

SHORT COMMUNICATION

Effects of domestic chemical stressors on expression of allergen genes in the European house dust mite

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Abstract. The expression of allergen genes in house dust mites is influenced by temperature and relative humidity, but little is known of the impacts of other environmental factors that may alter the repertoire of allergens released by mites in home microhabitats. Bioassays were conducted in concave microscope slides in combination with real-time quantitative polymerase chain reaction (RT-qPCR) to analyse gene expression of 17 allergens of *Dermatophagoides pteronyssinus* (Acariformes: Pyroglyphidae) exposed to three chemical stressors that can be present in domestic environments. Short-term exposure (5–12 days) to diesel exhaust particles (DEPs) (1 µg/cm²), bacterial lipopolysaccharide endotoxin (0.1 µg/cm²) and benzyl benzoate (3.2 µg/cm²), at concentrations exceeding those expected in homes, had no significant effect on allergen transcription. A significant increase in the transcription of allergens *Der p 3*, *Der p 8* and *Der p 21* was observed only after exposing mites to a higher concentration of DEPs (10 µg/cm²) over a whole generation. In combination, the present results suggest that the analysed factors have low impact on allergen production. The methodology described here offers a sound and rapid approach to the broad-spectrum study of factors affecting allergen-related mite physiology, and allows the simultaneous screening of different factors in a relatively short period with consideration of the full spectrum of allergen genes.

Key words. Acaricides, air pollution, allergy, bioassay, diesel exhaust particles, house dust mites, lipopolysaccharide, real-time quantitative PCR.

Prevalences of allergy are increasing worldwide with huge economic consequences (Pawankar *et al.*, 2013). Epidemiology studies have found a strong association between the growth in allergy and increased exposure to air pollution caused by urbanization and an increasingly Westernized lifestyle (D'Amato *et al.*, 2010). *In vitro* and animal studies have shown that simultaneous exposure to allergens and chemical pollutants may have synergistic effects at different levels of the immune response (Lambrecht & Hammad, 2014; Baldacci *et al.*, 2015). In addition, studies on the relationships between pollen allergens and pollutants have revealed different interactions that may have an impact on allergy, such as the induction of changes in the production of allergens by plants (Cortegano *et al.*, 2004; Chehregani

& Kouhkan, 2008). In relation to house dust mites (HDMs), little is known about the effects of the indoor environment on the production of allergens. A recent study demonstrated that environmental factors such as relative humidity (RH) may remarkably alter allergen gene expression in *Dermatophagoides pteronyssinus* (Vidal-Quist *et al.*, 2015). In that study, real-time quantitative polymerase chain reaction (RT-qPCR) was applied to analyse eight different *D. pteronyssinus* allergen genes. In the present work, this approach is extended to the analysis of nearly all *D. pteronyssinus* allergens [17 genes out of 19 reported allergens (www.allergen.org)] and combined with a bioassay method for the accurate and reproducible challenge of mites with exogenous compounds. The impact of three common domestic

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stressors on the expression of allergens by *D. pteronyssinus* was studied. The first set of tests involved diesel exhaust particles (DEPs), which account for the most abundant respirable particulate matter in urban environments and which, through their major components, the polycyclic aromatic hydrocarbons, are considered a major risk to respiratory health (D'Amato *et al.*, 2010). The second set of assays involved bacterial endotoxins, which are large lipopolysaccharide (LPS) molecules present in the outer membrane of Gram-negative bacteria that are ubiquitously found in the environment and elicit strong innate immune responses (Braun-Fahrlander *et al.*, 2002). The third compound analysed was benzyl benzoate (BnBzO), which is extensively used as an ingredient in fragranced personal care, household and laundry products that may cause contact allergy reactions in sensitive subjects and is subjected to specific labelling regulations in the European Union (EU Directive 2003/15/EC). In addition, BnBzO exhibits acaricidal activity and was formerly a common active ingredient of commercial HDM control products (Colloff, 2009), although it is no longer included among EU Annex 1 approved biocidal products (Regulation 528/2012).

