ORIGINAL ARTICLE

Allergen Profile of *Protophormia terraenovae*, Other Species of Calliphoridae, and *Lumbricus terrestris* in Anglers Allergic to Maggots in Cáceres, Spain

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Abstract

Background: Our group previously found that up to 7% of amateur anglers in Caceres, Spain may be allergic to the larvae of *Protophormia terraenovae* (order Diptera, family Calliphoridae) used as live bait for fishing.
Objective: To identify the pattern of major allergens in *P terraenovae* and other species of Calliphoridae.
Materials and Methods: Extracts of *P terraenovae*, *Calliphora vomitoria*, *Lucilia sericata* and *Lumbricus terrestris* were characterized using sodium dodecyl sulfate polyacrylamide gel electrophoresis and IgE-immunoblotting techniques in individual sera from 24 patients with a positive skin test result and/or specific IgE determination (enzyme-linked immunosorbent assay [ELISA]) to *P terraenovae*. ELISA and IgE-immunoblotting inhibition studies were also performed to identify potential cross-reactive allergens between these species.
Results: IgE-immunoblotting with *P terraenovae* showed a band of 15.3 kDa recognized by 15 patients, in addition to 2 further allergens of 22.8 kDa and 69 kDa. For *C vomitoria*, 5 bands of 73, 46, 40, 28, and 14 kDa were observed. For *L sericata*, 2 major allergens of 73 kDa and 14 kDa were observed. In the case of *L terrestris*, IgE from 13 patients recognized 1 allergen of around 15.5 kDa. IgE-immunoblotting and ELISA inhibition revealed the presence of cross-reactivity, mainly between *L terrestris* and *P terraenovae*.
Conclusions: P terraenovae appears to have species-specific allergens and allergens shared with *C vomitoria* and *L sericata*. Striking immunological cross-reactivity was observed between *P terraenovae* and *L terrestris*. An allergen of 15-16 kDa could be involved in this phenomenon.

Key words: *Protophormia terraenovae*. Maggot. Allergens. Allergy. Anglers. Fish bait.

Resumen

Antecedentes: La larva de *Protophormia terraenovae*, utilizada como cebo vivo para la pesca, es capaz de producir reacciones alérgicas en el 7% de la población de pescadores de agua dulce de Cáceres, según las observaciones previas de nuestro grupo.
Objetivo: Identificar el patrón de alérgenos en *P. terranovae* y otros Califóridos.
Materiales y métodos: Los extractos de *P. terraenovae*, *Calliphora vomitoria*, *Lucilia sericata* y *Lumbricus terrestris* se sometieron a técnicas de SDS-PAGE e IgE-immunoblotting utilizando sueros individuales de 24 pacientes sensibilizados a *P. terraenovae*. Se realizaron también técnicas de ELISA e IgE-immunoblotting inhibition para la identificación de posibles alérgenos comunes entre dichas especies.
Resultados: 15 pacientes reconocieron una banda entorno a los 15.3 kDa frente al extracto de *P. terraenovae*, además de otros 2 alérgenos de 22.8 and 69kDa. Con *C. vomitoria*, se observaron 5 bandas de 73, 46, 40, 28 y 14 kDa. Con *L. sericata* se observaron 2 alérgenos mayores de 73 y 14 kDa. Usando *L terrestris*, 13 pacientes reconocieron un alérgeno de unos 15.5 kDa. Los estudios de inhibición IgE demostraron la presencia de reactividad cruzada inmunológica principalmente entre *L. terrestris* y *P. terraenovae*.
Conclusiones: La larva de *P. terraenovae* parece tener alérgenos especie-específicos y alérgenos compartidos con *C. vomitoria* y *L. sericata*. Se ha observado una importante reactividad cruzada inmunológica entre *P. terraenovae* y *L. terrestris*. Un alérgeno entre los 15-16 kDa podría ser uno de los responsables de este fenómeno.

Palabras clave: *Protophormia terraenovae*. Gusano. Alérgenos. Pescadores. Cebo de pesca.

