Allergen-specific immunoglobulin E in sera of horses affected with insect bite hypersensitivity, severe equine asthma or both conditions

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Background: Genetic, epidemiologic, and clinical evidence suggests that, in horses, there are manifestations of hypersensitivity that can occur together.

Objectives: To investigate whether concurrent insect bite hypersensitivity (IBH) and severe equine asthma (EA) is associated with higher allergen-specific and total serum immunoglobulin E (IgE) concentrations than only EA or IBH.

Animals: Healthy control horses (C, n = 40), horses with IBH (IBH, n = 24), severe EA (EA, n = 18), and both conditions (IBH/EA, n = 23) were included.

Methods: In our retrospective comparative study, sera from horses with signs of severe EA, IBH, and control animals were used. IgE specific for 15 recombinant (r) allergens as well as total serum IgE concentrations were measured by enzyme-linked immunosorbent assay.

Results: Group IBH (median sum r-Culicoides IgE: optical density at 405 nm [OD405] = 3.54 [0.48-15.07]) and group IBH/EA (OD405 = 4.55 [0.46-17.15]) had significantly (P < .001) higher IgE against Culicoides r-allergens than groups C (OD405 = 0.44 [0.21-2.05]) and EA (OD405 = 0.6 [0.2-2.9]). There were no significant (P > .05) differences between group IBH and group IBH/EA. No significant differences among the groups were found for the other r-allergens or total serum IgE concentration. Compared to controls, horses with severe IBH had significantly increased IgE concentration to 5 Culicoides r-allergens (P < .05), whereas horses with moderate IBH had significantly increased IgE concentration to only 3 Culicoides r-allergens (P < .05).

Conclusions and Clinical Importance: Susceptibility of IBH-affected horses to develop EA is likely not associated with IgE-mediated immune reactions but with other immunopathological mechanisms.

KEYWORDS
equine asthma, horse, immunoglobulin E, insect bite hypersensitivity, multiple equine allergies

INTRODUCTION

Genetic, epidemiologic, and clinical evidence suggests that, in horses, as in other species, different manifestations of hypersensitivity occur concurrently.1,2 In humans, the "atopic march" manifests as a temporal sequence of hypersensitivities, with atopic dermatitis in the neonate preceding the development of allergic rhinitis and asthma.3 The atopic march and multiple allergies in humans can be associated with...
increased total serum immunoglobulin E (IgE), IgE cross-reactivity between different allergens, or both.3,4

In horses, multiple hypersensitivities can manifest as the combined occurrence of equine asthma (EA), insect bite hypersensitivity (IBH), and chronic recurrent urticaria. Particularly, the prevalence of IBH is increased in recurrent airway obstruction (RAO)-affected horses and vice-versa.3 Although longitudinal studies in affected horses are lacking, the typical earlier onset of IBH compared to RAO9 suggests that as in humans with multiple hypersensitivity disorder, a dysfunctional skin barrier serves as the site of allergen sensitization, resulting in a systemic T-helper-2 immunity which is a predisposing factor for the development of respiratory allergies.6

IBH is an allergic dermatitis provoked by Culicoides midge bites.5 Affected horses have intense pruritus, which results in typical skin lesions localized mainly along the dorsal midline and the base of the tail and the mane.5 The involvement of IgE-mediated reactions in the pathogenesis of this disease has been clearly established.5 The specific Culicoides allergens causing IBH have been identified at the molecular level and produced as pure recombinant (r) allergens.5,7,8 The use of these r-allergens compared to crude Culicoides nubeculosus whole-body extract improves the sensitivity and specificity of serologic IgE tests for IBH.9

EA encompasses inflammatory airway disease (the mild to moderate form of EA) and RAO, also known as heaves, the severe form.10 Dyspnea, coughing, and loss of performance are the most commonly observed clinical signs, caused mainly by the bronchoconstriction and the compromised gas exchange.11 The exact immunopathogenesis of severe EA remains unclear,12 and the involvement of IgE-mediated reactions is still controversial, but a part of horses with severe EA have increased IgE concentrations against r Asp f allergens.13 Even less is known about the pathogenesis and potential involvement of IgE in the milder forms of EA.10

Based on these findings, the aim of our study was to investigate allergen-specific IgE concentrations in the sera of healthy control (C) horses as well as horses with IBH, EA, and both conditions, using a panel of r-allergens including r Asp f and r-Culicoides allergens. A 2nd aim was to investigate whether allergic horses had increased serum IgE concentrations specific for r-allergens not previously tested in the horse (r-Cladosporium herbarum [r Cla h] 8, r-Glycophagus domesticus [r Gly d] 2, and r-Dermatophagoides pteronyssinus [r Der p] 23). Thus, a panel of different allergen classes that included midge, mold, and mite r-allergens was used to test the hypothesis that horses with both conditions, specifically IBH and EA, have more diverse and augmented reactions across the allergen classes compared to healthy horses and compared to horses with IBH or EA alone.

