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Allergens in Hymenoptera Venom

XIX. Allergy to *Vespa crabro*, the European Hornet

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Abstract. The European hornet, *Vespa crabro*, is a very large insect native to much of Europe and Asia and introduced into eastern North America. Four cases of allergic reactions to *V. crabro* stings are presented, one of which was fatal. *V. crabro* venom contains 2% protein and peptide. There are three major proteins: Phospholipase AB, antigen 5 and hyaluronidase. These proteins are structurally and antigenically related to those from other vespid wasps, especially *Vespula* yellow jackets. IgE antibodies from patients allergic to *Vespula* usually cross-react with *V. crabro* venom. In three of the four patients with known reactions to *V. crabro* venom antigen 5 appeared to be the most important allergen. Serum from one patient was most reactive with hyaluronidase. Phospholipase had relatively little IgE-binding activity, although it was the major protein in the venom. *V. crabro* venom is at least as closely related to *Vespula* venoms as *Dolichovespula* venoms are.

Introduction

Vespa crabro, the European hornet, is a very large species of wasp found in continental Europe, much of Asia and in eastern North America from Ontario south to Georgia and northern Alabama and west to the Dakotas [1]. *V. crabro* was introduced to North America accidentally in the 1850's, somewhere in the northeast coast, and has slowly spread to its present limits. It is easily recognizable by its large size, as shown in figure 1, and by its distinctive brown, orange and red colors. In the Old World it can be confused with queens of *Dolichovespula media*, which are very similar in pattern, although generally smaller in size. *V. crabro* generally makes aerial nests in very sheltered locations. Usually the nests are in a hollow tree or a similar location, in which cases there is usually no envelope. The combs removed from a nest in a hollow tree are illustrated in figure 2. When the nest is exposed, it is covered with brownish paper with an opening at the bottom. An exposed nest found in a tobacco barn is shown in figure 3.

In 1985 we reported on a death from *V. crabro* stings in North Carolina [2]. These hornets are generally not aggressive and only sting when provoked or

when their nest is threatened. We have encountered only a handful of reports of allergic reactions from *V. crabro* in the United States. In southern Europe reactions from *V. crabro* stings are more common. The three nonfatal cases of allergic reactions from *V. crabro* stings discussed in this paper were all from the area around Florence, Italy.

In addition to the rare cases of allergic reactions from *V. crabro* venom, this insect is also important because it allows the expansion of our basic knowledge of insect venom components and allergenic cross-reactivity of venom proteins. We have previously reported that *V. crabro* venom cross-reacts with *Vespula* (yellow jacket) venom using sera from patients who are from areas where *V. crabro* is not found [3]. *Vespa* is one of the four genera of Vespinae which also include *Vespula*, *Dolichovespula* and *Provespa*. In addition to *V. crabro* there are a large number of other species in this genus, several of which like *Vespa velutina*, which is found in east Asia, are known to be highly aggressive. The large size of *V. crabro* allows one to collect milliliter amounts of pure liquid venom for chemical analysis.

Argiolas and Pisano [4] have isolated two interesting peptides, mastoparan C and crabolin from *V. crabro* venom. Wheeler et al. [5] recently reported on the characterization of some semiterpenoid esters found

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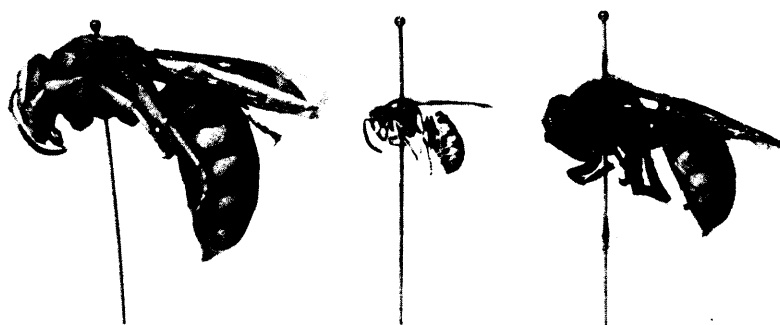


Fig. 1. European hornets, *V. crabro*. The largest insect on the left is a queen and the insect on the right is a worker. The small insect in the center is a *V. maculifrons*, yellow jacket, worker, to illustrate the large size of *V. crabro*. The color of *V. crabro* is usually light orange and brown, with red sometimes visible.