Introduction

In a previous study [1], we analyzed maggots sold as fishing bait in the city of Cáceres, Spain and detected the exclusive presence of *Protophormia terraenovae* larvae (an imported blowfly species). Furthermore, in vivo and in vitro studies in amateur anglers from Cáceres using *P terraenovae*, other species of blowfly, and the common earthworm emphasized the importance of *P terraenovae* larvae as the main sensitizing allergen (affecting 7% of anglers) and its clinical significance. We also showed that skin tests using *P terraenovae* extract in patients with allergic symptoms attributable to the handling of maggots had a high diagnostic yield.

The pattern of relevant allergens is unknown in most of the species studied. In Australia, Baldo et al [2] studied the allergenic characterization of an extract of *Lucilia cuprina* used by workers in an entomological research center. The same group also described different immunoglobulin (Ig) E recognition patterns against various species of flies (and other insects) in patients with suspected inhalant allergy to insects [3]. Other studies that mention a specific allergen generally include only 1 or 2 clinical cases, and thus provide information on allergens identified individually that cannot be compared owing to the differences in the species studied and the methodology used [4-6]. The aim of the present study was to identify the major allergens in Calliphoridae and earthworms used by anglers in Cáceres.

Materials and Methods

Diagnostic Allergenic Extracts

The method of obtaining allergenic extracts for diagnostic use was described in our previous study [1]. Local extracts of *P terraenovae* and common earthworm (*Lumbricus terrestris*) were used to perform skin prick tests, and allergenic extracts of imported *P terraenovae*, *Calliphora vomitoria*, *Lucilia sericata*, and *Lumbricus terrestris* were used for determinations of specific IgE and the allergen profile.

Selection of Sera

Sera were taken between 2000 and 2008 from 12 patients with a clinical history suggestive of allergy to live fish bait and positive results in a prick test, specific IgE determination, or both with the species studied. In addition, some of the patients

Table. Allergy Test in Patients Allergic to Live Fishing Bait

	Skin Prick Test		Mucosal Challenge Test		Specific Immunoglobulin E (ELISA), IU/mL			
	Protophormia terraenovae	*Lumbricus terrestris*	*Protophormia terraenovae*	*Lumbricus terrestris*	*Protophormia terraenovae*	*Calliphora vomitoria*	*Lucilia sericata*	*Lumbricus terrestris*
1	3 mm	3 mm	NCT + 0.5 mg/mL	CCT + 5 mg/mL	1.297	0.411	0.164	0.428
2	8 mm	9 mm	CCT + 0.05 mg/mL	CCT + 0.05 mg/mL	13.699	0.703	0.484	0.454
3	11 mm	9 mm	CCT + 0.05 mg/mL	CCT + 5 mg/mL	12.917	1.382	1.143	0.890
4	4 mm	–	CCT + 0.05 mg/mL	Not performed	0.387	–	–	–
5	15 mm	5 mm	CCT + 0.5 mg/mL	CCT + 5 mg/mL	12.675	1.202	1.304	0.494
6	8 mm	4 mm	CCT + 0.05 mg/mL	CCT + 5 mg/mL	11.167	0.910	0.287	0.562
7	13 mm	5 mm	CCT + 0.05 mg/mL	CCT + 5 mg/mL	2.692	–	–	0.459
8	4 mm	9 mm	CCT -	NCT + 0.5 mg/mL	0.457	–	0.346	0.418
9	10 mm	4 mm	CCT + 0.05 mg/mL	CCT + 0.5 mg/mL	7.834	0.145	0.355	0.573
10	8 mm	–	CCT + 5 mg/mL	Not perfomed	0.177	–	–	0.107
11	12 mm	5 mm	Not performed	Not performed	Not performed			
12	10 mm	11 mm	Not performed	Not performed	Not performed			

Abbreviations: CCT, conjunctival challenge test; ELISA, enzyme-linked immunosorbent assay; NCT, nasal challenge test.

