

Determination of 'irritant' threshold concentrations for intradermal testing with allergenic insect extracts in normal horses

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Abstract Sixteen healthy horses with no history of skin or respiratory disease were used for an intradermal testing (IDT) threshold study, in order to determine the concentrations of 13 commercial allergenic insect extracts most appropriate for IDT. Five dilutions of each extract were used, which included the manufacturer's recommended concentrations for equine IDT, plus one dilution higher and three lower than these standard concentrations. Allergens tested included caddisfly (*Trichoptera* spp.), mayfly (*Ephemeroptera* spp.), horsefly (*Tabanus* spp.), deerfly (*Chrysops* spp.), fire ant (*Solenopsis invicta*), black ant (*Camponotus pennsylvanicus*), cockroach mix (*Periplaneta americana* and *Blattella germanica*), mosquito (*Aedes aegypti*), house fly (*Musca domestica*), moth (*Heterocera* spp.), flea (*Ctenocephalides canis/C. felis*), *Culicoides variipennis* and *Culicoides nubeculosus*. Two separate methods were used to calculate the allergen concentration for each insect extract where the normal horses, as a group, ceased to show false-positive ('irritant') reactions. 'Irritant' threshold concentrations were determined for 9/13 of these allergens, whereas the other 4 were undetermined due to either insufficient reactivity (flea, *C. variipennis*) or excessive reactivity (black ant, moth) to the concentrations tested. Recommended concentrations for future use in equine patients with suspected insect hypersensitivity include: 125 pnu mL⁻¹ (mayfly); 250 pnu mL⁻¹ (caddisfly, horsefly, deerfly, fire ant, house fly); 500 pnu mL⁻¹ (cockroach); 1000 pnu mL⁻¹ (mosquito); and 1:10 000 w/v (*C. nubeculosus*).

Keywords: allergenic extracts, horse, insect hypersensitivity, intradermal allergy testing.

Type 1 hypersensitivity reactions to common environmental allergens have been implicated in the pathophysiology of several equine disease processes. Included in this group of diseases are atopic dermatitis (AD), recurrent urticaria (RU), chronic obstructive pulmonary disease (COPD) and reactive airway disease (RAD).^{1–4} Intradermal testing (IDT) is commonly used by veterinary dermatologists and equine practitioners to characterize specific allergen sensitivities for use in allergen avoidance or allergen-specific immunotherapy trials. It has been noted by several investigators that normal horses may also exhibit a moderate degree of allergen reactivity at the allergen concentrations commonly used for IDT.^{1,4–6} Two potential explanations for these reactions are typically provided: prior subclinical sensitization to the allergen, and testing of the allergen at a concentration that exceeds threshold for normal individuals.

Insect hypersensitivity is a common cause of pruritus in horses, and may also be involved in the aetiology of recurrent urticaria. Although a recent study casts doubt upon the role of allergens in COPD,⁵ insect allergens may be involved.^{7,8} Insect allergens are a

heterogenous group of antigens which may include venoms, salivary proteins, sloughed somatic cells and excrement of the insects. The allergenic species best known in horses are biting midges of the genera *Culicoides*.⁹ Salivary gland antigens, which have not been completely characterized, are the allergenic moiety in *Culicoides* hypersensitivity (CH).¹⁰

Insect allergens are problematic to use as appropriate concentrations for detecting true sensitization in affected horses are largely unknown. In addition, there is no standardization in the manufacturing of insect allergens and variability is inherent because most solutions are whole-body extracts that incorporate variable amounts of relevant allergens.

Testing with allergens at concentrations above the threshold for type-1 reactivity may result in non-specific false-positive (often referred to as 'irritant') reactions.¹¹ The purpose of this study was to determine the 'irritant' threshold concentrations for testing 13 commercial insect allergens, by IDT of normal horses using serial dilutions of the extracts.

MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee at the University of

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Pennsylvania. The primary author performed all IDT injections and interpreted all skin reactions.

