

# Occupational Allergy to Aquarium Fish Food: Red Midge Larva, Freshwater Shrimp, and Earthworm. A Clinical and Immunological Study

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## ■ Abstract

**Background:** Chironomids seem to be the main cause of occupational allergy to aquarium fish food.

**Objective:** The aim of this study was to investigate the pattern of occupational sensitization to 3 different arthropod species used as components of aquarium fish food.

**Methods:** The study sample comprised 8 workers from a fish food packing department. The control group comprised 40 atopic patients (20 of whom were allergic to mites). We performed prick tests with extracts of red midge larva (*Chironomus thummi*), freshwater shrimp (*Gammarus* species), earthworm (*Tubifex* species), and other arthropod species and a battery of common inhalant allergens. We measured peak expiratory flow rate (PEFR) and specific immunoglobulin (Ig) E and performed a methacholine challenge test, nasal challenge test, and immunoblotting. Cross-reactivity analyses were completed using immunoblotting and CAP inhibition.

**Results:** Prick test results were positive to red midge larvae in 7 patients (87.5%), *Gammarus* in 5 (62.5%), *Tubifex* in 3 (37.5%), and mites in 6 (75%). In the mite-allergic controls, 30% had positive prick test results to red midge larvae. PEFR decreased  $\geq 20\%$  during the packing process in all patients, and in 1 patient it indicated a dual asthmatic response. Methacholine challenge test results were positive in all participants. Nasal challenge tests were performed in 4 patients, and the results were positive. Specific IgE to red midge larvae was detected in 62.5%, *Gammarus* in 50%, and *Tubifex* in 16%. Bands of approximately 14-15 kDa and 31 kDa were observed in *Gammarus* and red midge larvae extracts. Cross-reactivity assays demonstrated that *Gammarus* totally inhibited red midge larvae, while *Tubifex* did so partially. *Dermatophagoides pteronyssinus* showed very low inhibitory capacity.

**Conclusions:** Aquarium fish food arthropods are potent allergens with an elevated prevalence of sensitization and variable degree of cross-reactivity. This is the first report of occupational allergy to *Tubifex*. More data are necessary to identify and characterize the responsible allergens.

**Key words:** Chironomids. *Gammarus*. *Tubifex*. Red midge larvae. Annelid. Fish food. Occupational allergy. Skin test. Cross-reactivity.

## ■ Resumen

**Introducción:** Los quironómidos parecen ser la principal causa de alergia ocupacional a la comida para peces de acuario.

**Objetivo:** El objetivo de este estudio fue investigar el patrón de sensibilización ocupacional a tres diferentes especies de artrópodos que componen la comida para peces de acuario en 8 de 10 trabajadores expuestos con rinitis y asma en el lugar del trabajo.

**Métodos:** Se realizaron prick-tests con extractos de *Chironomus thummi*, *Gammarus* y *Tubifex*, otras especies de artrópodos y batería de alérgenos inhalantes comunes. Cuarenta pacientes atópicos (20 de ellos alérgicos a ácaros) fueron utilizados como controles. Se realizaron mediciones de peak-flow, test de metacolina, pruebas de provocación nasal, determinación de IgE específica e inmunoblotting. El estudio de la reactividad cruzada se completó mediante inmunoblotting y CAP-inhibición.

**Resultados:** 87.5% de los pacientes presentaron prick-tests positivos a larva roja (*Chironomus thummi*), 62.5% a *Gammarus* y 37.5% a *Tubifex*. El prick-test fue también positivo a ácaros en el 75% de los pacientes. El 30% de los pacientes controles alérgicos a ácaros tuvieron prick-tests positivos a larva roja. El peak-flow cayó  $\geq 20\%$  en todos los pacientes durante el proceso de empaquetado. En un paciente el

peak-flow demostró una respuesta asmática dual. El test de metacolina fue positivo en todos. Las provocaciones nasales fueron realizadas en cuatro pacientes con resultados positivos. 62.5% de los pacientes tuvieron IgE específica positiva a larva roja, 50% a *Gammarus* y 16% a *Tubifex*. Se observaron bandas de 14-15 y 31 kDa en los extractos de *Gammarus* y larva roja. Las pruebas de reactividad cruzada demostraron que *Gammarus* inhibía totalmente a larva roja mientras que *Tubifex* solo lo hacía parcialmente y el extracto de *D. pteronyssinus* demostró muy baja capacidad de inhibición.

**Conclusiones:** Los artrópodos componentes de la comida para peces de acuario son potentes alérgenos con una elevada prevalencia de sensibilización. Presentan un grado variable de reactividad cruzada. Es el primer caso descrito de alergia ocupacional a *Tubifex*. Se necesitan más datos para identificar los alérgenos responsables y su naturaleza.

