

Immunological and clinical factors associated with adverse systemic reactions during the build-up phase of honeybee venom immunotherapy

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Summary

Background Adverse systemic reactions (SRs) are more common in honeybee venom immunotherapy (VIT) than in wasp VIT. Factors that might be associated with SRs during the honeybee VIT are poorly understood.

Objective Our aim was to evaluate risk factors for SRs during the build-up phase of honeybee venom immunotherapy.

Methods We included 93 patients who underwent ultra-rush honeybee VIT. The adverse SRs and their severity was compared to various immunological (sIgE, tIgE, basophil CD63 response, baseline tryptase, and skin tests), patient-specific (age, sex, cardiovascular conditions and medications, and other allergic diseases), and sting-specific factors (anaphylaxis severity, time interval to onset of symptoms, and absence of cutaneous symptoms). **Results** Twenty-three patients (24.7%) experienced mild SRs and 13 patients (14%) severe SRs. In five patients with severe SRs, the build-up was stopped. High basophil allergen sensitivity, evaluated as dose–response curve metrics of EC15, EC50, CD-sens, AUC, or the response to submaximal 0.01 µg/mL of venom concentration, was the most significant risk factor and only independent predictor of severe SRs and/or build-up stop. Time interval of <5 min after sting to onset of symptoms and lower specific IgEs to rApi m1 was also associated with severe SRs. There was no difference in other immunological, patient-specific, or sting-specific factors, including the baseline tryptase. None of the studied factors was associated with mild SRs.

Conclusion and Clinical Relevance High basophil allergen CD63 sensitivity phenotype was a major indicator of severe adverse SRs during the build-up phase of honeybee VIT. Possibly role was also showed for short latency to filed sting reaction and low sIgE to rApi m1. Before honeybee VIT, measurement of basophil allergen sensitivity should be used to identify patients with a high risk for severe side-effects.

Keywords adverse systemic reactions, basophils, CD63, honeybee venom allergy, IgE, immunotherapy, risk factors

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Introduction

Adverse systemic reactions (SRs) during the build-up phase of venom immunotherapy (VIT) frequently require emergency interventions and sometimes even a discontinuation of treatment. The major risk factor associated with SRs is honeybee venom allergy. Specifically, the frequencies of SRs during the build-up phase of VIT observed in large studies by Lockey et al. [1] were 40% for honeybee vs. 12% for wasp venom, Mueller et al. [2] 21% vs. 4%, Mosbech and Mueller [3] 24%

vs. 19%, and Rueff et al. [4] 16% vs. 5%, respectively. Apparently, the honeybee venom treatment-related SR frequencies are up to fivefold greater than for wasp venom. A variety of other factors that might be associated with SRs during VIT build-up were also studied, but only very few demonstrated some significance. One of them was an increased baseline serum tryptase. However, in the case of VIT-related SRs, Rueff et al. [4] showed that the increased baseline serum tryptase is associated with SRs only during wasp VIT but not during honeybee VIT.

The mast cells and basophils are considered the major effector cells in patients with allergic reactions. However, the mast cells are difficult to obtain, and although the basophil represents about 1% of the leucocytes, blood is more readily available for study. Beyond secretion assays, basophils can be also tested by activation markers, such as CD63 [5]. It has previously been demonstrated that the CD63 activation test could be an additional diagnostic tool in complex Hymenoptera cases with either double-negative [6, 7] or double-positive [8] standard diagnostic tests, and it might also be useful in monitoring VIT [9, 10]. Furthermore, some previous data suggest that the basophil CD63 allergen response may be associated with VIT-related SRs [10, 11], while others could not showed such associations [12]. Therefore, the aim of this prospective study was to determine the importance of the basophil CD63 allergen response and other immunological factors, such as sIgEs and baseline tryptase, and then the patient's specific clinical factors and details of the honeybee field sting-induced anaphylaxis as risk factors for adverse SRs and their severity during the build-up phase of ultra-rush honeybee VIT.

Material and methods

Study subjects

Ninety-three adult patients with a history of systemic allergic reaction after honeybee sting and indicated for VIT were included in this prospective single-centre study. The time period of recruitment was from January 2011 to May 2013. The study was approved by the Slovenian National Medical Ethics Committee, and all patients gave written informed consent for participation in this study.

Honeybee venom immunotherapy

All patients underwent honeybee ultra-rush VIT with honeybee venom allergen extract (HAL Allergy, Leiden, the Netherlands), with the type of build-up schedule as shown in the Supporting information and previously described in detail [13]. In case that build-up was stopped due to the SRs, a slower conventional dose increase was adopted. All patients were pre-treated with the antihistamine (desloratadine 10 mg), and substitution of antihypertensive medication was individually determined as recommended [14]. VIT was administrated in a setting that allowed prompt recognition and treatment of SRs. Diagnosis and grading of SRs that occurred during the build-up followed the World Allergy Organization classification with grading system for SCIT SRs from Cox et al. [15] (Table S1). The management of SRs followed established recommendations [16].

Clinical test variables

The patient-specific data collection included age and sex, concomitant cardiovascular, asthma, or allergic diseases, and concurrent beta-blocker or ACE inhibitor medication at the time of the VIT build-up. The honeybee field sting-induced anaphylaxis data collection included information on the severity of the most severe sting reaction according to the Ring and Meßmer [17] scale (Table S2), the anaphylaxis symptoms, the time interval between the most severe sting and onset of anaphylaxis symptoms, and the location of the sting.

Immunological test variables

Basophil CD63 testing was performed on heparinized whole blood taken just before starting of the treatment with serial dilutions of honeybee venom (1–0.001 µg/mL; Hal Allergie, the Netherlands) as previously described in detail [9–11, 18]. Basophil response was analysed as the CD63 up-regulation to different single allergen concentrations or as sensitivity metrics of allergen CD63 dose–response curve by including the allergen concentration at 50% (EC50 = half (50%) maximal effective concentration) [19] or 15% (EC15 = 15% maximal effective concentration) of maximal response, by CD-sens calculation [13, 18–20], which is the inverse value of EC50 concentration multiplied by 100, and as area under the curve (AUC) analysis (Supporting information).