Sixteen normal female horses housed at the Hoffman research centre at the University of Pennsylvania's New Bolton Center were utilized. The horses were housed on pasture with access to dry lot, and were utilized on a regular basis for reproductive tract examination labs by veterinary students. They were palpated and 'teased' on a regular basis and oestrus logs were maintained; none were known to be in oestrus at the time of this study. The horses had access *ad libitum* to pasture grass and water, with no additional dietary supplementation. All were free of skin lesions at the time of testing, and had no history of allergic skin or respiratory disease. The horses had received no antihistaminic, corticosteroid or analgesic drugs by any route of administration for longer than 60 days, and were dewormed on a rotational basis with ivermectin, pyrantel pamoate and oxbendazole every 3 months. All testing was performed on a single day in mid-June 2002.

Thirteen commercial insect allergens were utilized for the IDT threshold study. These included: caddisfly (*Trichoptera* spp.), mayfly (*Ephemeroptera* spp.), horsefly (*Tabanus* spp.), deerfly (*Chrysops* spp.), fire ant (*Solenopsis invicta*), black ant (*Camponotus pennsylvanicus*), cockroach mix (*Periplaneta americana* and *Blattella germanica*), mosquito (*Aedes aegypti*), house fly (*Musca domestica*), moth (*Heterocera* spp.), flea (*Ctenocephalides canis/C. felis*), *Culicoides variipennis* and *Culicoides nubeculosis* (aqueous allergens, Greer Laboratories, Lenoir, NC, USA). All allergens other than *Culicoides* spp. were supplied as 10 000 protein nitrogen unit (pnu) per mL stock concentrations, and were diluted for IDT to 2000, 1000, 500, 250 and 125 pnu mL⁻¹ concentrations, respectively. The manufacturer recommends routine testing with these allergens at 1000 pnu mL⁻¹. *C. variipennis* was supplied at a 1:5000 w/v concentration, while *C. nubeculosis* was supplied at 1:10 w/v. These extracts were diluted to 1:5000, 1:10 000, 1:15 000, 1:25 000 and 1:50 000 w/v concentrations for IDT. The manufacturer recommends testing *Culicoides* allergens at 1:25 000 w/v. Histamine phosphate (1:100 000 w/v) and phosphate-buffered saline (PBS) were used as positive and negative controls, respectively, at the beginning and end of each IDT procedure, for a total of 69 intradermal injections per horse.

All horses were sedated with xylazine hydrochloride 1.1 mg kg⁻¹ intravenously, immediately preceding IDT. A volume of 0.1 mL of each extract had been pre-measured into 0.5 mL syringes with 27 gauge × 13 mm needles (Becton Dickinson, Franklin Lakes, NJ, USA) for intradermal injection. Testing was performed after clipping of the hair with a no. 40 clipper blade on the lateral aspect of the neck, half way between the mandible and shoulder, and one hand width below the base of the mane. Templates were drawn with a permanent marking pen, with 2.5 × 2.5 cm squares in rows of 10.

Test site reactions were graded on a scale of 0–4+ using the positive and negative controls as reference points, where saline was assigned a standard reaction of 0 (negative) and histamine a standard reaction of 4+. Subjective grading was used only to record allergen responses, as precise measurement of wheal diameters does not account for depth and turgidity of the reactions. All reactions were evaluated at 30 min, 4 h, and 24 h after the final injection was administered. Medians of the reactions to each concentration of each allergen were calculated for the 16 horses. Two methods were used and compared to determine the threshold concentration of each allergen: the highest concentration at which the median response of 16 horses was < 1.0, and the highest concentration at which ≥ 75% of horses were nonreactive (receiving a score ≤ 1+).

RESULTS

The horses ranged in age from 5 to 25 years (mean 15.6 years). Breeds represented included: six Thoroughbreds, four Standardbreds, two Morgans, one Quarter Horse, one Holsteiner, one Dutch Warmblood, and one Warmblood–Paint cross.