had a positive result in specific conjunctival or nasal challenge with *P terraenovae* or common earthworm (Table). Sera were also taken from 12 patients with positive results included in our previous study [1] (skin test, IgE, or both) for *P terraenovae*, *L terrestris*, or both.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and IgE Immunoblotting

The bands of the different extracts of the imported species used as fishing bait were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (12.5% acrylamide gel) (SDS-PAGE) and transferred to a polyvinylidene fluoride membrane (Hybond P, Amersham Pharmacia Biotech) according to the method described by Towbin et al [7]. The membrane was then blocked with phosphate-buffered saline–Tween 0.1% (vol/vol) for 1 hour at room temperature. Sera were added to the membrane in a 1:1 ratio with blocking buffer and shaken overnight at 4°C. After a series of washes, the membrane was then incubated with an antihuman IgE antibody conjugated with horseradish peroxidase (DAKO) for 2 hours at room temperature. Immunodetection was performed using a chemiluminescent detection reagent according to the manufacturer's instructions (Western Lightning Plus, Perkin Elmer).

The molecular weight and intensity of the different bands was evaluated using an image processing system (GS 710 Calibrated Imaging Densitometer, Bio-Rad) equipped with a specific image analysis system (Bio-Rad Diversity Database).

Cross-reactivity Studies

Inhibition with enzyme-linked immunosorbent assay (ELISA): The solid phase allergen was fixed to the 96-well microtiter polystyrene plates at a concentration of 250 μg/mL. The free phase was prepared using a battery of tubes with different concentrations of inhibitor allergen. Each tube was filled with 200 μL of pooled sera (24 in total), except for 1 tube, which was filled with 200 μL of incubation buffer only. The solution was incubated at room temperature for 3 hours with orbital shaking. After addition of a blocking solution, 50 μL of the inhibiting free phase was added at different concentrations to selected wells, and the plate was left with constant agitation for 2 hours at room temperature. The IgE response was detected and absorbance measured as previously described in ELISA [8]. The curve obtained with the percentages of inhibition at different concentrations of each inhibitor allergen was calculated using the following formula: % INHIBITION = 1 – OD inhibitor allergen/OD without inhibitor × 100. Once the line of inhibition was obtained, the concentration of the allergen inhibitor capable of producing 50% inhibition (Ag 50) was calculated.

IgE-immunoblotting inhibition: A mixture formed from the sera of all the study patients was preincubated with different concentrations of the allergen extracts of the selected species before being added to the polyvinylidene fluoride membranes blotted with the proteins of the extract used as the solid phase. Thus, 3 lanes were obtained in each blot inhibition assay; a first lane, in which the pooled sera were added to the membrane without prior incubation, and 2 further lanes, in which preincubated serum was added at various concentrations of the free-phase inhibitor for 3 hours at room temperature at a ratio of 1:1. Immunodetection of the IgE response and evaluation of the allergenic bands were performed as described for ELISA (see above).

Results

SDS-PAGE and IgE Immunoblotting

Figure 1 shows the molecular weight profile obtained by SDS-PAGE and Coomassie blue staining of the protein components present in the extracts of *P terraenovae*, *C vomitoria*, *L sericata*, and *L terrestris*. The molecular weight pattern was very similar in all 3 Calliphoridae species. Twelve components were observed in the *L terrestris* extract, with molecular weights of between 13.2 kDa and 92.5 kDa. Nine proteins were identified in the extract of *P terraenovae* (12.8-85 kDa), 11 in *C vomitoria* (13.4-77.7 kDa), and 10 in *L sericata* (10.9-88.6 kDa). In the 4 species analyzed, more than 60% of the proteins were located in a range below 30 kDa.