**Palabras clave:** Quironómidos. *Gammarus*. *Tubifex*. Larva roja. Anélidos. Comida para peces. Alergia ocupacional. Prick-test. Reactividad cruzada.

## Introduction

Aquarium fish food contains a variety of arthropod species including red midge larva (*Chironomus thummi*, of the Chironomidae family), freshwater shrimp (*Gammarus* species, of the Gammaridae family), and the annelid *Tubifex tubifex*, a tubificid segmented earthworm (Figure 1).

Sensitization to *Gammarus* species is rare [1-3], in contrast with sensitization to chironomids, which are found all over the world and in nearly all types of inland waters. Environmental allergy to chironomids is common in some parts of Asia (Korea, Japan) [4-6], Africa (Sudan, Egypt) [7,8], Europe (Sweden) [9], and America (Mexico). In Europe, however, most cases of allergy to chironomids appear in patients who handle chironomid larvae, either at work [10] or as a hobby [11]. Several routes of sensitization have been reported, including cross-reactivity with other arthropods such as mites, cockroaches, crustaceans, and nematodes (*Anisakis simplex*) [9,12-15].

The principal manifestations of allergy to chironomids are urticaria, angioedema, anaphylaxis, rhinoconjunctivitis, asthma, contact urticaria [16], and contact dermatitis [17]. Findings from previous studies suggest that chironomids contain potent inhalant allergens and are responsible for human respiratory allergy [18]. The major allergens of *Chironomus thummi* have been associated with hemoglobin present during the larval stage and are structurally similar to the hemoglobins present in crustaceans [8,19-21]. Adult *Chironomus thummi*

do not have hemoglobins, although other allergens have been reported in chironomids [22]. In fact, tropomyosin could be the panallergen responsible for cross-reactivity between chironomids, arthropods (mites, cockroaches, shrimps), and *Anisakis* [23,24].

The objectives of this study were to investigate allergenic sensitization in individuals who were occupationally exposed to *Chironomus thummi*, *Gammarus* species, and *Tubifex* species and to study the immunochemical characteristics of the allergen extracts obtained.

## Material and Methods

### Patient Population

Two patients who worked in the same company came to the allergy department with rhinitis and asthma after handling fish food. As the company was small, we decided to find out whether other employees were also affected.

The company was situated in San Pedro del Pinatar, Murcia, Spain, a small town in the southeast of Spain. We visited the company on a working day to experience the work environment in situ. The staff comprised 10 employees, all of whom packed fish food in small and poorly ventilated premises with no protection against direct and continuous inhalation of aquarium fish food dust. The study sample comprised 8 of the 10 workers because 2 did not have rhinitis or asthma. All 8



Figure 1. Aquarium fish food species.

Table 1. Patient Population

Patient	Age, y	Symptoms	Time Working, mo	Onset of Symptoms, mo
1	24	Rhinitis and asthma	36	6
2	24	Rhinitis and asthma	48	31
3	22	Rhinitis and asthma <sup>a</sup> + Contact urticaria <sup>b</sup>	14	4
4	28	Rhinitis and asthma	96	72
5	21	Rhinitis and asthma	26	9
6	18	Rhinitis and asthma	7	Before starting work at the company <sup>c</sup>
7	20	Rhinitis and asthma	16	3
8	21	Rhinitis and asthma	12	7

<sup>a</sup>Immediate and late asthma.

<sup>b</sup>Induced by *Gammarus*.

<sup>c</sup>Previous allergy to dust mite.

were nonsmoking males aged between 18 and 28 (Table 1) and they all reported symptoms of rhinoconjunctivitis (sneezing, itching, rhinorrhea, nasal congestion, and eye symptoms) and cough, wheezing, and shortness of breathing during the canning process. One patient (Table 1) also reported having asthmatic symptoms after he had finished work (possible late asthma or dual asthmatic response) and contact urticaria whenever he was handling *Gammarus* species. The workers handled 3 species of arthropods: red midge larvae (*Chironomus* species), freshwater shrimp (*Gammarus* species), and 1 earthworm (*Tubifex tubifex*). The patients had been working in the fish food store for 7 months to 7 years (mean, 30.3 months). The latency period until onset of the first symptoms ranged from 3 months to 6 years (mean, 17.8 months). The patients had no history of rhinitis or asthma, except for 1 patient, who was allergic to dust mite (Table 1). No additional comorbid conditions were reported.

Once approval for the study was received from the local ethics committee, oral consent was obtained and serum samples were taken.