At 30 min, all histamine reactions were considered adequate (i.e. 4+) for use as positive controls, and allergen reactions of all 16 horses were evaluated. For 15/16 horses, a dose-dependent decrease was noted in wheal scores as the concentration of each allergen decreased (Fig. 1). The exception to this was a horse that had scores of '0' for all concentrations of all allergens except mosquito (scores of 1+ were noted for the two highest concentrations).

The threshold concentrations for 12/13 allergens were the same when calculated using either method, whereas the threshold of one allergen differed between methods by one dilutional factor (Table 1). For four allergens, the true threshold could not be determined based upon either method, as either the highest concentration used was insufficiently reactive (flea and *C. variipennis*) or the lowest concentration was excessively reactive (moth and black ant).

At 4 h, histamine wheals had faded considerably in all 16 horses, however, the subjective character of the allergen reactions had not changed. For example, many allergen test sites that had previously been graded 4+ remained the same size, making direct comparison with the waning histamine controls difficult. Although there were a few exceptions at individual injection sites, allergen reaction grades in general decreased or stayed constant in all horses but one (Fig. 2). This general reduction in wheal scores was responsible for a decrease in the calculated 4-h threshold concentration for three allergens, whereas the threshold for four allergens remained the same and that for two allergens increased (Table 1). A single horse was responsible for the increased threshold noted in two allergens: this horse developed strong (2+ to 4+) reactions to the higher concentrations of 7/13