IgE-immunoblotting analysis of *P terraenovae* extract (Figure 2A) showed a clearly identifiable allergen band around 15 kDa that was recognized by 15 patients, as well as an allergen of 22 kDa recognized by 8 patients and an allergen of 69 kDa recognized by 10 patients. For *C vomitoria*, an intense band was recognized between 14 kDa and 15 kDa by 15 patients and a band of around 73 kDa was recognized by 14 patients. Five major bands (73, 46, 40, 28, and 14 kDa)

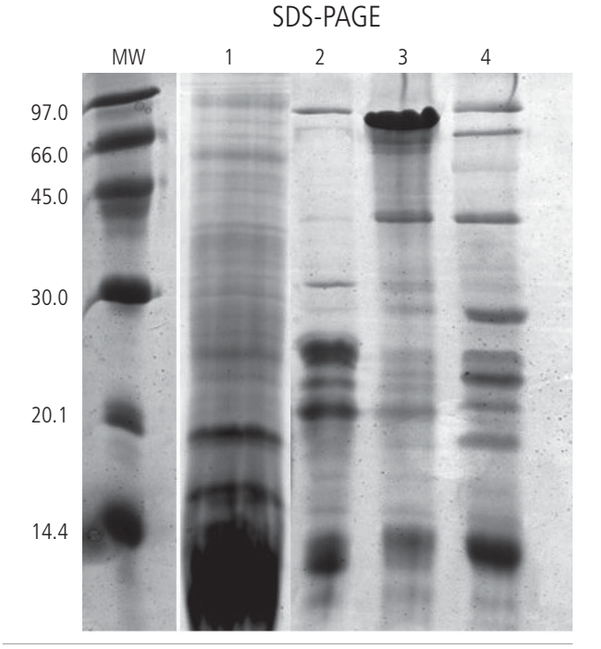

Figure 1. Electrophoresis using sodium dodecyl sulfate polyacrylamide gel electrophoresis. Lane 1, *Lumbricus terrestris*; lane 2, *Protophormia terraenovae*; lane 3, *Calliphora vomitoria*; lane 4, *Lucilia sericata*. MW indicates molecular weight.

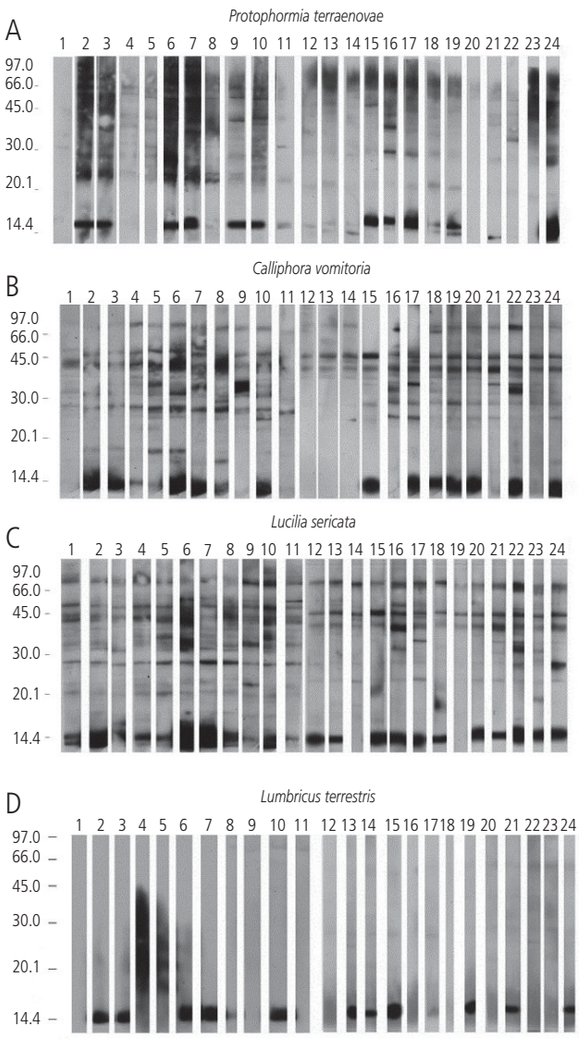

Figure 2. Immunoglobulin E–immunoblotting of extracts of *Protophormia terraenovae*, *Calliphora vomitoria*, *Lucilia sericata*, and *Lumbricus terrestris*.

were also recognized (Figure 2B). In the case of *L sericata*, major allergens of 73 and 14 kDa were recognized by 20 and 19 patients, respectively. A further 3 allergens (around 44, 39, and 27 kDa) formed an IgE recognition pattern that was very similar to that obtained with *C vomitoria* (Figure 2C). For *L terrestris*, we observed only 1 main allergen (15.5 kDa), which was recognized by 13 patients (Figure 2D).