2 | MATERIALS AND METHODS

2.1 | Study design

The serum used in our study was drawn from horses that were included in 3 previously published studies (Supporting Information Table S1).2,16,17 The studies were approved by the Animal Experimental Committees of the Cantons of Berne, Freiburg, Solothurn, and Geneva, Switzerland (BE10/13; 58/10; 118/16). Four groups were defined using questionnaire-based and clinical information: healthy controls (group C), IBH-affected horses (group IBH), severe EA-affected horses (group EA), and horses with both IBH and EA (group IBH/EA).

Samples from 69 horses included in Lanz et al (2017)2 formed group IBH (n = 24), group IBH/EA (n = 23), and 22 samples of group C. Because no horses affected with severe EA only were included in our study2 and increased concentrations of IgE specific for mold allergens had previously been described in horses with severe EA, samples from horses with severe EA and matched controls were drawn from 2 previous studies16,17 to form group EA (n = 18) and to complement group C with 18 controls. Samples were selected based on availability and on the Horse Owner Assessed Respiratory Signs Index (HOARSI).

2.2 | Serum samples, horse population structures, and group definitions

Blood was collected from the jugular vein using Serum Clot Activator-containing vacutainers (Vacuette; GreinerBioOne, St. Gallen, Switzerland). The blood was centrifuged for 10 minutes at 2000×g, and serum was separated and stored at −80°C until used.

For every horse, IBH severity and lung health status were determined using standardized questionnaires. All horses were withdrawn from medication for at least 2 weeks before clinical examination and blood sampling. Some of the horses with asthma had been treated with corticosteroids, bronchodilators, or both. Preventive measures such as blankets and repellents were used in some of the IBH-affected horses to decrease exposure to insects.

The standardized IBH score, based on the most severe clinical signs the horse had experienced (Supporting Information Table S1), was performed as described.2 Briefly, cutaneous lesions observed on the mane, tail, head, and ventral midline were classified according to 5 severity grades ranging from 0 (no signs of IBH) to 5 (bleeding from self-inflicted abrasions). Horses were classified as IBH-positive, if the IBH score was ≥0 and if they were seasonally affected (season consistent with exposure to insects, ie, spring to fall exacerbation). Because clinical signs of IBH are typical, 1 season of observed clinical signs was sufficient to qualify.

The HOARSI was used to grade EA severity and has been previously described and validated.16-18 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. Because increased allergen-specific IgE concentrations have only been described in the severe form of EA,12,14 horses for this group were selected based on the following parameters: HOARSI ≥3 and partial pressure of arterial oxygen <90 mm Hg, measured as described in the 3 previous studies.2,16,17

The control horses had no clinical signs or owner-reported history of IBH or severe EA. To help avoid false-negative control animals, all horses had to have spent at least 1 warm season (spring to fall) in Switzerland exposed to Culicoides and also had to be exposed previously to hay for at least 2 months.

2.3 | Allergens

The IgE concentrations specific to the 15 different r-allergens were measured in the horse sera. Detailed information about the allergens
used is given in Table 1. They consisted of 4 r-A. fumigatus allergens, expressed and purified as previously described.20 8 r-Culicoides allergens, 4 derived from Culicoides obsoletus, and 4 from C. nubeculosus, all also expressed in Escherichia coli and purified as described in a previous study.21 The Culicoides allergens were chosen based on unpublished data showing that these r-Culicoides allergens were the most relevant ones for horses in Switzerland. Additionally, in an exploratory study, 1 commercially available r-allergen from the mold C. herbarum (Cla h 8; Biomay AG, Vienna, Austria) and 2 r-mite allergens, D. pteronyssinus 23 (Der p 23; Biomay AG) expressed in Escherichia coli, and G. domesticus 2 expressed in Pichia pastoris (Gly d 2; Indoor Biotechnologies Inc, Charlottesville) were used.

