Insect Bite Hypersensitivity in Horses is Associated with Airway Hyperreactivity

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Background: Genetic and epidemiologic evidence suggests that in horses, as in other species, different manifestations of hypersensitivity may occur together.

Hypothesis: Horses affected with insect bite hypersensitivity (IBH) show airway hyperreactivity (AH) to inhaled histamine, even in the absence of overt clinical signs of equine asthma (EA).

Animals: Twenty-two healthy controls (group C), 24 horses suffering from IBH alone (group IBH), and 23 horses suffering from IBH and EA (group IBH/EA).

Methods: The clinical histories were assessed using 2 standardized questionnaires, the Horse Owner Assessed Respiratory Signs Index (HOARSI), and IBH scoring. Horses were classified as EA-affected if their HOARSI was >1 and as IBH-affected if IBH score was >0. Confounding disorders were excluded by clinical examination. The arterial partial pressure of oxygen (PaO_2) was measured and flowmetric plethysmography used to assess airway reactivity to increasing doses of inhaled histamine.

Results: The median histamine provocation concentration (PC) when Δ_{flow} values increased by 35% (PC35) was significantly higher in group C (5.94 [1.11–26.33] mg/mL) compared to group IBH (2.95 [0.23–10.13] mg/mL) and group IBH/EA (2.03 [0.43–10.94] mg/mL; $P < 0.01$). The PC50 and PC75 showed very similar differences between groups. Furthermore, PaO_2 was significantly lower in group IBH (84 ± 8 mmHg) and group IBH/EA (78 ± 11 mmHg) compared to group C (89 ± 6 mmHg; $P < 0.01$).

Conclusions and Clinical Importance: IBH is associated with AH and decreased PaO_2, even in the absence of overt respiratory clinical signs.

Key words: Equine asthma; Horse; Insect bite hypersensitivity; Multiple equine allergies.

Recent genetic and epidemiologic evidence suggests that different manifestations of hypersensitivity may occur together in horses. Specifically, horses with insect bite hypersensitivity (IBH) have been shown to have a higher risk of being concurrently affected by recurrent airway obstruction (RAO), and vice versa.[1] Although IBH is an allergic dermatologic disorder triggered by allergens from *Culicoides* biting midges,[2] RAO is thought to be the result of a hypersensitivity to inhaled allergens and represents the severe form of equine asthma (EA). Equine asthma, a recently introduced term, comprises the clinical severity spectrum from mild (inflammatory airway disease, IAD) to severe

Abbreviations:

AH	airway hyperreactivity
EA	equine asthma
HCS	histamine challenge score
HOARS	Horse Owner Assessed Respiratory Sings Index
IAD	inflammatory airway disease
IBH	insect bite hypersensitivity
IgE	serum immunoglobulin E
IL4RA	interleukin-4 receptor α chain
IL13	interleukin 13
MHS	multiple hypersensitivities
PaO_2	partial pressure of arterial oxygen
PC	provocation concentration
RAO	recurrent airway obstruction
Th2	T-helper-2
Th1	T-helper-1

forms (RAO).[3,4] In horses that are predisposed to EA, inhaled allergens and irritants play a major role in the development of cough, decreased performance, impaired gas exchange, bronchoconstriction, and airway hyperreactivity (AH).[4]

Human patients with asthma often also suffer from dermatologic allergic hypersensitivities, such as atopic dermatitis.[5] These cutaneous manifestations of atopy can mark the start of the so-called "atopic march" with approximately two-thirds of patients with atopic dermatitis later developing allergic rhinitis and half of them progressing to asthma.[5] Similar concurrences of different hypersensitivity manifestations in the same individuals also have been observed in cats with pruritic dermatosis and feline asthma,[6] in dogs with allergic dermatitis, conjunctivitis, rhinitis, and asthma-like clinical signs[7] and recently in horses with RAO, IBH, and

chronic recurrent urticaria.[1] These findings suggest a common immunogenetic background, route of sensitization, or both leading to these multiple hypersensitivities (MHS).

