

Allergen expression in the European house dust mite *Dermatophagoides pteronyssinus* throughout development and response to environmental conditions

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Abstract. House dust mites are a major source of allergy worldwide. While diagnosis and treatment based on mite extracts have remarkably advanced, little information exists on the expression of allergens in mites. We have studied gene expression of eight *Dermatophagoides pteronyssinus* (Trouessart) (Acari: Pyroglyphidae) allergens (*Der p* 1, 2, 3, 4, 5, 7, 10 and 21). All allergens showed higher transcription in nymphs compared with larvae or adults, with the only exception of *Der p* 10. The transcription of *Der p* 4 and *Der p* 10, together with the transcription and protein ratios *Der p* 1 to *Der p* 2, were higher in males than in females. One-week exposure of mite cultures to 16 or 35 °C (versus 24 °C) or low RH (44% versus 76%) significantly influenced the allergen gene transcription profile. Our results demonstrate that allergen expression is quantitatively and/or qualitatively influenced by mite development and sex, as well as by the environment. We suggest that monitoring allergen gene expression may be a useful tool to assist the optimization of mite cultures in the production of standardized allergenic extracts for clinical use.

Key words. Allergen profile, allergenic extracts, allergy, ELISA, HDM, mite culture, real-time quantitative PCR, tropomyosin.

Introduction

House dust mites (HDMs) are one of the most common sources of allergy worldwide and the major cause of allergic sensitization in asthmatics (Thomas *et al.*, 2010). *Dermatophagoides pteronyssinus* and *D. farinae* Hughes (Acari: Pyroglyphidae) are among the most medically relevant HDM species (Arlan *et al.*, 2002). Our knowledge of the medical effects and molecular processes underlying HDM allergy have grown extensively in the recent decades; as a result, diagnosis and immunotherapy have remarkably improved (Thomas, 2012). From the 17 allergens identified in *D. pteronyssinus* so far (<http://www.allergen.org>), only a limited number are responsible for most of the allergic reactions observed in patients, according to IgE-binding studies. Two major allergens, *Der p* 1 and *Der p* 2, collectively bind

50–60% of total anti-HDM IgE antibody; followed by a group of mid-potency but clinically relevant allergens (*Der p* 4, *Der p* 5, *Der p* 7 and *Der p* 21); and a third group of allergens with generally weak but detectable binding (*Der p* 3, *Der p* 8 and *Der p* 10) (Thomas *et al.*, 2010).

HDM allergy diagnosis and immunotherapy is at present based on natural allergen extracts produced in large-scale mite cultures, their standardization being one of the main concerns of the industry (Fрати *et al.*, 2012). Current routine monitoring of HDM extract production processes relies primarily on dosage of the two major allergens, *Der p* 1 and *Der p* 2, being their relative abundance (i.e. *Der p* 1 to *Der p* 2 ratio) an important quality parameter (Carreira *et al.*, 1994). Nevertheless, recent studies have stressed the great variability observed in the allergen composition of commercially available *D. pteronyssinus* extracts

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that lacked relevant allergens in some cases and produced heterogeneous results in allergy diagnostic tests (Brunetto *et al.*, 2010; Casset *et al.*, 2012). Production of standardized allergenic extracts from mite cultures is complex and, therefore, different studies are being conducted in order to find alternative systems for allergen production based on non-natural sources (Valenta *et al.*, 2011; Casset *et al.*, 2012; Thomas, 2012). However, the complexity of allergen–patient interactions together with the multi-allergen profile exhibited by HDM (Thomas *et al.*, 2010) may complicate their success. Furthermore, cross-reactivity with non-HDM allergens and geographical/socio-cultural factors may also contribute to variations in the above-mentioned hierarchy of HDM allergens (Thomas, 2012). Thus, there is a need to better understand the profile of allergens produced by mites, both quantitatively and qualitatively, and not only in mite cultures, but also at different home microhabitats. Noteworthy, efforts on understanding HDM allergy have mainly been focused on the patient's perspective, comparatively giving little attention to the mite's side. As a matter of fact, the physiological function of many allergens remains uncertain, or has been indirectly inferred from sequence/structural similarity to proteins of known function in other organisms (Cui, 2013). Studies directed towards the optimization of the cultural methods for production of allergenic extracts have shown that factors such as incubation temperature (Yella *et al.*, 2011) and diet (Avula-Poola *et al.*, 2012) may affect net allergen accumulation in whole cultures. However, allergen detection in those studies was restricted to Der p 1 and Der p 2 major allergens using specific antibodies. Thus, further investigation on allergen expression in HDM may be of great help in exploring their function and regulation, finally contributing to the optimization of standardization of allergenic extracts, as well as to a better understanding of mite-derived allergies.

In this paper, we report on the study of the expression of eight allergen genes in *D. pteronyssinus* using reverse transcription and real-time quantitative PCR (RT-qPCR). Our research aimed to address two key aspects of *D. pteronyssinus* physiology that have implications on allergen production and, therefore, with medical and industrial interest. First, we characterized baseline allergen expression levels of the different mite developmental instars in order to assess if it is likely that changes in population structure may affect allergen production. Second, we inspected whether short-term environmental changes (i.e. with low impact on population structure) may have a direct effect upon mite physiology that would alter allergen production.

Materials and methods

Mite cultures

The laboratory strain of *D. pteronyssinus* used in these studies originates from commercial cultures (Dermauw *et al.*, 2009) and has been kindly provided by Dr Mailleux (Catholic University of Louvain, Belgium). Mites were reared in 50-mL wide-mouth sterile Erlenmeyer flasks containing 2 g of a 1 : 1 mixture of fish food flakes (Vipan Sera, Heinsberg, Germany) and brewer's yeast flakes (Santiveri, Barcelona, Spain). A mite-rearing diet was pre-treated at 60 °C for 6 h in order to minimize the interference

of undesired biological contaminants. Air exchange was allowed by covering the flasks with sterile hydrophilic cotton caps. Culture flasks were maintained in a Sanyo MLR-350H incubator. The standard environmental conditions were 24 ± 0.3 °C and $76 \pm 2\%$ RH in the dark. RH was controlled by placing culture flasks inside sealed boxes containing a saturated NaCl solution (Winston & Bates, 1960). Subcultures (20-fold diluted inoculum) were performed approximately after the sixth week of growth.