### 2.4 | Allergen-specific IgE ELISA

Allergen-specific serum IgE against the different r-allergens was determined by ELISA, as described previously.22 Briefly, 96-well high-binding polystyrene plates (Millipore SA, Geneva, Switzerland) were coated with 2 μg/mL of the allergen in coating buffer (0.2 M carbonate-bicarbonate buffer pH 9.4) at 37°C for 2 hours. After washing the plates twice in washing buffer (phosphate-buffered saline [PBS] pH 7.4 containing 0.05% Tween 20), plates were blocked with blocking buffer (PBS pH 7.4, 5% milk powder, 0.05% Tween 20) for 1 hour at 37°C. All dilutions of serum samples and antibodies were made with blocking buffer. After washing, test serum samples were randomly added in duplicates to the plates at a dilution of 1 : 5 and incubated at 4°C overnight. After washing, a monoclonal mouse antibody-specific for equine IgE (1 μg/mL, clone 3H10)23 was added and incubated for 2 hours at room temperature (RT) on a shaker. After washing, alkaline phosphatase-conjugated, affinity-purified goat-anti-mouse IgG (1 : 2000; Jackson Immuno Research, Newmarket, UK) was added and the plates developed using 1.5 mg/mL phosphatase substrate (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) in 10% diethanolamine (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), pH 9.8. After 2 hours, absorbance was measured as optical density at 405 nm (OD405 nm). ELISA results were shown as OD405 nm values after a blank correction on the optical density and correction between plates, as described in our study.8

Because of the individual reaction patterns of the horses to the single r-allergens, the sum of IgE concentrations against all 8 tested r-Culicoides allergens were calculated for each horse and used for additional analyses. Accordingly, IgE concentrations to the mold or the mite allergens were also summed up for analysis.

### 2.5 | Determination of total serum IgE concentrations

Total IgE concentrations were determined in the sera of the horses as previously described.22,24 Briefly, plates were coated with 1 μg/mL monoclonal mouse anti-IgE 1C12 in coating buffer (0.05 M carbonate buffer, pH 9.4) and incubated overnight at 4°C. The plates were washed with wash buffer (PBS 0.05% Tween, pH 7.4) and then blocked with 100 μL blocking buffer (PBS Tween 0.5% BSA) for 30 minutes at RT. Dilutions of standard sera or horse sera (dilution 1 : 50) were made in blocking buffer, diluted serially 2-fold and incubated at RT for 2 hours and 30 minutes. After washing, a biotin conjugate monoclonal mouse anti- equine IgE mAb 3H1023 was added and incubated for a further 2 hours and 30 minutes. After a further wash, 100 μL of a 1 : 10 000 dilution Extravidin alkaline phosphatase (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) was added and plates incubated for 1 hour, on a shaker, at RT. After a final wash, the ELISA was developed with 1 mg/mL phosphatase substrate dissolved in 10% diethanolamine pH 9.8 (Carl Roth GmbH Co. KG, Karlsruhe, Germany). Plates were finally incubated in the dark, at RT, and were

### TABLE 1 | Recombinant (r) allergens used in the study

<table>
<thead>
<tr>
<th>Allergens</th>
<th>Name recombinant</th>
<th>Function</th>
<th>Genbank accession Number</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Molds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>r Asp f 6</td>
<td>MnSOD</td>
<td>U53561</td>
<td>Crameri et al (1999)20</td>
</tr>
<tr>
<td></td>
<td>r Asp f 7</td>
<td>Unknown</td>
<td>AJ223315</td>
<td>Crameri et al (1999)20</td>
</tr>
<tr>
<td><em>Cladosporium herbarum</em></td>
<td>r Cla h 8</td>
<td>Mannitol dehydrogenase</td>
<td>Commercial</td>
<td>Biomay</td>
</tr>
<tr>
<td><strong>Mites</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Glycophagus domesticus</em></td>
<td>r Gly d 2</td>
<td>NPC2 family</td>
<td>Commercial</td>
<td>Indoor biotechnologies</td>
</tr>
<tr>
<td><em>Dermatophagoides pteronyssinus</em></td>
<td>r Der p 23</td>
<td>Peritrophin-like proteins</td>
<td>Commercial</td>
<td>Biomay</td>
</tr>
<tr>
<td><strong>Culicoides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Culicoides obsoletus</em></td>
<td>r Cul o 1P</td>
<td>Kunitz BPI</td>
<td>JXS12273</td>
<td>Peeters et al (2013)8</td>
</tr>
<tr>
<td></td>
<td>r Cul o 2P</td>
<td>D7-related</td>
<td>JXS12274</td>
<td>Peeters et al (2013)8</td>
</tr>
<tr>
<td></td>
<td>r Cul o 3</td>
<td>Antigen-5 like</td>
<td>KC339673</td>
<td>Van der Meide et al (2013)7</td>
</tr>
<tr>
<td></td>
<td>r Cul o 6</td>
<td>D7-related</td>
<td>KC339676</td>
<td>Van der Meide et al (2013)7</td>
</tr>
<tr>
<td><em>Culicoides nubeculosus</em></td>
<td>r Cul n 3</td>
<td>Unknown</td>
<td>HM145951</td>
<td>Schaffartzik et al (2011)21</td>
</tr>
<tr>
<td></td>
<td>r Cul n 4</td>
<td>Unknown</td>
<td>HM145952</td>
<td>Schaffartzik et al (2011)21</td>
</tr>
<tr>
<td></td>
<td>r Cul n 5</td>
<td>Unknown</td>
<td>HM145953</td>
<td>Schaffartzik et al (2011)21</td>
</tr>
<tr>
<td></td>
<td>r Cul n 9</td>
<td>D7-related</td>
<td>HM145957</td>
<td>Schaffartzik et al (2011)21</td>
</tr>
</tbody>
</table>