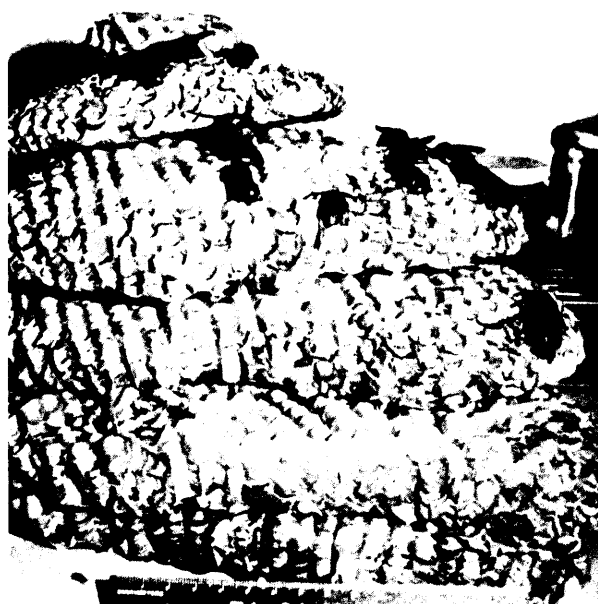


Fig. 2. Combs removed from a *V. crabro* nest found in a hollow tree. The number of cells reflects the maximum capacity for a single brood. The larger cells develop males and queens. A number of capped cells with pupae are easily visible. Cream-colored larvae are found in the rows of cells outside the capped cells.

in *V. crabro* venom. The venom also appears to contain a presynaptically acting neurotoxin analogous to those described from *Vespa mandarinia* [6] and *Vespa orientalis* [7].

In this study we describe the isolation of protein components from *V. crabro* venom, identify the protein components, and study their reactivity with IgE antibodies in sera from *V. crabro*-allergic patients. In addition we report on the cross-reactivity of *V. crabro* venom with *Vespula* venom and on the structural analogies among proteins from *V. crabro*, *Vespula* and *Dolichovespula* venoms.



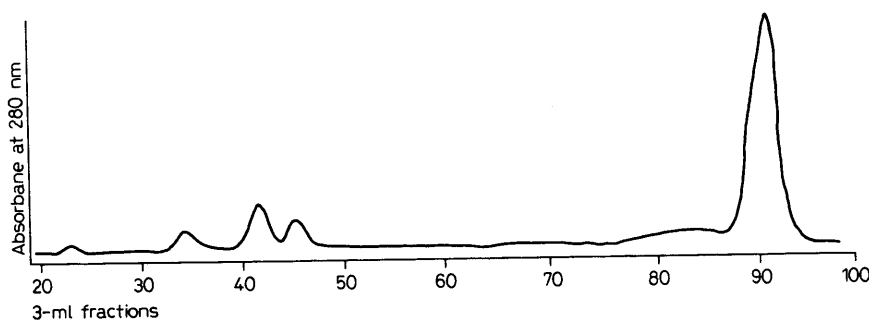
Fig. 3. Photo of an exposed nest of *V. crabro* found in a tobacco barn in eastern North Carolina. Note the paper envelope and the entrance on the bottom. The paper is much more brownish than that of *Dolichovespula* hornets.

Materials and Methods

Case Histories

Case No. 13604. A 47-year-old male senior engineer for an electric power company was stung by European hornets while spraying bushes in his yard in Cary, North Carolina, on July 17, 1984. He

Fig. 4. Gel filtration profile of *V. crabro* venom on a 1.6×160 cm column of Sephadex G-75 superfine. 50 μ l was applied in this experiment, giving a maximum absorbance of 1.66 at 280 nm (fraction 91). An identical separation was obtained for the 1.5-ml preparative run. The column is the same as was used by Hoffman [12]. The eluting buffer was 0.1 M ammonium formate, pH 5.0.



called the rescue squad and was found unconscious when they arrived 5 min later. He was noted to have swollen arms and legs and had great difficulty breathing. On the way to the hospital he stopped breathing and required cardiopulmonary resuscitation. He never regained consciousness and expired 5 days later. The victim positively identified the insect in his call to the rescue squad. He did not have a history of previous systemic reactions.

Case No. 3284. A 17-year-old Italian male was stung by *V. crabro* in September 1985. After 30 min he experienced generalized urticaria and intense itching and swelling of his face. He had no other history of reactions to hymenoptera stings. He was skin-test-positive to *V. crabro* at 0.01 μ g/ml and to *Vespula* at 0.1 μ g/ml. His leukocytes released histamine on exposure to *V. crabro* venom.