The outcome measures in the epidemiologic study of horses described above[1] were solely based on owner-reported medical history. No further examinations regarding the potential presence of respiratory abnormalities had been performed. Histamine bronchoprovocation testing using flowmetric plethysmography is an objective tool, available in equine medicine, that can be used to detect AH even in clinically normal horses.[8,9] We, therefore, aimed to specifically examine the respiratory status of IBH-affected horses with and without a history of EA. Respiratory status was evaluated using plethysmography with a bronchial histamine provocation test and arterial partial pressure of oxygen (PaO$_2$). Specifically, we hypothesized that IBH is accompanied by increased AH, even in the absence of overt respiratory clinical signs.

Materials and Methods

Study Design and Horses

This study was approved by the ethical committees of the Cantons of Berne, Freiburg, Solothurn and Geneva, Switzerland (BE10/13), and examinations were performed between March 2015 and July 2015.

The sample size calculation was based on raw data from research in our group.[10] To detect a decrease in provocation concentration (PC) when $\triangle_{flow}$ values increased by 35% compared to saline (PC35) from 7.1 mg/mL (healthy horses) to 3.6 ± 3.4 mg/mL (affected horses) at a 5% significance level and power of 80%, a sample size calculation[a] indicated that a minimal sample size of 15 per group would be required. The aim was therefore to recruit at least 20 horses per group to account for potential dropouts. Horses were recruited by way of an advertisement published in the "Bulletin," the official monthly publication of the Swiss equine sport and breeding associations. Additionally, the databases of the Swiss Institute of Equine Medicine (ISME) and the Department of Clinical Research of the University of Bern, Switzerland were screened for IBH-affected horses.

Potential candidates were examined either at their home stables or at the clinic of the ISME in Berne. Horses were included if they had a history compatible with IBH alone or IBH and EA (see below). Healthy individuals of similar breed and sex distribution also were recruited. Any animals showing signs of confounding disorders (see below) were excluded. It was required that at the time of examination, all horses were free of any medication that might influence the outcome measures. Specifically, a washout period of at least 3 weeks was required for any previously administered corticosteroids. Horses were divided into 3 subgroups: healthy controls (group C), IBH-affected animals (group IBH), and IBH and EA-affected animals (group IBH/EA) using a questionnaire-based classification (see below).

Questionnaires and Classification Criteria

For every horse, IBH severity and lung health status were determined using standardized questionnaires.

The IBH score was based on a previously described questionnaire[11] completed by the owner and based on the most severe clinical signs the horse had experienced. Briefly, cutaneous lesions observed on the mane, tail head, ventral midline, or some combination of these were classified in 5 severity grades, each with an additional characteristic compared to the preceding grade with (0) no signs of IBH; (1) increased skin flaking with visible epithelial debris; (2) areas of broken hairs or alopecia from scratching; (3) indurated skin folds; (4) obvious crusts from serous exudate, but without bleeding; and (5) bleeding from self-inflicted abrasions. Owners also were asked if a seasonal pattern (consistent with exposure to insects, that is, spring to fall exacerbation) regarding the clinical signs was observed. Horses were classified as IBH positive, if the IBH score was >0 and if they were seasonally affected. Because clinical signs of IBH are typical, 1 season of observed clinical signs was sufficient to qualify.

The Horse Owner Assessed Respiratory Signs Index (HOARSI) was used to grade EA severity, as described in detail in a previous study.[12] The HOARSI also referred to the most severe clinical signs in the horse's history. Horses graded HOARSI 1 were classified as healthy, whereas HOARSI 2 (mild), HOARSI 3 (moderate), and HOARSI 4 (severe) were consistent with EA.

To avoid false negative control animals as far as possible, all horses had to have spent at least 1 warm season (spring to fall) in Switzerland exposed to *Culicoides* and also, by definition, all horses that were classified according to the HOARSI had been exposed previously to hay for at least 2 months.