Collecting *D. pteronyssinus* instars and sexes

Samples of approximately 50 mg of thriving mite culture (5–6 weeks old, in the conditions described above) were spread onto a printer paper disk enclosed in a glass Petri dish. Mites were allowed to settle on the disk for 2–5 min at room temperature and the Petri dish was turned upside down. Crawling mites that remained attached to the paper disk were observed under the stereomicroscope and developmental stages and adult sexes were separated according to their morphological traits (Colloff, 2009). Samples were collected for RNA extraction or for ELISA test. For RNA extractions, the different mite instars/sexes were selectively collected using a vacuum pump connected to a 200-µL sterile filter tip. In order to reduce the stress caused by sampling, the collection time never exceeded 5 min to collect no more than 200–500 individuals. Mites were recovered from tips by gently tapping the tip, cutting the end with a sterile razor, pipetting 100–200 µL of ice-chilled TRIzol Reagent (Life Technologies, Carlsbad, U.S.A.) and pouring it immediately into a 1.5-mL microtube that was submerged in liquid N₂. Batches of 200–500 individuals were homogenized using a microtube pellet pestle and gathered together to complete 550–600 adult mites (males and females), 1200–1500 nymphs (proto- and tritonymphs together), 1500–1700 larvae, 200–250 adult females or 250–300 adult males. TRIzol-homogenates were kept at –80 °C. For ELISA assays, 20 mites per instar/sex were manually collected from paper disks using a very thin paintbrush and were transferred to 100 µL of phosphate buffer saline (PBS) solution pH = 7.3, samples were frozen into liquid N₂ and thawed in ice. Suspensions were homogenized using a microtube pellet pestle, followed by the addition of BSA solution (bovine serum albumin, BSA, 0.2% w/v in PBS pH = 7.3) to a final concentration of BSA 0.1% w/v, mixed and stored at –20 °C. Biological replicates for each developmental stage/sex were collected from independent culture flasks for either qPCR ($n = 3$) or ELISA test ($n = 7$).

Exposing mite cultures to different temperatures or RHs

Mite cultures were maintained as described above but, 5 weeks after seeding, some flasks were exposed during 1 week to a different temperature or RH, without a pre-conditioning step. Exposure for 7 days was preferred to longer periods in order to avoid a drastic change in mite populations during the experiments, thus emphasizing the analysis of changes at the physiological level of individual mites over effects on

whole populations. Three temperatures, 16, 24 and 35 °C, were assayed by transferring three flasks (biological replicates) to different climate chambers set at 16 or 35 °C, while maintaining the same number of flasks at the standard conditions (24 °C and 76% RH). Changes in the RH between treatments were minimized by keeping flasks into sealed boxes with a saturated NaCl solution. Thus, the RH inside the boxes was maintained at approximately 76% at the three temperature regimes (minimum throughout the experiment of 74.5%, and maximum 79.0%). In an independent experiment, two RH conditions were assayed at a constant temperature of 24 °C by transferring four culture flasks (biological replicates) to a sealed box containing a MgCl₂ saturated solution (equilibrium RH was approximately 44%, ranging from 43.3% to 44.2%), while maintaining the same number of flasks at the standard conditions described above. Experiments ended 6 weeks after seeding the flasks, and samples were collected after gentle homogenization of whole cultures (mixed mites and diet) with the aid of a laboratory spoon. Three samples were collected per flask to be used for (a) RNA purification and RT-qPCR (25–30 mg), (b) ELISA (50–60 mg), or (c) mite population counts (65–85 mg). Samples for RT-qPCR were immediately frozen in liquid N₂ and homogenization was performed with the aid of a microtube pellet pestle after adding 200 µL of ice-cold TRIzol in a first step, plus 600 µL in a second step. TRIzol-homogenates were kept at –80 °C. Samples for ELISA were homogenized in BSA solution (BSA 0.1% w/v, in PBS pH = 7.3) at 5% (w/v; culture/BSA solution), the supernatant was recovered after centrifugation (7500 g, 15 min, 4 °C) and stored at –20 °C. Samples collected for mite population counts were processed immediately.

Density and population structure in mite cultures

The instar/sex composition of the mite populations was checked at the sampling time. Samples were weighed and suspended in 25 mL of 75% glycerol. After gently mixing, 5 mL of the mite suspension was filtered through a paper disk grid using a Kitasato flask connected to a pump. The disk was designed as a concentric grid with shaded areas randomly placed to cover one-eighth of the surface. Mites lying on the shaded areas were counted using a stereomicroscope. Counts were averaged from two readings per sample and expressed as the number of mites per gramme of culture.

RNA extraction, reverse transcription and real-time quantitative PCR

Samples stored as TRIzol homogenates, as described above, were thawed on ice and RNA extraction was performed according to the TRIzol Reagent manufacturer's instructions (Life Technologies, Carlsbad, CA, U.S.A.). RNA precipitation was performed by adding 20 µg/µL UltraPure glycogen (Life Technologies) and RNA was resuspended in RNase-free Resuspension Solution (Life Technologies) followed by incubation at 60 °C for 10 min. RNA quantity was assessed using the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.), and quality was verified by the Experion Automated Electrophoresis System (Bio-Rad, Hercules,

CA, U.S.A.). Prior to reverse transcription, 2 µg of total RNA was treated with RQ1 DNase (Promega, Madison, WI, U.S.A.) by incubation at 37 °C for 35 min followed by 10 min at 75 °C. Reverse transcription was carried out using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions, with minor modifications: 0.5 µg of oligo (dT)₁₈ primer was added to the sample and incubated at 70 °C for 5 min; followed by addition of the 5× reaction buffer, RiboLock RNase inhibitor and dNTP mix, contained in the kit, and incubated for 5 min at 37 °C; the reaction was completed by adding 200 U of reverse transcriptase and incubating for 1 h at 42 °C, followed by 10 min at 70 °C. The resulting cDNA was diluted in ultrapure water to a final concentration of 50 ng/µL (equivalent to the starting amount of total RNA), it was then aliquoted and stored at –80 °C.