Case No. 3828. A 42-year-old Italian female had experienced several large local reactions following stings of various bees and wasps. In October 1984 she developed a large local reaction from a *V. crabro* sting. Intradermal skin tests were positive to *V. crabro* at 0.01 μ g/ml, *Vespula* at 0.01 μ g/ml and *Polistes* at 0.1 μ g/ml. Skin tests were negative to bee.

Case No. 3388. A 29-year-old Italian male experienced several systemic reactions to stings from wasps and bees. In September 1985 he was stung by *V. crabro* and suffered a generalized reaction that was primarily cutaneous. Intradermal skin tests were positive to *V. crabro* at 0.01 μ g/ml, bee at 0.1 μ g/ml and to *Vespula* and *Polistes* at 1 μ g/ml.

Venom Collection

Nests of *V. crabro* were collected in Eastern North Carolina and brought back to the laboratory. After chilling in a cold room at 4°C the insects were separated into mature workers, immature workers (teneral), queens and males. All studies were performed with mature worker venom. The insects were then frozen. Immediately after thawing the venom sacs were removed from the bodies, and then the venom was expressed into 1.5-ml centrifuge tubes by gently squeezing the sac. The tubes of venom were stored frozen. *V. crabro* venom sac extract was purchased from Vespa Laboratories, Division of Pharmacia (Spring Mills, Pa.). The Italian patients were skin-tested with venom and sac extracts from the United States.

Radioallergosorbent Test and Inhibition Tests

Radioallergosorbent test (RAST) testing was performed by the methods we have previously described [8]. RAST disks were coupled to purified allergens at limiting concentration to minimize effects of impurities [9, 10]. RAST inhibition tests were performed as we have previously described [11].

Isolation and Purification of Venom Allergens

V. crabro venom was separated into fractions by gel filtration on a 1.6×160 cm column of Sephadex G-75® superfine, as we have described for other vespid venoms [12].

The fractions were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme activity tests before pooling. The major pools were further purified by cation-exchange chromatography on a Pharmacia Mono-S column (Pharmacia, Piscataway, N.J.) and gel filtration on Sephadex G-75 [12]. Final purities were established by enzyme assays and SDS-PAGE.

Protein and Enzyme Analysis

Concentrations of protein in venom and fractions were determined by the dye-binding assay that we have previously reported [13]. Hyaluronidase, phospholipase A and B and proteases were all measured using the methods we have previously described [14].

Limited Hydrolysis Peptide Maps

Proteins isolated from *V. crabro* venom were compared to the corresponding proteins from other vespid venom by limited hydrolysis peptide mapping with digestion by trypsin, chymotrypsin, staphylococcal protease V8 and cyanogen bromide using the methods we have described for vespid phospholipases [15].

SDS-PAGE

SDS-PAGE was performed in minigels of 10% total acrylamide concentration and 2.8% cross-linker, as previously described [12]. Peptide map gels were 15% total acrylamide concentration and 2.8% cross-linker. Gels were stained with Rapid-Ag silver stain purchased from ICN Radiochemicals (Irvine, Calif.).

Results

V. crabro venom was obtained from workers removed from five nests collected during a single season; two of the nests are illustrated in figures 2 and 3. The venom from mature workers was found to have a protein content of 2.05% and that from teneral workers to contain 0.8% protein by dye-binding assay. By similar assays honey bee venom has a protein content of 40–60%. No data on the protein content of liquid

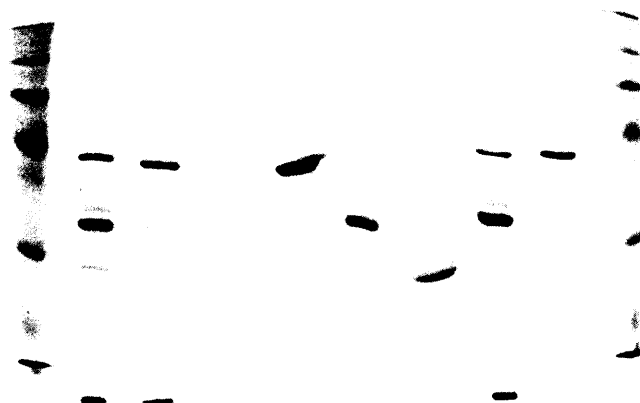


Fig. 5. SDS-PAGE of *V. crabro* venom and its purified fractions. The two end patterns are molecular weight markers at 97,400, 68,000, 43,000, 25,700, 18,400 and 14,300 daltons. Lanes 2 and 8 contain 0.025 μ l of venom from adult workers; lanes 3 and 9 contain 0.05 μ l of venom from teneral workers. The purified fractions are contained in the center lanes: lane 4, G-75 exclusion peak; lane 5, hyaluronidase; lane 6, phospholipase, and lane 7, antigen 5. The gel was stained with ICN Rapid-Ag stain.

