

Longitudinal analysis of allergen-specific IgE and IgG subclasses as potential predictors of insect bite hypersensitivity following first exposure to *Culicoides* in Icelandic horses

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Background – Insect bite hypersensitivity (IBH) is an allergic dermatitis of horses caused by bites of *Culicoides* spp. IBH does not occur in Iceland because of the absence of *Culicoides*, but the prevalence is high in horses imported from Iceland to environments where *Culicoides* are present.

Hypothesis/Objective – Test, in a longitudinal study before and after *Culicoides* exposure, whether a primary sensitizing *Culicoides* allergen can be identified and if an increase of allergen-specific immunoglobulin (Ig)E or IgG subclasses precedes clinical signs of IBH.

Animals – Thirty two horses imported from Iceland to Europe; 16 developed IBH and 16 remained healthy.

Methods – Determination of IgE and IgG subclasses against recombinant (r)-*Culicoides* allergens and *Culicoides* extract in sera taken before first exposure to *Culicoides* and yearly over a period of 3–4 years.

Results – Before *Culicoides* exposure, there were no significant differences in *Culicoides*-specific serum IgE levels between horse that developed IBH or remained healthy. *Culicoides* exposure induced an individual IgE response pattern (to a median of 4.5 r-allergens) in the IBH but not in the healthy end-point group. The increase in serum IgE levels to *Culicoides* r-allergens was concurrent with the initial onset of clinical signs of IBH. IBH-affected horses displayed significantly higher allergen-specific IgG1 and IgG5 levels than healthy controls. Recombinant *Culicoides obsoletus* 1 (Cul o1) and Cul o3-specific IgG5 was significantly higher in the IBH compared to the healthy end-point group, before clinical signs of IBH.

Conclusion/Clinical relevance – Allergen-specific serum IgE cannot be used as predictor for IBH, whereas allergen-specific IgG5 levels may have a predictive value.

Introduction

Insect bite hypersensitivity (IBH) is an allergic, pruritic dermatitis of horses caused mainly by immunoglobulin (Ig)E-mediated reactions to bites of insects of the genus *Culicoides* (midges), affecting 5–50% of horses, depending on the horse breed, family and environment.¹ During summer, when the insects are present in the

environment, horses with IBH suffer from severe pruritic dermatosis affecting the mane and the tail area, and sometimes also the ventral midline and the face. Absence of the insects in winter leads to temporary remission. The causative allergens for IBH, salivary gland proteins from *Culicoides*, have been characterized at the molecular level and produced as recombinant proteins.^{2–6} These recombinant allergens (r-allergens) bind serum IgE from IBH-affected animals, but only rarely from healthy control horses.^{3–7} Affected horses have been shown to react to antigens of many different indigenous, as well as nonindigenous *Culicoides* spp. in intradermal tests,^{8,9} therefore suggesting shared common allergens between species.¹⁰ In mainland Europe, midges from the *Culicoides obsoletus* (*C. obsoletus*) complex are the most common species;¹¹ however, IBH-affected horses have serum IgE antibodies binding to various salivary gland proteins from laboratory-bred *C. nubeculosus*.^{3,4,12}

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IBH, insect bite hypersensitivity; OD, optical density; REU, relative ELISA units.

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Nevertheless, studies suggest that allergens derived from *C. obsoletus*, the most frequent *Culicoides* spp. found in the environment of horses, are more immune-reactive for IBH than those from laboratory-bred, non-indigenous species.^{5,6} However, not all allergens from *C. obsoletus* corresponding to previously described *C. nubeculosus* allergens have been identified.

Insect bite hypersensitivity does not occur in horses born and living in Iceland due to the lack of antigen exposure because *Culicoides* spp. are absent. After importation of horses born in Iceland to continental Europe, where *Culicoides* spp. are present, $\geq 50\%$ of the imported horses develop IBH when exposed to *Culicoides*. Conversely, the prevalence of IBH in Icelandic horses born in Europe, and thus exposed to *Culicoides* already as foals, is at the same level as in other breeds (3–10%).¹ Even though first exposure to *Culicoides* spp. at an adult age was found to be the main risk factor for developing IBH in exported Icelandic horses,¹³ the exact reasons for the high prevalence of IBH in these horses are still subject to speculation. Although previous studies had demonstrated that IBH-affected horses of the Icelandic breed often have high IgE levels against one or more recombinant *C. nubeculosus* allergens,⁴ the time point and possible predictive value of the initial increase in allergen-specific serum IgE are unknown.

The aims of the study were to determine the IgE response pattern of horses imported from Iceland to Europe in a longitudinal study and to test whether – out of a panel of recombinant allergens – a first sensitizing allergen can be identified. Then, in the next part of the study, allergens that seemed to be the most relevant were selected and the IgG subclass response measured in order to determine which IgG subclasses are associated with IBH and whether allergen-specific IgE or IgG antibodies can possibly be used as predictor for the development of IBH.

Material and methods

Horses

Thirty two horses with a well-defined clinical diagnosis regarding occurrence of IBH (16 horses with and 16 horses without IBH) were selected out of a group of Icelandic horses born in Iceland and imported to Europe and monitored over a period of 3–5 years following import.¹⁴ The mean age at time of import of the 32 horses was 7.7 years (range 6–11 years). At time of import the horses had no signs of pruritus. Following importation, the horses (20 geldings, 12 mares) lived in different stables throughout Switzerland, where *Culicoides* were known to be present, based on the presence of at least one IBH-affected individual in each of the stables. The horses were examined clinically over a period of four to five years, at the time of import and then every year in autumn, at the end of the *Culicoides* season. The clinical history was assessed; to be assigned to the IBH-affected group the typical clinical signs of IBH had to recur the following summer, after a remission period in winter. After clinical signs became apparent, nearly all affected horses were treated by insect avoidance through covering with blankets and stabling, as well as local application of various lotions for insect and pruritus control. The horses assigned to the healthy outcome (H) group had no history of skin problems during the four year period of observation and no preventive measures such as blankets in summer were taken.

The power of the study to detect differences in mean antibody levels between horses in the IBH and H group was evaluated with the software WinEpiscope 2.0 (EpiDecon, Wageningen University,

NL). The available sample size of 16 horses per group was sufficient to detect a difference between means of 1.1 times the standard deviation with a power of 80%, and a significance level of 5%.

Blood samples

Blood samples were obtained before first exposure to *Culicoides* allergens and after exposure at the end of each subsequent *Culicoides* season. Blood was collected from the jugular vein using Serum Clot Activator-containing vacutainers (Vacuette®; Greiner BioOne, St. Gallen, Switzerland). Serum was separated and stored at -80°C until used. The study was approved by the Animal Experimental Committee of the Canton of Berne, Switzerland (No. BE 121/05 and BE 51/13).

Allergens and antigens

Ten recombinant *C. nubeculosus* salivary gland allergens (rCul n 1–rCul n 10)^{3,4} and three recombinant *C. obsoletus* salivary gland allergens rCul o 1,⁵ rCul o 2⁵ and rCul o 3,⁶ were used in the study. They were all expressed in *Escherichia coli* and purified, as described previously.⁴

Additionally, rCul o 3 was expressed in insect cells (Bac-rCul o 3) and used for comparison. Briefly, rCul o 3 was expressed in insect cells with the Bac-to-Bac Baculovirus expression system (Invitrogen, Waltham, MA, USA, <https://www.thermofisher.com>) as a SUMOstar fusion protein (Life Sensors, Malvern, PA, USA, <https://www.lifesensors.com>) in accordance with manufacturers' protocol. The baculovirus was amplified in Sf-9 insect cells and the protein expressed in High five insect cells. The protein was purified under denaturing conditions with the HIS-select™ nickel affinity gel (Sigma, St. Louis, MO, USA, <https://www.sigmaaldrich.com>) according to protocol and dialysed against 4 M urea. After dialysis, Bac-rCul o 3 was analysed with SDS-PAGE and the gel stained with Coomassie blue, (PhastGel Blue R, Sigma) using standard protocols (Figure S1) or transferred to a PVDF membrane (Millipore, Hellerup, Denmark, <http://www.merckmillipore.com>) and detected with mouse anti-His antibody (Bio-Rad, Hercules, CA, USA, <https://www.bio-rad-antibodies.com>), diluted 1:1,000, followed by the standard procedure of Western blot, as described previously.¹⁵

Thorax extract from *C. nubeculosus* (NubTE) and whole body extract from *C. obsoletus* complex midges (ObsWBE) were prepared as described previously.⁵

Equine Influenza A Fontainebleau 79 inactivated virus (APHA, Weybridge, Surrey, UK; EURL Influenzavirus), was used as a control antigen in the IgG-subclasses enzyme-linked immunosorbent assay (ELISA), because Icelandic horses are not vaccinated against equine Influenza in Iceland, whereas they are vaccinated following import into Switzerland.