ELISA Inhibition

P terraenovae extract was chosen as the solid phase. The inhibiting allergens were extracts of *P terraenovae*, *C vomitoria*, *L sericata*, and *L terrestris*. The tracer element was the mixture of the 24 sera used for the IgE-immunoblotting studies.

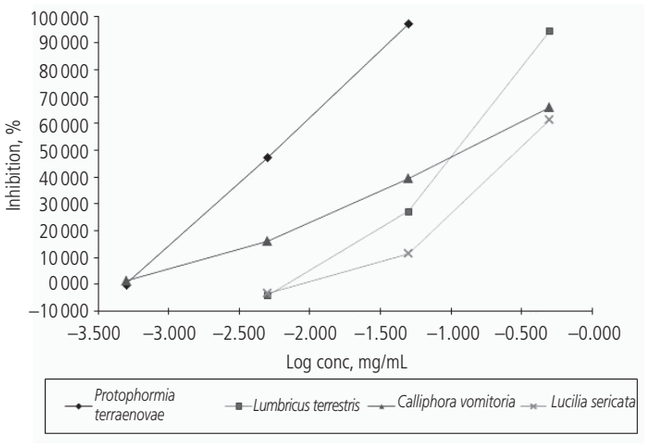

Figure 3. Enzyme-linked immunosorbent assay. Solid phase: *Protophormia terraenovae*. Free phase: *Protophormia terraenovae*, *Lumbricus terrestris*, *Calliphora vomitoria*, and *Lucilia sericata*.

Figure 3 shows that the homolog achieved the greatest inhibition: 97% at a concentration of 0.05 mg/mL. *L terrestris* was the second most powerful allergen in inhibiting the response to *P terraenovae*, reaching almost 95% inhibition at the highest concentration used (0.5 mg/mL); in other words, it required a concentration 10-fold higher than the homolog to achieve almost complete inhibition. The other 2 species of Calliphoridae, *C vomitoria* and *L sericata*, achieved an inhibition of 66% and 61%, respectively, at the maximum concentration of 0.5 mg/mL. The Ag50 values were 5 μg/mL for *P terraenovae*, 81 μg/mL for *L terrestris*, 117 μg/mL for *C vomitoria*, and 286 μg/mL for *L sericata*.

IgE-Immunoblotting Inhibition

In assay 1, *P terraenovae*, *C vomitoria*, *L sericata*, and *L terrestris* extracts were used as the solid phase and *L terrestris* extract as the free-phase inhibitor. In assay 2, *L terrestris* was used as the solid phase, and *L terrestris*, *P terraenovae*, *C vomitoria*, and *L sericata* as the free-phase inhibitor. We performed 3 analyses using the pooled sera without (lane 1) and after preincubation with the allergen in free phase at 0.5 mg/mL and 5 mg/mL (lanes 2 and 3, respectively).

Figure 4A shows that, in assay 1, *L terrestris* completely inhibited the response to its homolog at a concentration of 5 mg/mL. It also completely inhibited the specific IgE bands against *L sericata* extracts. In the case of *C vomitoria*, *L terrestris* at 5 mg/mL inhibited bands with a molecular weight of approximately 129, 16.5, and 15.1 kDa, with the recognition of 3 bands of 56, 40, and 32 kDa persisting (although with less intensity). At a concentration of 5 mg/mL, *L terrestris* inhibited several bands of IgE recognition to *P terraenovae* extract. In this case, 2 bands of 124 kDa and 82 kDa persisted, and the bands with an approximate molecular weight of 65, 55, 48, 40, 30, 22, 19, 16.8, and 14.5 kDa disappeared. In assay 2 (Figure 4B), *L terrestris* completely inhibited its homolog. However, except for *L sericata*, the larvae of the Calliphoridae studied