### Extract Preparation

Dead red midge larvae, *Gammarus*, and *Tubifex* were supplied by the workers. Invertebrates were macroscopically examined under a stereoscope to detect any contamination by other organisms, such as mites. The samples were individually homogenized, diluted 1/20 in phosphate buffered saline (PBS) 0.01 M, and extracted overnight at 4°C under continuous magnetic stirring. Next, extracts were centrifuged at 16000g and the supernatant collected. After filtration, the extracts were dialyzed against bidistilled water, sterile filtered, frozen, and freeze-dried.

Absence of mite allergens (Der p 1) was confirmed using enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies (Indoor Biotechnologies).

The protein content of the extracts was measured using the Lowry-Biuret method (Bio-Rad).

Skin prick test solutions were prepared with each extract at a final concentration of 1 mg and 5 mg of frozen dried material per milliliter. Nasal challenge tests were prepared at 2 mg/mL after diluting freeze-dried material in nasal solution containing 0.9% saline solution and albumin.

### Skin Prick Tests

All the individuals underwent skin prick testing with 2 concentrations of extracts from the 3 species (1 and 5 mg/mL). The skin prick test result was considered positive when the wheal size was 3 mm or larger. Histamine dihydrochloride (10 mg/mL) and saline solution were used as positive and negative controls, respectively. The test battery used comprised standardized common inhalant allergens (including mites, molds, epithelia, pollens, mosquito, and cockroach), *Anisakis simplex*, and shrimp. The control group comprised 20 atopic patients not previously exposed to the 3 fish food species with a positive skin prick test result to mites. The controls also underwent skin prick testing with red midge larvae, *Gammarus*, and *Tubifex* extracts, as well as mosquito (*Culex* and *Aedes* species). Another 20 unexposed outpatients, who were only allergic to pollens and had a negative skin prick test result with mites, also underwent skin prick testing with extracts of red midge larvae, *Tubifex*, and *Gammarus*.

### Measurement of Peak Flow and Bronchial Hyperresponsiveness

All patients recorded morning and evening peak expiratory flow rates (PEFR) both at work and outside work. They were given instructions on how to perform the test and asked to make 3 attempts. The highest reading was recorded as the PEFR. Mini Wright peak flow meters were used (Clement Clarke International).

All patients underwent methacholine challenge tests with increasing concentrations of methacholine to confirm bronchial responsiveness. The tests were performed using a previously described protocol [25]. The procedure ended when the forced expiratory volume in the first second (FEV<sub>1</sub>) decreased by more than 20% of the postsaline value or when the highest methacholine concentration (25 mg/mL) was reached.

### Nasal Challenge Test

Nasal challenge tests were performed in 4 of the 8 individuals with positive skin prick test results to fish food. The other 4 patients refused to undergo the test. A nasal challenge test with red midge larvae and *Gammarus* extracts was conducted in 3 and 2 patients, respectively, while challenge with *Tubifex* extract was only performed in 1 patient with a large wheal and high levels of specific IgE.

Serial dilutions of extract (up to 1/10 000) were prepared. Increasing doses were administered every 30 minutes in alternate nostrils. Sneezing, pruritus, and nasal secretion were measured. Peak nasal inspiratory flow (PNIF) was analyzed in order to measure the extent of nasal congestion [26]. The challenge test was considered positive when there was a  $\geq 40\%$  decrease in PNIF [27] with respect to baseline and a positive clinical score was observed. The control group comprised 4 individuals with nonallergic rhinitis, 2 individuals with no rhinitis symptoms, and 8 individuals with allergic rhinitis to pollen, molds, or epithelia and negative skin prick test results with mites.

### Specific IgE Determination

Specific IgE against red midge larvae, *Gammarus*, and *Tubifex* extracts was measured using direct enzyme-linked immunosorbent assay. Briefly, 10  $\mu\text{g/mL}$  of protein was dissolved in carbonate/bicarbonate buffer (pH 9.6) and coated onto plastic microtiter plates (Immulon IV, Dynex Technologies). Each serum sample was diluted 1:2 vol/vol in 0.01 M PBS and incubated (100  $\mu\text{L}$ ) for 2 hours in the wells. The plates were washed and incubated for 2 hours with antihuman IgE conjugated with peroxidase. After 5 washes, the reaction was developed for 30 minutes and stopped with 1N

sulfuric acid. Three individual sera from nonallergic patients were used as negative controls. Optical densities  $\geq 3$  times the mean value of the negative control were considered positive.

### Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the protein profile of the extracts. Samples were run in electrophoresis gel with 2.67% C and 15% acrylamide as described by Laemmli [28]. Samples (100  $\mu\text{g}$  of protein) were diluted in sample buffer, denatured at 100°C for 10 minutes, and centrifuged for 1 minute at 10000 rpm; 200  $\mu\text{g}$  of freeze dried material of extracts 1, 2, and 3 were loaded in each lane. After electrophoresis, the gel was fixed for 45 minutes and stained with Coomassie (Bio-Rad).