Bioassays were conducted in concave microscope slides in an adaptation of a previously described methodology (Sánchez-Ramos *et al.*, 2004). In brief, mites were collected from standard thriving cultures (Vidal-Quist *et al.*, 2015), placed in the cavity of a slide containing a 6-mm diameter disc of fish-food diet [approximately 2.4 mg per disc (Vipan; Sera GmbH, Heinsberg, Germany)], and confined using a cover slip that allowed gas exchange. Before mites were transferred, diet discs were treated with the addition of a 5- μ L drop of the treatment solution/suspension, and air dried for 5–10 min. Mites were exposed to DEPs (SRM 2975; National Institute of Standards and Technology, Gaithersburg, MD, U.S.A.) suspended in phosphate-buffered saline (PBS) (pH 7.3) after 5 min sonication, LPS (*Escherichia coli* LPS-EB Ultrapure; InvivoGen, Inc., San Diego, CA, U.S.A.) diluted in the same buffer, and BnBzO (Sigma-Aldrich Corp., St Louis, MO, U.S.A.) diluted in ethanol. The slides were placed on methacrylate stackable racks designed in-house, confined in sealed plastic boxes containing a saturated NaCl solution to maintain a constant RH of $76 \pm 2\%$, and kept in a climatic chamber at $24 \pm 0.3^\circ\text{C}$ in the dark. Dose–response assays were conducted in order to determine the appropriate test concentration for gene expression analysis. Five slides (with 30 tritonymphs per slide) were prepared per concentration of DEPs (four 10-fold serial dilutions, up to $10\text{ }\mu\text{g}/\text{cm}^2$) or LPS (three 100-fold serial dilutions, up to $1\text{ }\mu\text{g}/\text{cm}^2$). Two weeks after initial exposure, all treatments were assessed for increased mortality or any delay in nymphal development in comparison with the control treatment showing no differences. Five concentrations of the acaricide BnBzO were tested (0.2, 0.8, 3.2, 12.8 and $51.2\text{ }\mu\text{g}/\text{cm}^2$) on groups of 10 male and 10 female adult mites (10 replicated slides). Mortality was recorded at 2 days and 5 days after treatment and the number of eggs laid per group was counted at 5 days. Treatments at 0.2 and $0.8\text{ }\mu\text{g}/\text{cm}^2$ had no effect on mortality or egg laying (0% corrected mortality; 26.7 ± 5.4 eggs and 30.4 ± 5.2 eggs, respectively, vs. 23.0 ± 4.7 eggs in the control treatment). However, treatment at $3.2\text{ }\mu\text{g}/\text{cm}^2$ produced low mortality (1.6 and 7.6% corrected mortality at 2 days and 5 days, respectively) and a reduction in the number of eggs laid (12.2 ± 3.5 eggs).

Treatments over this concentration caused over 80% mortality at 2 and 5 days.

For the analysis of gene expression in the absence or presence of pollutants, 4–10 slides, each containing 10–30 individuals, were prepared per biological replicate to collect a pool of 100–200 adults (male:female ratio: 1:1) at the time of sampling, with three or four biological replicates per treatment. Hence, four experiments were performed: (a) exposure of tritonymphs to $1\text{ }\mu\text{g}/\text{cm}^2$ of DEPs for 12 days to sample emerging adults; (b) long-term exposure of adults to $10\text{ }\mu\text{g}/\text{cm}^2$ of DEPs for 44 days to obtain adults of the second generation; (c) exposure of tritonymphs to $0.1\text{ }\mu\text{g}/\text{cm}^2$ of LPS (approximately 150 endotoxin units/ cm^2) for 12 days to obtain emerging adults, and (d) exposure of adults to $3.2\text{ }\mu\text{g}/\text{cm}^2$ of BnBzO for 5 days. Total RNA was purified using the TRIzol reagent (Life Technologies Corp., Carlsbad, CA, U.S.A.) and stored in 75% ethanol at -80°C until further use. Gene expression was studied by RT-qPCR as previously described (Vidal-Quist *et al.*, 2015) using three reference genes (*RPL13a*, *RPS4*, *EF1a*). Primers targeted 17 *D. pteronyssinus* allergen genes, nine of which were specifically designed for this study (Table 1). Raw qPCR data (*C_q* values) were efficiency-corrected and transformed to normalized relative quantities (NRQs). Statistical analysis was performed on *C_q*' values ($\log_2$ transformation of NRQs) using Student's *t*-test. To demonstrate the current experimental conditions, the *C_q* values recorded for all genes under study in four replicated control samples are shown in Figure S1.