Clinical Examination

Horses underwent a thorough clinical examination that included respiratory tract and cardiac auscultation, rectal temperature, mucous membrane color, capillary refill time, submandibular lymph node palpation, and lung auscultation at rest and during rebreathing to exclude confounding disorders. Furthermore, blood was collected from the common carotid artery before the rebreathing examination for determination of PaO$_2$ (i-STAT® Portable Clinical Analyze[b]).

Histamine Bronchoprovocation with Plethysmography

To characterize airway reactivity, a pulmonary function test, with flowmetric plethysmography (Open Pleth System[c])[9] with histamine bronchoprovocation[13] was used. The horses were sedated with detomidine[d] IV (Equisedan, 0.01–0.02 mg/kg) and fitted with an airtight mask and a pneumotachograph. In addition, 2 respiratory inductance bands were placed at the 11th intercostal space and just behind the last rib, respectively. Subsequently, calibration of the system was performed, after the manufacturer's instructions. Baseline measurements were recorded for 2 minutes, and then sterile 0.9% saline solution was nebulized (negative control) with the compressor and nebulizer provided with the system to generate a particle size <4 μm, followed by another 3-minute recording. If baseline $\triangle_{flow}$ exceeded 3.5 L/s ($\triangle_{flow}$ is the maximum difference between the flows measured by the pneumotachograph compared to the calibrated flow from the bands) or the $\triangle_{flow}$ increased more than 50% compared to baseline after saline administration, measurements were stopped, and no histamine was inhaled. Otherwise, histamine (histamine diphosphate monohydrate)[e] in saline then was nebulized in doubling concentrations (2, 4, 8, 16, 32 mg/mL). After each dose of histamine, measurements were recorded for 3 minutes. This procedure was continued until 1 of the following conditions was achieved: (1) the highest concentration (32 mg/mL) of histamine was reached; (2) an increase of > 50% in $\triangle_{flow}$ values from saline measurement was registered; (3) the horse showed markedly increased abdominal breathing; or (4) the horse started to cough repeatedly. The histamine challenge score (HCS) referred to the concentration of histamine when the inhalation was stopped with (1) =32 or 16 mg/mL, (2) =8 mg/mL, (3) =4 mg/mL,

(4) =2 mg/mL, and (5) =no histamine inhalation possible. No histamine inhalation was possible when airway obstruction with increased breathing effort, excessive coughing, or both were observed at baseline measurements or after saline inhalation. When the inhalation protocol was completed, the software of the Open Pleth System also provided the PC35, PC50, and PC75 using log-linear interpolation of the dose–response curve.

Statistics

Analyses were performed with the statistical package NCSS 2007.[f] Descriptive statistics are reported as mean ± standard deviation (SD) or median (range) as appropriate. Normality was assessed with the Skewness and Kurtosis (Omnibus) test. For comparisons among the 3 groups, one-way ANOVA was used for normally distributed data (PaO_2) or Kruskal–Wallis one-way ANOVA on ranks for non-normally distributed and ranked data (PC35, PC50, PC75; HCS). The α-level of statistical significance was set at $P < 0.05$. For post hoc testing, Kruskal–Wallis multiple-comparison z-value test (Dunn's test) with z-value > 1.96 indicating a P-value < 0.05 (Bonferroni test) was used. To assess group differences regarding the categoric values sex and breed (Icelandic versus non-Icelandic), Fisher's exact test was used.