### Allergen Profile

Separated bands of the 3 extracts were electrophoretically transferred to Immobilon-P membranes (Millipore). After transfer, the membrane was dried for 4 hours and incubated overnight with the 6 individual sera diluted in 0.01 M PBS Tween 2%. Specific IgE binding was detected with peroxidase-conjugated monoclonal antihuman IgE (Ingenasa). Immunoblots were visualized using chemiluminescence.

### Immunoblotting Inhibition

A specific pool of sera was prepared from aliquots of the 6 individual sera with positive specific IgE to the allergen extracts and stored at  $-20^\circ\text{C}$  for the inhibition studies.

Immunoblot inhibition was performed to identify the cross-reactive bands in the 3 extracts. Red midge larvae extract was used in the solid phase (100  $\mu\text{g}$  of protein), electrophoresed, and electro-transferred to an Immobilon-P membrane following the procedure described above (see *Allergen Profile*). *Tubifex* and *Gammarus* extracts and *Dermatophagoides pteronyssinus* (500  $\mu\text{g}$ , negative control) were used as inhibitors and incubated for 2 hours at room temperature with the serum pool (dilution 1/2). The inhibited sera were then added to the membranes and incubated overnight.

Table 2. Skin Prick Test and Specific IgE Results

Patients	Skin Prick Test								Specific IgE		
	Red midge larvae	<i>Tubifex</i>	<i>Gammarus</i>	<i>Anisakis</i>	<i>Aedes</i>	<i>Culex</i>	Shrimp	Mites	Red midge larvae	<i>Tubifex</i>	<i>Gammarus</i>
1	+	+	+	-	-	-	-	+	+	-	+
2	+	+	+	-	+	+	-	+	+	-	+
3	+	-	+	-	-	-	-	-	+	-	+
4	+	+	-	-	+	-	-	-	-	+	-
5	-	-	-	-	-	-	-	+	-	-	-
6	+	-	-	-	-	-	-	+	-	-	-
7	+	-	+	-	-	-	-	+	+	ND	-
8	+	-	+	-	-	-	-	+	+	ND	+

Abbreviations: Ig, immunoglobulin; ND, not determined.

### CAP Inhibition Experiments

Red midge larvae extract (700 µg) was previously labelled using a biotin kit (Roche Diagnostics) and used in the solid phase. Aliquots of 50 µL of biotin-labeled red midge larvae extract were incubated in streptavidin uniCAP disks (Phadia) for 30 minutes. The serum pool was preincubated (2:3 dilution) with *Tubifex*, *Gammarus*, and *D pteronyssinus* extracts at a concentration of 5 mg of freeze-dried material per milliliter for 2 hours at room temperature. The preincubated extracts were then placed on the previously red midge larvae-coated uniCAP disks. The experiment was performed using the ImmunoCAP 100E system (Phadia). A standard curve was obtained with red midge larvae. Results were expressed as the percentage of inhibition of each extract with respect to red midge larvae.

## Results

### Skin Test Sensitization

Seven of the 8 patients had a positive skin prick test result to 1 or more aquarium fish food arthropod species (Table 2). The skin prick test results were positive to the red midge larvae extract, *Gammarus*, and *Tubifex* in 7 (87.5%), 5 (62.5%), and 3 (37.5%) patients, respectively (Table 2). The mean (SD) wheal sizes induced by the different extracts were 37 (12) mm<sup>2</sup>, 28 (12) mm<sup>2</sup>, and 30 (14) mm<sup>2</sup>, respectively. Six individuals (75%) also had positive prick test results to mites and 2 to mosquitoes (25%). The patient with the dual asthmatic response and contact urticaria by *Gammarus* had a positive skin prick test result to red midge larvae and *Gammarus* and a negative result to *Tubifex* and mites (Table 2, patient 3). The patient with a previous history of rhinitis and asthma had a positive prick test result to dust mites and red midge larvae (Table 2, patient 6). One patient was positive only to mites. Therefore, 87.5% of patients were sensitized to fish food and mites or mosquitoes. All individuals had negative skin tests to other arthropod species (*Anisakis simplex*, shrimp, cockroach) (Table 2), pollens, molds, and dander.

Of the 20 atopic patients used as controls (positive skin prick test to mites), 6 (30%) had a positive skin reaction to red midge larvae and 3 (15%) to mosquitoes. In the other 20 controls (allergic only to pollens), the skin prick test results with red midge larvae, *Tubifex*, and *Gammarus* extracts were negative.