IgE and IgG subclasses enzyme-linked immunosorbent assay

Allergen-specific serum IgE levels against the ten recombinant *C. nubeculosus* allergens and NubTE, as well as against the three *C. obsoletus* r-allergens and ObsWBE were determined by indirect ELISA. Levels of serum IgG subclasses against three of the most relevant rCulicoides allergen, rCul n 4, rCul o 1, rCul o 3, and NubTE and equine Influenza, were determined by ELISA, as described previously.¹⁵ Briefly, microtitre plates were coated with 2 µg/mL allergens, followed by blocking with blocking buffer (Phosphate buffered saline containing 5% dry milk powder and 5% Tween-20). All dilutions of serum samples and antibodies were made in blocking buffer. Following washing, test serum samples were added in duplicates to the plates at a dilution of 1:5 for IgE and 1:100 for IgG subclasses and incubated at 4°C overnight. After washing, monoclonal antibodies specific for equine IgE (1 µg/mL, clone 3H10)¹⁶ or for equine IgG1 (1 µg/mL, CVS45), IgG1/3 (1 µg/mL clone 159),¹⁷ IgG4/7 (1 µg/mL; CVS 39) and IgG5 (1 µg/mL; clone 416)¹⁷ were added and incubated for 2 h at room temperature on a shaker. After washing, alkaline phosphatase conjugated, affinity purified goat anti-mouse IgG (Jackson Immuno Research; Newmarket, UK) was added and the plates developed

using phosphatase substrate (Sigma) in 10% diethanolamine (Sigma), pH 9.8. Absorbance was measured at 405 nm after 2 h for IgE and 30 min for the IgG subclasses.

Optical density (OD) values of the IgE ELISAs were converted to relative ELISA units (REU). For this purpose, a horse serum with a high allergen-specific IgE level was used as a reference serum and assigned a value of 100 REU. Serial dilutions of the reference serum were used to generate a standard curve. The REU values of the test serum samples were calculated from the standard curve using an ELISA software programme (Softmax PRO® 2.31 for Windows, Molecular Devices Co.; Sunnyvale, CA, USA). Due to the very individual reaction patterns of the horses to the single recombinant allergens, the sum of IgE levels against the ten rCul n [SumIgE (Cul n)] allergens and the three rCul o [SumIgE (Cul o)] allergens, respectively, was calculated for each horse and was used for analysis (Figure 1).

In order to further analyse IgE reactivity to the single allergens, an arbitrary cut-off value was used to distinguish between positive and negative reactions in the allergen-specific IgE ELISA. The cut-off was defined as the mean plus two standard deviation values of the ELISA values from all horses at time T0, before exposure to *Culicoides*.

Enzyme-linked immunosorbent assay results of the IgG subclasses were shown as OD₄₀₅ values after a blank correction on the optical density and correction between plates, as described previously.⁵

Statistical analyses

Statistical analyses were carried out using the software programme NCSS 10 (NCSS; Kaysville, UT, USA). Descriptive statistics were run on the IgE and IgG data, and Shapiro–Wilk W test showed that the data were not normally distributed.

In order to assess factors influencing the antibody response, data were analysed by function of the clinical end-point, namely horses that remained healthy until the fourth summer after import (H group) or developed IBH at any time point within this time period (IBH group), and by function of time. Time point T0 corresponded to the first blood sample taken before exposure to *Culicoides* midges. T1, T2 and T3 represented the times of blood withdrawals and clinical examination at the end of each subsequent summer, following exposure to *Culicoides*. In this analysis, the horses were grouped according to the clinical end-point only and not to the diagnosis at the actual time point.

In order to assess effects of the clinical end-point (IBH or H) in function of time (summer after import, T0–T3) and potential interactions thereof on allergen-specific IgE and IgG subclasses, a repeated measures ANOVA was performed (Table 1). The Shapiro–Wilk W test showed that after log-transformation of the data, there was no severe violation of normality (Shapiro–Wilk $W \geq 0.96$) for the Sum (IgECul n) and Sum (IgECul o) and for IgG1, IgG1/3, IgG4/7, IgG5 and IgE antibody levels to rCul n 4, NubTE, rCul o 1, rCul o 3, Bac-Cul o 3 and influenza, whereas the distribution of the remaining antibody levels remained non-normal after log transformation and repeated measures ANOVA could thus not be performed. Separate models were run for each antigen, with levels of IgG1, IgG1/3, IgG4/7, IgG5 and IgE as outcome variables, respectively. Interaction of time (=summer after import) and clinical end-point was included in the models. Mauchly's test was used to check for the assumption of circularity of within-subject covariance matrices. Because this assumption was not always fulfilled, the Geisser-Greenhouse correction was used for the within-subject F test. For significant factors in any of the repeated measures ANOVA models, significant subgroup differences were identified correcting for multiple testing with the Tukey–Kramer method (Table 1). Fisher's exact test was used to compare the proportion of end-point IBH and end-point H horses with IgE levels above cut-off (=positive) to the single allergens at T0, T1, T2 and T3 (Table S1).

In order to further investigate IgE reactivity to the various *Culicoides* allergens at the time of first clinical signs of IBH, serum antibody levels were compared between end-point IBH horses at the time of onset of clinical signs (T_{IBH}) and end-point H horses matched for the corresponding time points. To investigate changes in

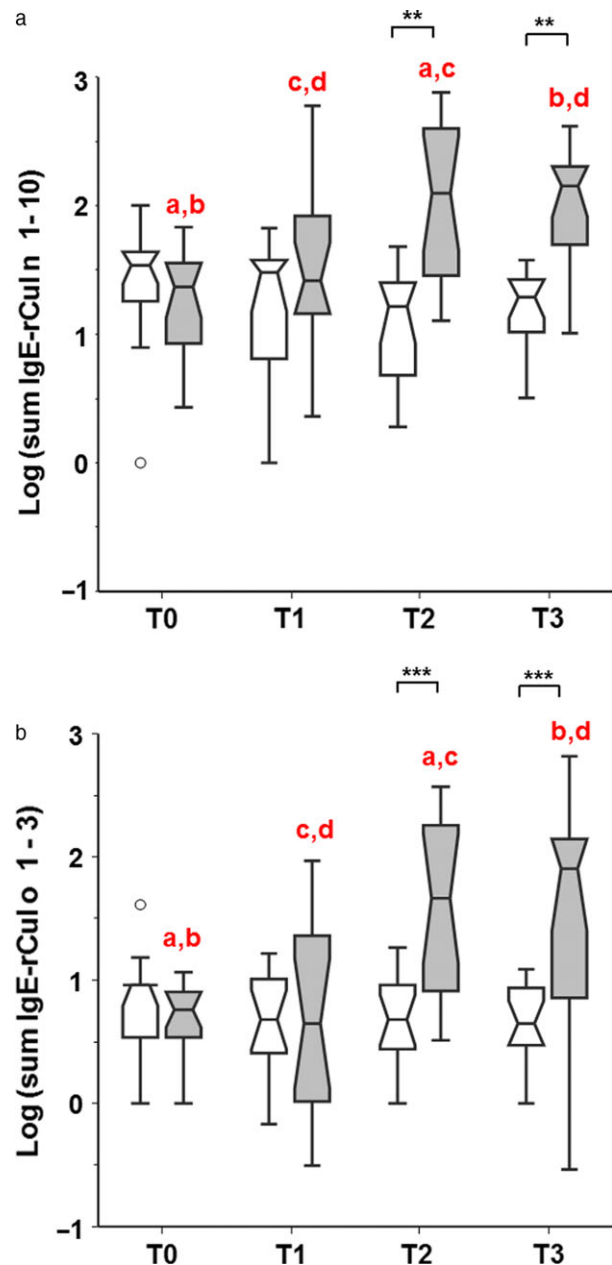


Figure 1. Comparison of the sum of allergen-specific serum immunoglobulin (IgE) levels against rCul n 1–10 (a) and rCul o 1–3 (b), respectively, between the end-point insect bite hypersensitivity (IBH) (shaded box) and the end-point healthy (H) (white box) horse groups at time points T0–T3.