Prick and intradermal skin tests with honeybee venom were performed as previously described [9]. Intradermal tests were performed only in the case of negative prick tests. Serum honeybee and wasp venom-specific IgE and total IgE were determined with Immulite (Simens, Erlangen, Germany), and IgE to rApi m1 and baseline tryptase were measured with ImmunoCAP-FEIA (Phadia, Uppsala, Sweden). Tryptase concentrations exceeding 11.4 µg/L were considered elevated.

Data analysis

The end-point of the study was to evaluate the relation between different immunological test variables and clinical variables to the occurrence and severity of SRs during VIT. The descriptive statistics were presented as medians and interquartile ranges for measurement data and percentages for categorical data. Differences between groups were assessed by Mann–Whitney U-test for all continuous and categorical data. Firth's bias-reduced multiple logistic regression was used to evaluate the relative contribution of all measured variables [21]. Additionally, another logistic regression model was created where variables were selected using the stepwise approach, and only variables significantly

contributing to the logistic regression model were retained. A receiver operating characteristics (ROC) curve was used to evaluate sensitivity and specificity of the selected variables. All statistical analyses were performed using GraphPad Prism (version 5), IBM SPSS Statistics (version 21.0), R Statistical Computing (version 3), and R package logistf: Firth's bias-reduced logistic regression (version 1.21) [22].

Results

Patient characteristics and adverse SRs

A total of 93 patients were analysed. Their demographic, clinical, and laboratory characteristics are summarized in Table 1. The majority of patients were male (70%), and the mean age was 48 years. Most patients had grade 2 anaphylactic reaction after a field sting (51.6%). Anaphylactic reactions occurred without any cutaneous signs in 24.7% of patients, and in 46 patients (49.5%), the anaphylactic reaction started <5 min after the sting. The sting was most often located on the head or neck (51.6%). According to anamnestic data, honeybee was the culprit insect in 86 patients (92.5%); for three patients (3.2%), the culprit insects were both the honeybee and wasp; and four patients (4.3%) did not recognized the culprit insect. Skin prick and intradermal tests confirmed sensitization to honeybee in 96.5% of patients. The vast majority of patients (97.8%) had positive specific IgE for honeybee and 67.7% for rApi m1. In one patient, sensitization to honeybee was confirmed only with BAT [6]. Furthermore, 47.3% of patients had positive wasp-specific IgE. Baseline serum tryptase was elevated in five patients (5.4%). Almost one-third of the patients (32.3%) had concomitant cardiovascular disease, and 10.8% had been diagnosed with asthma or other allergic disease. Before the start of the VIT, 14 patients (15.1%) were being treated with ACE inhibitors and four patients (4.3%) were on beta-blockers. Among these, the beta-blocker treatment was discontinued in three of them prior to VIT. More than three-quarters of patients (77.4%) started VIT between 2 and 12 months after the last sting-induced anaphylaxis.

Systemic reactions during VIT build-up are characterized in detail in Table 2. Patients were classified into three groups: the first group included 57 patients (61.3%) without SRs, the second group included 23 patients (24.7%) who experienced mild SRs (Cox grade 1), and the third group included 13 patients (14%) who experienced severe SRs. The group with severe SRs was composed of 11 patients who experienced Cox grades 2–4 SRs (in three patients, VIT build-up was stopped) and of two patients who experienced repeated Cox grade 1 SRs (one had four and the second eight SRs) in whom VIT build-up was also stopped.

Table 1. Demographic, clinical, and laboratory characteristics

	<i>n</i>	%
No. of patients	93	
Age (years). mean (range)	48	(18–78)
Gender (male/female)	65/28	(70/30)
Severity of anaphylaxis		
Grade 1	13	14.0
Grade 2	48	51.6
Grade 3	32	34.4
Absence of cutaneous signs during anaphylaxis	23	24.7
Sting localization*		
Head/neck	48	51.6
Hand/arm	37	39.8
Foot/leg	18	19.4
Trunk	1	1.1
Latency†		
<1 min	4	4.3
1–5 min	42	45.2
6–15 min	34	36.6
16–30 min	10	10.8
>30 min	2	2.2
Culprit insect		
Honeybee	86	92.5
Honeybee and wasp	3	3.2
Not known	4	4.3
Diagnostic evaluation		
Positive honeybee SPT or IDT‡	82	96.5
Positive honeybee sIgE	91	97.8
Positive wasp sIgE	44	47.3
Positive rApi m1 sIgE	63	67.7
Positive OSR sIgE	23	24.7
Elevated baseline serum tryptase (≥ 11.4 µg/L)	5	5.4
Other diseases		
Cardiovascular disease	30	32.3
Asthma or allergic disease	10	10.8
Medication		
Beta-blocker§	4	4.3
ACE inhibitor	14	15.1
Time from last sting to VIT. median (IQR)		
<2 months	3	3.2
2–12 months	72	77.4
>12 months	18	19.4

SPT, skin prick test; IDT, intradermal test.

*Some patients had stings on multiple locations; sting location for two patients is not known.

†Data for one patient are missing.

‡Data for eight patients are not available; we first performed SPT and, if negative, then IDT.

§Beta-blocker treatment was discontinued in three of them prior to VIT.

Risk factors for adverse systemic reactions during build-up of honeybee venom immunotherapy

Table 2 depicts the demographic, clinical, and immunological variables for the three distinct groups of patients categorized according to the severity of SRs, and Fig. 1

Table 2. Systemic reactions (SRs) during the build-up phase of honeybee venom immunotherapy in relation to demographic, clinical, and laboratory data