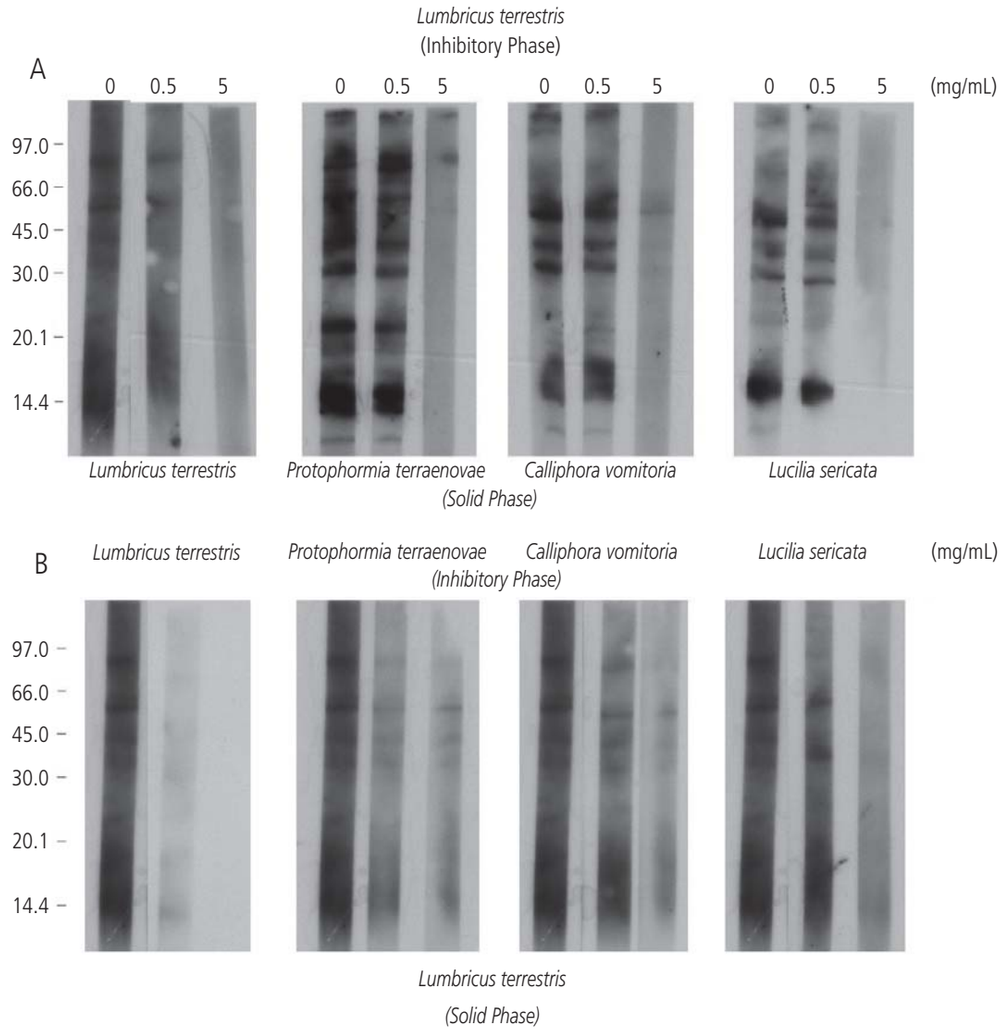

Figure 4. Immunoglobulin E–immunoblotting inhibition. A, *Lumbricus terrestris, Protophormia terraenovae, Calliphora vomitoria,* and *Lucilia sericata* as the solid phase and *L terrestris* as the inhibitory phase. B, *Lumbricus terrestris* as the solid phase and *Protophormia terraenovae, Lumbricus terrestris, Calliphora vomitoria*, and *Lucilia sericata* as the inhibitory phase.

only inhibited the area of recognition between 15 kDa and 20 kDa, with the recognition of bands around 85, 57, 44, and 36 kDa persisting.