Results are shown as log of sum IgE Cul n 1–10 (a) and log sum IgE Cul o 1–3 (b) in Relative ELISA Units (REU). T0–T3 correspond to the yearly blood samples, with T0 representing the blood sample collected before exposure to *Culicoides* and T1–T3 representing the blood samples collected at the end of each subsequent summer. Within the horse group that developed IBH during the follow-up study (=end-point IBH group) the cumulative percentages of horses with IBH at each time point were 0% at T0, 25% at T1, 69% at T2 and 88% at T3. The last two horses (12%) developed IBH at T4. IgE levels are not shown for this time point because too many horses had to be withdrawn from the study at T4 because of treatment with corticosteroids. A repeated measures ANOVA was performed. Asterisks indicate significant differences between the groups (** $P \leq 0.01$; *** $P \leq 0.0001$). IgE levels at time points with the same superscript letter are significantly different ($P \leq 0.05$) within each horse group. rCul n, recombinant *Culicoides nubeculosus* antigen; rCul o, recombinant *Culicoides obsoletus* antigen.

Table 1. Influence of the end-point (H remained healthy, IBH developed insect bite hypersensitivity) in relation to number of summers after import from Iceland to Europe on antigen-specific immunoglobulin IgG and IgE levels

Allergens	Time	Log IgG1			Log IgG1/3			Log IgG4/7			Log IgG5			Log IgE		
		IBH	H	P	IBH	H	P	IBH	H	P	IBH	H	P	IBH	H	P
rCul o 1	T0	3.12 (0.17)	2.84^a (0.09)	≤0.0001	3.04 (0.28)	2.66^{a,b} (0.14)	≤0.001	3.25 (0.15)	3.28 (0.12)		2.85 (0.39)	2.38 (0.46)	≤0.001	1.86 ^a (0.25)	2.03 (0.34)	
	T1	3.09 (0.26)	2.99 (0.17)		2.99 (0.36)	2.92 ^a (0.21)		3.22 (0.23)	3.30 (0.19)		3.10 (0.38)	2.66 (0.60)	≤0.01	1.87 ^b (0.54)	1.90 (0.31)	
	T2	3.12 (0.23)	2.96 (0.20)		3.04 (0.33)	2.86 (0.41)		3.31 (0.17)	3.29 (0.18)		3.12 (0.22)	2.54 (0.57)	≤0.00001	2.53^{a,b} (0.67)	1.85 (0.41)	≤0.00001
	T3	3.09 (0.18)	3.05 ^a (0.23)		3.03 (0.28)	2.99 ^b (0.40)		3.26 (0.19)	3.34 (0.14)		3.12 (0.26)	2.50 (0.73)	≤0.00001	2.57^{a,b} (0.70)	1.77 (0.41)	≤0.00001
rCul o 3	T0	2.19 ^a (0.38)	1.89 (0.57)		0.78 ^{a,b} (0.77)	0.73 (0.64)		2.73	2.62		2.56 (0.47)	2.09 (0.26)	≤0.001	1.17 ^{a,b} (0.55)	1.13 (0.47)	
								2.38–2.89	2.53–2.67							
	T1	2.36 (0.58)	1.87 (0.71)		1.23 (1.00)	0.89 (0.91)		2.77 2.44–2.97	2.69 2.28–2.91		2.78 (0.34)	2.38 (0.33)	≤0.01	1.62 ^a (0.83)	1.12 (0.66)	
	T2	2.76^a (0.47)	1.61 (0.91)	≤0.00001	1.63 ^a (1.00)	0.86 (0.93)	≤0.05	2.90 2.56–3.38	2.57 1.92–2.93		2.82 (0.37)	2.11 (0.42)	≤0.00001	2.14^a (0.92)	1.10 (0.71)	≤0.0001
Bac-rCul o 3	T0	2.54 (0.67)	1.76 (0.81)	≤0.0001	1.54 ^b (0.88)	0.95 (0.82)		3.06 2.85–3.20	2.68 2.20–2.97		2.85 (0.25)	2.29 (0.41)	≤0.00001	2.29^{b,c} (0.71)	0.96 (0.71)	≤0.00001
	T1	3.16 (0.31)	3.03 (0.23)		3.34 (0.13)	3.27 (0.11)		3.41 (0.12)	3.37 (0.08)		2.45^{a,b,c} (0.54)	1.95 (0.59)	≤0.01	1.60 ^{a,b} (0.75)	1.60 (0.61)	
	T2	3.17 (0.39)	2.95 (0.32)		3.30 (0.23)	3.17 (0.21)		3.37 (0.22)	3.30 (0.20)		2.84^a (0.40)	2.30 (0.52)	≤0.001	1.96 ^a (0.78)	1.43 (0.67)	
	T3	3.35 (0.28)	2.91 (0.47)	≤0.00001	3.41 (0.14)	3.13 (0.32)	≤0.00001	3.47 (0.15)	3.23 (0.31)	≤0.0001	2.96^b (0.31)	2.24 (0.44)	≤0.00001	2.60^a (0.85)	1.30 (0.85)	≤0.00001
rCul n 4	T0	3.31 (0.30)	3.01 (0.45)	≤0.01	3.39 (0.17)	3.23 (0.24)		3.44 (0.17)	3.31 (0.24)		3.04^a (0.21)	2.47 (0.38)	≤0.0001	2.57^{b,c} (0.71)	1.57 (0.52)	≤0.0001
	T1	2.75 ^a (0.21)	2.78 (0.10)		3.14 ^a	3.11		3.25 (0.17)	3.39^a (0.09)	≤0.01	2.87 (0.28)	2.66 (0.25)		1.68 ^{a,b} (0.42)	1.92 (0.23)	
	T2	2.81 (0.25)	2.73 (0.22)		3.17	3.14		3.22 ^a (0.21)	3.25 ^a (0.21)		2.87 (0.35)	2.73 (0.38)		1.87 ^{a,d} (0.79)	1.92 (0.35)	
	T3	2.94^a (0.17)	2.75 (0.15)	≤0.05	3.39 ^a	3.22		3.35 ^{a,b} (0.17)	3.31 (0.16)		3.04 (0.31)	2.76 (0.43)	≤0.05	2.52^{a,e} (0.67)	1.77 (0.32)	≤0.00001
Nub TE	T0	3.10 ^a (0.20)	3.00 (0.13)		2.99 (0.22)	2.79 (0.18)	≤0.05	3.11 (0.16)	3.25^{a,b,c} (0.10)	≤0.001	2.76 (0.31)	2.61 (0.34)		1.88 ^{a,b} (0.39)	2.10	
	T1	3.17 (0.23)	3.04 (0.15)		3.06 (0.29)	2.84 (0.23)	≤0.01	3.10 (0.13)	3.15 ^a (0.16)		2.85 (0.26)	2.64 (0.35)		1.82 ^{a,d} (0.78)	1.93	
	T2	3.30^a (0.17)	3.02 (0.19)	≤0.00001	3.17 (0.23)	2.89 (0.28)	≤0.001	3.17 (0.13)	3.15 ^b (0.14)		2.88 (0.24)	2.61 (0.32)	≤0.01	2.36^{a,e} (0.57)	1.85	≤0.05
	T3	3.21 (0.17)	3.05 (0.18)	≤0.05	3.12 (0.16)	2.88 (0.28)	≤0.01	3.12 (0.16)	3.14 ^a (0.15)		2.87 (0.19)	2.68 (0.32)		2.54^{b,d} (0.46)	2.04	≤0.05
Influenza	T0	2.42 ^{a,b} (0.54)	1.92 ^{a,b,c} (0.61)	≤0.05	2.31 ^a (0.65)	2.10 ^a (0.23)		2.76 ^{a,b,c} (0.37)	2.66 ^{a,b,c} (0.24)		1.62 ^a (0.72)	1.40 (0.84)		1.20 (0.62)	1.32 (0.54)	
	T1	2.87 ^a (0.42)	2.50 ^a (0.53)		2.76 (0.54)	2.43 (0.67)		3.07 ^a (0.34)	2.98 ^a (0.35)		1.92 (1.13)	1.64 (1.27)		1.49 (0.60)	0.93 (0.68)	
	T2	2.83 (0.46)	2.70 ^b (0.50)		2.70 (0.54)	2.59 (0.70)		3.11 ^b (0.30)	3.12 ^b (0.36)		1.86 (0.98)	1.60 (1.40)		1.26 (0.77)	0.82 (0.82)	
	T3	3.06 ^a (0.31)	2.65 ^a (0.87)		2.95 ^a (0.36)	2.63 ^a (0.84)		3.26 ^a (0.36)	3.14 ^a (0.34)		2.60 ^a (0.64)	1.87 (1.09)		1.29 (0.62)	0.80 (0.67)	