	No SR		Mild SRs (Cox grade 1 ¹⁵)		Severe SRs (Cox grades 2–4 ¹⁵ or venom immunotherapy (VIT) build-up stopped [§])	
No. of patients	57	61.3%	23	24.7%	13	14.0%
Age (years). mean (range)	49	(23–78)	45	(18–77)	49	(24–77)
Gender (male/female)	39/18	68.4%/31.6%	16/7	69.6%/30.4%	10/3	76.9%/23.1%
No. of SR during VIT per patient median (IQR)	0		1	(1–2)	3	(2–4)
No. of patients treated for SR during VIT (antihistamines and/or epinephrine)	0		18	78.3%	13	100.0%
Total no. of SR in a group with corresponding symptoms and signs			41		34	
Cutaneous	–	–	37	90.2%	28	82.4%
Conjunctival	–	–	1	2.4%	6	17.6%
Upper respiratory	–	–	3	7.3%	9	26.4%
Lower respiratory	–	–	0	0.0%	2	5.9%
Cardiovascular	–	–	0	0.0%	6	17.6%
Gastrointestinal	–	–	0	0.0%	5	14.7%
VIT build-up stopped	0		0	0.0%	5	38.5%
	Median	IQR	Median	IQR	Median	IQR
Immunological factors						
BAT AUC	0.478	0.357–0.607	0.486	0.377–0.605	0.674	0.569–0.746
BAT EC15	0.011	0.008–0.033	0.020	0.005–0.030	0.002	0.001–0.010
BAT EC50	0.050	0.014–0.140	0.050	0.011–0.102	0.010	0.004–0.023
BAT CD-sens	1910.435	657–6161	2199	979–6700	10052	4872–25500
BAT BV 0.001 µg/mL	3.16	1.67–4.72	3.01	2.27–4.92	3.66	2.88–6.34
BAT BV 0.01 µg/mL	8.70	3.14–23.64	6.72	4–23.45	42.11	20.86–63.99
BAT BV 0.1 µg/mL	63.69	22.47–87.19	59.30	31.8–86.39	88.84	65.82–94.6
BAT BV 1 µg/mL	87.65	68.47–94.96	80.99	61.9–90.57	94.78	85.88–96.94
Honeybee sIgE	6.700	2.09–14.8	4.710	1.96–11.3	3.370	1.785–9.745
Wasp sIgE	0.310	0–1.165	0.350	0–1.11	0.000	0–0.49
rApi m1 sIgE	1.000	0.305–3.04	0.400	0–1.76	0.390	0–0.81
Total IgE	54.000	25.6–141.5	45.000	20.1–113	45.700	22.8–91.2
Baseline serum tryptase	4.910	3.335–6.105	3.840	3.2–5.74	3.830	3.31–9.58
Elevated baseline serum tryptase (≥11.4 µg/L)	4	7.0%	0	0.0%	1	7.7%
Positive honeybee SPT or IDT*	50	96.2%	22	100.0%	10	90.9%
Details of sting reaction						
Anaphylaxis severity						
Grade 1	8	14.0%	5	21.7%	0	0.0%
Grade 2	26	45.6%	14	60.9%	8	61.5%
Grade 3	23	40.4%	4	17.4%	5	38.5%
Reaction latency <5 min [†]	25	44.6%	11	47.8%	10	76.9%
Absence of cutaneous symptoms	16	28.1%	3	13.0%	4	30.8%
Head or neck sting [‡]	32	56.1%	8	36.4%	5	41.7%
Other diseases						
Cardiovascular disease	18	31.6%	6	26.1%	6	46.2%
Asthma or allergic disease	4	7.0%	5	21.7%	1	7.7%

Table 2 (continued)

	Median	IQR	Median	IQR	Median	IQR
Medication						
Beta-blocker [‡]	4	7.0%	0	0.0%	0	0.0%
ACE inhibitor	8	14.0%	3	13.0%	3	23.1%

BAT, basophil activation test; AUC, area under the curve; EC15, 15% maximal effective concentration; EC50, half (50%) maximal effective concentration; CD-sens, inverse value of EC50 multiplied by 100; BV, honeybee venom; SPT, skin prick test; IDT, intradermal test.

*Data for eight patients are missing (five in no SR, one in mild SR, and two in severe SR group).

[†]Data for one patient in no SRs group are missing.

[‡]Sting location for two patients is not known (one in mild SR, one in severe SR group).

[§]Two patients experienced several Cox grade 1 reactions, which resulted in halting the build-up; for that reason they were also included in the severe group.

[¶]beta-blocker treatment was discontinued in three of them prior to VIT.