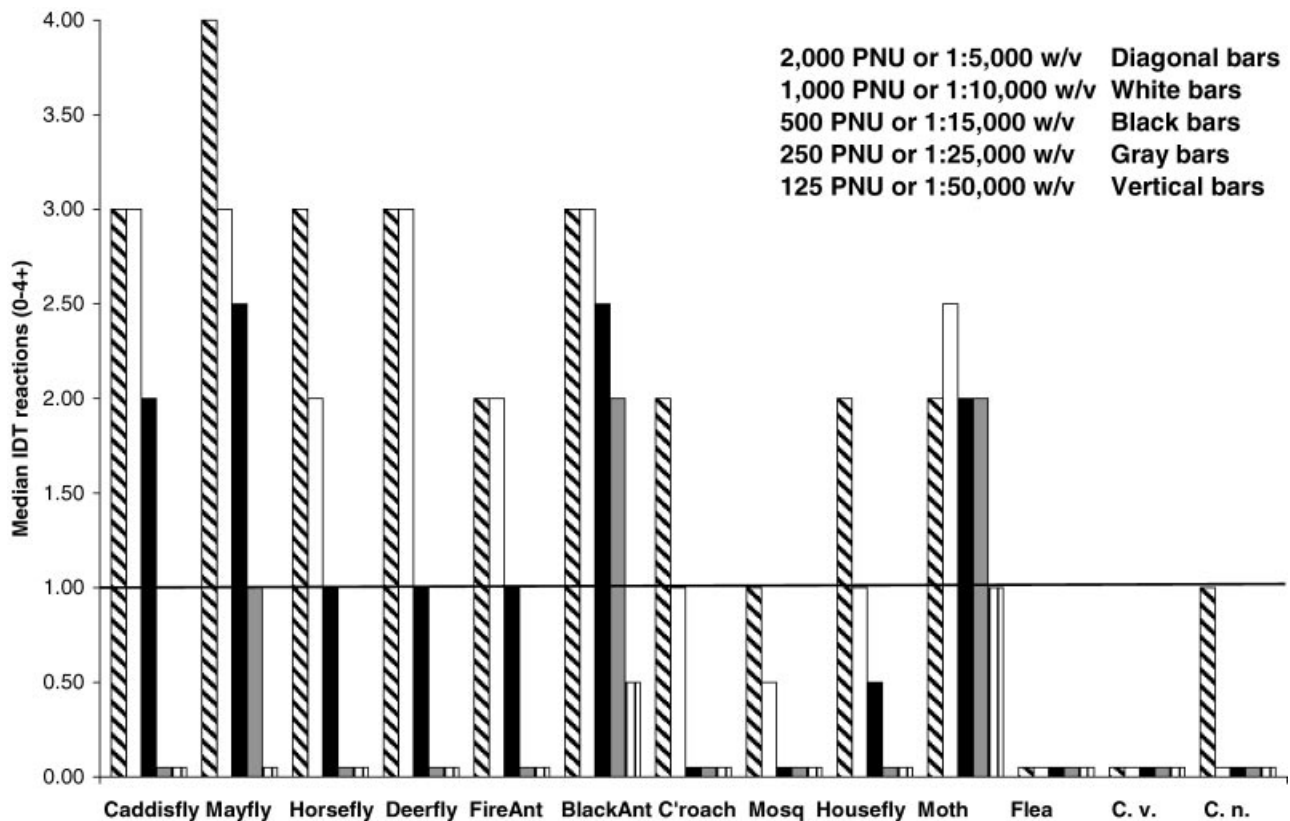


Figure 1. Median 30-min IDT reactions of 16 normal horses to 13 insect allergens. All insect allergen concentrations are expressed in pnu mL⁻¹ except *Culicoides variipennis* (C.v) and *Culicoides nubeculosis* (C.n) which are expressed in w/v.

Table 1. Threshold concentrations of insect extracts for 16 normal horses when evaluated at 30 min and 4 h

Allergen	30 min		4 h	
	A	B	A	B
Caddisfly	250	250*	500	500
Mayfly	125	125*	250	250
Horsefly	250	250*	250	250
Deerfly	250	250*	250	500
Fire ant	250	250*	250	250
Cockroach	500	500*	250	250
Mosquito	1000	1000*	125	250
House fly	500	250*	125	125
<i>C. nubeculosis</i>	1:10 000	1:10 000*	1:10 000	1:10 000
<i>C. variipennis</i>	> 1:5000	> 1:5000	> 1:5000	> 1:5000
Flea	> 2000	> 2000	> 2000	> 2000
Moth	< 125	< 125	< 125	< 125
Black ant	< 125	< 125	< 125	< 125

All allergen concentrations are expressed in pnu/mL except for the two *Culicoides* species, which are expressed in w/v. The thresholds in the 'A' columns were calculated as the concentrations where the median of 16 horses was < 1.0. The thresholds in the 'B' columns are the concentrations where ≥ 75% of horses were nonreactive. Recommended concentrations for future IDT are marked with an asterisk (*).

allergens to which it had previously been completely nonreactive. There were no other horses that developed 4-h reactions where 30-minute reactions had been absent. The calculated threshold of two allergens differed between methods by one dilutional factor.

At 24 h, 14/16 horses were available for evaluation. Twelve horses had no detectable reactions, whereas two had areas with marked coalescence of oedema that spanned all serial dilution injection sites of several individual allergens. For one horse, this occurred in the areas of mayfly, horsefly and deerfly injections; for the other it occurred in the area of *C. nubeculosis*. These horses had exhibited only moderate reactions at 30 min and 4 h that had tapered as the allergen concentrations decreased.

DISCUSSION

Our results indicate that concentrations other than those recommended by the manufacturer for IDT should be utilized for most insect allergens, and we were unable to establish 'irritant' threshold recommendations for 4 of 13 allergens.