Results

Signalment and Clinical Examination

A total of 69 horses (41 geldings, 25 mares, 3 stallions) was recruited. There were 22 horses assigned to group C, 24 to group IBH, and 23 to group IBH/EA. Sex distribution (male [stallions and geldings combined] versus female; $P = 0.98$) and age of group IBH (11 [4–24] years), group IBH/EA (14 [3–27] years), and group C (13 [5–25] years; $P = 0.36$) did not significantly differ between groups. There were no differences between the groups regarding the distribution of Icelandic horses versus other breeds ($P = 0.36$). A total of 29 Icelandic horses was included. The other breeds included Shetland Ponies (n = 7), Swiss Warmbloods (n = 6), Freibergers (n = 6), Arabians (n = 3), Friesians (n = 2), Polo Ponies (n = 2), Tinkers (n = 2), a Connemara Pony, a Pinto, a Purebred Spanish Horse, an American Quarter Horse, a Westphalian horse, and mixed breeds (n = 7). Group C and group IBH contained 11 Icelandic horses each, whereas group IBH/EA included of seven Icelandic horses. No horses were excluded based on clinical signs suggestive of confounding disorders. Overall, the severity grades of the cutaneous lesions of IBH-affected horses (distribution was similar between IBH alone and IBH/EA) were as follows: 1 (n = 2); 2 (n = 18); 3 (n = 3); 4 (n = 4); and, 5 (n = 20).

Histamine Bronchoprovocation with Plethysmography

Baseline Δ_{flow} measurements did not differ among group C (n = 21; 1.01 [−0.25–3.29] L/s), group IBH (n = 17; 0.77 [−1.26–7.61] L/s), and group IBH/EA (n = 12; 1.15 [−0.45–3.90] L/s; $P = 0.43$). However, no histamine provocation was performed in 3 horses in group IBH and another 8 in IBH/EA, because the maximal Δ_{flow} values at baseline exceeded 3.5 L/s or because Δ_{flow} increased >50% compared to baseline after

administration of saline. In 1 additional horse in group C, 4 horses in IBH, and 3 in IBH/EA, bronchoprovocation measurements were not possible because of technical problems (eg, software failure to calculate PC values), repeated coughing or both.

The PC35 values were higher in group C (n = 21; 5.94 [1.11–26.33] mg/mL) compared to group IBH (n = 17; 2.95 [0.23–10.13] mg/mL) and group IBH/EA (n = 12; 2.03 [0.43–10.94] mg/mL; $P < 0.01$). Comparison among the 3 groups showed that group C was different from group IBH (z-value: 2.10) and group IBH/EA (z-value: 2.74). Group IBH and group IBH/EA did not differ (z-value: 0.81). However, group size for IBH/EA in this comparison was below the calculated n = 15 necessary to detect a difference between the IBH group and the IBH/EA group with a power of 80%.

Figure 1 illustrates the PC35 values of the different groups. The PC50 and PC75 values showed results similar to those of PC35 (see Figures S1 and S2).

Distribution of Maximal Doses Reached

The median histamine challenge score (HCS) of group C was significantly lower (n = 22; 1 [1–3]) than that of group IBH (n = 24; 2.5 [1–5]) and group IBH/EA (n = 23; 4 [1–5]). Comparison among the three groups (z-value > 1.96 = P < 0.05) indicated that group C was different from group IBH (z-value: 3.24) and group IBH/EA (z-value: 5.06). Group IBH and group IBH/EA did not show a difference (z-value:
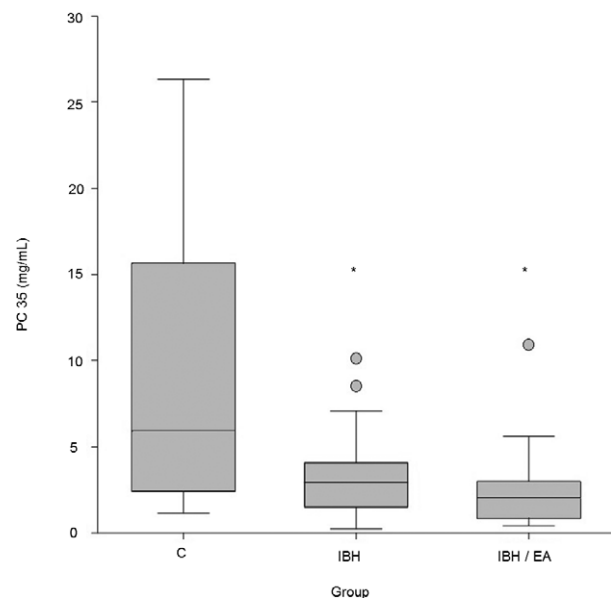