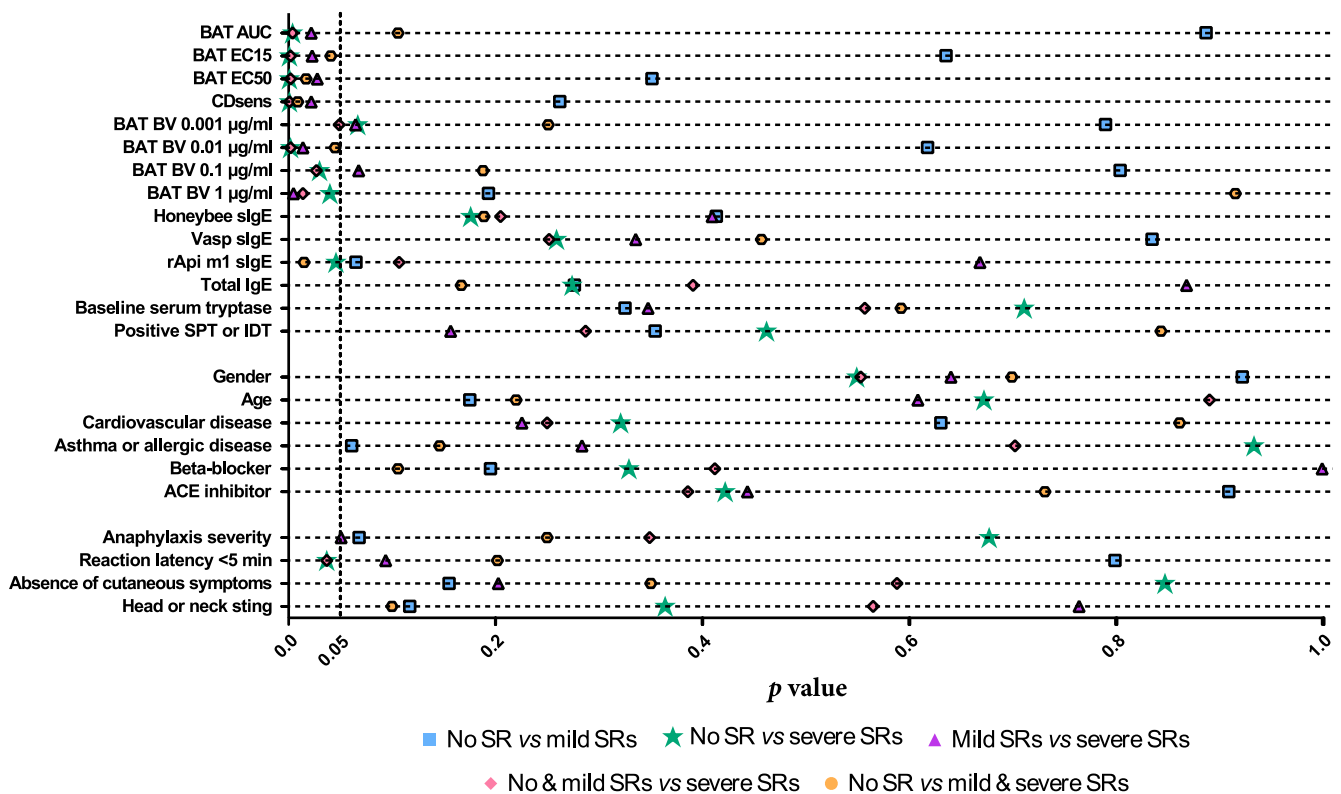


Fig. 1. Factors correlating with SRs during the build-up of honeybee VIT. The significance line corresponds to $P = 0.05$. VIT, venom immunotherapy; SRs, systemic reactions; BV, honeybee venom; BAT, basophil activation test; AUC, area under the curve; EC15, 15% maximal effective concentration; EC50, half (50%) maximal effective concentration; CD-sens, inverse value of EC50 concentration multiplied by 100; SPT, skin prick test; IDT, intradermal test.

presents the significance of those factors obtained by statistical comparison between groups.

The most significant factor associated with severe SRs during VIT build-up was increased basophil allergen CD63 activation: the sensitivity metrics of the allergen CD63 dose-response curve such as EC15, EC50, CD-sens and AUC, and the response to 0.01 of allergen concentration were markedly higher when compared to the group without SRs and the group with mild SRs

(Figs 1–3). Moreover, the highest CD63 basophil sensitivity was demonstrated in individuals in whom VIT build-up had to be stopped (Fig. 3). Similar, but smaller, differences were evident for CD63 response to 0.1 and 1 $\mu\text{g/mL}$ of allergen concentrations. The EC15, EC50, CD-sens, and response to 0.01 $\mu\text{g/mL}$ of allergen were also higher when combined groups of patients with mild and severe SRs were compared with patients without SRs. However, there was no difference in any

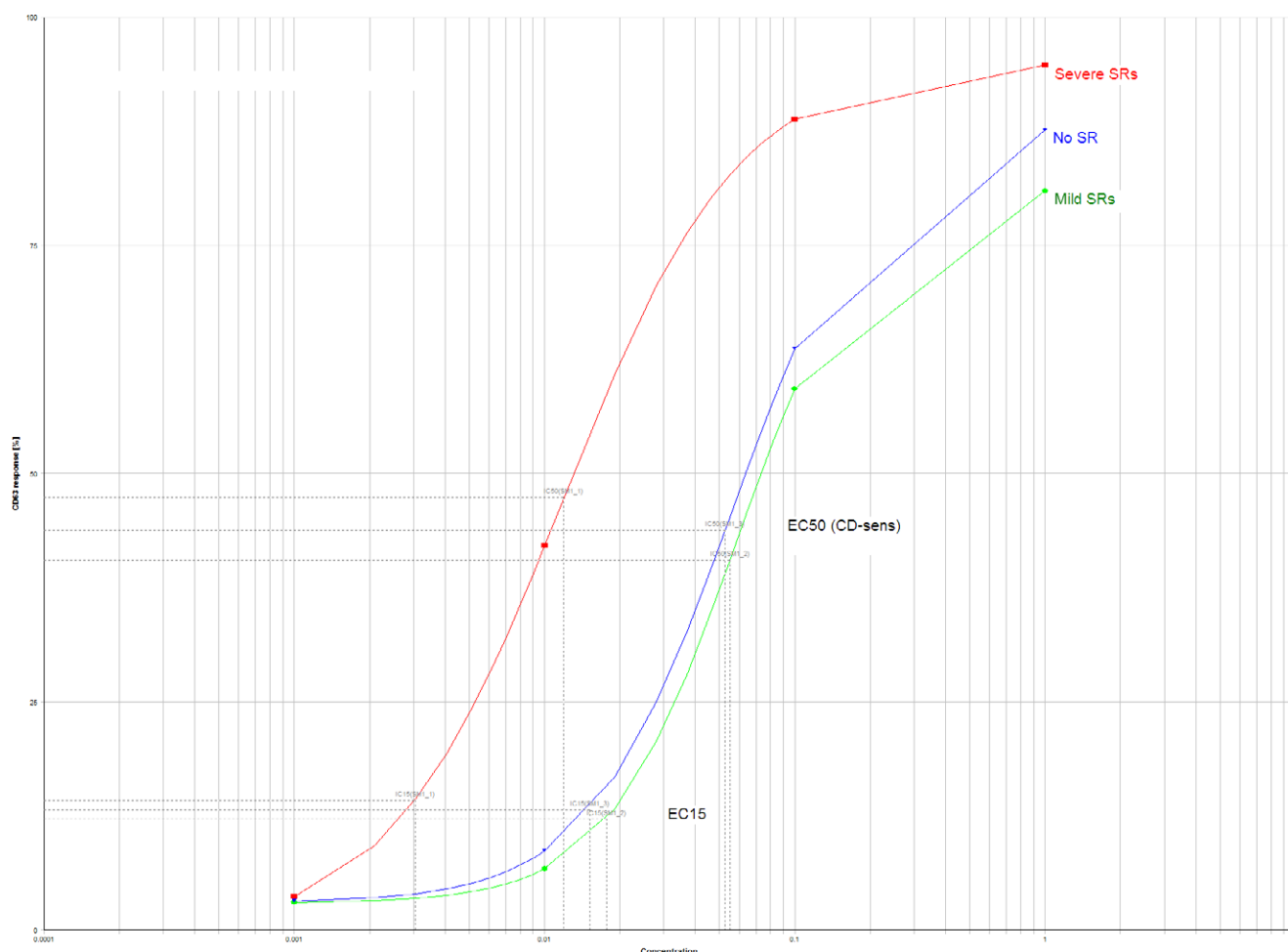


Fig. 2. Plotted median CD63 basophil dose-response curves for patients with severe, mild, or no SRs during the build-up of honeybee VIT. SRs, systemic reactions; EC15, 15% maximal effective concentration; EC50, half (50%) maximal effective concentration.

BAT variables between the group of patients without SRs and the group with mild SRs (Figs 1–3). In addition, no differences were found in response to control BAT stimuli (Table S3).