Time points T0–T3 correspond to the yearly blood samples, with T0 representing the blood sample collected before exposure to *Culicoides* and T1–T3 representing the blood samples collected at the end of each subsequent summer. Mean OD₄₀₅ values (standard deviations) and P-values of repeated measures ANOVAs are depicted. Significant differences between the time points within the end-point IBH or H groups are indicated with the same superscript letter. P indicates significant differences at the same time point between IBH and H control horses. Values in italics were non-normally distributed (Shapiro-Wilk test) and therefore a nonparametric Wilcoxon–Mann–Whitney U-test with Bonferroni correction for multiple comparisons was performed. Bold values are significantly different between the groups. rCul o, recombinant *Culicoides obsoletus* antigen; Bac, insect cells with the Bac-to-Bac Baculovirus expression system; Bac rCul o 3, rCul o 3 expressed in insect cells using the Bac-to-Bac expression system; rCul n, recombinant *Culicoides nubeculosus* antigen; Nub TE, Thorax extract from *C. nubeculosus*; Influenza, Equine Influenza A Fontainebleau 79 inactivated virus antigen.

sensitization in relation to onset of clinical signs, results of the IBH-affected horses one year before ($T_{IBH}-1$) and one year after clinical onset ($T_{IBH}+1$) were compared. The nonparametric Wilcoxon signed rank paired t-test was used to compare the sum of positive (i.e. above cut-off) IgE reactions of each individual horse against the *Culicoides* r-allergens (rCul n 2–n 10, rCul o 1–o 3) in the IBH group between the time points T_{IBH} , $T_{IBH}-1$ and $T_{IBH}+1$, respectively. To avoid duplication, rCul n 1 was omitted for this analysis as it is the homologous allergen of rCul o 3. Both are antigen-5 like proteins and have an amino acid identity of 70%. The number of positive reactions at the three time points was also compared with the H control horses (Figure 2).

A Fisher's exact test was used to compare the number of IBH-affected and control horses with positive allergen-specific IgE results. This was performed for each of the single allergens in the 16 IBH-affected horses at T_{IBH} and the H control horses (Figure 3). A nonparametric Wilcoxon–Mann–Whitney U two sample t-test was performed to compare median IgE (Table S2) and median IgG subclasses (Table 2) levels against individual *Culicoides* r-allergens between IBH-affected horses at T_{IBH} and H horses. The level of significance was set at $P \leq 0.05$.

Results

Incidence of clinical signs of IBH

In the end-point IBH group ($n = 16$), the median number of summers between importation and onset of clinical signs of IBH was two summers (range 1–4 summers). Four of the 16 end-point IBH horses developed clinical signs during the first summer after import, nearly half of the horses (seven of 16) after the second summer, three after the third and the remaining two horses after the fourth summer following import. Accordingly, within the IBH end-point group the cumulative frequencies of horses with IBH at each time point were 0% at T0, 25% at T1, 69% at T2, 88% at T3 and 100% at T4.

Time course of allergen-specific IgE levels in relation to clinical end-point

Figure 1 shows that before first exposure to *Culicoides* spp. (T0), IgE levels to the *Culicoides* allergens were low and there were no differences in the Sum IgE(rCul n 1–10) or Sum IgE(rCul o 1–3) between the IBH and H groups. This was also true for the single allergens (Table S1). After the first summer (T1) there was no significant increase in IgE in either group. It was after the second summer (T2), when 11 out of the 16 IBH horses had developed clinical signs of IBH, that a significant increase was observed in the IBH but not in the H group (Figure 1). Accordingly, there was a highly significant difference between the H and the IBH end-point group at T2 and T3. Within the IBH group, the significant increase in Sum IgE(Cul n) and Sum IgE(Cul o) was associated with the prevalence of clinical IBH at the different time points (Figure 1). This was also reflected in the results for the single allergens (Table S1). After the second summer, significant differences between both groups were seen for seven of the 13 tested r-allergens and the two extracts (Table S1).

Allergens sensitization in relation to clinical signs

At $T_{IBH}-1$, one summer before occurrence of clinical signs, the IBH group had IgE above cut-off to a median number of one allergen (Figure 2). This was not significantly different from the control group. There was a significant increase between $T_{IBH}-1$ and T_{IBH} (i.e. the summer of onset of clinical signs), with IgE levels above cut-off to a median number of 4.5 allergens in the IBH group. This also differed significantly from the control group. At T_{IBH} , 12 of 16 IBH-affected horses exhibited

positive IgE responses against two or more r-allergens. Only one IBH horse did not show any positive reactions to the tested r-allergens. The following summer ($T_{IBH}+1$), nine of the 11 IBH horses that were assessed reacted against an equal or higher number of recombinant allergens than at T_{IBH} . In contrast, only four healthy horses of 16 showed an IgE reaction towards one recombinant allergen.

In order to determine which *Culicoides* allergens were of highest relevance at onset of the disease, the results at T_{IBH} are presented for each single *Culicoides* allergen (percentage of positive reactions in Figure 3 and median values in Table S2). Nine of the ten r-Cul n allergens and NubTE bound allergen-specific IgE in at least three IBH horses (Figure 3a). In contrast, a positive IgE response could be observed only for one H horse each for the allergens rCul n 2 and rCul n 6. For the allergens rCul n 4, n 5, n 8 and NubTE, the differences between the IBH and H groups were statistically significant. Only rCul n 4 and rCul n 8 bound IgE from $\geq 50\%$ of the tested horses and thus seemed to be major allergens for IBH (Figure 3a). All of the tested *C. obsoletus* allergens bound IgE in the sera from $\geq 50\%$ of the IBH horses (Figure 3b).

Time course of IgG subclasses responses

In order to assess whether the *Culicoides*-specific IgG subclass antibody response following import from Iceland to Europe differed between horses that developed IBH or remained healthy, a repeated measures ANOVA was performed. This was performed for the IgG subclasses, IgG1, IgG1/3, IgG4/7 and IgG5, as well as for IgE as comparison (Table 1 and illustrated for rCul o 3 in Figure 4).

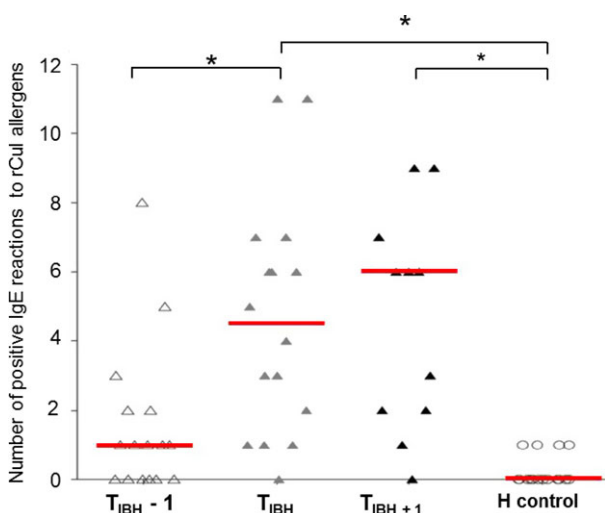


Figure 2. Cumulative number of positive serum immunoglobulin (Ig) E levels per horse for the recombinant allergens rCul n 2–10 and rCul o 1–3 taken together, at time of clinical onset of IBH (T_{IBH}), one year before ($T_{IBH} - 1$) and one year after ($T_{IBH} + 1$) and in healthy control horses.