Abbreviations: r Asp f, recombinant *Aspergillus fumigatus*; r Cla h, recombinant *Cladosporium herbarum*; r Cul n, recombinant *Culicoides nubeculosus*; r Der p, recombinant *Dermatophagoides pteronyssinus*; r Gly d, recombinant *Glycophagus domesticus*. 
read after 30 minutes, 1 hour, and until the OD_{405} nm of the highest concentration of the standard curve reached 1.00. As a standard, a 6-fold dilution of a horse serum with a known IgE concentration ranging from 4 to 128 ng/mL was used. This sample was tested in duplicate. The OD of the sera was introduced from the standard curves to get their corresponding IgE concentration expressed as ng/mL value and was subsequently multiplied by the dilution factor. The concentration of the sera is shown as the mean of the values from those dilutions where the concentration was within the standard curve. All samples were randomly distributed on 8 plates.

2.6 Statistical analyses

Statistical analyses were carried out using the statistical package NCSS 2011 (NCSS Statistical Software, Kaysville, Utah). A 1st analysis indicated that allergen-specific IgE concentrations were not distributed normally; therefore, medians and ranges were used for descriptive statistics.

The nonparametric Kruskal-Wallis Multiple-Comparison Z-Value test (Dunn’s test) with Bonferroni correction for multiple comparisons was used to analyze differences in allergen-specific IgE concentrations among the 4 groups of horses and for total serum IgE concentrations in IBH, IBH/EA, EA, and control horses used in our study. A 1st analysis showed that IgE concentrations in the sera of the group C from the study by Lanz et al (2017) did not differ significantly from those of group C horses derived from the studies by Rettmer et al (2014) and Laumen et al (2010), except for r-Asp f 8, where group C horses from the older studies had higher r-Asp f 8-specific IgE (OD_{405} = 0.10 nm [0.03-0.33 nm] versus 0.06 nm [0-0.36 nm], P = .04, Mann Whitney U test). Thus, for this allergen, the results from the samples from Lanz et al (2017) were analyzed separately from those of the 2 other studies. The results are communicated accordingly.

Associations between EA or IBH and allergen-specific IgE concentrations were also tested in the Kruskal-Wallis Multiple-Comparison Z-Value test by comparing horses with IBH to horses without IBH, independently of a concomitant affection with EA. The same was done for EA. Similarly, EA-affected horses were also compared to horses without EA (Table 3).

To analyze a possible effect of the IBH severity score on specific IgE, horses from Lanz et al (2017) were used. Because the number of IBH-affected horses in each score group was too small for statistical analysis, the horses with scores from 1 to 3, moderate IBH (see Supporting Information Table S1), were pooled. Accordingly, horses with more severe IBH (scores 4 and 5, severe IBH) were also pooled for the analysis. Consequently, r-Culicoides allergen-specific IgE concentrations in the sera of horses without IBH (score = 0, n = 22), with moderate IBH (score 1-3, n = 23), or severe IBH (score 4-5, n = 24) were compared using a Kruskal-Wallis Multiple-Comparison Z-Value test (Dunn’s test) with Bonferroni correction for multiple comparisons.

The level of significance was set at P ≤ .05. Results are presented as median (range).

3 RESULTS

The total sample set included 105 horses of various breeds and sexes (4 stallions, 54 mares, and 47 geldings) aged 3-27 years (Supporting Information Table S1, Figure 1). There were no significant differences in breed, age, or sex distribution among the 4 groups of horses (IBH, IBH/EA, EA, and control horses) used in our study. A 1st analysis showed that IgE concentrations in the sera of the group C from the study by Lanz et al (2017) did not differ significantly from those of group C horses derived from the studies by Rettmer et al (2014) and Laumen et al (2010), except for r-Asp f 8, where group C horses from the older studies had higher r-Asp f 8-specific IgE (OD_{405} = 0.10 nm [0.03-0.33 nm] versus 0.06 nm [0-0.36 nm], P = .04, Mann Whitney U test). Thus, for this allergen, the results from the samples from Lanz et al (2017) were analyzed separately from those of the 2 other studies.