The concentration of rApi m1-specific IgE was lower in combined groups with mild and severe SRs and in severe SRs group compared to the group without SRs (Fig. 1).

A sting-induced anaphylactic reaction latency of less than 5 was associated with severe SRs compared to the group of patients without SRs and also to the combined group of patients without SRs and with mild SRs during VIT (Fig. 1).

There was no difference in the following immunological factors: honeybee-specific IgE, wasp-specific IgE, total IgE, baseline serum tryptase, and SPT or IDT. Moreover, we also did not identify any differences in of the following clinical variables: gender, age, concomitant cardiovascular diseases, asthma or other allergic diseases, medication with beta-blockers or ACE inhibitors, severity of the sting-induced anaphylaxis, absence

of cutaneous symptoms, and location of the sting that led to anaphylaxis (Fig. 1).

The evaluation of the potential risk factors for severe SRs vs. mild and no SRs during VIT build-up was made using the multivariable Firth's bias-reduced logistic regression analysis (Table 3). Logistic regression analysis demonstrated that only the BAT response (AUC) was an independent predictor of severe SRs. Additionally, another Firth's bias-reduced logistic regression model was created where variables were selected using the stepwise approach. This approach yielded a logistic regression model with only two variables: BAT response and honeybee sIgE (Table 4). The stepwise approach similarly demonstrated that AUC was highly significant and independent predictor of severe SRs.

The ROC analysis for the AUC and CD-sens is presented in Fig. 4, respectively. The ROC area was up to 0.81 ($P \leq 0.0006$) for the severe SR group vs. no SR and/or mild SR group. A smaller area was found (up to 0.66) when we compared the combined mild and severe SR groups to the no SR group. No significance with an

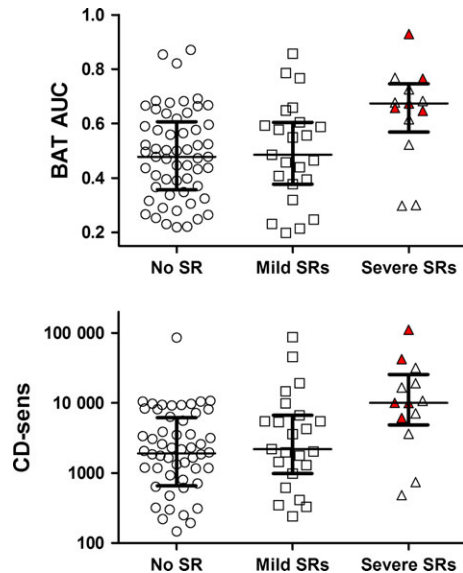


Fig. 3. BAT AUC and CD-sens dot plots for patients with severe, mild, or no SRs during the build-up phase of honeybee VIT. The red dots represent patients in whom build-up was stopped. Horizontal bars represent median and IQR. SRs, systemic reactions; BAT, basophil activation test; AUC, area under the curve; CD-sens, inverse value of EC50 concentration multiplied by 100.

area of up to 0.58 was found when we compared the no SR group to the mild SR group.

Discussion

Our study was designed to evaluate the importance of a variety of immunological and clinical factors in predicting

SRs and their severity during the build-up phase of honeybee VIT, namely severe SRs are experienced in up to 15% [1–4] of honeybee venom-treated patients, and thus, it would be important to assess the risk for their development before starting the treatment. The key finding of this study is that high basophil allergen sensitivity is a major and independent predictor of severe SRs during the build-up phase of honeybee VIT. Severe SRs were also associated with short latency of the sting reaction and low sIgE to rApi m1.

The median basophil allergen CD63 response in our study subjects was 3%, 9%, 69%, and 88%, for stimulation from a minimum of 0.001 to a maximum of 1 µg/mL of honeybee venom. This optimum allergen concentration dependence increase in CD63 dose-response curves allowed us that the individual curves were also analysed as allergen sensitivity curves. Johansson was the first to derive a curve metric of the basophil sensitivity approach [20]. This was the concentration of allergen needed for 50% of the maximum response, which was reversed, multiplied by 100, and termed 'CD-sens'. Johansson proposed that CD-sens measures the degree of allergen sensitivity and may relate the basophil *in vitro* functionality to the clinical presentation. This approach was initially applied to monitor the efficacy of anti-IgE therapy [23] or pollen immunotherapy [24, 25] and was recently also applied to monitor long-term or short-term effects of VIT [9, 13, 18]. In our study, we compared not only 50% of the maximal response and its derivative CD-sens, but also a lower curve part of 15% of the maximal response and the area under the dose–

Table 3. Multivariable Firth's bias-reduced logistic regression of risk factors for severe systemic reactions during the build-up phase of honeybee venom immunotherapy

	Log OR (95% CI)	OR* (95% CI)	P value
BAT AUC	4.37 (0.33 to 13.22)	79.31 (1.4 to 553682.39)	0.03
Honeybee sIgE	−0.09 (−0.58 to 0.02)	0.91 (0.56 to 1.02)	0.15
Vasp sIgE	0.05 (−0.11 to 0.28)	1.05 (0.89 to 1.32)	0.21
rApi m1 sIgE	0.13 (−0.14 to 1.03)	1.14 (0.87 to 2.81)	0.39
Total IgE	0 (−0.01 to 0.01)	1 (0.99 to 1.01)	0.85
Baseline serum tryptase	0 (−0.22 to 0.06)	1 (0.8 to 1.06)	0.95
Positive SPT or IDT	−1.15 (−4.13 to 1.9)	0.32 (0.02 to 6.66)	0.43
Gender	0.65 (−1.2 to 2.55)	1.92 (0.3 to 12.85)	0.46
Age	−0.04 (−0.12 to 0.03)	0.96 (0.89 to 1.03)	0.26
Cardiovascular disease	1.57 (−0.56 to 3.86)	4.82 (0.57 to 47.67)	0.14
Asthma or allergic disease	0.38 (−4.69 to 4.29)	1.46 (0.01 to 73)	0.83
Beta-blocker	0.07 (−5.74 to 4.42)	1.08 (0 to 82.75)	0.97
ACE inhibitor	−0.09 (−2.7 to 2.6)	0.91 (0.07 to 13.48)	0.94
Anaphylaxis severity	0.39 (−1.18 to 2.09)	1.48 (0.31 to 8.08)	0.61
Reaction latency <5 min	1.03 (−0.6 to 3.34)	2.8 (0.55 to 28.24)	0.22
Absence of cutaneous symptoms	0.35 (−1.84 to 2.79)	1.42 (0.16 to 16.23)	0.75
Head or neck sting	0.94 (−0.71 to 3.67)	2.55 (0.49 to 39.26)	0.27
(Constant)	−3.18 (−8.64 to 1.45)	0.04 (0 to 4.25)	0.18

BAT, basophil activation test; AUC, area under the curve; IDT, intradermal test; SPT, skin prick test; OR, odds ratio.