Each symbol represents a separate horse and red lines represent the medians. A nonparametric paired-sample Wilcoxon signed rank test was used to compare between the time points or with the matched healthy control horses. Asterisks indicate significant differences between the time points ($P \leq 0.01$). rCul n, recombinant *Culicoides nubeculosus* antigen; rCul o, recombinant *Culicoides obsoletus* antigen.

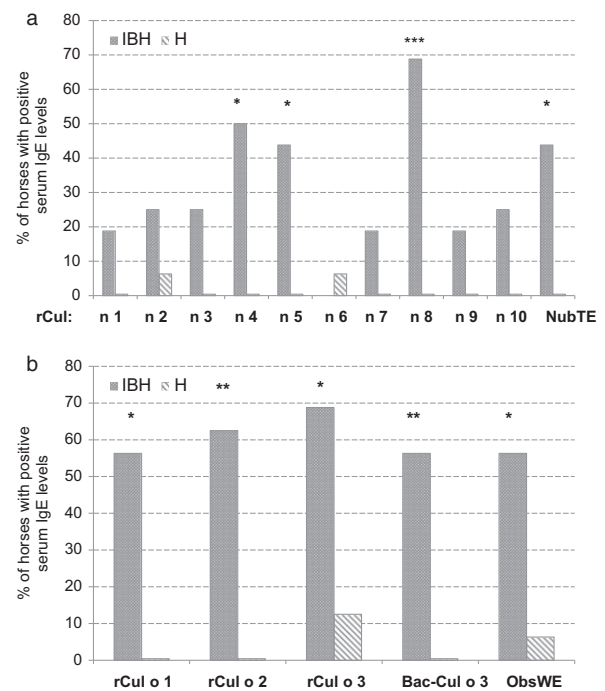


Figure 3. Percentage of horses with serum immunoglobulin (Ig) E levels above cut-off value binding rCul n 1–10 and NubTE (a) and rCul o 1–3, bac-Cul o 3 and ObsWBE (b).

Dotted bars represent results from the insect bite hypersensitivity IBH-affected horses at time of onset of clinical signs (T_{IBH}), whereas striped bars represent healthy control horses at time points matched to the IBH group. Asterisks indicate a significant difference between the “endpoint IBH” and “endpoint H” group in the Fisher’s exact test. * $P \leq 0.01$. ** $P \leq 0.001$. *** $P \leq 0.0001$. rCul n, recombinant *Culicoides nubeculosus* antigen; Nub TE, thorax extract from *C. nubeculosus*; rCul o, recombinant *Culicoides obsoletus* antigen; Bac-rCul o 3, rCul o 3 expressed in insect cells; ObsWBE, whole body extract from *C. obsoletus* complex midges.

For the IgG subclasses against the *C. nubeculosus* allergens (Table 1), we found only few significant differences between the horse groups. At T2, IgG1 as well as IgG5 levels were significantly higher in the IBH group than in the controls, both for rCul n 4 and NubTE and there was a significant increase in IgG1 between T0 and T2 in the IBH-group for both rCul n 4 and NubTE. Furthermore, horses from the IBH group had significantly higher IgG1/3 against NubTE than the controls at all time points. Surprisingly, IgG4/7 levels were significantly higher in the controls at T0, both for rCul n 4 and NubTE.

The most striking differences for IgG subclasses were found with the rCul o allergens (Table 1). The end-point IBH horses had significantly higher IgG5 levels against rCul o 1 and rCul o 3 (Figure 4) than the control horses at all time points and, surprisingly, already at T0. Significant differences between both groups of horses were also found for IgG1 and IgG1/3. Higher antibody levels were always observed in the IBH group compared to the controls, but the time points when these differences reached significance varied. As expected, there was a significant increase in IgE levels for each of the tested single allergens only in the IBH but not in the H end-point group. Significant differences between both horse groups were observed at T2 and T3.

Table 2. Allergen-specific immunoglobulin (Ig)G subclass levels at time of onset of clinical signs of insect bite hypersensitivity (IBH) in the end-point IBH group and in time point-matched healthy (H) controls

Allergen	Subclass	IBH	H	P-value
rCul n 4	IgG1	2.98 (2.57–3.37)	2.75 (2.48–2.98)	≤0.01
	IgG1/3	3.35 (2.97–3.61)	3.1 (2.84–3.47)	≤0.05
	IgG4/7	3.28 (2.96–3.56)	3.33 (2.91–3.57)	ns
	IgG5	3.07 (2.29–3.54)	2.9 (1.9–3.55)	ns
rCul o 1	IgG1	3.16 (2.86–3.49)	2.99 (2.56–3.27)	≤0.05
	IgG1/3	3.09 (2.31–3.53)	2.98 (1.92–3.42)	ns
	IgG4/7	3.35 (2.84–3.53)	3.31 (2.8–3.54)	ns
	IgG5	3.2 (2.38–3.53)	2.52 (1.19–3.53)	≤0.01
rCul o 3	IgG1	2.88 (1.7–3.43)	1.84 (0–2.93)	≤0.001
	IgG1/3	2.03 (0–2.94)	1.14 (0–2.4)	ns
	IgG4/7	2.89 (1.91–3.48)	2.55 (0–3.18)	ns
	IgG5	3.04 (2.06–3.31)	2.16 (1.55–3.46)	≤0.001
NubTE	IgG1	3.34 (2.75–3.55)	3.13 (2.55–3.27)	≤0.01
	IgG1/3	3.22 (2.51–3.44)	3.02 (2.16–3.23)	≤0.01
	IgG4/7	3.19 (2.81–3.4)	3.2 (2.89–3.35)	ns
	IgG5	2.87 (2.53–3.32)	2.66 (1.97–3.31)	≤0.05
Influenza	IgG1	3.08 (2.15–3.53)	2.76 (1.81–3.54)	ns
	IgG1/3	2.95 (1.98–3.65)	2.71 (1.37–3.56)	ns
	IgG4/7	3.34 (2.57–3.57)	3.21 (2.64–3.54)	ns
	IgG5	2.17 (0–3.51)	1.75 (0–3.52)	ns

The median (range) log(OD₄₀₆) values are depicted. P-values ≤ 0.05 in the nonparametric Wilcoxon–Mann–Whitney U two sample t-test indicate significant differences between the IBH (n = 16) and H (n = 16) groups.

rCul n, recombinant *Culicoides nubeculosus* antigen; rCul o, recombinant *Culicoides obsoletus* antigen; Nub TE, Thorax extract from *C. nubeculosus*; Influenza, Equine Influenza A Fontainebleau 79 inactivated virus antigen.

Finally, the same analysis was performed for IgE and IgG subclasses against equine influenza as control. No significant differences were observed between the groups for any antibody class at any time point, except for higher IgG1 at T0 in the IBH group compared to the control group. In contrast to the results obtained with the *Culicoides* allergens, there was no increase in IgE but a significant increase in IgG4/7 in both horse groups (Table 1). Furthermore, significant increases in IgG1 and IgG1/3 levels were observed in both groups, whereas the increase in IgG5 only reached significance in the IBH-group.

Allergen-specific IgG subclasses in horses with clinical signs of IBH and H control horses

In order to further analyse differences in IgG subclass levels between healthy and IBH-affected horses, serum IgG subclass levels were compared between end-point IBH horses at time of onset of clinical signs (T_{IBH}) and H control horses matched for time since import (Table 2). There was a significant difference between IBH-affected and nonaffected control horses in IgG1 levels against all tested allergens (rCul n 4, rCul o 1, rCul o 3, NubTE). Furthermore, IgG5 levels against rCul o 1, rCul o 3 and NubTE, but not rCul n 4, were significantly higher in the IBH-affected compared to the H control horses. No significant difference between the groups was found for IgG4/7. As expected, no significant differences in IgG subclass levels against equine influenza (used as a control) were found between the IBH and the H control group.