Table I. Enzyme contents and yields of *V. crabro* venom fractions after gel filtration, cation-exchange chromatography and a second gel filtration

Fraction	Hyaluronidase, %	Phospholipase, %	Protein, mg	Venom protein, %
G-75 exclusion	0.04	0.03	0.52	1.7
Hyaluronidase	100	< 0.01	0.82	2.7
Phospholipase	0.01	100	6.76	22.0
Antigen 5	< 0.01	< 0.01	1.54	5.0
Starting venom protein and peptide			30.75	

venom have been published for *Vespula* and *Dolichovespula* species because of the small amounts of liquid venom normally obtained. *V. crabro* venom was found to contain hyaluronidase and phospholipase A and B activities, but several tests for protease were all negative.

The *V. crabro* venom was separated on a Sephadex G-75 column, as shown in figure 4. This is the same column as was used for separation of the other vespid venoms that we have reported [12]. Three major protein peaks were found: one containing hyaluronidase in fractions 34–39, one containing phospholipase A and B in fractions 40–45 and one at fractions 46–49 which contained a protein that exhibited cross-reactivity on nitrocellulose membrane with rabbit antisera to *Vespula* antigen 5. A small exclusion peak was present at fractions 22–28 as well as at lower molecular weight peaks. The three major protein peaks were each further purified by ion-exchange chromatography and gel filtration. SDS-PAGE of the venom and purified fractions is shown in figure 5. The yields and purities of the fractions are given in table I.

RAST disks were prepared from the pure *V. crabro* venom and the three purified fractions; and sera from

the four patients with *V. crabro* allergy described in Materials and Methods were tested for IgE-binding activity. The results are shown in figure 6. All of the sera had higher binding to *V. crabro* venom than to *Vespula* venom. The three patients who were skin-tested reacted with *V. crabro* venom at a lower concentration than they did with *Vespula* venom. Three of the sera reacted most strongly with antigen 5, which was identified by molecular weight and antigenic cross-reactivity with *Vespula maculifrons* antigen 5. One serum reacted most strongly with hyaluronidase. All of the reactions with phospholipase were minimal.

Cross-reactivity of *V. crabro* and *Vespula* venoms was studied using sera from yellow-jacket-allergic patients, since sufficient quantities of sera were not available from the four *V. crabro*-allergic patients. We previously reported on the results of testing a panel of 39 *Vespula*-allergic sera to *V. crabro* venom sac extract [3]. Only 2 of the sera were RAST-negative to *V. crabro*. The mean percent specific binding $\pm$ standard deviation was 16.7 ± 6.2 to *V. maculifrons*, 13.2 ± 8.4 to *Vespula squamosa* and 7.2 ± 6.0 to *V. crabro*. The linear correlation coefficient between *V. maculifrons*

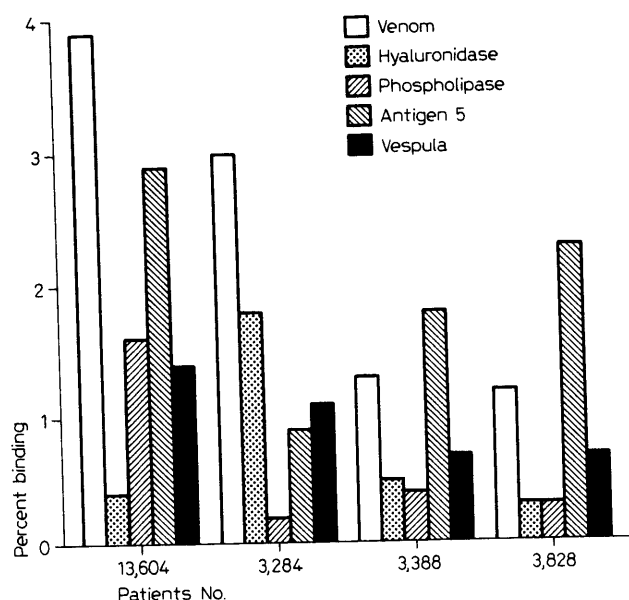


Fig. 6. RAST results with *V. crabro* and *Vespula* venoms and with purified antigens from *V. crabro* venom. The sera are from the four patients who experienced allergic reactions to *V. crabro* stings. Results are expressed as percent binding after subtraction of nonspecific binding for each kind of coupled disk. Values of greater than 0.7% would be scored as positive.