*Corresponds to a one-unit increase.

Table 4. Multivariable Firth's bias-reduced logistic regression analysis with stepwise approach where only variables significantly contributing to the logistic regression model were retained

	Log OR (95% CI)	OR* (95% CI)	<i>p</i> value
BAT AUC	6.42 (2.6 to 11.01)	611.86 (13.42 to 60209.91)	<0.001
Honeybee sIgE	−0.12 (−0.29 to −0.01)	0.89 (0.75 to 0.99)	0.02
Constant	−4.4 (−7.08 to −2.31)	0.01 (0 to 0.1)	<0.001

BAT, basophil activation test; AUC, area under the curve; OR, odds ratio.

*Corresponds to a one-unit increase.

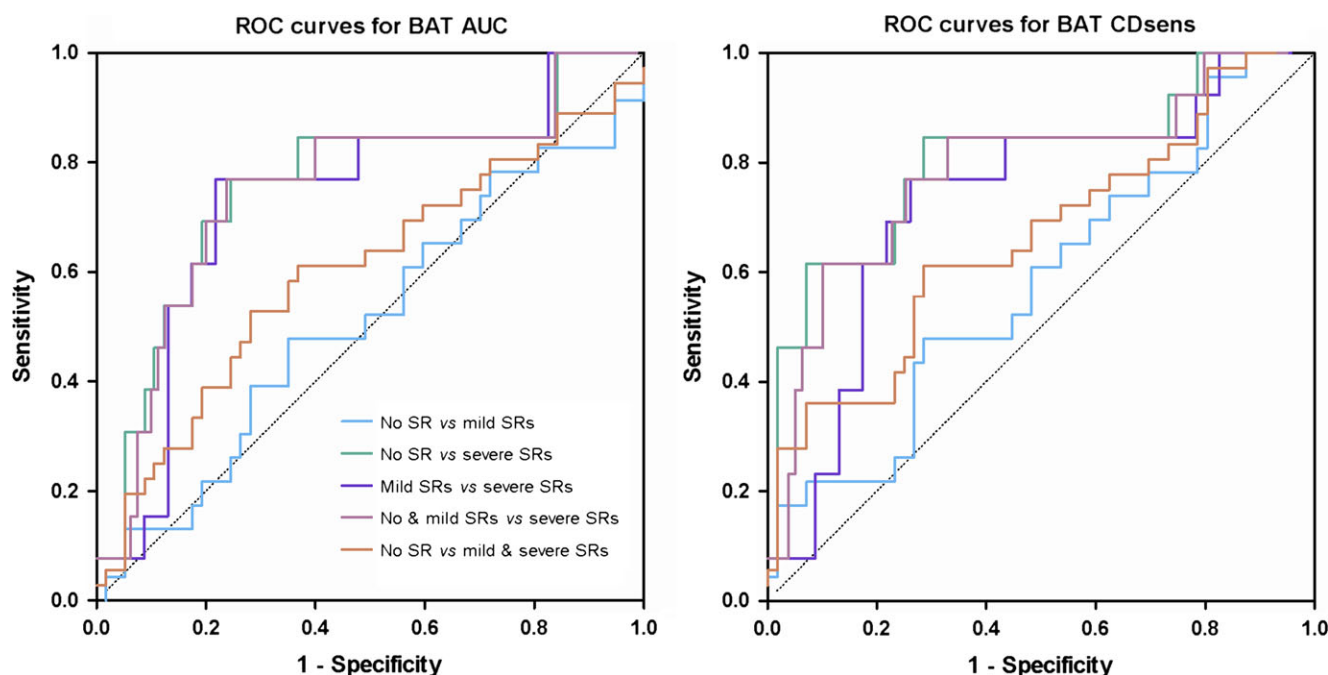


Fig. 4. BAT AUC and CD-sens ROC curve analyses in patients characterized according to the severity of SRs during the build-up phase of honeybee VIT. SRs, systemic reactions; BAT, basophil activation test; AUC, area under the curve; CD-sens, inverse value of EC50 concentration multiplied by 100.

response curve (AUC), which represents the full response curve. We showed that increased basophil allergen sensitivity evaluated with any one of the three curve metrics is comparably and highly significantly associated with the severe SRs. On the other hand, the basophil allergen sensitivity was not associated with mild SRs. Nevertheless, the association of those parameters with severe SRs was of such significance that even for analysis of the entire SR cohort (mild plus severe SRs patients), the corresponding associations could still be recognized. We also clearly showed that the basophil response is an independent predictor of severe SRs and that a rise in AUC for 0.1 (the median for the no SR and mild SRs groups was 0.5 and for the severe SR group was 0.7) will increase the risk for severe SRs by a factor of 7.8–61. Additionally, the logistic regression model with the stepwise approach had the lowest Akaike information criterion (AIC) value of all the examined models (data not shown),

indicating that this model was the best among all examined logistic regression models. Furthermore, the ROC analysis showed that, if AUC is higher than 0.6 and if CD-sens is higher than 5000, the sensitivity and specificity to distinguish between patients with severe SRs vs. mild and/or no SRs is about 80%. These findings provide further evidence for the concept that the individual basophil allergen sensitivity might be a very important predictor for the severity of allergic reactions. In addition, it was recently suggested that distinct parameters of the basophil CD63 activation test reflect the severity and threshold of allergic reactions to peanut [19].

The importance of basophil response for side-effects during VIT build-up was already suggested previously [10, 11]. However, in previous reports, we did not evaluate the severity of adverse reactions, and cellular sensitivity was estimated only by the comparison of the response between two log allergen concentrations.

It is important that in the current report, we applied a standard WAO classification system for grading SCIT-associated SRs [15]. From the clinical end-point, this was a major step as we upgraded previous observations by demonstrating that the increased basophil sensitivity is associated only with the severe SRs. Next, from the laboratory point of view, a major step forward was a successful application of the metrics of honeybee allergen CD63 dose-response curve and we strongly believe that this basophil phenotyping is robust enough for possible daily clinical routine use.