Results are summarized in Table 2. Short-term exposure (within one mite generation) to DEPs, LPS and BnBzO had no significant effect on the transcription of any of the analysed allergen genes in comparison with controls. Bioassays were designed to allow different ways of exposing mites to the tested compounds (i.e. by ingestion and/or contact), and concentrations were selected to considerably exceed the levels expected in domestic environments. Diesel exhaust particles originate from outdoor activities, such as road traffic, but may penetrate indoors, where HDMs will be exposed primarily after DEP deposition. To the present authors' knowledge, there are no data available on actual levels of DEP deposits in homes, and hence a concentration of $1\text{ }\mu\text{g}/\text{cm}^2$ was initially assayed because this is expected to greatly exceed actual exposures according to the findings of a previous study which estimated elemental carbon concentrations (a surrogate for DEPs) in indoor air at a heavy-traffic urban site to be about $0.6\text{ pg}/\text{cm}^3$ (Baxter *et al.*, 2008). In light of the results of the current study, and with the aim of imposing a further challenge to *D. pteronyssinus* mites, a 10-fold higher concentration of DEPs was assayed for a longer period (mites were exposed to the pollutant from the formation of eggs at F0 to the adult stage at F1). Even under these extreme conditions (black DEPs were easily visible within the mite digestive tract and in faecal pellets), none of the major allergens were affected, and significant changes in the transcription of only three allergen genes, *Der p 3*, *Der p 8* and *Der p 21*, were recorded (maximum 1.4-fold increase). The implications of this result in a natural scenario are limited, given the conditions imposed in the present study; however, they highlight the power of the method described herein to resolve small transcription

Table 1. Primers used in quantitative polymerase chain reaction (qPCR) of *Dermatophagoides pteronyssinus* genes.

Gene	Gene function	GenBank accession nos.	qPCR primers (5'–3')	Amplicon length, bp	Efficiency, %
<i>Der p 1*</i>	Cysteine protease	U11695, DQ185508, FM177224	F: GCCGTCATTATTGGCATCAAAG R: TCGACACCTTGTGCGTTAC	137	93.77
<i>Der p 2*</i>	ML domain, lipid-binding	AF276239, FM177223, AM263560	F: ACCAGGATGCCATGGTTCAG R: ACCATCGATTGAGGCTTTGATT	127	95.11
<i>Der p 3*</i>	Trypsin	U11719	F: TGATTCTGGTGGACCGGTTG R: CACCTGGATAACCTTTACGTGC	95	92.31
<i>Der p 4*</i>	α -amylase	AF144060	F: GGGCCGCATTATTGAATTCCG R: TCACCACTGGGAACCAATCC	117	90.15
<i>Der p 5*</i>	Unknown	X17699, S76340	F: ACGTGGTGTACTTGATCGTCT R: TCACGTTCCAAAATACACCAGA	102	90.84
<i>Der p 6</i>	Chymotrypsin	EX162270, EX162310, EX163598, EX163814, EX163851	F: TCCGGGTATGATTGTGCATTG R: TGGTCCCCATGATACAATACCG	112	107.00
<i>Der p 7*</i>	Lipid-binding protein	U37044	F: CCGCAGCTTTTGTGGCCG R: ATTGCAGCGACGGCTTCATC	94	94.02
<i>Der p 8</i>	Glutathione S-transferase	S75286, AY825938, AY825939	F: TGCCCGACTGCTTGAAATTGA R: CGGGCACCATCACCTTGAC	126	90.50
<i>Der p 9</i>	Serine protease	AY211952, AF409111	F: ACTTGGGGCTCTGTCAATGC R: ACCAAAGGACCACCAGAATCAC	95	101.10
<i>Der p 10*</i>	Tropomyosin	Y14906	F: ACGTGCCGAAACTGGTGAAT R: TGCTTCTTCACGTTGTTGTGC	118	95.68
<i>Der p 11</i>	Paramyosin	AY189697, EX163198	F: TGCTTTGGCCGGTGGTAAAC R: TCCGTTTCAGCATGTCGTCG	102	96.30
<i>Der p 14</i>	Apolipoprotein	AF373221	F: GCATCGCTGGTCAATCCACG R: AGCAATGTCGGATGATCGCA	105	92.40
<i>Der p 15</i>	Chitinase	DQ078741, DQ078740, EX162804, EX162854, EX163241	F: TTTCGGCCATAATGCTCCGT R: TCGGGTAGCGCCATTGTTC	112	90.30
<i>Der p 18</i>	Chitin-binding protein	DQ078739	F: TGTACCGACATTCCAGGTGT R: CGGGCAATGATGAACGGTTTG	130	95.20
<i>Der p 20</i>	Arginine kinase	EU684970, EU106619	F: TGGACGTTTGGTCAAGGCTG R: ACCGAAGCACGGATTGTGT	120	90.00
<i>Der p 21*</i>	Unknown	DQ354124	F: ACCCTTTTCGCTGCCATTGT R: ACGATCGAATGCCATACGCC	93	90.70
<i>Der p 23</i>	Peritrophin-like protein	EU414751, EX162598	F: CCGTTCATCCAACAACAACCGA R: TGTGGATCTTTGGATCGGCA	88	93.70
<i>RPL13a*</i>	Ribosomal protein L13a	—	F: AACGTGGTGATTGGCACTC R: GTAATGCAGCCGGTACAACC	96	97.17
<i>RPS4*</i>	Ribosomal protein S4	KF964488	F: ACCGGCCGAAAGGTATC R: TTCCCGTATCCATTTCATAGCG	112	97.45
<i>EF1a*</i>	Elongation factor 1- α	JQ000858	F: ACCCGTGAACATGCTTTGCT R: ACGAGCTTGCGAGAATGGTG	102	92.10