The detailed data of total and allergen-specific IgE concentration are presented in Table 2. The following results are summarized by allergen classes.

3.1 Allergen-specific IgE against midges

Comparison of r-Culicoides allergen-specific IgE concentrations in the 4 groups of horses showed that groups IBH and IBH/EA had
TABLE 2  Results of allergen-specific IgE concentrations in horses sera

<table>
<thead>
<tr>
<th>Allergens</th>
<th>EA (N = 18)</th>
<th>IBH (N = 24)</th>
<th>IBH/EA (N = 23)</th>
<th>Control (N = 40)</th>
<th>P-value 1-way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>r Cul o 1P</td>
<td>0.089±b (0.001-2.29)</td>
<td>0.65±c (0.003-3.11)</td>
<td>0.76±b (0.01-3.096)</td>
<td>0.073±c (0.001-0.41)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>r Cul o 2P</td>
<td>0.033±d (0-0.086)</td>
<td>0.23±d (0-0.003-1.19)</td>
<td>0.13±d (0-0.003-2.25)</td>
<td>0.047±d (0-0.98)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>r Cul o 3</td>
<td>0.074 (0.01-0.19)</td>
<td>0.22±c (0-3.22)</td>
<td>0.087b (0-3.42)</td>
<td>0.04±b (0-0.81)</td>
<td>≤.001</td>
</tr>
<tr>
<td>r Cul o 6</td>
<td>0.025±b (0-0.087)</td>
<td>0.11c (0-3.22)</td>
<td>0.09±c (0-3.22)</td>
<td>0.04±c (0-0.27)</td>
<td>≤.001</td>
</tr>
<tr>
<td>r Cul n 3</td>
<td>0.12 (0.027-1.29)</td>
<td>0.24±c (0.022-3.022)</td>
<td>0.12 (0.115-1.69)</td>
<td>0.11±c (0.012-0.75)</td>
<td>.01</td>
</tr>
<tr>
<td>r Cul n 4</td>
<td>0.08 (0-0.18)</td>
<td>0.151 (0-1.12)</td>
<td>0.13 (0-1.17)</td>
<td>0.095 (0-0.55)</td>
<td>≤.001</td>
</tr>
<tr>
<td>r Cul n 5</td>
<td>0.044 (0.027-0.35)</td>
<td>0.086 (0.003-2.67)</td>
<td>0.088 (0-1.13)</td>
<td>0.057 (0-0.37)</td>
<td>.089</td>
</tr>
<tr>
<td>r Cul n 9</td>
<td>0.08 (0.016-0.206)</td>
<td>0.105 (0.006-1.96)</td>
<td>0.16 (0-3.35)</td>
<td>0.082 (0-0.69)</td>
<td>NS</td>
</tr>
<tr>
<td>Sum Culicoides</td>
<td>0.6±b (0.2-2.9)</td>
<td>3.54±c (0-15-1067)</td>
<td>4.548±c (0.46-17.15)</td>
<td>0.44±c (0.21-2.052)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>r Asp f 4</td>
<td>0.067 (0-0.28)</td>
<td>0.023 (0-0.19)</td>
<td>0.038 (0-0.29)</td>
<td>0.043 (0-0.33)</td>
<td>NS</td>
</tr>
<tr>
<td>r Asp f 6</td>
<td>0.098 (0-0.51)</td>
<td>0.17 (0-0.74)</td>
<td>0.12 (0-0.47)</td>
<td>0.16 (0-0.55)</td>
<td>NS</td>
</tr>
<tr>
<td>r Asp f 7</td>
<td>0.057±b (0.033-0.18)</td>
<td>0.031 (0-0.2)</td>
<td>0.016±b (0-0.36)</td>
<td>0.04±b (0-0.23)</td>
<td>≤.01</td>
</tr>
<tr>
<td>r Asp f 8 Lanz et al2</td>
<td>NA</td>
<td>0.045 (0-1.3)</td>
<td>0.069 (0-1.4)</td>
<td>0.057 (0-0.36)</td>
<td>NS</td>
</tr>
<tr>
<td>r Asp f 8 Rettermer et al16; Laumen et al17</td>
<td>0.123 (0.04-0.36)</td>
<td>NA</td>
<td>NA</td>
<td>0.102 (0.033-0.33)</td>
<td>NS</td>
</tr>
<tr>
<td>r Cla h 8</td>
<td>0 (0-0.054)</td>
<td>0 (0-0.18)</td>
<td>0 (0-0.17)</td>
<td>0 (0-0.18)</td>
<td>NS</td>
</tr>
<tr>
<td>Sum molds</td>
<td>0.35 (0.06-1.29)</td>
<td>0.333 (0.052-1.84)</td>
<td>0.36 (0.109-1.93)</td>
<td>0.338 (0.022-1.00)</td>
<td>NS</td>
</tr>
<tr>
<td>r Der p 23</td>
<td>0.086 (0-0.31)</td>
<td>0.046 (0-0.72)</td>
<td>0.035 (0-0.74)</td>
<td>0.067 (0-0.27)</td>
<td>NS</td>
</tr>
<tr>
<td>r Gly d 2</td>
<td>0.066 (0-0.34)</td>
<td>0.15 (0-0.31)</td>
<td>0.098 (0-0.69)</td>
<td>0.088 (0-0.33)</td>
<td>NS</td>
</tr>
<tr>
<td>Sum mites</td>
<td>0.16 (0-0.66)</td>
<td>0.21 (0-0.38-0.93)</td>
<td>0.13 (0-0.04-0.9)</td>
<td>0.16 (0-0.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Total IgE*</td>
<td>NA</td>
<td>14.19 (0-56.62)</td>
<td>10.81 (0-63.13)</td>
<td>6.88 (0-63.13)</td>
<td>NS</td>
</tr>
<tr>
<td>Sum all IgE</td>
<td>1.011±b (0.33-4.36)</td>
<td>3.97±c (0.96-16.77)</td>
<td>3.78±d (0.84-20.67)</td>
<td>1.24±d (0.43-2.86)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