Discussion

The Western blot studies revealed an almost mirror-like pattern of recognition against the extracts of *C vomitoria* and *L sericata*. Five major allergens with a molecular weight of around 14, 26-27, 39-40, 44-45, and 72 kDa were identified. With *P terraenovae*, we observed a major allergen of around 15 kDa and another 2 allergens of 22 kDa and 69 kDa, which initially did not correspond to those observed for *C vomitoria* and *L sericata*.

Finally, only 1 major allergen was identified in the *L terrestris* extract; it was recognized by 13 patients and had an approximate molecular weight of 15.5 kDa, which is very similar to that of the allergen identified in *P terraenovae* (15.3 kDa). The presence of allergens of similar molecular weights in the species of Calliphoridae studied and in *L terrestris* does not mean they are the same proteins, but suggests a high degree of structural homology. In the only previous case report of allergy to *P terraenovae*, Western blot revealed the presence of an allergen of 80 kDa and another of 22 kDa [9], which may correspond to that observed in this study, although it does not appear to be relevant in the other 2 Calliphoridae.

The study by Baldo et al [2] on the identification of allergens in *L cuprina* and other species of flies showed that *L cuprina* could produce sensitization at any stage of development, from the egg through the larval stage to the adult, although it appeared that the more advanced the degree

of development, the greater the degree of antigen recognition, with specific allergens detected at each stage. The results of Baldo et al are comparable to ours, since their study included patients exposed to a particular species of fly. The study also provided data on the identification of allergens to flies of the genera *Lucilia* and *Calliphora*. The main allergen (67 kDa), both in extracts of adult flies and in larvae of *L cuprina*, may correspond to the 69-kDa allergen we observed in *P terraenovae* (range, 65-72 kDa). More analogs might be identified if the results obtained by Baldo et al [2] were compared with the individual allergens of each of our patients.

Our previous results [1] showed the possible existence of cross-reactivity between *L terrestris* and *P terraenovae*. Only 1 of our patients suggested earthworm as being possibly responsible for their symptoms. However, 10 patients had a positive skin test result to *P terraenovae* and *L terrestris*, and 7 patients had a positive specific challenge result to both. The existence of an IgE recognition band of 15 kDa with a similar morphology in extracts of both *L terrestris* and *P terraenovae* confirmed the suspicion. Inhibition ELISA studies finally confirmed the existence of cross-reactivity. *L terrestris* almost completely inhibited the specific IgE response to *P terraenovae*, with an inhibitive power even greater than that of the other 2 species of Calliphoridae. *L terrestris* required a concentration 1.4 times and 3.5 times lower than *C vomitoria* and *L sericata*, respectively, to achieve 50% inhibition. The same phenomenon was observed in the immunoblotting inhibition studies. Acting as the free phase, *L terrestris*, significantly inhibited the IgE response to protein extracts from the 3 species of Calliphoridae.