Real-time quantitative PCR (qPCR) primers were designed for eight allergen genes and five reference genes (Table 1) using the online application Primer-BLAST (Ye *et al.*, 2012). Sequences used for primer design were previously available at the GenBank Database, with the exception of those for *RPL13a*, *RPS4* and *aTub* that were obtained by PCR amplification with degenerate primers, cloning and sequencing (Table 1). The degenerate primers used were: for *RPL13a*, FDEGR13a (5'-TG GCGNACNGTNMGNGGNATG) and RDEGR13a (5'-TGRT AYTTCANCCNACYTCRTG); for *RPS4*, FDEGRS4 (5'-AA YTAYCCNGCNGGNTTYATGGA) and RDEGRS4 (5'-TTR TGNCNCNCNGTNACCATRCA); for *aTub*, FDEGATub (5'-GA RCAYTCNGAYTGYGCNTTYATGG) and RDEGATub (5'-TC CATNCCYTCNCCNACRTACCA). Non-specific binding of each primer pair was inspected by running the BLASTn functionality incorporated in Primer-BLAST against the *nr* NCBI nucleotide sequence database. When possible, primers were designed in sites with no reported nucleotide variation and/or in regions flanking intronic sequences. Primer efficiency was estimated on five-fold serial diluted cDNA and standard curves were obtained using the Rotor-Gene 6000 Series Software v1.7 (Qiagen, Hilden, Germany). Efficiency values were averaged from up to three independent standard curves. qPCR was carried out in a Corbett Rotor Gene 6000 real-time cycler (Qiagen) using the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, U.S.A.). The reactions contained 1× master mix, 0.3 µM primers, 10 ng cDNA template and ultrapure water to a total volume of 15 µL. Cycling conditions consisted of one initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s (with data acquisition at the end on this step). A final melt curve step was included to evaluate dissociation of amplification products: a pre-melting step for 3 min at 50 °C and a temperature ramp of 50–99 °C, acquisition at 1 °C intervals. Melting curves corresponding to cDNA samples of different origins (purified mite bodies or whole culture) amplified with the same primers showed identical profiles. Two technical replicates were performed per sample in all the experiments. The distribution of biological and technical replicates in the thermal cycler along successive qPCR runs (70 reactions per run) was designed to achieve a statistical balance in order to minimize eventual bias resulting from inter-run variation (Rieu & Powers, 2009). Raw qPCR data (*C_q* values) were efficiency corrected and allergen gene transcription was normalized to the reference

Table 1. Primers used in qPCR of *Dermatophagoides pteronyssinus* genes.

Gene	Gene function	GenBank accessions	qPCR primers (5'-3')	Amplicon length (bp)	Efficiency (%)
Der p 1	Cysteine protease	U11695; DQ185508; FM177224	F: GCCGTCATTATTGGCATCAAAG R: TCGACACCTTGTGCGTTAC	137	93.77
Der p 2	ML domain, lipid-binding	AF276239; FM177223; AM263560	F: ACCAGGATGCCATGGTTTCAG R: ACCATCGATTGAGGCTTTGATT	127	95.11
Der p 3	Trypsin	U11719	F: TGATTCTGGTGGACCGGTTG R: CACCTGGATAACCTTTACGTGC	95	92.31
Der p 4	Alpha-amylase	AF144060	F: GGGCCGCATTATTGAATTCCG R: TCACCACTGGGAACCAATCC	117	90.15
Der p 5	Unknown	X17699; S76340	F: ACGTGGTGTACTTGATCGTCT R: TCACGTTCCAAAATATCACCAGA	102	90.84
Der p 7	Lipid-binding protein	U37044	F: CCGCAGCTTTTGTGCGCG R: ATTGCAGCGACGGCTTCATC	94	94.02
Der p 10	Tropomyosin	Y14906	F: ACGTGCCGAACTGGTGAAT R: TGCTTCTTCACGTTGTTGTGC	118	95.68
Der p 21	Unknown	DQ354124	F: ACCCTTTTCGCTGCCATTGT R: ACGATCGAATGCCATACGCC	93	90.70
RPL13a*	Ribosomal protein L13a	—†	F: AACGTGGTGATTGGCACTC R: GTAATGCAGCCGGTACAACC	96	97.17
RPS4*	Ribosomal protein S4	KF964488	F: ACCGGGCCGAAAGGTATC R: TTCCCGTATCCATTTCATAGCG	112	97.45
<i>α</i>Tub*	Alpha-tubulin	KF964489	F: TCAATCACTGCTTCGCTTCG R: CCGAAATAACCGGTGCATAGG	124	94.66
EF1a	Elongation factor 1- α	JQ000858	F: ACCCGTGAACATGCTTTGCT R: ACGAGCTTGCAGAAATGGTG	102	92.10
Actin	Beta-actin	EX163181; EX163263; EX163476	F: GCCGGTGATGATGCTCCA R: ATCTTTGTTGCCCATACCAACC	90	101.18

*Sequences obtained *de novo* in this study.

†The known sequence is shorter than 200 bp, thus, it has not been submitted to the GenBank database.

genes to obtain normalized relative quantities (*NRQ*), as previously described (Hellems *et al.*, 2007). The group of reference genes used in data normalization were independently selected for each experiment in the basis of their expression stability; as estimated by three different algorithms, namely GeNorm, Normfinder and Bestkeeper (Vandesompele *et al.*, 2002; Andersen *et al.*, 2004; Pfaffl *et al.*, 2004). Relative gene transcription was graphically displayed with respect to a fixed factor/treatment in the form of *NRQ* ratios; standard errors were propagated accordingly (Rieu & Powers, 2009).

Enzyme-Linked ImmunoSorbent Assay (ELISA)

Der p 1 and Der p 2 content was determined by ELISA, based on allergen-specific monoclonal antibodies (Barber *et al.*, 2012). The assays were calibrated against the WHO/IUIS *D. pteronyssinus* standard (NIBSC 82/518) as containing 12.5 μ g of Der p 1 and 0.3 μ g of Der p 2 per ampoule.