Table II. Inhibition of RAST to *V. crabro* by *V. maculifrons*

Serum, no.	Concentration for 50% inhibition, $\mu\text{g}/\text{ml}$
2762	25
2788	2.0
2794	0.5
3526	8.0
3644	20
4092	<0.3
5673	34
5675	0.9
5747	30
6738	10

V. maculifrons homologous inhibition: 0.1–0.9; *V. squamosa* by *V. maculifrons*: 0.1–28; *V. maculifrons* by *V. squamosa*: 0.9–>100.

and *V. crabro* was 0.53, not statistically significant. RAST inhibition was performed with 10 individual sera. The results are presented in table II. The concentrations of *V. maculifrons* venom sac extract that gave 50% inhibition of binding to *V. crabro* disks ranged from less than 0.3 to 30 $\mu\text{g}/\text{ml}$. For comparison using the same sera homologous inhibition of *V. maculif-*

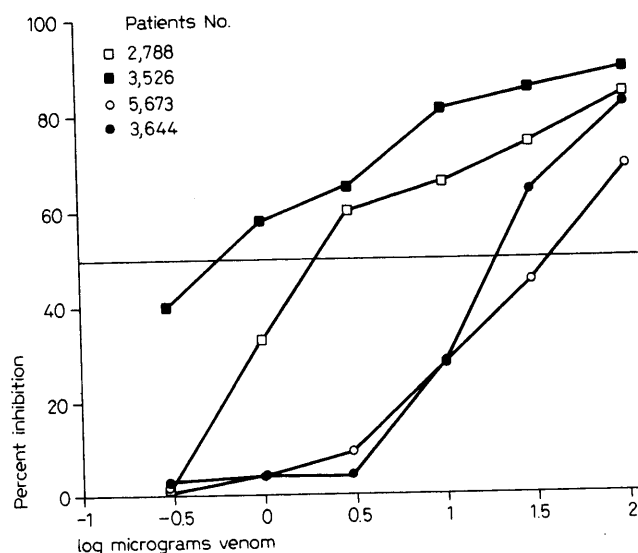


Fig. 7. Inhibition of IgE antibody binding to *V. crabro* venom by *V. maculifrons* venom for four typical sera from patients allergic to *Vespula*. Patients No. 2788 and 5673 were natives of California, an area in which *V. crabro* is not known. The heavy line marks 50% inhibition.

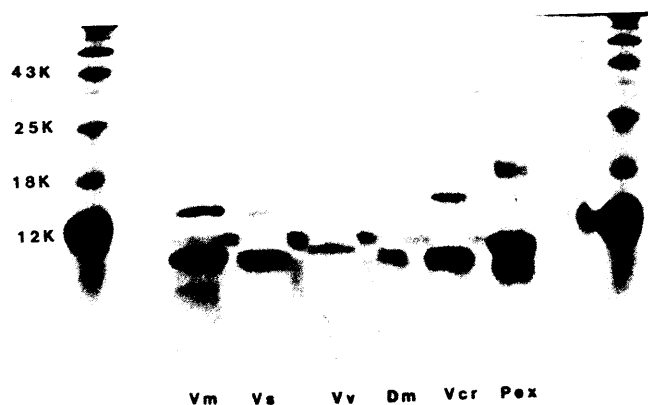


Fig. 8. SDS-PAGE gel of the cyanogen bromide digest fragments of antigen 5 isolated from venoms of vespid wasps. The molecular weight markers are labeled on the photograph. Species are: Vm = *V. maculifrons*; Vs = *V. squamosa*; Vv = *Vespula vidua*; Vcr = *V. crabro*; Dm = *D. maculata*; Pex = *Polistes exclamans*.

rons binding required concentrations of 0.1–0.9 $\mu\text{g}/\text{ml}$. Inhibition curves for four of the sera tested are shown in figure 7. Sera No. 2788 and 3526 are highly cross-reactive and sera No. 5673 and 3644 are less cross-reactive.