*Originally designed by Vidal-Quist *et al.* (2015).

changes. Thus, the reported overexpression of *Der p 3*, encoding a trypsin endoprotease, may be indicative of an alteration in the mite digestive process after ingestion of this high dose of toxic polycyclic aromatic hydrocarbons. Similarly, the increase of *Der p 8* transcription under such conditions is consistent with the expected role in detoxification of its protein, glutathione

S-transferase (GST) (Colloff, 2009). *Der p 21* is a mid-tier allergen of potential clinical relevance for some allergic patients; however, a link between the observed overexpression and challenge with DEPs cannot be established because this gene has been found only in mites and its function remains unknown (Thomas, 2015).

Table 2. Gene transcription of *Dermatophagoides pteronyssinus* allergen genes in response to exposure to domestic chemical stressors, estimated by real-time quantitative polymerase chain reaction (RT-qPCR).

Gene	DEPs		DEPs long-term		LPS		Benzyl benzoate	
	RT*	P-value†	RT*	P-value†	RT*	P-value†	RT*	P-value†
<i>Der p 1</i>	-1.1	0.753	1.3	0.075	-1.1	0.866	1.2	0.333
<i>Der p 2</i>	1.0	0.745	1.1	0.258	1.1	0.504	-1.0	0.777
<i>Der p 3</i>	1.1	0.737	1.2	0.049	1.2	0.262	-1.0	0.935
<i>Der p 4</i>	-1.4	0.365	-1.1	0.363	1.1	0.308	-1.4	0.110
<i>Der p 5</i>	-1.0	0.912	1.2	0.129	-1.1	0.190	1.0	0.953
<i>Der p 6</i>	-1.1	0.697	1.0	0.836	1.0	0.700	-1.0	0.802
<i>Der p 7</i>	-1.2	0.509	-1.3	0.262	1.2	0.258	-1.1	0.770
<i>Der p 8</i>	-1.0	0.443	1.2	0.038	-1.1	0.084	-1.0	0.864
<i>Der p 9</i>	1.0	0.889	1.1	0.148	-1.0	0.832	1.0	0.881
<i>Der p 10</i>	1.0	0.894	1.1	0.438	1.0	0.765	-1.1	0.684
<i>Der p 11</i>	-1.2	0.177	1.1	0.234	-1.2	0.187	-1.2	0.402
<i>Der p 14</i>	-1.4	0.432	-1.3	0.239	-1.0	0.866	-1.2	0.351
<i>Der p 15</i>	-1.4	0.352	-1.1	0.295	1.0	0.904	-1.3	0.287
<i>Der p 18</i>	-1.1	0.765	1.2	0.098	-1.1	0.621	1.2	0.231
<i>Der p 20</i>	-1.1	0.399	-1.0	0.897	-1.1	0.649	-1.1	0.475
<i>Der p 21</i>	-1.2	0.442	1.4	0.013	-1.2	0.278	1.1	0.799
<i>Der p 23</i>	-1.1	0.671	1.2	0.534	-1.0	0.910	-1.1	0.356