Abbreviations: EA, equine asthma; IBH, insect bite hypersensitivity; IgE, immunoglobulin E; NS, nonsignificant; NA, not analyzed; r Asp f, recombinant Aspergillus fumigatus; r Cla h, recombinant Cladosporium herbarum; r Cul n, recombinant Culicoides nubeculosus; r Der p, recombinant Dermatophagoides pteronyssinus; r Gly d, recombinant Glycophagus domesticus.

Median OD405 (range) of IgE concentrations against each of the tested r-Asp f, r-Gly d, r-Der p, r-Cla h, r-Cul o and r-Cul n allergens, including the Cul n r-allergens, were significantly higher in horses with IBH compared to horses without IBH, independently of the presence or absence of EA (Supporting Information Table S2).

3.2 | Allergen-specific IgE against mold and mite allergens

No significant differences among the 4 groups of horses were detected for IgE concentrations against the mold allergens r-Cla h 8, r-Asp f 4, r-Asp f 6, and r-Asp f 8 or the mite allergens r-Der p 23 and r-Gly d 2. Group IBH/EA had significantly lower r-Asp f 7-specific IgE than groups EA and C. No significant differences among the 4 horse groups were found for r-Asp f 8.

3.3 | Total serum IgE concentrations

There was a high variation of total IgE concentrations among the horses, with values ranging from 0 to 147.74 μg/mL (Table 2, Supporting Information Figure 4). However, a clinical diagnosis of IBH or of severe EA was not associated with the total IgE concentrations observed in our population. Group IBH (14.2 μg/mL [0-56.62 μg/mL]) and group IBH/EA (10.8 μg/mL [0-147.74 μg/mL]) did not have significantly different values when compared to group C (6.9 μg/mL [0-63.13 μg/mL]; P = .37).