Fig 1. Box plot illustrating the provocation concentrations (PC) when Δflow values increased by 35% (PC35) compared to baseline of healthy controls (C) (n = 21; 5.94 [1.11–26.33] mg/mL), horses suffering from insect bite hypersensitivity (IBH) (n = 17; 2.95 [0.23–10.13] mg/mL], and horses diagnosed with both IBH and equine asthma (IBH/EA) (n = 12; 2.03 [0.43–10.94]). *Indicates significant difference compared to C.
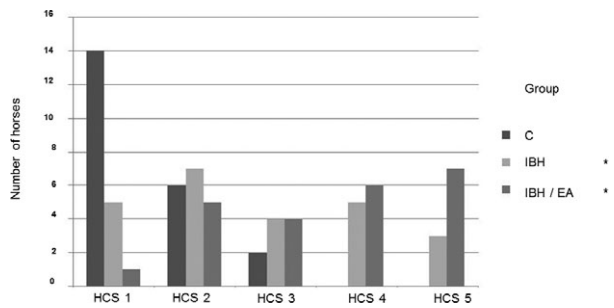
Fig 2. Bar graph illustrating the number of horses assigned a particular histamine challenge score (HCS). The histamine challenge score (HCS) refers to the dose of histamine when the inhalation was stopped with HCS1 = 32 or 16 mg/mL, HCS2 = 8 mg/mL, HCS3 = 4 mg/mL, HCS4 = 2 mg/mL, and HCS5 = no histamine inhalation possible. *indicates significant difference compared to group C.

1.89). The numbers of horses achieving the different HCS in each group are illustrated in Figure 2.

Partial Pressure of Arterial Oxygen

Group C had a higher resting PaO_2 (89 ± 6 mmHg) than both group IBH (84 ± 8 mmHg) and group IBH/EA (78 ± 11 mmHg). Comparison among the 3 groups (z-value > 1.96 = P < 0.05) indicated that group C was different from group IBH (z-value: 2.05) and group IBH/EA (z-value: 4.16). Group IBH and group IBH/EA also were different (z-value: 2.16, Fig 3).

Discussion

Our results indicate that IBH is associated with AH. Although this finding confirms our hypothesis, it is surprising, because these 2 hypersensitivity disorders affect separate organ systems. However, previous epidemiologic investigations in 2 distinct populations showed that horses suffering from the dermatologic disorders IBH or urticaria had an increased risk of also being affected by RAO, and vice versa.[1]

Histamine provocation clearly separated the IBH/EA group from the control group. Most interestingly, group IBH, the horses of which did not show overt clinical signs of EA, also displayed significantly decreased PC35, 50, and 75 as well as lower PaO_2 values than the control group. Our study thus demonstrates that an allergic skin disorder can be accompanied by AH indicative of a subclinical effect on the lower respiratory tract.

However, PC values varied considerably within the groups and overlapped among groups as illustrated in Figure 1. Therefore, isolated PC values from single horses cannot accurately predict the EA or the IBH status of an individual animal.

Equine asthma had not been identified by the owners in this group because the inclusion criteria required that the owners had not noted any coughing, nasal discharge, or increased respiratory effort (HOARSI 1). The HCS, which was possible to assess in all horses,
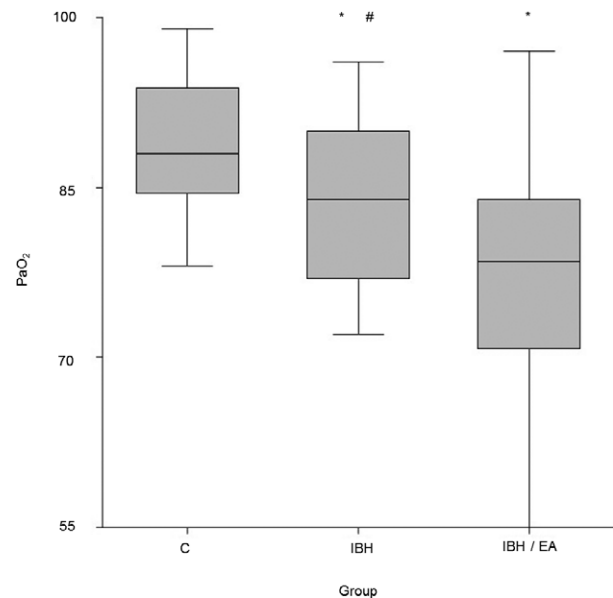

