sylvestre</td>
</tr>
<tr>
<td>horse for 12-18 hr/day</td>
<td></td>
<td></td>
<td>41 Acarus immobilis</td>
</tr>
<tr>
<td>4. Corn bucket inside barn</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>5. Round hay bales in 3-sided shed</td>
<td>35 Tyrophagus neiswanderi</td>
<td>None</td>
<td>25 Tyrophagus putrescentiae</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11 Lepidoglyphus destructor</td>
</tr>
<tr>
<td>6. Square hay bales in 3-sided shed</td>
<td>29 Lepidoglyphus destructor</td>
<td>None</td>
<td>3 Lepidoglyphus destructor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 Acarus farris</td>
</tr>
<tr>
<td>8. Hay feeder inside gelding barn</td>
<td>20 Tyrophagus putrescentiae</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>9. Hay feeder in outside front pasture</td>
<td>None</td>
<td>6 Tyrophagus putrescentiae</td>
<td>None</td>
</tr>
</tbody>
</table>

Locations given as corresponding number as well as description of location site. Number of actual mites with corresponding genus and when applicable species found at each location during each season provided.
Table 8. Mite Genera and Number in Nine Stable Locations Collected via BT Mite Traps™

<table>
<thead>
<tr>
<th>Location</th>
<th>Spring</th>
<th>Late Summer</th>
<th>Winter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Feeder bin in indoor mare run-in shed</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>2. Straw bedding in indoor mare run-in</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>3. Wood shavings inside stall housing horse for 12-18 hr/day</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>4. Corn bucket inside barn</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>5. Round hay bales in 3-sided shed</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>6. Square hay bales in 3-sided shed</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>7. Sawdust pile stored inside barn</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>8. Hay feeder inside gelding barn</td>
<td>2 <em>Tyrophagus putrescentiae</em></td>
<td>7 <em>Tyrophagus putrescentiae</em></td>
<td>None</td>
</tr>
<tr>
<td>9. Hay feeder in outside front pasture</td>
<td>6 <em>Tyrophagus putrescentiae</em></td>
<td>7 <em>Tyrophagus putrescentiae</em></td>
<td>None</td>
</tr>
</tbody>
</table>

Locations given as corresponding number as well as description of location site. Number of actual mites with corresponding genus and when applicable species found at each location during each season provided.
Table 9. Average Relative Humidity and Temperature Across Three Seasons

<table>
<thead>
<tr>
<th></th>
<th>Spring</th>
<th>Late Summer</th>
<th>Winter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average relative humidity (%)</td>
<td>43.5</td>
<td>48.7</td>
<td>47.4</td>
</tr>
<tr>
<td>Average temperature</td>
<td>68.0°F (20.0°C)</td>
<td>79.5°F (26.4°C)</td>
<td>32.8°F (0.44°C)</td>
</tr>
</tbody>
</table>

Temperature measured in degrees Celsius. Average relative humidity and temperature measured using RH300 Dual Input Hygro-Thermometer Psychrometer® (Extech Instruments, Nashua, NH, USA) over 4 days at 9 locations during 3 seasons.
Figure 1: Exact Intradermal Threshold Concentrations

Figure 1: Probability representative of percentage of clinically normal horses with positive (≥2+) intradermal reaction. Concentration measured in weight/volume (w/v).
Chapter 4

Conclusion and Future Direction

In this study, we found that the current suggested concentrations for house dust mites (HDMs) and storage mites (SMs) in equine intradermal (IDT) are too potent and may lead many false positive, irritant reactions. Our recommended equine IDT concentration are as follows: Dermatophagoides farinae (D. farinae) at 1:80,000 w/v, Dermatophagoides pteronyssinus (D. pteronyssinus) at 1:160,000-1:80,000 w/v, Acarus siro (A. siro) at 1:40,000-1:20,000 w/v, Lepidoglyphus destructor (L. destructor) at 1:20,000 w/v, and Tyrophagus putrescentiae (T. putrescentiae) at 1:20,000-1:10,000 w/v. A statistical seasonal variation from spring to late summer was only noted for D. pteronyssinus based on our subjection and statistical findings.

Of the healthy, nonallergic horses tested, 45% in the spring and 34% in the late summer had positive serum allergen-specific IgE results to at least one mite species. These findings most likely represent false positives rather than subclinical sensitization since such a large proportion of healthy animals were affected. When comparing allergy testing methods, a relationship was found between SAT and subjective IDT for D. farinae in the spring and D. pteronyssinus in both seasons. Significance between SAT and exact statistical IDT was detected for A. siro in the later summer only. Because of the suspected high number of false positives and lack
of agreement between the two testing methods, IDT remains the preferred method of allergen testing in the equine species over SAT.

For collection of mites in the equine environment over the three seasons, 35 genera of mites were collected. At least one mite from each of the 4 genera, *Dermatophagoides, Acarus, Lepidoglyphus* and *Tyrophagus*, were found. Of these, *Tyrophagus* was the most numerous while *Dermatophagoides* was the least numerous. Only two of the five species, *T. putrescentiae* and *L. destructor*, relevant to the study were detected using both the flotation and mite trap techniques. Both of these mite species were found in all three seasons in various locations. Because these SMs were found year round, they may correlate with nonseasonal allergic disease in horses. House dust mites may also contribute to disease although only one *Dermatophagoides* mite was found in this study. It is possible that we did not collect in the correct locations or seasons. More samples in more locations would be needed to definitively confirm that HDMs are not present in the equine environment. Finally, the flotation method collected more mites in more locations when compared to BT Mite Traps™. Therefore, the flotation method is the recommended method of mite collection for this setting.

While we were primarily concerned with the two species of HDMS and three species of SMs, we did collect several other genera, which may be of significance. *Orbidtida, Cheyletus, Glycyphagus* and *Tarsonemidae* mites were some of the most numerous genera collected, which may be of biologic importance in the horse. Their exact role in equine allergic is unknown at this time. Future work would be warranted to assess their significance in equine medicine and disease.
Additional studies with more horses across different US locations in all 4 seasons would be needed to confirm our TCs. The next step in evaluating the two species of HDM and three species of SMs studied would be to test our intradermal TCs in a population of allergic horses and evaluate the results. To develop a better understanding of determinants and main species of the equine mite fauna for this region, samples from additional locations and different types of farms over all four seasons are be necessary. Additionally detection of the presence of a mite’s specific allergen, if known and diagnostic reagents are available, in the environment would be of use to distinguish the level of allergen present vs. the number of mites present.


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