3.4 | Influence of IBH severity on allergen-specific serum IgE concentrations

Grouping of the horses according to their IBH severity score (Table 3) showed that horses with severe IBH had significantly higher serum IgE concentrations against r-Cul o 1p, r-Cul o 2p, r-Cul o 3, r-Cul n 3, and r-Cul n 4, and sum r-Cul compared to horses without IBH, whereas horses with moderate IBH only had significantly higher IgE against r-Cul o 1p, r-Cul o 6, r-Cul n 9, and sum r-Cul compared to the C group. Horses with severe IBH did not have significantly higher IgE concentrations to any of the tested allergens than horses with moderate IBH.
FIGURE 2  Serum immunoglobulin E (IgE) concentrations (presented as optical density [at 405 nm (OD405 nm)] values) specific for recombinant Culicoides obsoletus (Cul o; A-D) or Culicoides nubeculosus (Cul n; E) allergens and the sum of Cul o–specific and Cul n–specific IgE (sum Cul; F) in the sera of horses affected with severe equine asthma (group EA), insect bite hypersensitivity (group IBH), or both EA and IBH (group IBH/EA) and in healthy control horses (group C). Each dot represents the value of a single horse, and the red lines representing the median. * indicates significant differences among the groups in the Kruskal-Wallis Multiple-Comparison Z-Value test (Dunn's test) with Bonferroni correction.
Abbreviations: IBH, insect bite hypersensitivity; IgE, immunoglobulin E; NS: nonsignificant; r Asp f, recombinant previous studies, which demonstrate that IBH-affected horses did not influence these IgE concentration, independently of a concomitant affection with EA. Contrarily, tested r-allergens, compared to horses affected with IBH only or with IBH and EA do not have higher IgE concentrations to any of the tested r-allergens, compared to horses affected with IBH only or with EA. The presence or absence of EA did not influence these IgE concentrations against r-Asp f 7 were extremely low in all groups; therefore, this finding is probably not biologically relevant (Supporting Information Figure S1A). The difference of our findings to these previous studies might be because of the different horse populations and possibly also environmental factors, that is, exposure to moldy hay. Furthermore, in a previous study, the horses were all living in the same stable, that is, environmental effects were more homogenous than in the present study. Although IgE concentrations to r-Asp f 7 were very low in all tested samples, a few horses in groups IBH (n = 2) and IBH/EA (n = 3) had high r-Asp f 8-specific IgE in their sera (OD$_{405} > 0.5$; Supporting Information Figure S1B). This could be explained by the development of IgE autoimmunity, as described in severe allergic diseases in human patients. The presence of autoimmune reactions against phylogenetically highly conserved intracellular proteins such as the ribosomal P2 protein have been described in severe atopic diseases and is the result of cross-reactivity. r-Asp f 8 is a ribosomal P2 protein from Aspergillus fumigatus. Human patients with severe allergic bronchopulmonary aspergillosis have high serum IgE concentrations and positive skin tests both to A. fumigatus and Dermatophagoides pteronyssinus; r Gly d, recombinant Glycophagus domesticus.

**4 | DISCUSSION**

The major findings of our study are that horses affected both with IBH and EA do not have higher IgE concentrations to any of the tested r-allergens, compared to horses affected with IBH only or with EA only, whereas horses with IBH had significantly higher IgE concentrations against r-Culicoides allergens than horses without this condition, independently of a concomitant affection with EA. Contrarily, the presence or absence of EA did not influence these IgE concentrations (Table 2, Supporting Information Table S2). Our findings confirm previous studies, which demonstrate that IBH-affected horses have increased IgE concentrations against r-Culicoides allergens. However, the impact of additional hypersensitivity disease on serum IgE concentrations in horses has so far not been investigated. In line with previous studies, IBH-affected horses display individual IgE-binding patterns to r-Culicoides allergens. For some allergens, only a few horses with IBH had increased serum IgE concentrations (eg, to r-Cul o 6 and r-Cul n 3), whereas for other allergens such as r-Cul o 1p, a majority had increased specific IgE concentrations. Horses in group EA, with the exception of 1 individual (Figure 2), were not sensitized to r Culicoides allergens. Conversely to earlier studies, we did not find increased IgE concentrations specific for the 2 r-Asp f allergens, r-Asp f 7 and r-Asp f 8, in the EA-affected group. Surprisingly, group IBH/EA had significantly lower r-Asp f 7-specific IgE than groups EA and C. We cannot explain these results, but the IgE concentrations against r-Asp f 7 were extremely low in all groups; therefore, this finding is probably not biologically relevant (Supporting Information Figure S1A). The difference of our findings to these previous studies might be because of the different horse populations and possibly also environmental factors, that is, exposure to moldy hay. Furthermore, in a previous study, the horses were all living in the same stable, that is, environmental effects were more homogenous than in the present study. Although IgE concentrations to r-Asp f 7 were very low in all tested samples, a few horses in groups IBH (n = 2) and IBH/EA (n = 3) had high r-Asp f 8-specific IgE in their sera (OD$_{405} > 0.5$; Supporting Information Figure S1B). This could be explained by the development of IgE autoimmunity, as described in severe allergic diseases in human patients. The presence of autoimmune reactions against phylogenetically highly conserved intracellular proteins such as the ribosomal P2 protein have been described in severe atopic diseases and is the result of cross-reactivity. r-Asp f 8 is a ribosomal P2 protein from A. fumigatus. Human patients with severe allergic bronchopulmonary aspergillosis have high serum IgE concentrations and positive skin tests both to A. fumigatus ribosomal protein P2 and to its human counterpart. As these cytoplasmic proteins are unlikely to be accessible for antigen-antibody interactions under normal circumstances, this leads to the hypothesis that IgE autoreactivity could be a consequence of tissue damage and release of autoantigens at the site of inflammation. Indeed, skin integrity is often markedly compromised in IBH, which would favor this pathophysiological mechanism.
Testing of novel, commercially available r-allergens from molds and mites not previously investigated in the horse did not indicate that they are relevant for EA or IBH. The limited number of potential allergens for EA tested is certainly a limitation of this exploratory part of our study. More efficient methods such as allergen chip arrays, which allow for testing hundreds of allergens simultaneously, should be used in the future for such purposes. To further test whether equine multiple hypersensitivity disorder was not associated with a higher IgE response, total serum IgE concentrations were then determined in the sera of EA/IBH-affected horses in comparison to IBH-affected horses and the corresponding healthy controls. Again, no significant differences between the EA/IBH and IBH groups were found. Total IgE concentrations were not determined in the sera from the horses derived from the 2 oldest studies because of these negative results and because previous studies had demonstrated that RAO-affected horses do not have significantly different total IgE concentrations than control horses. The high variation of IgE concentrations in the sera of the tested horses, independent of the clinical diagnosis, suggests that these horses were probably infected to varying degrees with endoparasites. Parasite infection increases total serum IgE concentrations in the horse as in other species. Unfortunately, no data on endoparasite infestation were available for the horses included in our study. And thus the data on total IgE should be interpreted with caution.