The factors that were also associated with severe SRs during the build-up phase of honeybee VIT were a short time interval between a field sting and the onset of symptoms and low sIgE to mayor honeybee venom allergen, rApi m1. The latency has not yet been described as a factor associated with SRs; however, it was previously associated with the severity of anaphylaxis [14]. The significance of low sIgE to rApi m1 was a surprising observation because previous reports did not find any correlation between SR and specific or total IgEs [26, 27]. Nevertheless, a recent study identified additional major honeybee allergens, such as Api m3 and Api m10, and possibly sensitizations to those novel allergens might be important for SRs during the build-up phase of honeybee VIT [28]. Furthermore, rApi m1 was positive only in 67% of honeybee venom-allergic patients, and thus, its diagnostic sensitivity was similarly limited as demonstrated previously [28, 29]. Although both the latency time and rApi m1 showed significant association with SRs, they both did not show any significance in logistic regression analysis and thus seem not to be independent predictors of SRs. What is also important to stress is that higher levels of allergen-specific IgE were not associated with SRs. Moreover, stepwise logistic regression analysis even suggested that low sIgE to whole venom might be important for SRs, but this should be further confirmed.

None of the other immunological parameters examined (baseline serum tryptase, SPT, or IDT), patient-specific factors (age, sex, cardiovascular conditions and medications, and other allergic diseases), or other details of the honeybee field sting-induced anaphylaxis (anaphylaxis severity, absence of cutaneous symptoms, and head or neck sting) were not associated with SRs during the build-up phase of honeybee VIT. For basal tryptase, it was previously shown that it is significantly associated with SRs during the build-up phase of wasp VIT [4], but neither the previous nor the current report was able to show any importance of baseline serum tryptase for SRs during honeybee VIT. Another significant factor previously observed was the long interval that had passed since the most recent sting anaphylactic reaction [4]. This factor was not

applicable in our study because a large majority of our patients started immunotherapy within 1 year after the sting reaction.

In five of our patients, ultra-rush VIT build-up was stopped due to the SRs. This was not an unexpected observation as some of those individuals showed the highest basophil sensitivities, and also overall in great majority of severe SRs patients, the sensitivity was indeed very high, for example, the median basophil *in vitro* response to very low 0.01 µg/mL of allergen concentration in the severe SRs group was 42% in comparison with 7% in the mild SRs group and 9% in the no SRs group. Furthermore, in four patients, the treatment was restarted with a conventional slower dose increase, lasting up to 3 months. Yet in three patients, the slower types of build-up schedules also induced SRs, and consequently, the treatment was again stopped. For such difficult cases, the anti-IgE pretreatment might be beneficial because some reports suggested that their use may decrease the incidence of severe SRs [30]. Moreover, it was previously suggested that already after 1 week after starting anti-IgE treatment, a significant and clinically relevant decrease in allergen basophil sensitivity became evident and that after 2 months also the decrease in mast cell response became evident [31]. Future research to clarify the mechanisms leading to high basophil sensitivity is also needed.

A complicated course of VIT (i.e. SRs during VIT) is beside increased baseline tryptase [32, 33], an important risk factor for relapse. Thus, we can speculate that an increased basophil allergen sensitivity, which was shown to be a major risk factor for severe SRs during build-up of honeybee VIT, might also be associated with increased risk of relapse after completing VIT. Such assumptions are supported by a recent long-term sting challenge study that showed that a decrease in basophil sensitivity is associated with the induction of tolerance after VIT, and that in case of failed VIT, the basophil sensitivity remains high [9]. Another report, which cross-sectionally monitored the response to field re-sting after VIT discontinuation, also demonstrated that increased sensitivity is associated with treatment failure [34]. We can speculate at this point that, if the initial allergen sensitivity is very high, the VIT treatment might not change allergen sensitivity to achieve long-term protection. Obviously, further studies, which would first accurately evaluate cellular sensitivity and then monitor the patients longitudinally during and after VIT, are obviously needed to clarify these assumptions.

We conclude that high basophil allergen sensitivity is a major factor associated with severe adverse SRs during build-up phase of honeybee VIT or even build-up stop. Measurement of basophil CD63 allergen

response should be helpful in identifying patients with a high risk for severe adverse SRs during the build-up phase of honeybee VIT.

Conflict of interests

The authors declare no conflict of interest.