### Specific IgE

Five individuals (62.5%) had positive specific IgE to red midge larvae, 4 (50%) to *Gammarus*, and only 1 (16.6%) to *Tubifex* (Table 2). The highest levels of specific IgE to *Gammarus* were observed in patient 3, the only one with a dual asthmatic response and contact urticaria by *Gammarus*. The highest levels of specific IgE to *Tubifex* extract were observed in patient 4 (Table 2, Figure 2).

### Measurement of Peak Expiratory Flow and Bronchial Hyperresponsiveness

A decrease in PEF<sub>R</sub> ≥20% with respect to baseline was observed in all participants within a few minutes of starting the canning process (Figure 3). PEF<sub>R</sub> also indicated a late asthmatic response in the patient sensitized to *Gammarus* (he also presented contact urticaria by *Gammarus*) and red midge larvae (Table 1, patient 3; Figure 4).

The methacholine challenge test was positive (FEV<sub>1</sub> decreased by >20%) in all patients.

### Nasal Challenge Tests

Nasal challenge tests with the 3 extracts induced a significant fall (≥40%) in the PNIF and a positive symptoms score in the 4 individuals analyzed. In the case of the patient with the dual asthmatic response and contact urticaria, the strongest response was to *Gammarus* extract; in addition, the lowest concentration used induced a fall of 80% in PNIF. The results are summarized in Table 3. Negative results were obtained in all controls.

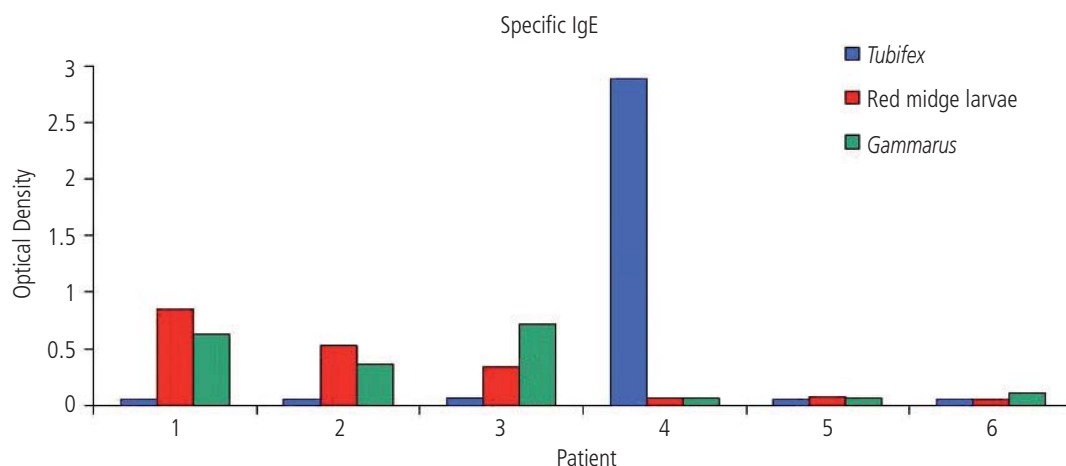


Figure 2. Specific IgE determinations to the allergen extracts. Ig indicates immunoglobulin.

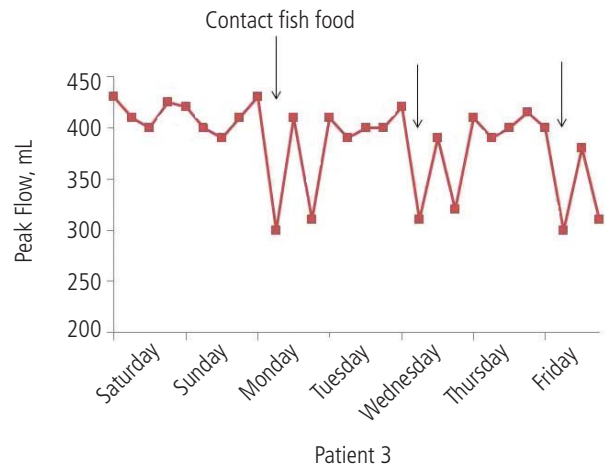
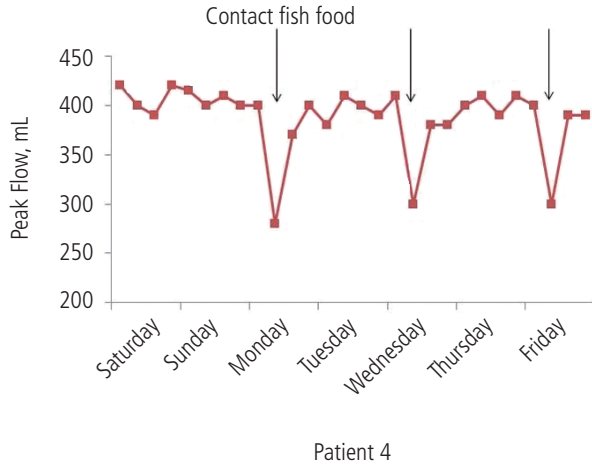


Figure 3. Daily peak flow values in patient 4, who was sensitized to *Tubifex*.