Fig 3. Box plots revealing the partial pressure of arterial oxygen (PaO_2) of healthy horses (C) (n = 22; 89 ± 6 mmHg), horses suffering from insect bite hypersensitivity (IBH) (n = 24, 84 ± 8 mmHg), and horses suffering from both IBH and equine asthma (IBH/EA) (n = 23, 78 ± 11 mmHg). *Indicates a significant difference compared to group C. # indicates a significant difference between group IBH and IBH/EA.

confirmed the calculated PC values, where a number of measurements had to be discarded. From our data, it was not possible to draw sound conclusions on the effect of IBH severity on airway reactivity, because of the small numbers of horses in some of the IBH score groups.

In humans, investigations into the immunogenetic background of these MHS disorders showed evidence for specific genetic determinants as a potential underlying reason for a general allergic phenotype.[14] One candidate gene involved in different allergic diseases, specifically asthma, atopic dermatitis, and rhinitis, is interleukin-4 receptor a-chain (IL4RA). In EA, a genetic association between a region on equine chromosome (ECA) 13, containing the gene for IL4RA, and RAO was described in a high-prevalence RAO family.[15,16] A linkage between RAO and a single nucleotide polymorphism (SNP BIEC2-224511) on ECA 13 near IL4RA was confirmed in unrelated horses.[17] However, this polymorphism was not associated with MHS in horses in a recent study.[1]

Additional genetic variants that might be associated with both airway hypersensitivities and skin allergies are polymorphisms in IL13, a gene playing the same role in the T-helper-2 (Th2)-type response as IL4RA, and a mutation in a profilaggrin/filaggrin gene.[18,19] The IL13 polymorphisms have been associated with both asthma and rhinitis, suggesting a common etiologic pathway for their development.[18,20] Furthermore, these studies also showed an interaction between IL13 and IL4R polymorphisms in asthma. However, even though the RAO-associated quantitative trait locus on equine

chromosome 13 has triggered these investigations into MHS in horses, RAO and IBH exhibit important immunopathologic differences that must be considered in the context of genes such as IL13 or IL4RA. Importantly, IBH is characterized as a type I allergy with IgE- and Th2-type driven immunopathogenesis, and lesional eosinophil and mast cell involvement.[2]

The immunopathology of RAO, in contrast, is still unclear. Many contradictory results regarding a Th1-Th2- Th17 or mixed immune response have been reported.[3]

A further interesting candidate gene in humans with MHS is filaggrin,[21] which is not directly involved in immune function, but rather in integrity of epithelial barrier function.[5] Cutaneous manifestations of atopy often represent the beginning of the atopic march in humans,[21] and data from experimental animals as well as from clinical studies in humans suggest a role for the skin as an important route of sensitization to asthma-inducing allergens.[22] These findings support the concept that skin barrier dysfunction increases the risk of sensitization to allergens by facilitating crossing of the epidermal barrier by environmental allergens and subsequent induction of sensitization. In horses, there is presently no published data on a possible involvement of filaggrin mutations in IBH or RAO.