Reports of IDT with insect allergens are limited in the equine literature, and have been part of larger allergen panels which have included pollens, moulds and epidermals for testing horses with clinical diagnoses of AD, COPD, RU and RAD.¹⁻⁶ In a study published in 1992, several insect allergens (mosquito, horsefly, blackfly) were tested in serial dilutions in normal horses and horses with RU and COPD.¹ It was suggested that *Aedes* sp. (mosquito) should be tested at a concentration ≤ 250 pnu mL⁻¹ to avoid false-positive

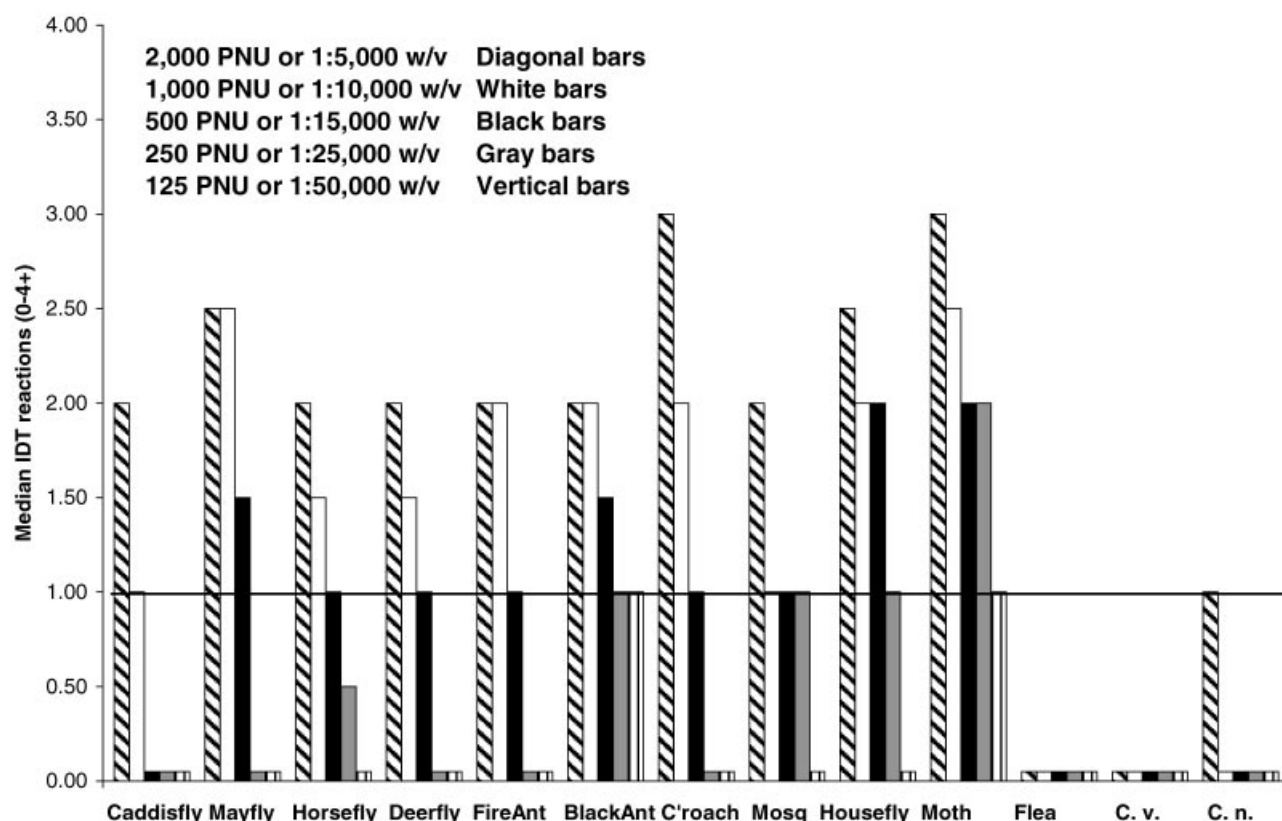


Figure 2. Median 4-h IDT reactions of 16 normal horses to 13 insect allergens. All insect allergen concentrations are expressed in pnu mL⁻¹ except *Culicoides variipennis* (C.v) and *Culicoides nubeculosus* (C.n) which are expressed in w/v.

reactions. In our study, mosquito (*A. aegypti*) had one of the highest threshold concentrations for normal horses (Table 1). Allergens for the former study were obtained from two different companies; no mention was made of which company manufactured the individual insect extracts. It may be difficult therefore to compare results from 10 years ago with currently available extracts, as standardization is lacking not only between allergen producers, but also between individual batches of allergen.¹¹