References

- Lockey RF, Turkeltaub PC, Olive ES, Hubbard JM, Baird-Warren IA, Bukantz SC. The Hymenoptera venom study III: safety of venom immunotherapy. *J Allergy Clin Immunol* 1990; **86**: 775–80.
- Mueller U, Helbling A, Berchtold E. Immunotherapy with honeybee venom and yellow jacket venom is different regarding efficacy and safety. *J Allergy Clin Immunol* 1992; **89**:529–35.
- Mosbech H, Mueller U. Side-effects of insect venom immunotherapy: results from an EAACI multicenter study. European Academy of Allergology and Clinical Immunology. *Allergy* 2000; **55**:1005–10.
- Rueff F, Przybilla B, Bilo MB *et al*. Predictors of side effects during the build-up phase of venom immunotherapy for Hymenoptera venom allergy: the importance of baseline serum tryptase. *J Allergy Clin Immunol* 2010; **126**:105–11, e5.
- Knol EF, Mul FPJ, Jansen H, Calafat J, Roos D. Monitoring human basophil activation via CD63 monoclonal antibody 435. *J Allergy Clin Immunol* 1991; **88**:328–38.
- Korošec P, Eržen R, Šilar M, Bajrović N, Kopač P, Košnik M. Basophil responsiveness in patients with insect stinging allergies and negative venom-specific immunoglobulin E and skin prick test results. *Clin Exp Allergy* 2009; **39**:1730–7.
- Korošec P, Šilar M, Eržen R *et al*. Clinical routine utility of basophil activation testing for diagnosis of hymenoptera-allergic patients with emphasis on individuals with negative venom-specific IgE antibodies. *Int Arch Allergy Immunol* 2013; **161**:363–8.
- Eberlein B, Krischan L, Darsow U, Ollert M, Ring J. Double positivity to bee and wasp venom: improved diagnostic procedure by recombinant allergen-based IgE testing and basophil activation test including data about cross-reactive carbohydrate determinants. *J Allergy Clin Immunol* 2012; **130**:155–61.
- Eržen R, Košnik M, Šilar M, Korošec P. Basophil response and the induction of a tolerance in venom immunotherapy: a long-term sting challenge study. *Allergy* 2012; **67**:822–30.
- Žitnik SE, Vesel T, Avčin T, Šilar M, Košnik M, Korošec P. Monitoring honeybee venom immunotherapy in children with the basophil activation test. *Pediatr Allergy Immunol* 2012; **23**:166–72.
- Košnik M, Šilar M, Bajrović N, Mušič E, Korošec P. High sensitivity of basophils predicts side-effects in venom immunotherapy. *Allergy* 2005; **60**:1401–6.
- Eberlein-König B, Schmidt-Leidescher C, Behrendt H, Ring J. Predicting side-effects in venom immunotherapy by basophil activation? *Allergy* 2006; **61**:897.
- Čelesnik N, Vesel T, Rijavec M *et al*. Short-term venom immunotherapy induces desensitization of FcεRI-mediated basophil response. *Allergy* 2012; **67**:1594–600.
- Bonifazi F, Jutel M, Bilo BM, Birnbaum J, Muller U, EAACI Interest Group on Insect Venom Hypersensitivity. Prevention and treatment of hymenoptera venom allergy: guidelines for clinical practice. *Allergy* 2005; **60**:1459–70.
- Cox L, Larenas-Linnemann D, Lockey RF, Passalacqua G. Speaking the same language: the World Allergy Organization subcutaneous immunotherapy systemic reaction grading system. *J Allergy Clin Immunol* 2010; **125**:569–74, 574.e1–574.e7.
- Kemp SF, Lockey RF, Simons FE. Epinephrine: the drug of choice for anaphylaxis: a statement of the World Allergy Organization. *Allergy* 2008; **63**:1061–70.
- Ring J, Meßmer K. Incidence and severity of anaphylactoid reactions to colloid volume substitutes. *Lancet* 1977; **1**:466–9.
- Čelesnik Smodiš N, Šilar M, Eržen R, Rijavec M, Košnik M, Korošec P. Down-regulation of FcεRI-mediated CD63 basophil response during short-term VIT determined venom-nonspecific desensitization. *PLoS ONE* 2014; **9**:e94762.
- Santos AF, Du Toit G, Douiri A *et al*. Distinct parameters of the basophil activation test reflect the severity and threshold of allergic reactions to peanut. *J Allergy Clin Immunol* 2015; **135**:179–86.
- Johansson SG, Nopp A, van Hage M *et al*. Passive IgE-sensitization by blood transfusion. *Allergy* 2005; **60**:1192–9.
- Firth D. Bias reduction of maximum likelihood estimates. *Biometrika* 1993; **80**:27–38.
- Heinze G, Ploner M, Dunkler D, Southworth H. *Logistf: Firth's bias reduced logistic regression. R package version 1.2.1*. Vienna: Medical University of Vienna, 2013.
- Nopp A, Johansson SG, Ankerst J *et al*. Basophil allergen threshold sensitivity: a useful approach to anti-IgE treatment efficacy evaluation. *Allergy* 2006; **61**:298–302.
- Nopp A, Cardell LO, Johansson SG, Oman H. CD-sens: a biological measure of immunological changes stimulated by ASIT. *Allergy* 2009; **64**:811–4.
- Lalek N, Košnik M, Šilar M, Korošec P. Immunoglobulin G-dependent changes in basophil allergen threshold sensitivity during birch pollen immunotherapy. *Clin Exp Allergy* 2010; **40**:1186–93.
- Birnbaum J, Ramadour M, Magnan A, Vervloet D. Hymenoptera ultra-rush venom immunotherapy (210 min): a safety study and risk factors. *Clin Exp Allergy* 2003; **33**:58–64.
- Roll A, Hofbauer G, Ballmer-Weber BK, Schmid-Grendelmeier P. Safety of specific immunotherapy using a four-hour ultra-rush induction scheme in bee and wasp allergy. *J Investig Allergol Clin Immunol* 2006; **16**:79–85.
- Köhler J, Blank S, Müller S *et al*. Component resolution reveals additional major allergens in patients with honeybee venom allergy. *J Allergy Clin Immunol* 2014; **133**:1383–9.
- Korošec P, Valenta R, Mittermann I *et al*. Low sensitivity of commercially available rApi m 1 for diagnosis of honeybee venom allergy. *J Allergy Clin Immunol* 2011; **128**:671–3.
- Galera C, Soohun N, Zankar N, Caimmi S, Gallen C, Demoly P. Severe anaphylaxis to bee venom immunotherapy: efficacy of pretreatment and concur-

- rent treatment with omalizumab. *J Investig Allergol Clin Immunol* 2009; **19**:225–9.
- 31 Eckman JA, Sterba PM, Kelly D *et al*. Effects of omalizumab on basophil and mast cell responses using an intranasal cat allergen challenge. *J Allergy Clin Immunol* 2010; **125**:889–895.e7.
- 32 Rueff F, Przybilla B, Bilo MB *et al*. Predictors of severe systemic anaphylactic reactions in patients with Hymenoptera venom allergy: importance of baseline serum tryptase—a study of the European Academy of Allergology and Clinical Immunology Interest Group on Insect Venom Hypersensitivity. *J Allergy Clin Immunol* 2009; **124**:1047–54.
- 33 Niedoszytko M, de Monchy J, van Doormaal JJ, Jassem E, Oude Elberink JN. Mastocytosis and insect venom allergy: diagnosis, safety and efficacy of venom immunotherapy. *Allergy* 2009; **64**:1237–45.
- 34 Peternelj A, Silar M, Erzen R, Kosnik M, Korosec P. Basophil sensitivity in patients not responding to venom immunotherapy. *Int Arch Allergy Immunol* 2008; **146**:248–54.

Supporting Information

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

Data S1. Ultra-rush honeybee venom build-up immunotherapy protocol.

Data S1. Basophil activation test.

Table S1. Cox WAO subcutaneous immunotherapy systemic reaction grading system¹⁵.

Table S2. Classification of severity of the anaphylactic reaction after a honeybee field sting according to Ring and Meßmer¹⁷.

Table S3. BAT response to control stimuli.