However, both RAO and IBH are genetically complex diseases. The heritability of IBH susceptibility has been reported in several breeds including Icelandic horses, Friesian horses, Shetland ponies, and Belgian Warmblood horses with a wide range from 0 to 0.36 on the observed scale.[15,23–27] Additional studies may explore some of the candidate genes such as filaggrin. It is very likely that multiple, even numerous genes are involved in these disorders and it must be determined which genes overlap and play a role in both hypersensitivity manifestations. Other potential causes for multiple allergies include epigenetic regulation as well as the gastrointestinal, respiratory tract and skin microbiome, which are postulated to play important roles in allergies in humans.[28]

Group classifications in our study were based on owner-reported history using standardized scores. Previous studies have extensively described and validated the HOARSI scoring system in several populations with different degrees of EA.[10,12,29] The results of these experiments suggest that horses with HOARSI 3 and 4 (regular to permanent coughing, increased breathing effort after work and, in HOARSI 4, also at rest) suffer from RAO, whereas HOARSI 2 corresponds best with IAD and HOARSI 1 indicates a clinically healthy lower respiratory tract. The latter 2 categories are less distinct however, making HOARSI a valuable, but imperfect surrogate marker for EA. Accordingly, in future studies, it might be interesting to investigate if the difference in hyperreactivity between IBH and IBH/EA is more marked if a larger group of severely EA (RAO)-affected horses (ie, HOARSI 3 and 4) is studied.

It would have been useful to perform endoscopy to assess the mucus quantity in the trachea and collect bronchoalveolar fluid (BALF) for cytology. This is likely the main limitation of our study. Although the HOARSI provides a validated scoring system, it is still only a surrogate marker for EA. Current definitions of RAO and IAD are based on cytologic variable, and the relationship between hypersensitivity and cytology is still unclear.[4,30] Some studies have reported AH predominantly in mast cell-type or eosinophilic IAD,[4] which might fit with the atopic, Th2-primed immune response observed in IBH,[2,31] but a recent report did not confirm these associations.[32]

Endoscopy and BALF cytology were not performed because owner compliance was particularly challenging, and the number of participating horses would have been insufficient if these slightly more invasive procedures would have been required. Furthermore, most horses were examined at their home stables, making additional examinations logistically challenging. However, we aimed to characterize horses as comprehensively as possible under the given conditions. The HOARSI scoring system was complemented by a thorough clinical examination, resting arterial oxygen tension measurements, baseline lung function measurements, and bronchoprovocation with histamine. The IBH scoring system used worked well in our hands and, as far as we are aware, there are no other published scoring systems for IBH. For an additional more in-depth classification of IBH, future studies could use a validated cellular antigen stimulation test, and the period between the most severe historic clinical signs and time of examination could be more standardized.[33] Finally, we only examined horses for allergic reactions involving the skin and the lungs (ie, IBH and EA). No other organ systems were evaluated for potential hypersensitivity disorders. Additional investigations concerning MHS in horses, therefore, are needed. For instance, it would be interesting to explore whether horses with urticaria, which also was shown to be associated with RAO,[1] also suffer from hyperreactive airways or if food-related allergies, such as the recently described gluten hypersensitivity in horses,[34] play a role in MHS.

In conclusion, the airways of horses with IBH alone showed hyperreactivity similar to airways of horses with EA. Although future studies may identify the immunogenetic basis for this phenomenon, our results already suggest that horses suffering from IBH have a higher risk for AH and therefore might be predisposed to develop EA in the future. These findings should be taken into account when evaluating IBH-horses presented for subtle respiratory signs.

Footnotes

Acknowledgment

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Box plot illustrating the provocation concentrations (PC) when Δflow values increased by 50% (PC50) compared to baseline of healthy controls (C) (n = 21; 6.71 [1.59–38.79] mg/mL), horses suffering from insect bite hypersensitivity (IBH) (n = 17; 3.11 [0.32–12.53] mg/mL) and horses diagnosed with both IBH and equine asthma (IBH/EA) (n = 12; 2.45 [0.61–11.53]). *Indicates significant difference compared to C.

Figure S2. Box plot illustrating the provocation concentrations (PC) when Δflow values increased by 75% (PC75) compared to baseline of healthy controls (C) (n = 20; 8.77 [2.53–32.60] mg/mL), horses suffering from insect bite hypersensitivity (IBH) (n = 17; 4.28 [0.48–16.54] mg/mL) and horses diagnosed with both IBH and equine asthma (IBH/EA) (n = 12; 3.24 [0.0.91–12.50]). *Indicates significant difference compared to C.