Total soluble protein content in mites

As the bodies of larvae, nymphs, adult males and adult females exhibit substantial differences in size, and being challenging to

accurately weigh mites, the total soluble protein content was estimated for each *D. pteronyssinus* instar/sex and used for the normalization of allergen quantification data. Groups of a counted number of mites (50 to 200 individuals, depending on the instar) were suspended in 100 μ L of PBS pH = 7.3, flash frozen and thoroughly homogenized. After spinning down large debris (20 s at 2000 $\times$ g), protein content of the soluble fraction was estimated by the Lowry method using a BSA standard curve and the DC Protein Assay Kit (Bio-Rad), according to manufacturer's instructions. Three replicates were analysed per instar/sex and data were averaged as micrograms of soluble protein per mite. The soluble protein content of each instar/sex was assumed to be proportional to the mite fresh weight and, therefore, it was used as the reference to normalize mite allergen content.

Statistical analysis

Statistical analysis of gene transcription data was performed on *Cq'* values ($\log_2$ transformation of *NRQs*) and groups were compared using, alternatively, one-way analysis of variance (ANOVA) with multiple comparisons by Bonferroni's post-test or *t*-test, depending on the experiment. For inherent reasons of the qPCR method, expression ratios between target genes

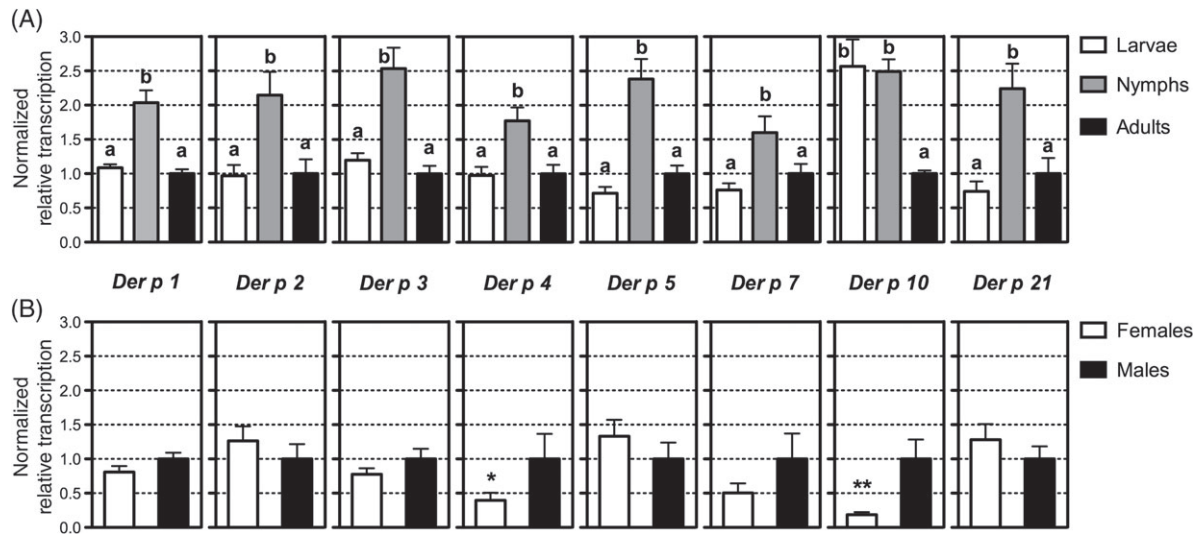


Fig. 1. Gene transcription of *Der p 1*, *Der p 2*, *Der p 3*, *Der p 4*, *Der p 5*, *Der p 7*, *Der p 10* and *Der p 21* according to mite developmental stage or sex, estimated by real-time quantitative PCR (RT-qPCR). Plotted values represent relative gene expression ratios between developmental stages (A; in relation to the adult stage) or between adult sexes (B; in relation to males) for each allergen gene, in two independent experiments. Data were normalized with the reference genes *RPL13a*, *RPS4* and *alpha-Tubulin* (A) or *RPL13a*, *RPS4* and *EF1a* (B); and are means from three biological replicates. Error bars show the propagated standard error of the ratio. (A) Different letters above relative expression ratios of each gene denote significant differences between developmental stages assessed by one-way ANOVA with Bonferroni's post-test ($P < 0.05$). (B) Asterisks show significant differences between sexes assessed by the t -test (* $P < 0.05$, ** $P < 0.01$).

(i.e. *Der p 1* : *Der p 2* expression ratio) are methodologically biased and not quantitatively meaningful, as relative gene expression data for each gene is obtained from different primer pairs (Rieu & Powers, 2009). However, regardless of actual relative values, qualitative changes can be addressed statistically on ordinal (ranked) $NRQ_{Der p 1} : NRQ_{Der p 2}$ values using either Kruskal–Wallis (coupled with Dunns post-test) or Mann–Whitney non-parametric tests. Statistical analysis of mite culture counts was carried out using one-way ANOVA, mortality rates were compared using non-parametric tests (Kruskal–Wallis or Mann–Whitney test) and differences in population structure were assessed by Pearson's chi-squared test of the pooled counts from all biological replicates (larvae, nymphs, adult males and adult females mite counts corresponding to 2 mg of culture). ELISA experiments were analysed by either one-way ANOVA coupled with Bonferroni's post-test or t -test. Statistical analyses were computed using GraphPad PRISM v5.00 (GraphPad Software, La Jolla, CA, U.S.A.).

Results

Allergen transcription levels comparing developmental instars

It is worth remarking that all allergen genes studied were expressed in all developmental instars studied at the optimal range for detection under our RT-qPCR conditions. However, the transcription level of seven of the eight allergen genes studied (*Der p 1*, 2, 3, 4, 5, 7 and 21) was significantly higher in the nymphal instar compared with larvae or adults (Fig. 1A).