Discussion

Horses imported from Iceland, where *Culicoides* are absent, to *Culicoides*-rich environments, represent a unique model population to study the natural course of sensitization to new environmental allergens, because of the known time point of first *Culicoides* exposure and because of the high incidence of the disease in these horses. Previously, such studies were limited due to the lack of pure *Culicoides* allergens.¹² The expression of highly purified *Culicoides* r-allergens^{2–6} has opened up new possibilities to investigate the natural sensitization process in this horse population.

At the time of import, before first exposure to *Culicoides*, allergen-specific IgE levels were absent or very low in the sera of the Icelandic horses. This is to be expected and confirms previous studies showing a low reactivity in the intradermal test by Icelandic horses living in Iceland.¹⁸ Before exposure to *Culicoides*, there were no differences in allergen-specific IgE between end-point IBH and H groups (Figure 1). A highly significant increase in IgE levels against *Culicoides* allergens was observed in the second summer after import in the IBH group, when the majority of horses had developed clinical signs of IBH, whereas IgE levels in the H group remained on a consistently low level throughout the study period. Induction of allergen-specific IgE production seems therefore to be linked to the occurrence of clinical signs. Although allergen-specific IgE closely reflects the clinical situation of the horses and may be useful for diagnostic purposes, it holds no predictive value as a biomarker for future development of IBH in horses. However, as antibody levels were only tested once a year in our study, we can only say that IgE does not increase in the *Culicoides* season preceding first occurrence of IBH signs. Monitoring the course of antibody levels more closely, with more frequent blood samplings during the IBH season would be helpful to more specifically pinpoint the exact timing of the increase in allergen-specific IgE.

Identification of primary sensitizing allergens and of the major allergens in the course of IBH-pathogenesis is a prerequisite for development of preventive and therapeutic vaccines, respectively. However, the present study revealed a complex sensitization pattern at the time of onset of clinical signs of IBH and confirms an earlier study using immunoblots with salivary gland extract.¹² Although sensitization against a maximum of one r-allergen was observed in the H control horses, IBH horses displayed positive IgE-levels against a median number of 4.5 allergens at time of first signs of IBH (Figure 2). Furthermore, a tendency for horses to show IgE reactivity against an increasing number of *Culicoides* allergens in the subsequent *Culicoides* seasons after first onset of IBH was observed in the IBH group. We suspect that this increase would be even more distinct if no preventive measures against *Culicoides* exposure, such as use of blankets, had been taken once IBH diagnosis was established. Additionally, the r-allergens that IBH horses were sensitized to varied widely between individual horses. Nevertheless, some r-allergens, notably rCul o 1 (a Kunitz-protease inhibitor), rCul o 2 (a D7-related protein), rCul o 3 (an antigen-5 like protein), as well as the *C. nubeculosus* allergens

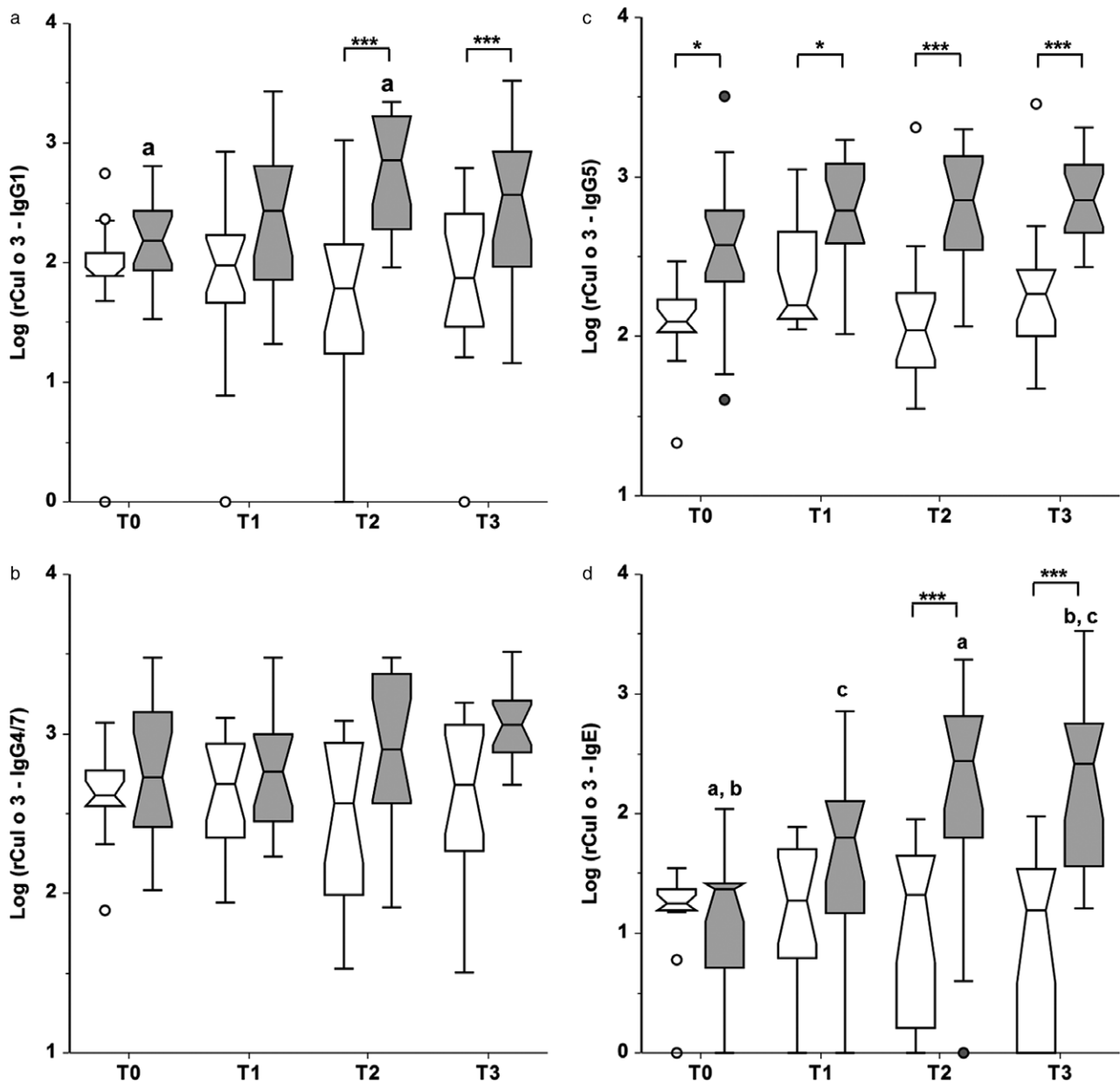


Figure 4. Comparison of rCul o 3-specific serum immunoglobulin (IgG1 (a), IgG4/7 (b), IgG5 (c) and IgE (d) levels, respectively, between the end-point IBH (shaded box) and the end-point H (white box) horse group at time points T0–T3.

Results are displayed as log OD₄₀₅. T0–T3 correspond to the yearly blood samples, with T0 representing the blood sample collected before exposure to *Culicoides* and T1–T3 representing the blood samples collected at the end of each subsequent summer. This figure illustrates the results of the repeated measures ANOVA, also described in Table 1. Asterisks indicate significant differences between the groups (** $P \leq 0.001$, *** $P \leq 0.0001$). Time points with the same superscript letter are significantly different ($P \leq 0.05$) within each horse group.

rCul n 4 and rCul n 8, elicited IgE responses in $\geq 50\%$ of IBH horses and could therefore be classified as major allergens (Figure 3). The other tested r-allergens bound IgE antibodies from a smaller number of horses. To further elucidate the role of individual r-allergens as potential primary sensitizing allergens, studies using a larger number of horses and a complete panel of all known *Culicoides* allergens is required.