In a more recent study, serial dilutions of mosquito, deerfly, horsefly and black fly (500, 250, 125 pnu mL⁻¹) were used to test horses with clinical diagnoses of COPD, RU and AD compared with a healthy control group. The authors state that responses to the 500 pnu mL⁻¹ concentrations were used for statistical analysis, and do not further discuss observations with the serial dilutions. However, horses with RU and AD had a significantly higher mean number of immediate reactions to insect allergens than the healthy control group, and horses with COPD and RU had a higher mean number of late-phase reactions to insect allergens than the control group.⁴ The authors suggest that because black fly and horsefly caused positive reactions in 3 or more of 16 healthy horses, reactions to these allergens should be interpreted with caution.

In two other recent studies, insect allergens have been tested at 500 pnu mL⁻¹ (with the exception of black ant, which was tested at 1000 pnu mL⁻¹).^{5,6} In these two studies, the control group of 22 nonatopic

horses was the same. Black ant produced a positive reaction in 33/88 observations (37.5%) in nonatopic horses, 27/64 observations (42.2%) in COPD horses, 7/28 observations (60.7%) in horses with AD, and 24/40 (60%) in horses with RU. Other common insect reactions included black fly with 16/64 observations (25%) in horses with COPD, and horsefly with 13/28 observations (46.4%) in horses with AD.

We obtained our allergens from the same manufacturer utilized by the investigators of the latter three studies. Interestingly, we also found that black ant was highly reactive in normal horses, and did not test with a concentration low enough to identify the true 'irritant' threshold of the allergen. This was also the case with moth allergen. However, we cannot exclude the possibility that one or more of our 'normal' horses was subclinically sensitized to these allergens. For example, one horse showed 4+ reactions to all dilutions of moth allergen, which could indicate prior sensitization. However, several other horses had 2+ reactions to even the most dilute concentration of moth allergen. Furthermore, when thresholds were recalculated excluding the highly reactive horse's data, only one of the two threshold calculations for moth was changed. Therefore, we do not feel that it would be appropriate to exclude this data and suggest that we have identified the correct 'irritant' threshold for moth allergen.

For flea and *C. variipennis*, thresholds could not be established owing to insufficient reactivity at the highest concentrations used. Only 2/16 horses showed a 1+

reaction to the highest concentration (1:5000 w/v) of *C. variipennis*, which was unexpected. This extract is currently available only for research purposes from Greer Laboratories. We had also used it previously in seven horses with clinical diagnoses of *Culicoides* hypersensitivity. As per the manufacturer's recommendations, concentrations of 1:25 000 and 1:50 000 w/v were used for testing. None of these seven horses reacted to either concentration with a significant wheal score (i.e. $\geq 2+$). We cannot, therefore, recommend an appropriate concentration for the use of this extract, but its antigenicity appears to be quite low. Although we were able to establish a threshold for *C. nubeculosis*, it should be stressed that 6/16 horses were completely nonreactive to the extract, which may indicate low antigenicity for it as well. *C. nubeculosis* is not known to be a native species in our region.

Although thresholds were calculated for both the 30-min and 4-h evaluations periods, our recommendation for future IDT is to use the 30-min values. Use of late-phase reactions for determining skin testing threshold concentrations has not been reported in any species to the knowledge of the authors, and most practising veterinarians will more reliably assess 30-min reactions. As recommended previously,⁶ we also suggest that evaluation of 4-h reactions is important in order to avoid missing late-phase reactions in clinical cases, as one of the normal horses in this study showed predominantly late-phase reactions. Although it is possible that the late-phase reactions in this one horse represented prior (i.e. subclinical) sensitization, it is unknown if the immediate and late-phase reactions to allergens share common threshold concentrations; therefore explanation of these late-phase reactions by either mechanism would be completely speculative.