In another study by Baldo et al [3], patients diagnosed with inhalant allergy to insects recognized allergens in Diptera (including *Musca domestica*, *Calliphora stygia*, *Calliphora augur*, *L cuprina*, *Chrysomya bezziana*, and *Parasarcophaga* species), Lepidoptera, Dermestidae, and cockroach. Immunoblotting studies revealed a 37-kDa allergen common to all the species of flies. Tropomyosin, with a molecular weight of 34-38 kDa and which is found in many arthropods and other invertebrates, has been implicated as an important cause of cross-reactivity, even among species with no phylogenetic relation. Martinez et al [10] used immunoblotting studies with a mixture of sera from patients with allergy to household arthropods and demonstrated the presence of tropomyosin in extracts from insects, mites, crustaceans, mollusks, and even parasites such as *Anisakis simplex* and *Ascaris lumbricoides* (nematodes). This cross-reactivity between nematodes and Diptera was also demonstrated by Pascual et al [11] in patients sensitized to *Anisakis* species, *Blattella germanica* (cockroach), and Chironomidae and is possibly due to tropomyosin. In addition, Cabrera [12] obtained a higher specific IgE response to extracts of *Calliphora vicina* and *P terraenovae* in patients sensitized to *Ascaris*, with evidence of cross-reactivity in ELISA inhibition studies. It is suggested that, in areas where *A lumbricoides* infection is endemic, sensitization to tropomyosin may predispose to an allergic response to aeroallergens from insects such as mites and cockroaches, which have very similar tropomyosins and can thereby induce inflammation and asthma [13]. However, in spite of this evidence, it does not seem that the most relevant allergens against species of Calliphoridae and *L terrestris* in anglers from Cáceres belong to the tropomyosins. To date, no cross-reaction has been reported between Diptera and annelids in any of the studies on allergic reactions due to Calliphoridae larvae or in studies describing any type of allergic reaction to Diptera. However, as mentioned above, cross-reactivity between arthropods and nematodes has been reported, and De Castro et al [14] showed cross-reactivity between *A simplex* (nematode) and *L terrestris* (annelid), although, in this case, the allergen responsible for the cross-reactivity, with a molecular weight of 20 kDa, is not within the known range for the various tropomyosins. The results of our study suggest the possible amplification of panallergens to the phylum of annelids, which would close the circle between annelids, arthropods, and nematodes. Cross-reactivity between *L terrestris* and Calliphoridae does not seem to have a clear phylogenetic basis. In 1812, Cuvier [15] established a classification of Articulata, which encompassed arthropods and annelids, based on a common segmented structure; however, modern authors have rejected this theory. A phylogenetic relationship has been described between arthropods and nematodes (thus justifying the cross-reactivity reported in other studies) and between annelids and mollusks [16,17]. A possible explanation for this cross-reactivity derives from the existence of a very intense band of IgE recognition in Western blot studies of around 15-16 kDa in *P terraenovae* and *L terrestris* extracts. We suggest that this allergen could correspond to a hemoglobin, for several reasons. The earthworm possesses extracellular hemoglobins, called erythrocruorins, which are involved in the gas exchange between oxygen and carbon dioxide. These proteins of the globin family are composed of 12 subunits that form a hexagonal structure. Each subunit is formed from the union of a trimer (A, B, and C chains) and a monomer (D chain). The amino acid sequence of both fractions has been identified. The trimer (extracellular globin-2) represents a fraction of 145 aa with a molecular weight of 16 254 Da [18] and the monomer (extracellular globin-1), a fraction of 142 aa with a molecular weight of 16 130 Da [19]. Moreover, the extracellular hemoglobins of the Chironomidae (nonbiting mosquitoes, Diptera, and Nematocera) have been described and characterized as major environmental and occupational allergens [20]. Thus, the major allergen of *Chironomus thummi thummi*, Chi t 1, is an erythrocruorin with a molecular weight of 16.3 kDa (UniProtKB/Swiss-Prot taxonomy database). Although the existence of hemoglobins in Diptera has traditionally gone unnoticed (except for Chironomidae) owing to the existence of a well-developed tracheal system, recent studies with various species of Drosophila (Diptera, Drosophilidae) have shown the existence of hemoglobin genes that are expressed primarily in the tracheal cells in both the larvae and the adults of these species [21]. In addition, the same authors found a phylogenetic relationship between globin1 of Drosophila, the intracellular hemoglobin of *Gasterophilus intestinalis* larva (Diptera, Oestridae), and the extracellular hemoglobin of the Chironomidae. The role of hemoglobin in Drosophila is not yet known, although presumably it serves to facilitate the transport of oxygen from the tracheoles to the tissues. In the case of Chironomidae, hemoglobin allows the larvae to live in zones with very poor oxygen concentrations,

such as the depths of lakes. The presence of hemoglobin in Drosophila and *Giardia intestinalis* (which belong to the same superfamily, Oestroidea, as the Calliphoridae in our study) suggests that it may also be present in other Diptera. The indirect suspicion of hemoglobin in *P terraenovae* larvae could explain the cross-reactivity we observed with *L terrestris*. Future studies involving techniques of purification, isolation, and sequencing of these allergens may help us confirm whether these proteins are hemoglobin and whether they are actually responsible for such cross-reactivity.

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