A further aim of our study was to investigate IgG subclasses in IBH, using two of the most relevant *C. obsoletus* allergens, rCul o 1 and rCul o 3, as well as one of the most relevant *C. nubeculosus* allergens, rCul n 4. At time of onset of clinical signs, the IBH group had significantly

higher IgG1 and IgG5 levels against all tested allergens than the control group. This is in accordance with some studies in the human allergy field, showing that IgG responses are more frequent and stronger in individuals with current IgE sensitization¹⁹ and that some IgG subclasses (i.e. human IgG4) are increased in atopic compared to nonatopic individuals.²⁰ However, the IgG response to allergenic molecules is very complex and highly dependent on the route of allergen exposure.²¹ Our data strongly suggest that IgG5 is the subclass associated with allergy in horses. Previous studies had shown that IgG3 and IgG5 responses are mainly induced by extracellular pathogens;^{22,23} whereas IgG4, IgG7 and

probably IgG1 protect against various intracellular infections.^{17,24,25} Our data indicate that IgG1 but not IgG4/7 levels increase following exposure to *Culicoides*, mainly in the IBH group. IgG5 also increased over time but this increase only reached statistical significance for Bac-rCul o 3 in the IBH group (Table 1). Horses from the IBH group thus had usually significantly higher allergen-specific IgG1 and IgG5 than the H group. Furthermore, these results using *Culicoides* r-allergens confirm previous studies with immunoblots of *Culicoides* salivary gland extract where horses with IBH had IgE as well as IgGa (corresponding to IgG1) and IgG(T) (corresponding to IgG3 & 5) against a higher number of protein bands than healthy controls, whereas no significant difference was seen for IgG(b), which corresponds to IgG4/7.¹² On the other hand, the present study shows that vaccination with equine influenza resulted in a strong increase in IgG1 and IgG4/7 against equine influenza and a weak increase in equine influenza-specific IgG5 in the IBH group, whereas no IgE was induced. No differences between the IBH and H horse groups was found except for higher IgG1 levels at T0 in the IBH group. This is due to the fact that pre-*Culicoides* exposure blood samples in a few of these horses were taken in the winter months and several weeks after import to Europe and when they had already received the first vaccination against equine influenza.

Most surprisingly, our study revealed that horses that developed IBH after import to Europe already had significantly higher IgG5 levels against rCul o 1 and rCul o 3 before *Culicoides* exposure. A previous study had demonstrated that the antigen 5-like allergen from *Simulium vittatum* (*S. vittatum*) and *C. nubeculosus* is cross-reactive.³ Furthermore, a Kunitz Protease Inhibitor from *S. vittatum* had been identified as an IgE-binding protein, binding IgE from sera of IBH-affected horses significantly more frequently than from healthy controls.²⁶ High IgG5 levels to rCul o 1 and rCul o 3 in Icelandic horses before exposure to *Culicoides* could thus be due to cross-reactivity between *Simulium* and *Culicoides* salivary proteins, as *S. vittatum* are present in Iceland.²⁷ Further studies are needed to elucidate the role of rCul o 1- and rCul o 3-specific IgG5 antibodies in IBH and, in particular, why IgG5 but not IgE antibodies specific for these proteins can be detected in the sera before exposure to *Culicoides*.

In conclusion, the present study shows that first exposure of adult Icelandic horses to *Culicoides* induces an individual IgE response pattern and that the increase in serum IgE levels to *Culicoides* allergens is concurrent with the initial onset of clinical signs of IBH. Allergen-specific serum IgE can thus be used to confirm the clinical diagnosis but not as a predictor for IBH. Furthermore, IgG5 levels to the *C. obsoletus* allergens rCul o 1 and rCul o 3 were already increased year(s) before the first clinical signs of IBH were noted. Further studies are needed to test whether increased allergen-specific IgG5 levels may have a predictive value for IBH.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Horses within group end-point H (=remained healthy) and end-point insect bite hypersensitivity IBH (=developed IBH) with serum IgE levels above cut-off per *Culicoides* r-allergen for each time point.

Table S2. Allergen-specific IgE levels in relative ELISA units (REU) at time of onset of clinical Insect bite hypersensitivity (IBH) signs in the end-point IBH group ($n = 16$) and in time point-matched H (healthy) controls ($n = 16$).

Figure S1. Purification and detection of Bac-rCul o 3 produced in High five insect cells.

Résumé

Contexte – L’hypersensibilité aux piqûres de moustiques (IBH) est une dermatite allergique des chevaux causée par des piqûres de *Culicoides* spp. L’IBH n’existe pas en Islande en raison de l’absence de *Culicoides* mais la prévalence est élevée chez les chevaux importés d’Islande dans les environnements où *Culicoides* est présent.

Hypothèses/Objectifs – Tester, dans une étude longitudinale avant et après exposition à *Culicoides*, si une sensibilisation primaire à l’allergène de *Culicoides* peut être identifiée et si une augmentation d’IgE spécifique d’allergène ou de sous-classe d’IgG précède les signes cliniques d’IBH.

Sujets – Trente deux chevaux importés d’Islande en Europe ; 16 avaient une IBH et 16 étaient sains.

Méthodes – Déterminer les sous classes d’IgE et d’IgG contre les allergènes recombinants (r)-*Culicoides* et des extraits de *Culicoides* dans des séra avant la première exposition à *Culicoides* et une fois par an pendant une période de 3-4 ans.

Résultats – Avant l’exposition à *Culicoides*, il n’y avait aucune différence significative dans les niveaux d’IgE sériques spécifiques de *Culicoides* entre les chevaux qui ont développés une IBH ou ceux qui restaient sains. L’exposition à *Culicoides* induisait une réponse IgE individuelle en patron (à une moyenne de 4.5 r-allergènes) chez les IBH mais pas dans le groupe de fin sain. L’augmentation des niveaux sériques d’IgE aux allergènes-r *Culicoides* était concomitante avec l’apparition initiale des signes cliniques d’IBH. Les chevaux atteints d’IBH présentaient des taux d’IgG1 et IgG5 spécifiques d’allergènes plus élevés que les contrôles sains. Les IgG 5 spécifiques de Cul o1 et Cul o3 (recombinant *Culicoides* obsoletus) de *Culicoides* étaient significativement plus élevés dans l’IBH comparé au groupe sain avant les signes cliniques d’IBH.

Conclusion/Signification clinique – Les IgE sériques spécifiques d’allergènes ne peuvent être utilisés comme indicateur de l’IBH tandis que les taux d’IgG5 spécifiques d’allergènes peuvent avoir une valeur prédictive.

Resumen

Introducción – La hipersensibilidad a picaduras de insectos (IBH) es una dermatitis alérgica en caballos causada por picaduras de *Culicoides* spp. IBH no se produce en Islandia debido a la ausencia de *Culicoides*, pero la prevalencia es alta en caballos importados de Islandia a otros ambientes donde *Culicoides* están presentes.

Hipótesis / Objetivo – Probar si en un estudio longitudinal antes y después de la exposición a *Culicoides*, se puede identificar un alérgeno sensibilizante primario de *Culicoides* y si se produce un aumento de las subclases de inmunoglobulina (Ig)E o IgG específicas de alérgeno antes de los signos clínicos de IBH.

Animales – Treinta y dos caballos importados de Islandia a Europa; 16 tenían IBH y 16 eran aparentemente sanos.

Métodos – Determinación de las subclases de IgE e IgG frente a alérgenos recombinantes (r) de culicoides y extractos de culicoides en suero tomados antes de la primera exposición a culicoides y anualmente durante un periodo de 3-4 años.

Resultados – Antes de la exposición a culicoides no hubo diferencias significativas en los niveles de IgE séricos específicos de culicoides entre caballos que desarrollaron IBH o permanecieron sanos. La exposición a culicoides indujo en los animales afectados una respuesta de IgE (con una mediana de 4,5 alérgenos-r) en la IBH, pero no en el grupo de animales sanos. El aumento de los niveles séricos de IgE a alérgenos recombinantes de culicoides fue concomitante con el inicio de los signos clínicos de IBH. Los caballos afectados por IBH mostraron niveles significativamente mayores de IgG1 e IgG5 específicas de alérgeno que los controles sanos. La IgG5 específica para los antígenos recombinantes *Culicoides obsoletus* 1 (Cul o1) y Cul o3 fue significativamente mayor en los animales con IBH en comparación con el grupo sano, antes de observarse signos clínicos de IBH.