The higher relative expression observed in nymphs ranged from 1.60-fold (*Der p 7*) to 2.54-fold (*Der p 3*). Transcription of *Der p 10* did not significantly differ between larvae and nymphs, but it was significantly lower in adults (approximately 2.5-fold reduction). In addition, comparing adult males and females revealed that *Der p 4* and *Der p 10* expression varied significantly between sexes, being lower in females than in males: 2.52-fold for *Der p 4* and 5.57-fold for *Der p 10* (Fig. 1B). As noted in the *Materials and Methods*, caution should be taken when comparing expression values obtained by RT-qPCR from different genes. Consequently, we have compared the variation of *Der p 1* to *Der p 2* ratios among different developmental instars at an ordinal scale (values can be defined as greater, equal or less than each other). The comparison revealed that the *Der p 1* to *Der p 2* expression ratio significantly differed between sexes of the adult mite (being higher in males compared with females), but not among the different developmental stages (Table 2).

Der p 1 and *Der p 2* proteins content comparing developmental instars

The content of *Der p 1* and *Der p 2* proteins in mite bodies was estimated by ELISA. As the number of mites analysed per sample was known, raw values could be expressed as picograms of allergen per mite (Table 3). Besides, the total soluble protein was additionally estimated for each instar and adult sex in order to normalize *Der p 1* and *Der p 2* concentrations in accordance with the varying size of the different instars or sexes. Thus, *Der p 1* and *Der p 2* content was also expressed as parts per million (p.p.m.) in relation to the total soluble

Table 2. Effect of developmental stage, sex, temperature or relative humidity on the *Der p 1* : *Der p 2* transcription ratio.

	Significance*(<i>P</i> -value)	Test statistic	<i>n</i>
Developmental stage	NS (<i>P</i> = 0.4298)	<i>H</i> (2) = 1.689	3
Adult sex	*(<i>P</i> = 0.0500)	<i>U</i> ≤ 0.001	3
Temperature	*(<i>P</i> = 0.0390)	<i>H</i> (2) = 6.489	3
Relative humidity	NS (<i>P</i> = 0.5000)	<i>U</i> = 8.000	4

*Statistical significance of changes in the *Der p 1* : *Der p 2* gen expression ratio were assessed by comparison of the $NRQ_{Der p 1} : NRQ_{Der p 2}$ ratio of each biological replicate using non-parametric tests (Kruskal–Wallis or the Mann–Whitney test; NS *P* > 0.05; **P* < 0.05).

protein content. As expected, mites with larger bodies generally contained significantly higher total amounts of both *Der p 1* and *Der p 2* allergens (i.e. adult females). When the allergen content was normalized to the total soluble protein content (p.p.m.), significant differences were reported between instars for both allergens. The normalized *Der p 1* and *Der p 2* contents were significantly higher in nymphs than in any other instar, except for *Der p 2* in adult females. By contrast, *Der p 1* and *Der p 2* normalized contents did not significantly differ between adult sexes. Finally, the *Der p 1* to *Der p 2* ratio was not significantly different when comparing developmental stages but was significantly higher in adult males than in females.

Allergen transcription levels at different environmental conditions

Mite cultures were exposed to a change in temperature, from 24 to 16 °C or 35 °C (keeping RH between 74.5 and 79.0%), or a change in RH, from 76% to 44% (keeping temperature at 24 °C). The alteration and maintenance of a different rearing temperature for 1 week did not significantly affect mite population abundance (number of larvae, nymphs, adults and total mites), mortality or population structure [$\chi^2_{(6)} = 5.690$; *P* = 0.4588]. By contrast, even although mite population structure was not significantly affected by 1-week exposure at low RH [$\chi^2_{(3)} = 1.765$; *P* = 0.6225], the total number of individuals and the number of nymphs were significantly lower compared with cultures at the standard conditions. In addition, the low RH had a significant effect on mortality, which attained 25% (Table 4).

Rearing at a different temperature for 1 week had a slightly but significant effect on the transcription of two of the eight genes studied, *Der p 3* (1.32- and 1.46-fold reduction under 16 and 35 °C, respectively) and *Der p 10* (1.68-fold reduction under 16 °C) (Fig. 2A). By contrast, low RH influenced the transcription level of all allergens studied with the exception of *Der p 3* and *Der p 7*. Thus, low RH caused a significant reduction in the expression of *Der p 1*, 2, 4, 5 and 21; ranging from 2.54 (*Der p 5*) to 3.68 (*Der p 4*), and a significant 3.50-fold increase in the expression of *Der p 10* (Fig. 2B). In addition, rearing temperature, but not RH, significantly influenced the *Der p 1* to *Der p 2* expression ratio (Table 2). Further Dunn's post-test for multiple comparisons of ratios at the three tested temperatures revealed that only the exposure at 16 °C produced a significant variation (higher values at an ordinal scale) in the *Der p 1* to *Der p 2* expression ratio (*P* ≤ 0.05).

Der p 1 and *Der p 2* proteins accumulation in cultures subjected to environmental changes

Whole culture samples (mite and diet) were collected from the same flasks and at the same time points selected for gene expression studies in order to simultaneously estimate *Der p 1* and *Der p 2* content in cultures subjected to environmental changes. Data obtained are shown in Table 5. The contents of group 1 and group 2 allergens were significantly affected by temperature. *Der p 1* content was significantly lower in cultures placed for 1 week at 16 or 35 °C than in cultures maintained at the standard culture conditions (24 °C), whereas *Der p 2* content was only significantly lower in cultures placed at 35 °C. Consequently, the *Der p 1* to *Der p 2* ratio varied between treatments, with significantly lower ratios in cultures placed at 16 °C than at 35 °C. *Der p 1* culture content was significantly lower in cultures placed for 1 week at low RH (44%) compared with cultures maintained at a standard condition; whereas *Der p 2* content did not significantly differ between treatments. In spite of this, the *Der p 1* to *Der p 2* ratio did not significantly change when comparing both RH conditions.

Table 3. *Der p 1* and *Der p 2* allergen content in bodies of *Dermatophagoides pteronyssinus* at different developmental instars.