Figure 4. Daily peak flow values in patient 3 (asthmatic) with immediate and late response.

Table 3. Nasal Challenge Tests Results in 4 Patients Sensitized to Fish Food Components.

Patient	Red Midge Larvae			<i>Gammarus</i>			<i>Tubifex</i>		
	Dilution	Reduction in PNIF	Symptoms	Dilution	Reduction in PNIF	Symptoms	Dilution	Reduction in PNIF	Symptoms
1	1/1000	60%	Yes	1/100	80%	Yes		ND	
3	1/1000	50%	Yes	1/10000	80%	Yes		ND	
4		ND			ND		1/10	40%	Yes
6	1/100	40%	Yes		ND			ND	

Abbreviations: ND, not determined; PNIF, peak nasal inspiratory flow.

**Protein Profile**

SDS-PAGE revealed the presence of different protein bands in a molecular weight range of 10 kDa to 70 kDa in *Gammarus* and *Tubifex* and a range of 10 kDa to 45 kDa in red midge larvae. Three prominent bands were observed at 14, 28, and 30 kDa in red midge larvae; several bands were seen at approximately 14, 20, 30, and 60 kDa in *Gammarus* and at 14, 18, 30, and 35 kDa in *Tubifex* (Figure 5).

**Allergen Profile**

Immunoblotting confirmed IgE-binding to red midge larvae in 4 patients and *Gammarus* extract in 5 (Figure 6). No bands were recognized extract in the solid phase with *Tubifex*.

The most prominent band identified in *Gammarus* extract had a molecular weight of 31 kDa. A band of 14 kDa was also detected in both *Gammarus* and *Tubifex*.

**Immunoblot Inhibition**

*Gammarus* and *Tubifex* had significant inhibitory capacity. Red midge larvae extract was completely inhibited by *Gammarus* and partially inhibited by *Tubifex*,

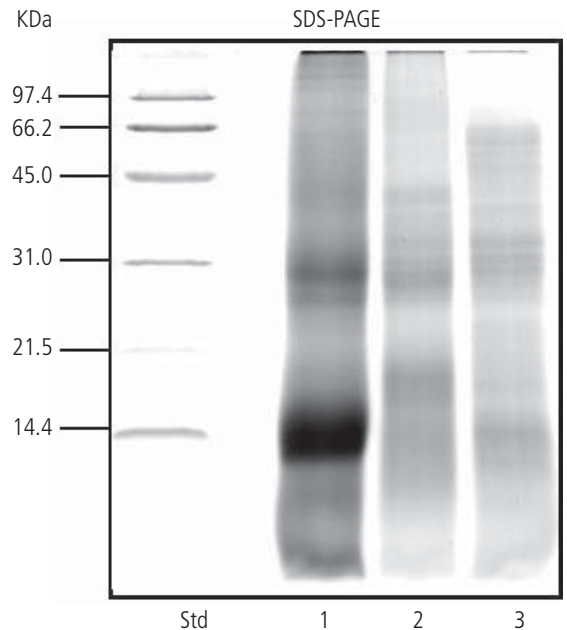


Figure 5. SDS-PAGE. Lane 1, red midge larvae; lane 2, *Tubifex*; lane 3, *Gammarus*. SDS-PAGE indicates sodium dodecyl sulfate polyacrylamide gel electrophoresis.