*Relative gene transcription (RT) expressed as fold-change in relation to the control treatment, calculated from normalized relative quantities (NRQs) ratios; negative values indicate lower transcription values than control.

†P-value of the *t*-test performed on *C_q* values (log₂-transformed NRQs). Data displayed in bold indicate statistically significant differences in relation to control values (*P* < 0.05).

DEPs, diesel exhaust particles; LPS, lipopolysaccharide.

Lipopolysaccharide recognition is a widespread mechanism for sensing bacteria and eliciting innate immune responses in mammals and invertebrates (Stokes *et al.*, 2015). No previous study has investigated the effect of ingested LPS on mites, although orally administered LPS is known to be to some extent adsorbed by the intestinal epithelium of mammals, to interact with the innate immune system, and potentially to exert beneficial effects in diseases such as allergy (Inagawa & Soma, 2011). House dust mites are constantly exposed to LPS endotoxins in their home microhabitats as significant amounts of endotoxins can be found in mattresses (1.4 units/cm²) and dust (22.8 units/mg) in non-farming households (Braun-Fahrlander *et al.*, 2002), as well as in mass-rearing cultures. Indeed, as has been revealed by genomic approaches, the gut microbiota of HDMs is especially rich in LPS-containing Gram-negative bacteria (Chan *et al.*, 2015). Thus, it was intriguing to investigate whether the ingestion of LPS by mites might affect allergen gene expression, particularly that of the group 2 allergens, which show structural similarity to the MD-2 protein of the mammal immune system and, like the latter, can bind LPS (Thomas, 2015). The exogenous application of LPS at a concentration about 100-fold higher than the typical concentrations found in household mattresses (Braun-Fahrlander *et al.*, 2002) did not induce any change in the expression of allergen genes. However, as this assay only partially represents the complexity of mite–bacteria interactions, the eventual impact of the microflora present in mattresses or dust on allergen expression remains to be elucidated.

Similar results were obtained in treatments with the acaricide BnBzO. The assayed concentration exceeds the cut-off level declared on the product label as stipulated for cosmetic products in the EU (10 p.p.m.; EU Directive 2003/15/EC) by almost 40-fold and therefore the physiological effects observed in the present bioassay (lower egg laying, little mortality) were by far greater than the residual activity expected for BnBzO as an ingredient of household products. This acaricide acts by ingestion, although its mechanism of action is not known (Colloff, 2009). However, none of the HDM allergens of which expression could potentially be altered, such as digestive enzymes [i.e. endoproteases (groups 1, 3, 6 and 9) and α -amylase (*Der p 4*)] (Table 1), or the detoxification enzyme *Der p 8*, were affected.

The methodological approach described in this paper (i.e. highly controlled bioassays conducted in concave microscope slides in combination with RT-qPCR) will be of use in studies of the impact of other factors, such as rearing diet or additional environmental exogenous compounds, on the expression of HDM allergen genes. The lack of commercial antibodies specific for most HDM allergens constrains study of their production in a mass-rearing context. However, the conducting of bioassays in slides and study of gene expression offer the advantages of simultaneous screening of different factors in a relatively short period and consideration of the full spectrum of allergen genes. Gene expression is not always directly related to protein production, but studying short-term transcriptional responses of mites under different experimental conditions will contribute to understanding of the allergen-related physiology of HDMs and thus to allergy research.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: DOI: 10.1111/mve.12200

Figure S1. Real-time polymerase chain reaction quantification cycles (*C_q*) determined for allergen and reference genes in four biological replicates corresponding to controls (not exposed to chemical stressors) in the experiment involving long exposure to diesel exhaust particles.

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