IBH severity can affect Culicoides-specific IgE reactivity. In the present study, horses suffering from severe IBH have, compared to healthy controls, significantly increased IgE concentrations to more r-Culicoides allergens (5 allergens) compared to horses with moderate IBH that only had significantly higher IgE to 3 r-Culicoides allergens. Nevertheless, no significant differences in allergen-specific IgE concentrations between horses with severe and moderate IBH were found (Table 3). This could be because the IBH score was based on the most severe clinical signs the horse had experienced in life and not on the IBH severity at the time of blood sampling. Furthermore, contrarily to the C group, where the variance of Culicoides r-allergens was low, the variance was much higher in the moderate and severe IBH groups (ie, in both IBH groups, horses with high IgE levels to Culicoides r-allergens were found). Given this large variation in the affected horses and the moderate numbers in these groups, insufficient power may have led to type II errors, that is, failure to detect differences between groups of moderate and severe IBH. Most IBH horses were protected from insect bites by blankets or use of repellents. This reduced exposure to the causative allergens likely resulted in a decrease of specific IgE concentrations. Interestingly, only the horses with moderate but not with severe IBH had significantly higher IgE to r-Cul o 6 and r-Cul n 9 compared to controls. This might indicate differences in the allergic skin response depending on the allergen to which an individual is sensitized. However, this finding would need to be confirmed in a larger set of horses. The allergens r-Cul o 6 and r-Cul n 9 both belong to the D7 protein family, expressed in salivary glands of Diptera. They have been shown to bind serotonin, histamine, and norepinephrine and thus antagonize platelet aggregation, vascular contraction, and pain. The fact that sera were drawn from 3 different studies, which was because of the availability of the IBH/EA group (a valuable collection of rare multiple hypersensitivity phenotypes), which had to be complemented by a severe EA group, entails several limitations and potential pitfalls. This includes potential confounders regarding case and group definitions, as well as bias through differences in allergen exposure between the groups that were compared. The potential confounder by differences in exposure is already discussed above, specifically regarding comparisons with studies using more homogeneous exposure conditions. However, the 3 studies from which samples were used were all performed in the same restricted geographical area of central and Western Switzerland within a 10-year period. Thus, relevant differences in allergen exposure between the populations used in our study are unlikely. Furthermore, although the serum samples originated from horses from different studies, the same questionnaire was consistently used in all horses, specifically the well-validated HOARSI classification as well as thorough clinical and complementary examinations. Importantly, assessment for occurrence of other hypersensitivity diseases such as IBH or recurrent urticaria was performed in all 3 studies (Supporting Information Table S1).

In conclusion, our study shows that r-Culicoides-specific serum IgE in IBH is not associated with concurrent severe EA. Thus, the clinically documented increased risk of IBH horses to develop severe EA does not seem to be influenced by a concomitant increase in total or allergen-specific IgE concentrations. Further studies using a larger panel of r-allergens, for example, by using an allergen chip array and larger numbers of horses to reduce type 2 errors are warranted.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.