The inherent risk of basing threshold recommendations purely upon results in normal horses is that the 'irritant' threshold concentration for normal skin may not be the same as the 'allergy detection' threshold concentration for hypersensitive individuals. Ideally, a group of hypersensitive horses should be tested with the same dilutions of allergens.¹¹ However, although identifying a group of horses with clinical allergy to *Culicoides* spp. may not be difficult due to the well-described clinical syndrome it creates, identifying a group of caddisfly allergic horses might be quite difficult as there is nothing specifically known about the clinical effects of this allergen.¹² Considering the sheer number of insect allergens to be analysed, the task of establishing 'allergy detection' thresholds for each is quite daunting.

There is also the inherent risk of including subclinically sensitized subjects in any threshold study. We did not rule out subclinical COPD in this group of horses, and relied solely on the historical lack of clinical signs in this very closely monitored group of horses. It is unclear at this time what role, if any, allergens play in COPD as one group of investigators found that there is no difference in allergen reactivity between normal horses and those with clinical diagnoses of COPD.⁵

However, evidence suggests that more proactive testing modalities, such as bronchoalveolar lavage and auscultation after exercise, are necessary to exclude a diagnosis of subclinical COPD.¹³

In conclusion, we can only recommend 'irritant' threshold concentrations of insect allergens for use in IDT of hypersensitive horses at this juncture. Further studies to characterize 'allergen detection' thresholds in hypersensitive horses would be a welcome addition to the practice of equine allergy diagnosis, and will probably be most easily achieved with *Culicoides* spp. owing to the well-defined clinical features of CH. As it appears that currently available serological tests are not reliable diagnostic tools for equine allergy,¹⁴ IDT may remain the gold standard for the foreseeable future.

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Résumé Seize chevaux sains, sans historique de maladie cutanée ou respiratoire ont été inclus dans une étude sur le seuil de concentration des extraits pour intradermoréactions (IDT), afin de déterminer les concentrations optimales de 13 extraits commerciaux d'insectes. Cinq dilutions de chaque extrait ont été utilisées: la concentration recommandée par le fabricant pour tester les chevaux, 1 dilution plus forte et 3 dilutions plus faibles que les concentrations standard. Les allergènes testés étaient: *Trichoptera* spp., *Ephemeroptera* spp., *Tabanus* spp., *Chrysops* spp., *Solenopsis invicta*, *Camponotus pennsylvanicus*, *Periplaneta americana*, *Blattella germanica*, *Aedes aegypti*, *Musca domestica*, *Heterocera* spp., *Ctenocephalides canis/C. felis*, *Culicoides variipennis*, et *Culicoides nubeculosus*. Deux méthodes différentes ont été utilisées pour calculer la concentration allergénique de chaque extrait pour laquelle aucune réaction faussement positive ('irritante') n'est observée. Des concentrations 'irritantes' ont été déterminées pour 9/13 allergènes, mais pas pour les 4 autres allergènes à cause d'une réactivité insuffisante (puce, *C. variipennis*) ou d'une réactivité excessive (black ant, moth) aux concentrations testées. Les concentrations recommandées pour l'utilisation chez les chevaux suspects d'hypersensibilité aux insectes sont: 125 pnu/ml (mayfly); 250 pnu/ml (caddisfly, horsefly, deerfly, fire ant, house fly); 500 pnu/ml (cockroach); 1000 pnu/ml (moustique); et 1:10 000 w/v (*C. nubeculosus*).