Stage/sex	pg/mite*		Total soluble protein (µg/mite)	p.p.m. †		Ratio‡ <i>Der p 1</i> : <i>Der p 2</i>
	<i>Der p 1</i>	<i>Der p 2</i>		<i>Der p 1</i>	<i>Der p 2</i>	
Larvae	77 ± 18 (a)	88 ± 15 (a)	0.64 ± 0.17 (a)	120 ± 28 (a)	137 ± 23 (a)	0.86 ± 0.12 (ab)
Nymphs	2251 ± 285 (bc)	2461 ± 556 (a)	1.74 ± 0.07 (b)	1294 ± 164 (c)	1414 ± 319 (c)	1.02 ± 0.11 (ab)
Adult males	1005 ± 236 (ab)	923 ± 351 (a)	2.42 ± 0.26 (b)	415 ± 98 (ab)	382 ± 145 (ab)	1.66 ± 0.39 (a)
Adult females	3859 ± 782 (c)	5443 ± 1124 (b)	5.63 ± 0.11 (c)	685 ± 139 (b)	967 ± 200 (bc)	0.75 ± 0.05 (b)

*Absolute ELISA estimates per mite.

†Allergen content normalized to the average total soluble protein content per mite, expressed as parts per million (p.p.m.).

‡Ratio of the absolute estimates for *Der p 1* and *Der p 2*.

Different letters in the same column indicate significant differences (*P* < 0.05) between data assessed by one-way ANOVA coupled with Bonferroni's post-test. All values in the table are expressed as means ± standard error of the mean (*n* = 3 for total soluble protein; *n* = 7 for ELISA).

Table 4. Composition of *Dermatophagoides pteronyssinus* cultures subjected to different environmental conditions for 1 week.

	Temperature				Relative humidity		
	16 °C ($\times 10^3$ mites/g)	Standard * ($\times 10^3$ mites/g)	35 °C ($\times 10^3$ mites/g)	P†	Standard * ($\times 10^3$ mites/g)	44% ($\times 10^3$ mites/g)	P†
Larvae	22.87 $\pm$ 0.84	21.67 $\pm$ 2.09	17.50 $\pm$ 1.02	NS	24.53 $\pm$ 4.20	14.34 $\pm$ 3.72	NS
Nymphs	54.50 $\pm$ 5.03	54.97 $\pm$ 3.00	49.83 $\pm$ 5.43	NS	45.33 $\pm$ 6.20	25.68 $\pm$ 5.29	*
Adult females	8.30 $\pm$ 2.65	5.09 $\pm$ 0.51	5.38 $\pm$ 1.40	NS	4.14 $\pm$ 0.52	3.10 $\pm$ 0.76	NS
Adult males	7.40 $\pm$ 1.61	7.15 $\pm$ 0.61	5.86 $\pm$ 2.19	NS	4.55 $\pm$ 0.56	3.20 $\pm$ 0.69	NS
Total	93.00 $\pm$ 9.50	88.90 $\pm$ 3.92	78.57 $\pm$ 8.15	NS	78.50 $\pm$ 9.16	46.33 $\pm$ 9.86	*
Mortality (%)	9.3 $\pm$ 2.7	10.3 $\pm$ 0.7	19.7 $\pm$ 1.9	NS	5.5 $\pm$ 1.5	25.3 $\pm$ 3.7	**

*Standard growth conditions (24 °C, 76% RH).

†Statistical differences between treatments (NS $P > 0.05$; * $P < 0.05$; ** $P < 0.01$) assessed by one-way ANOVA (comparison of larvae, nymphs, adult stages and total number of mites) or the Kruskal–Wallis/Mann–Whitney test (for mortalities).

All values in the table are expressed as mean $\pm$ standard error of the mean ($n = 3$, temperature; $n = 4$, RH).

Discussion

We report for first time the study of the gene expression of eight of the most hierarchically relevant allergens produced by *D. pteronyssinus* (Thomas *et al.*, 2010). Quantification of allergens produced by HDM in previous studies has been limited to monitor accumulation of major allergens Der p 1 and Der p 2 in cultures, using monoclonal antibodies (Yella *et al.*, 2011; Avula-Poola *et al.*, 2012) and, in a few cases, to detect the presence of other allergens in allergenic extracts, although using semi-quantitative techniques (Batard *et al.*, 2006; Casset *et al.*, 2012). The comprehensive methodological approach presented in this paper has demonstrated that the allergen gene expression profile in *D. pteronyssinus* is influenced by development and

by the environment. Consequently, changes in the population structure in a culture, as well as variations in the climatic regime (temperature and relative humidity) may quantitatively and/or qualitatively affect final allergen production.

First, we demonstrate that *D. pteronyssinus* actually expresses the eight allergens studied in all post-embryonic stages, although with differences during development. Interestingly, the maximum transcription was reported at the nymphal stage for seven of the eight allergen genes under study (Fig. 1A). Consistent with these results, the quantification of Der p 1 and Der p 2 proteins in mite bodies, as assessed by ELISA and normalized for the different developmental instars (Table 3), showed that these allergens are also produced at a higher rate in nymphs. Considering that little information exists on the