Conclusión / Importancia clínica – La IgE sérica específica de alérgeno no puede utilizarse para predecir animales con IBH, mientras que los niveles de IgG5 específicos de alérgeno pueden tener un valor predictivo.

Zusammenfassung

Hintergrund – Die Hypersensibilität auf Insektenstiche (IBH) ist eine allergische Dermatitis der Pferde, die durch *Culicoides* spp. verursacht wird. Eine IBH kommt in Island nicht vor, da es keine *Culicoides* Mücken gibt, aber die Prävalenz bei Pferden, die aus Island importiert werden, ist in Gegenden mit *Culicoides* Mücken hoch.

Hypothese/Ziele – Ein Versuch im Rahmen einer longitudinalen Studie herauszufinden, ob vor und nach Exponierung zu *Culicoides* ein primäres sensibilisierendes *Culicoides* Allergen identifiziert werden kann und ob eine Zunahme an Allergen-spezifischen Immunglobulinen (Ig) der Subklassen E oder IgG den klinischen Anzeichen der IBH vorausgehen.

Tiere – Von zweiunddreißig Pferden, die aus Island nach Europa importiert wurden, hatten 16 IBH und 16 waren gesund.

Methoden – Eine Bestimmung der IgE und IgG Subklassen gegen rekombinante (r)- *Culicoides* Allergene und *Culicoides* Extrakt in Sera, die vor einer ersten Exponierung zu *Culicoides* und jährlich über eine Zeit von 3-4 Jahren genommen wurden.

Ergebnisse – Vor der Exponierung zu *Culicoides* gab es keine signifikanten Unterschiede bei den *Culicoides*-spezifischen IgE Werten im Serum zwischen den Pferden, die IBH entwickelten oder gesund blieben. Die Exponierung zu *Culicoides* induzierte ein individuelles IgE Antwortmuster (ein Median von 4,5 r-Allergenen) in der IBH Gruppe, jedoch nicht in der gesunden Endgruppe. Die Zunahme an Serum IgE Werten zu *Culicoides* r –Allergenen lief gleichzeitig mit dem ersten Auftreten von klinischen Zeichen einer IBH. Pferde, die eine IBH entwickelten, zeigten signifikant höhere Allergen-spezifische IgG1 und IgG5 Werte als die Kontrolltiere. Die Werte für rekombinantes *Culicoides obsoletus* 1 (Cul o 1) und Cul o 3-spezifische IgG5 Werte waren bei Pferden mit IBH vor dem Auftreten von klinische Anzeichen einer IBH signifikant höher als bei der gesunden Endpunktgruppe.

Schlussfolgerungen/Klinische Relevanz – Allergen-spezifisches IgE im Serum kann nicht als Vorhersagewert für eine IBH genommen werden während Allergen-spezifische IgG5 Werte einen Vorhersagewert darstellen könnten.

要約

背景 – 昆虫刺咬性過敏症(IBS)は、*Culicoides*種の咬傷によって引き起こされる馬のアレルギー性皮膚炎である。アイスランドでは*Culicoides*が存在しないため、IBHは発生しないが、アイスランドから*Culicoides*が存在する環境下に輸入された馬での発生率は高い。

仮説/目的 – *Culicoides*暴露前後の縦断研究により、初期感作*Culicoides*アレルゲンが同定できるかどうか、およびアレルゲン特異的免疫グロブリン(Ig)EまたはIgGのサブクラスの増加がIBHの臨床症状発現に先行するかどうかを評価する。

供与動物 – アイスランドからヨーロッパに輸入された32頭の馬。16頭はIBH、16頭は健康であった。

方法 – *Culicoides*に最初に暴露される前、およびその後3～4年の期間にわたり毎年採取された血清中の組換え(r)-*Culicoides*アレルゲンおよび*Culicoides*抽出物に対するIgEおよびIgGサブクラスの測定。

結果 – *Culicoides*への暴露前には、IBHを発症した馬と健康馬との間に*Culicoides*特異的血清IgEレベルに有意差はなかった。*Culicoides*暴露は、IBH馬にそれぞれのIgE応答パターンを引き起こしたが(中央値4.5 r-アレルゲン)、健康群では認められなかった。*Culicoides* r-アレルゲンに対する血清IgEレベルの増加は、IBHの臨床症状の発症と同時に認められた。IBH発症馬は、健康馬よりも有意に高いアレルゲン特異的IgG1およびIgG5レベルを示した。組換え*Culicoides obsoletus* 1(Cul o1)およびCul o3特異的IgG5は、健康馬群と比較してIBHでは臨床症状の発症前に有意に高かった。

結論および臨床的な重要性 – アレルゲン特異的血清IgEは、IBHの予測因子として使用することはできないが、アレルゲン特異的IgG5レベルは予測因子となる可能性が示唆された。

摘要

背景 — 昆虫叮咬过敏(IBH)是一种由库蠓叮咬引起的马过敏性皮炎,由于没有库蠓,IBH不会出现在冰岛,但从冰岛进口的马,来到有库蠓存在环境中,有很高的患病率。

假设/目的 — 在接触库蠓之前和之后的纵向研究中,鉴定主要的致敏库蠓过敏原,以及过敏原特异性免疫球蛋白IgE或IgG亚类,其增加是否先于IBH的临床症状。

动物 — 从冰岛进口到欧洲的32匹马;16匹患有IBH,16匹健康。

方法 — 在3到4年的时间内,测定血清重组(r)-库蠓过敏原和库蠓提取物的IgE和IgG亚型。

结果 — 接触库蠓前,在发生IBH或保持健康状态的马中,库蠓血清IgE水平没有显著差异。接触库蠓诱发出IBH的独特IgE反应模式(达到4.5 r-过敏原中值),而末端健康组则没有这种反应。对库蠓 r-过敏原的血清IgE水平升高,与IBH临床症状同时出现。IBH患马的过敏原特异性IgG1和IgG5水平,显示高于健康对照组。在IBH发生临床症状之前,IBH的重组库蠓 1(Cul o1)和Cul o3特异性IgG5显著高于末端健康组。

结论/临床相关性 — 过敏原特异性血清IgE不能用于预测IBH,而特异性IgG5水平可能具有预测价值。

Resumo

Contexto — A hipersensibilidade à picada de insetos (HPI) é uma dermatite alérgica de cavalos causados pelas picadas de *Culicoides* spp. A HPI não ocorre na Islândia devido à ausência de *Culicoides*, mas a prevalência é alta em cavalos importados da Islândia para locais onde *Culicoides* estão presentes.

Hipótese/Objetivos — Testar, em um estudo longitudinal antes e após exposição a *Culicoides*, se a sensibilização por alérgeno de *Culicoides* pode ser identificada e se um aumento de imunoglobulina das subclasses E (IgE) ou IgG precede os sinais clínicos de HPI.

Animais — Trinta e dois cavalos importados da Islândia para a Europa; 16 tinham HPI e 16 eram saudáveis.

Métodos — Determinação de subclasses de IgE e IgG contra alérgenos de *Culicoides* recombinantes e extrato de *Culicoides* no soro retirado antes da primeira exposição a *Culicoides* e anualmente por um período de 3-4 anos.

Resultados — Antes da exposição a *Culicoides*, não havia diferenças significativas no nível de IgE específicos para *Culicoides* entre os cavalos que desenvolveram HPI e os que permaneceram saudáveis. A exposição a *Culicoides* induziu um padrão de resposta IgE individual (para uma média de 4,5 r-alérgenos) no HPI, mas não nos animais saudáveis ao final. O aumento no nível sérico de IgE a r-alérgenos de *Culicoides* foi concomitante com o início dos sinais clínicos de HPI. Os cavalos afetados por HPI apresentaram níveis de IgG1 e IgG5 alérgeno-específicos significativamente maiores que os controles saudáveis. Os níveis de IgG5 específico para *Culicoides obsoletus* 1 (Cul o1) e Cul o3 foi significativamente maior no grupo HPI comparado aos cavalos saudáveis, antes dos sinais clínicos de HPI.

Conclusão/relevância clínica — Imunoglobulina E (IgE) alérgeno específicos não podem ser usados como um preditor de HPI, enquanto IgG5 alérgeno específicos podem ter um valor preditivo.