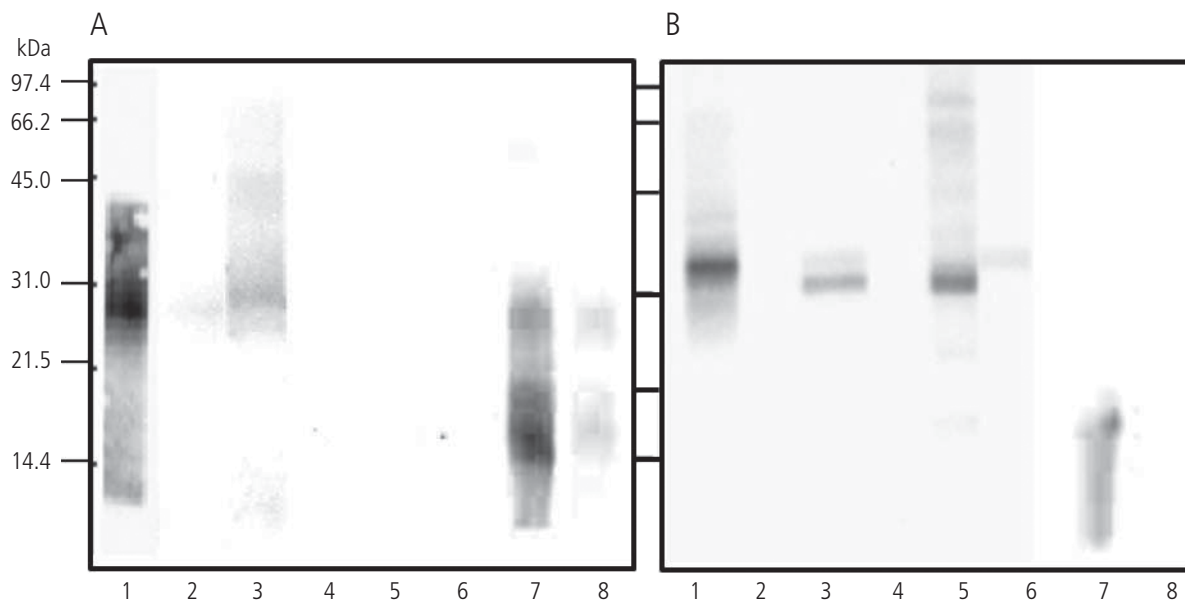


Figure 6. Allergenic profile of individual serum samples (diluted 1:2). A, Solid phase: 100 µg red midge larvae. B, 100 µg *Gammarus*.

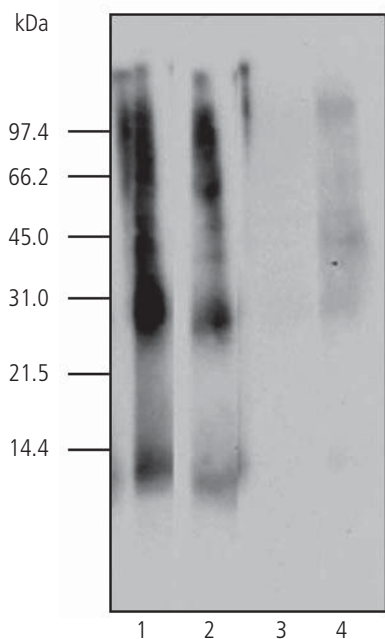


Figure 7. Immunoblot inhibition. Solid phase: red midge larvae (100 µg). Lane 1, no inhibition; lane 2, *Dermatophagooides pteronyssinus*, 0.5 mg/mL; lane 3, *Gammarus*, 0.5 mg/mL; lane 4, *Tubifex*, 0.5 mg/mL.

especially in bands with a low molecular weight. In contrast, *D pteronyssinus* extract had a very limited inhibitory capacity (Figure 7).

#### CAP Inhibition

Inhibition rates were high between the extracts. *Gammarus* extract inhibited red midge larvae in 58% of cases, while *Tubifex* inhibited red midge larvae in 45%. *D pteronyssinus* inhibited the red midge larvae in 12%.

#### Discussion

The objective of this study was to examine the sensitization pattern and the immunological relationships between 3 arthropod species present in aquarium fish food. Based on clinical symptoms, PEFr, and methacholine challenge test result, we found that 8 out of 10 exposed workers experienced rhinitis and immediate asthma while they were handling fish food. One patient also had a late asthmatic response (Figure 3) and contact urticaria after handling *Gammarus* (Table 1). The positive results in skin prick tests, nasal challenge tests, and specific IgE determinations demonstrated the existence of IgE-mediated hypersensitivity.

Seven out of 8 patients (87.5%) were sensitized to 2 or more of the fish food components analyzed. Moreover, sensitization was also detected to 2 or more arthropod species, including mites and mosquitoes (Table 2). Most patients were sensitized to red midge larvae (positive prick test results in 7 of 8), as reported elsewhere [29].

Our results showed that testing with red midge larvae extract revealed approximately 25% more patients sensitized to aquarium fish food than *Gammarus*, thus demonstrating the high diagnostic ability of this extract. Surprisingly, the results of skin prick tests with *Tubifex* were positive in 3 of the workers (37.5%), while only 1 serum sample showed

specific IgE against this extract. These results and those of the in vitro inhibition studies suggest that the red midge larvae extract shared cross-reactive allergens with the other extracts, although each extract may have its own allergens, which we were unable to detect. Therefore, extracts should be further compared using inhibition assay.