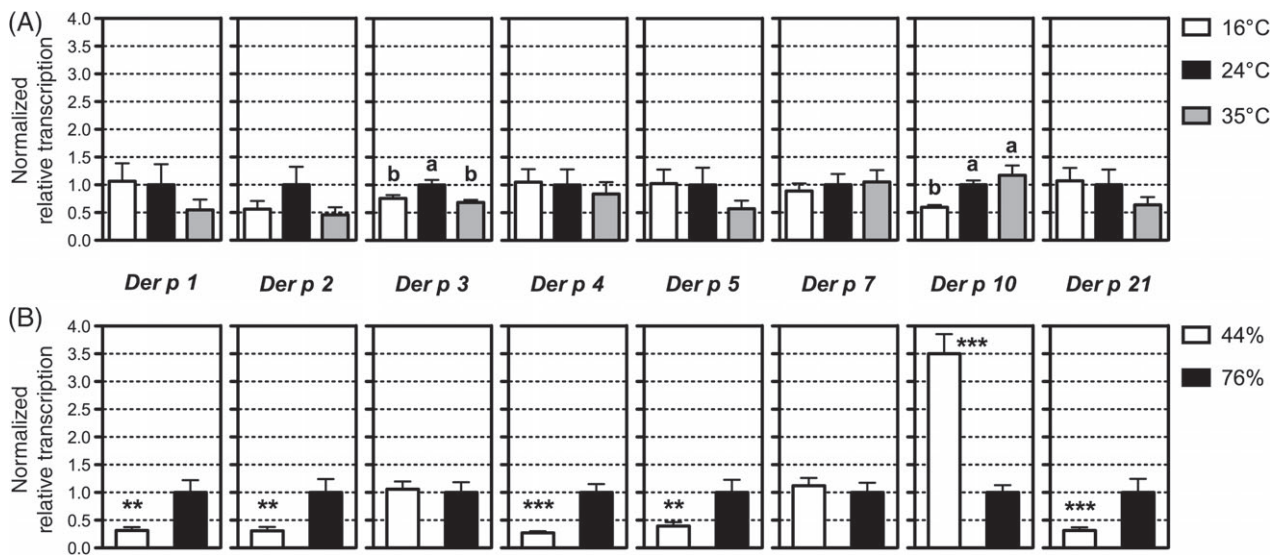


Fig. 2. Gene transcription of *Der p 1*, *Der p 2*, *Der p 3*, *Der p 4*, *Der p 5*, *Der p 7*, *Der p 10* and *Der p 21* in cultures subjected to different environmental conditions, estimated by RT-qPCR. Samples were obtained from whole cultures maintained at different temperature (A) or relative humidity (B) during the last week of culture, in two independent experiments. Plotted values represent relative gene expression ratios in relation to the standard condition (24 °C in A; 76% RH in B) for each allergen gene, normalized with the reference genes *RPL13a*, *RPS4* and *Actin* (A) or *RPL13a*, *RPS4* and *EF1a* (B); and are means from three (A) or four (B) biological replicates. Error bars represent the propagated standard error of the ratio. (A) Different letters above relative expression ratios of each gene denote significant differences between temperatures assessed by one-way ANOVA with Bonferroni's post-test ($P < 0.05$). (B) Asterisks show significant differences between RHs assessed by the *t*-test (** $P < 0.01$; *** $P < 0.001$).

Table 5. Der p 1 and Der p 2 allergen content in whole cultures of *Dermatophagoides pteronyssinus pteronyssinus* subjected to different environmental conditions for 1 week.

	Temperature*			Relative humidity*	
	16 °C	24 °C	35 °C	76%	44%
Der p 1 (µg/g culture)	669 ± 14 (a)	1062 ± 31 (b)	767 ± 45 (a)	838 ± 145	433 ± 90 (*)
Der p 2 (µg/g culture)	811 ± 62 (a)	919 ± 17 (a)	513 ± 26 (b)	774 ± 71	552 ± 102 (NS)
Der p 1 : Der p 2	0.83 ± 0.05 (a)	1.16 ± 0.03 (ab)	1.51 ± 0.17 (b)	1.06 ± 0.12	0.77 ± 0.02 (NS)

*Cultures were subjected to either a different temperature or a different RH for 1 week, in two independent experiments. Different small letters in the same row indicate significant differences ($P < 0.05$) of allergen content between temperatures estimated by ELISA, assessed by one-way ANOVA coupled with Bonferroni's post-test. Statistical differences between RH treatments were assessed by the t -test (NS $P > 0.05$; * $P < 0.05$). All values in the table are expressed as means ± standard error of the mean ($n = 3$, temperature; $n = 4$, RH).

exact physiological role of some of the allergens identified in *D. pteronyssinus*, and in HDM in general, any explanation to our findings should be taken with caution. However, the higher expression of allergens observed in the nymphal stage could be related to their higher growth rate compared with larvae (Arlian *et al.*, 1990). Similarly, adults could have a lower feeding activity than immature stages, as it occurs in some insects (Scriber & Slansky, 1981), and this could eventually lead to lower expression of metabolism-related allergens in this stage, as we observed. We also demonstrate that allergen transcription is influenced by the sex of the adult mite, with females exhibiting lower transcription levels of *Der p 4* and *Der p 10* (Fig. 1B). In addition, we show that the relative Der p 1 and Der p 2 level is likely influenced by sex, based on the significance of the differences of their respective transcription and protein ratios (Tables 2 and 3). Altogether, our results stress that the evolution of population structure in mite cultures should be taken into account when optimizing the production of allergenic extracts, as it may influence the relative composition of the different allergens in the final product. As a matter of fact, HDM population dynamics and structure might be influenced by different factors, both in natural and laboratory populations. Remarkably, shifts in the sex ratio of mite populations have been observed throughout the establishment of cultures in the laboratory (Hodgson, 1976). In addition, the HDM population structure at homes may suffer significant fluctuations on a seasonal basis or in response to control methods (e.g. acaricide treatment) (Colloff, 1992) which, according to our findings, could in turn influence the repertoire of the allergen being released to the environment.

We have additionally shown that the gene expression profile of *D. pteronyssinus* allergens is influenced by short-term changes in the temperature or RH regimes, suggesting that both culture rearing conditions or microhabitat fluctuations at homes would probably have an impact on the repertoire of allergens found in allergenic extracts or in natural mite populations. Rearing at a different temperature for 1 week significantly affected the gene expression of *Der p 3* and *Der p 10* (Fig. 2A), as well as the Der p 1 to Der p 2 ratio, a commonly used parameter to monitor large-scale cultures (Carreira *et al.*, 1994). It is worth noting that the results obtained by the two methods used to monitor this ratio, ELISA or RT-qPCR, were rather divergent. While the Der p 1 to Der p 2 ratio was higher in cultures at 35 °C than at 16 °C according to ELISA (Table 5), the corresponding gene expression ratio was lower at 35 °C (and 24 °C) than at 16 °C. The

higher ratio reported at 35 °C by ELISA could be explained by a significant drop of the total Der p 2 culture content, not observed for Der p 1 (Table 5). However, this result could not be supported by an equivalent drop of *Der p 2* transcription (Fig. 2A). The apparent lack of consistency of these results may be explained by the inherent differences of both quantification methods to monitor whole cultures; while RT-qPCR provides normalized data reflecting the actual physiological status of mites and at the transcription level, detection by ELISA of an allergenic protein that is accumulated in a culture is likely influenced by additional factors, from post-transcriptional regulation to population dynamics or protein stability.