Resumen Dieciséis caballos sanos, sin historia de enfermedades de piel o respiratorias, fueron utilizados para un estudio de umbral en un test intradérmico (TID) con el fin de determinar las concentraciones más adecuadas de 13 extractos de insectos comerciales alergénicos para el TID. Se utilizaron cinco diluciones de cada extracto, en las cuales se incluyeron las concentraciones recomendadas por el fabricante para el TID equino, más una dilución más alta y tres diluciones inferiores a estas diluciones estándar. Los alérgenos incluidos fueron: tricóptero (*Trichoptera* spp.), mosca de mayo (*Ephemeroptera* spp.), tábano (*Tabanus* spp.), mosca del ciervo (*Chrysops* spp.), hormiga roja (*Solenopsis invicta*), hormiga negra (*Camponotus pennsylvanicus*), mezcla de cucarachas (*Periplaneta americana* y *Blattella germanica*), mosquito (*Aedes aegypti*), mosca doméstica (*Musca domestica*), polilla (*Heterocera* spp.), pulga (*Ctenocephalides canis/C. felis*), *Culicoides variipennis*, y *Culicoides nubeculosus*. Se utilizaron dos métodos separados para calcular la concentración de alérgeno necesario para cada extracto de insecto, donde los caballos normales, como grupo, no mostraron una reacción positiva ('irritante') falsa. La concentración umbral 'irritante' fue determinada para 9/13 de estos alérgenos, mientras que en los cuatro restantes no pudo determinarse debido a una reactividad insuficiente (pulga, *C. variipennis*) o excesiva (hormiga negra, polilla) en las concentraciones utilizadas. Las concentraciones recomendadas para su uso futuro en pacientes equinos con una hipersensibilidad a insectos son: 125 pnu/ml (mosca de mayo); 250 pnu/ml (tricópteros, tábano, mosca del ciervo, hormiga roja, mosca doméstica); 500 pnu/ml (cucaracha); 1000 pnu/ml (mosquito); y 1:10 000 w/v (*C. nubeculosus*).

Zusammenfassung Sechzehn gesunde Pferde, die bisher keine Anzeichen von Hautkrankheiten oder Erkrankungen des Respirationstraktes gezeigt hatten, wurden fuer eine Studie zur Bestimmung des Schwellenwerts beim Intradermaltest (IDT) verwendet, um die fuer IDT geeignetste Konzentration von 13 kommerziellen Insektextraktallergenen zu bestimmen. Fuenf Verduennungen von jedem Extrakt, die vom Hersteller empfohlene Konzentration, eine hoehere und drei kleinere Verduennungen mit eingeschlossen, kamen zum Einsatz. Koecherfliege (*Trichoptera* spp.), Eintagsfliege (*Ephemeroptera* spp.), zwei Bremsenarten (*Tabanus* spp. und *Chrysops* spp.), zwei Ameisenarten (*Solenopsis invicta* und *Camponotus pennsylvanicus*), eine Kuechenschabenmischung (*Periplaneta americana* und *Blattella germanica*), Stechmuecke (*Aedes aegypti*), Stubenfliege (*Musca domestica*), Motte (*Heterocera* spp.), Floh (*Ctenocephalides canis/C. felis*), *Culicoides variipennis*, und *Culicoides nubeculosus* Allergene wurden verwendet. Zwei unterschiedliche Methoden wurden verwendet, um die Allergenkonzentration fuer jeden Insektenextrakt zu bestimmen, bei der die Gruppe von normalen Pferden keine falsch-positiven ('irritierenden') Reaktionen mehr zeigte. 'Irritierende' Schwellenwertkonzentrationen wurden fuer 9/13 dieser Allergene bestimmt, in den anderen vier Extrakten war das auf Grund von mangelnder Reaktivitaet (Floh, *C. variipennis*) oder ueberschiessender Reaktivitaet (*Camponotus pennsylvanicus*, Motte) der getesteten Konzentrationen nicht moeglich. Die empfohlenen Konzentrationen fuer zukuenftige Verwendung beim Pferd mit Vverdacht auf Insektallergie sind 125 pnu/ml (Eintagsfliege); 250 pnu/ml (Koecherfliege, Bremsen, *Solenopsis invicta*, Stubenfliege); 500 pnu/ml (Kuechenschabe); 1000 pnu/ml (Stechmuecke) und 1:10 000 w/v (*C. nubeculosus*).