In Europe, most cases of allergy to chironomids have been reported in patients who handle *Chironomus* larvae professionally or nonprofessionally (they are components of certain aquarium fish foods) [30,31]. The high percentage of sensitization we observed in the symptomatic individuals indicates that prevalence could be higher than previously published [10,11,32]. In addition, the short period between starting to work for the company and onset of symptoms (3 to 9 months in most patients) highlights the potent sensitizing capacity of these agents. The particular characteristics of the working environment (direct exposure to high amounts of aquarium fish food dust and poor ventilation) might also account for the high allergen levels and, consequently, the increased risk of sensitization. It would have been interesting to quantify allergen levels in the workplace, although the appropriate device to do so was unavailable at the moment of the study. Positive prick tests to mites were observed in 71.4% of patients with positive prick test results to red midge larvae (Table 2). In addition, 30% of the nonexposed mite-allergic control patients had positive results in prick tests with red midge larvae. This finding could indicate that cross-reactivity between mites and chironomids might be responsible for sensitization, as described elsewhere [12-15,33]. However, this hypothesis was almost refuted by the immunoblotting-inhibition results, which showed that *D pteronyssinus* extract had a poor capacity to inhibit the red midge larvae extract (Figure 7). The panallergen tropomyosin has been reported to be responsible for cross-reactivity between chironomids, *Anisakis*, mites, and other arthropods [23,24]. In this study, and according to other authors [1,31], tropomyosin did not seem to be responsible, since all patients had negative prick tests to shrimp, cockroach, and *Anisakis*. Nevertheless, it is noteworthy that we could not rule out true double sensitization to mites and aquarium fish food owing to the high environmental exposure observed. Therefore, we think that cross-reactivity with other arthropods cannot account for the elevated prevalence of sensitization to red midge larvae, *Gammarus*, and *Tubifex*. Although 30% of control mite-allergic patients were sensitized to red midge larvae, we could not rule out hypothetical subclinical environmental exposure to chironomids.

We found a good correlation between symptoms and the results of prick tests, specific IgE determinations, and nasal challenge tests to all 3 extracts. Although the gold standard for diagnosing occupational asthma is allergen-specific bronchial challenge, we opted not to perform this test because of the potential danger of triggering severe asthmatic reactions. Nevertheless, the positive results in the methacholine challenge tests confirmed asthma in all 8 patients. In addition, the causal relationship between allergen exposure and onset of asthmatic symptoms was proven by the presence of variable airflow limitation at work, as determined by PEF and positive specific nasal challenge results.

As expected, skin tests revealed higher sensitivity to the 3

extracts than specific serum IgE determination. The sensitivity of specific IgE to *Tubifex* was especially poor. Although *Chironomus* larva extracts can induce reactions when used in skin testing [34], none of our patients developed local or systemic adverse reactions to them.

We found that 3 workers were sensitized to *Tubifex* (positive prick test results), and that 1 of these individuals (Figure 2, patient 4) also had the highest levels of specific IgE and a positive nasal challenge test result (Table 3). This patient experienced the most intense symptoms (rhinoconjunctivitis and asthma) on contact with *Tubifex* (Figure 3). To our knowledge, this is the first report of occupational sensitization to this annelid. None of the patients had positive prick test results to *Anisakis* species, which is taxonomically close to *Tubifex*.

The protein profile of the 3 species assessed proved to be similar: a band of approximately 31 kDa was observed in all cases. Low-molecular-weight bands (approximately 8 and 15 kDa) were also identified, mainly in red midge larvae; high-molecular-weight bands were observed in *Gammarus*. Immunoblot inhibition analysis confirmed the presence of bands at 14 and 31 kDa in *Gammarus* and red midge larvae, weights compatible with those of monomeric and dimeric hemoglobins [20,21], respectively, while no bands were identified in *Tubifex*. Both allergens could play a significant role in the sensitization pattern of both extracts.

Cross-reactivity was analyzed using ELISA inhibition and immunoblot inhibition, which showed that the protein profile of red midge larvae (solid phase) was more similar to that of *Gammarus* (totally inhibited) than that of *Tubifex* (partially inhibited). Similar results confirming these data were obtained when inhibition was analyzed using CAP.

In summary, our results demonstrate the presence of allergens in aquarium fish food components and the high capacity of these allergens to sensitize exposed workers in a short period. We have demonstrated a variable degree of cross-reactivity between red midge larvae, *Gammarus*, and *Tubifex*, although more data are needed to identify the responsible allergens and their nature.

To our knowledge, this is the first report of occupational allergy to *Tubifex*.

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### Conflicts of Interest

The authors declare that they have no conflicts of interest.

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