One week at 44% RH had a higher impact on mite cultures than exposition at 16 or 35 °C for the same period of time at 76% RH, having a significant effect on the expression of all genes studied except for *Der p 3* and *Der p 7* (Fig. 2B). While *D. pteronyssinus* can survive a broad spectrum of temperatures, RH is more critical for both growth and survival (Pike *et al.*, 2005). Mites keep their water balance primarily by actively extracting water from unsaturated air (Arlian, 1992). Accordingly, reducing the ambient RH below 51% is regarded as the most effective measure to control mite populations and reduce allergen exposure in homes (Arlian *et al.*, 2002). From previous works, it is known that the *D. pteronyssinus* feeding rate dramatically declines under sub-optimal RH (< 65–75%) and, as a result, also does the production of major allergens (Arlian, 1992). Our results support this evidence as low RH produced a decline in the accumulation of Der p 1 and Der p 2 in whole cultures, of about 50 and 30%, respectively (Table 5). It is worth noting that the mortality caused by low RH in our experiment, accompanied by significantly lower total number of mites per gramme of culture compared with the standard condition, may have conditioned these results, although the lower expression of *Der p 1* and *Der p 2* genes was additionally supported by RT-qPCR analysis (Fig. 2B). Underexpression of genes involved in digestion and metabolism in response to dehydrating conditions, as reported in our study for *Der p 1* (cysteine protease) and *Der p 4* (alpha-amylase), leads to metabolic depression, which is a known adaptive mechanism that contributes to minimize respiratory water loss (Teets *et al.*, 2012). Similarly, consistent with our findings, the exposure of *Psoroptes ovis* (Hering) (Acari: Psoroptidae) mites to starvation, a different but related type of stress, has also reported to decrease transcription of the *Der p 1* and *Der p 2* gene orthologues (Burgess *et al.*, 2012).

Cross-linking the results of the different experiments shows that Der p 10 suffered the highest and most divergent regulation of the eight allergens evaluated in the range of conditions tested (Figs 1 and 2). Although IgE-binding studies class tropomyosin as a low-potency allergen (Thomas *et al.*, 2010), the relevance of this allergen relies on the great cross-reactivity exhibited to tropomyosins from other organisms, mostly but not only, from insects, molluscs and crustaceans (sea-food) (Reese *et al.*, 1999). Changes in Der p 10 transcription were reported on the basis of developmental stage, sex or incubation temperature. Furthermore, Der p 10 was significantly upregulated upon dehydration stress while the remaining allergens were mostly downregulated (Fig. 2B). The observed flexibility of Der p 10 regulation in our experiments is consistent with the discussed multifunctional role attributed to tropomyosins as actin-binding proteins (Gunning *et al.*, 2008), that are ubiquitously distributed in mites (Nisbet *et al.*, 2006) and have been quantified as the 23rd most abundant protein in *D. pteronyssinus* extracts (Batard *et al.*, 2006). Previous functional genomics and proteomics studies conducted on arthropods have shown that different cytoskeletal proteins, including tropomyosin, are upregulated in response to desiccation-related stressors, like freezing (Li *et al.*, 2009; Zhang *et al.*, 2011; Storey & Storey, 2012; Teets *et al.*, 2012). House dust mites are adapted to the hydration/dehydration cycles derived from the daily fluctuations in RH that typically occur in domestic environments (e.g. beds) (Pike *et al.*, 2005), in such a way that they contract their body as they lose internal water during dehydration (Arlian, 1992). This body contraction mechanism has been described in other species, and has been suggested to be an active process mediated by the cytoskeleton to prevent mechanical damage during desiccation (Li *et al.*, 2009).

Interestingly, the use of qPCR-specific primers for either Der p 5 or Der p 21 revealed strikingly parallel transcription patterns for both allergens in all the experiments conducted in this study, including during development or in response to environmental changes (Figs 1 and 2). Group 5 and 21 allergens are related proteins that have only been found in mites so far, they share about 40% sequence similarity and their predicted secondary structures are similar (Thomas, 2012). Our findings provide a further relation between these two genes that, as suggested previously, might share a similar or related function, although still unknown (Thomas, 2012).

Studies carried out by Casset *et al.* (2012) and Brunetto *et al.* (2010) comparing different commercial allergenic extracts remarked the great heterogeneity existing in the market regarding allergen composition, even when comparing different batches from the same manufacturer, and pointed out the need to properly monitor and standardize allergenic extracts to avoid risks of inadequate diagnosis or treatment. Such advances may require an even greater refinement in the quality control of both the production process and the final product (allergenic extracts). The first one of these two could be accomplished by, among other measures, extending the range of tools used to monitor allergen production. As discussed earlier, allergen gene expression and allergen detection by ELISA do not offer equivalent or substitutive information but rather complementary. While ELISA is very handy in assessing actual allergen composition, and therefore may be of particular interest in the quality control of allergenic extract products; gene expression

studies deliver information that is potentially more specific, precise and immediate to reflect the physiological status of a mite, or a whole mite population, thus it can be particularly powerful assisting both applied (e.g. development of new allergenic extract production systems) and fundamental purposes. In addition, application of ELISA to accurately quantify HDM allergen contents is limited by the availability of specific antibodies (commercially available only for groups 1, 2 and 5; and in few HDM species) whereas the RT-qPCR approach can be virtually developed for any allergen of any species as long as their gene sequence is available (<http://www.allergen.org>). Thus, we suggest that monitoring allergen gene expression is an upstream control that could be very useful to study the impact of changes in the mite culture conditions and, consequently, to assist the process of optimization of cultures for the production of standardized allergenic extracts. Finally, our knowledge on mite's allergenicity would greatly improve if we better understand the underexplored area of mite physiology.

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