Structural homologies between *V. crabro* venom proteins and those from other vespid wasp venoms

were investigated by limited hydrolysis peptide mapping. The results for phospholipase were previously illustrated [15]. Digestions by cyanogen bromide, chymotrypsin and trypsin all showed significant homologies between *V. crabro* and *V. maculifrons* phospholipases. The antigen 5 proteins exhibited a high degree of structural homology, as shown in figure 8, and the hyaluronidases were also closely related. The *V. crabro* venom proteins were all reactive with hyperimmune rabbit antisera raised against purified *V. maculifrons* venom proteins [14] in immunoblot tests.

Discussion

The giant European hornet, *V. crabro*, can cause severe allergic reactions in man. Of the four cases of *V. crabro* venom allergy described in this paper, one was fatal and another life-threatening. There is one other report in the literature of death following *V. crabro* stings [16]; the patient died with hyperfibrinolysis after multiple stings. The victim of the fatal reaction in this paper did not have a previous history of *V. crabro* stings, but, according to his wife, may have had a yellow jacket allergy. Two of the three cases of *V. crabro* allergy that we report from Italy had previous histories of reactions from other vespid stings.

V. crabro venom contains about 2% protein and large peptide that can bind dye. Three major proteins were isolated: hyaluronidase, phospholipase AB, and antigen 5. These proteins are of comparable molecular weights to those isolated from other vespid venoms [13, 14, 17]. The *V. crabro* proteins demonstrated antigenic cross-reactivity with homologous vespid proteins in immunoblot studies, and were structurally homologous to the corresponding proteins from other vespids by limited hydrolysis peptide mapping.

All but 2 of 39 sera from yellow-jacket-allergic patients demonstrated significant IgE antibody binding to *V. crabro* venom. Fourteen of these positive sera were from patients who resided in areas where *V. crabro* is not found. *V. maculifrons* venom could inhibit RAST to *V. crabro* venom at concentrations similar to those needed to inhibit binding to *V. squamosa* venom [3]. As can be seen in figure 7, maximum inhibition is over 80% at 100 µg/ml for three of the four sera illustrated. It appears that the allergens in *V. crabro* venom are closely related to those in *Vespula* venom, causing a high degree of cross-reactivity, as is

also seen with *Dolichovespula* venom [11, 18–21]. In the case of *Dolichovespula* hornets it is probable that some instances of clinical reactivity are due to cross-reactive antibodies induced by *Vespula* stings. The three genera of Vespinae that have been studied all appear to have venom proteins that are closely related. *Polistes* belong to a related subfamily of Vespidae, Polistinae. Venom proteins from *Polistes* are less closely related to those of *Vespula*, both structurally [15] and allergenically [11, 18]. Using venom sac extracts, Mueller et al. [22] investigated the cross-reactivity of *Dolichovespula maculata*, *Dolichovespula arenaria*, *V. crabro* and *V. orientalis*. They found a high degree of cross-reactivity between the *Dolichovespula* hornet venoms and *V. crabro* venom using both hyperimmune rabbit antisera and sera from allergic patients. The two *Vespa* species also showed strong cross-reactivity. The venom sac extract from *V. orientalis*, however, did show significant differences with *Dolichovespula* venoms.

Some biochemical and pharmacologic studies have been carried out on substances isolated from *V. crabro* venom. Two biologically active peptides, mastoparan C, which degranulates mast cells, and crabrolin, which facilitates phospholipase activity, have been isolated and sequenced [4]. The structures of some of the low-molecular weight esters found in *V. crabro* venom have been determined [5]. Two groups have isolated presynaptic neurotoxins from the venoms of the related species *V. mandarinia* [6] and *V. orientalis* [7]. These materials are proteins which appear to correspond in size and properties with antigen 5. A number of other studies have been reported with venom sac extract and/or venom (not usually clear from the papers) of related species, especially *V. orientalis* [reviewed in ref. 23]. Substances isolated from *V. mandarinia* include a bradykinin analogue, vespakinin M [24], and a mast-cell-degranulating peptide, mastoparan M [25]. In 1961, a kinin-like activity was reported in *V. crabro* venom along with the presence of acetylcholine, 5-hydroxytryptamine and histamine [26].

V. crabro is found in much of Europe and Asia, as well as having been introduced into eastern North America. Although it is generally not an aggressive species, it will sting man when provoked or when the nest is threatened. It can cause allergic reactions either by direct sensitization by previous *V. crabro* stings or by cross-reactions with *Vespula* yellow jacket venom and probably also with *Dolichovespula* hornet venom.

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