

Dietary shifts have consequences for the repertoire of allergens produced by the European house dust mite

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Abstract. Products manufactured from mass-cultured house dust mites, currently commercialized for the diagnosis and immunotherapy of allergy, are heterogeneous in terms of allergen composition and thus present concerns to regulatory authorities. The most abundant species, *Dermatophagoides pteronyssinus* (Trouessart) (Astigmata: Pyroglyphidae), produces 19 allergenic proteins. Many of these are putatively involved in mite digestive physiology and metabolism. This study aimed to evaluate the effects of mite-rearing media on allergen production. Mites were adapted to feed on culture media supplemented with proteins, lipids, carbohydrates or beard shavings, and collected to quantify major allergens (Derp 1 and 2) by immunodetection, transcription of allergen genes by real-time quantitative polymerase chain reaction, and allergen-related enzymatic activities. All culture media significantly affected the content of major allergens. Modification of macronutrients in the diet produced minor effects on the transcription of allergen genes, but significantly altered mite allergen-related activities. The most remarkable impacts were detected in mites feeding on beard shavings and were reflected in reductions in the content of major allergens, alterations in the transcription of nine allergen genes, and changes in eight allergen-related activities. These results demonstrate the importance of culture media to the quality and consistency of mite extracts used for pharmaceuticals, and highlight the need to further elucidate allergen production by mites in the laboratory and in domestic environments.

Key words. *Dermatophagoides pteronyssinus*, culture, human skin, macronutrients, physiology, protease inhibitors, RT-qPCR, standardization.

Introduction

Allergy-causing house dust mites (HDM) are mass-reared to provide active pharmaceutical ingredients (API) used for diagnosis and immunotherapy of allergy. *Dermatophagoides pteronyssinus* is the most abundant and widespread HDM species (Calderón *et al.*, 2015). To date, 19 different allergens eliciting immunoglobulin E (IgE) antibodies in susceptible subjects have been described in *D. pteronyssinus* (www.allergen.org). As different allergens contribute distinctively to the IgE

response in human allergic populations, there is a generally accepted hierarchical pattern of allergenicity in which Derp 1, 2 and 23 are referred as major and serodominant allergens (Thomas, 2015, 2016). The exact physiological function of most *D. pteronyssinus* allergens has still to be elucidated. Many of them are found in faecal pellets and/or in the mite gut (Colloff, 2009; Batard *et al.*, 2016), which suggests they may directly or indirectly participate in the digestive physiology and metabolism of the mite. It is noteworthy that almost half of these allergens belong to known enzyme families, such as

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endoproteases Der p 1 (cysteine protease), Der p 3 (trypsin), Der p 6 (chymotrypsin) and Der p 9 (elastase, collagenolytic enzyme), and other metabolic enzymes, including Der p 4 (α -amylase), Der p 15 (chitinase), Der p 20 (arginine kinase) and Der p 8 [glutathione S-transferase (GST)].

Culture systems and manufacturing methods for each mite species are not harmonized within the industry. This scenario, coupled with the inherent variability associated with biological materials, explains the heterogeneity of products available on the market, which can differ greatly in terms of allergen composition and allergenicity (Brunetto *et al.*, 2010; Casset *et al.*, 2012; Moreno Benítez *et al.*, 2015). Standardization is a main concern for regulatory agencies, as exemplified by the new monographs of the European Pharmacopoeia (01/2017:1063 and 01/2017:2625). However, most allergens are not specifically monitored in the routine component-based standardization of API, which generally relies on the quantification of Group 1 and Group 2 allergens and its ratio (Carreira *et al.*, 1994; Larsen *et al.*, 2014). Improving the quality and consistency of HDM products would require both ensuring the reproducibility of the upstream process of production (Henmar *et al.*, 2016) by understanding the factors that determine the expression of allergens under rearing conditions, and implementing routine methods to monitor the complexity of API containing a whole set of clinically relevant allergens during downstream processes. In this regard, the present group has shown how the expression of several allergen genes is influenced by factors such as mite development, sex, temperature and relative humidity (RH) (Vidal-Quist *et al.*, 2015) and has recently proposed a method for the quality control of native extracts by the analysis of allergen-related enzymatic activities (Vidal-Quist *et al.*, 2017a). The enzymatic analysis conducted in *D. pteronyssinus* cultures at different ages, different fractions (mite bodies or faecal pellets), and on international *D. pteronyssinus* reference standards demonstrated how crucial the production process is to obtaining homogeneous allergen extracts.

The ingredients used in mite-rearing media must follow general safety guidelines for pharmaceuticals, and are recognized as a critical factor in the manufacture of HDM products (Fрати *et al.*, 2012; Fernández-Caldas, 2013). House dust mites are adapted to home microhabitats (mattresses, upholstered furniture, carpets, dust, etc.) in which they feed on skin scales rich in keratin and lipids, other biological debris and associated microflora (Arlian *et al.*, 2002). In the laboratory, mites have been successfully adapted to grow on diverse media from different sources, such as cereals, baker's yeast, fishmeal and beard shavings, among others. Rearing on such a wide range of foodstuffs can affect their performance in terms of growth rate, fertility and mortality (Colloff, 2009). In addition, previous studies have demonstrated that the accumulation of Group 1 and 2 allergens in whole HDM cultures and body fractions can be affected by the rearing medium (Avula-Poola *et al.*, 2012), and that certain of its components (i.e. gluten from wheat) can specifically deplete the immunoreactivity of Der p 1 (Barber *et al.*, 1996). However, the impact of food quality on the production of HDM allergenic proteins is still largely unknown and needs to be further addressed.

The purpose of this research is to inspect how changes in the composition of a standard culture medium enriched with the three main macronutrients (proteins, lipids and carbohydrates),

or supplemented with a food source present in the domestic habitat (human skin from beard shavings), affect the physiology and the allergen repertoire of *D. pteronyssinus*. Using purified mite body fractions, the authors investigated the content of the major allergens Der p 1 and Der p 2, the expression of 17 allergen genes, and nine enzymatic activities related to allergens.

Materials and methods

Mite cultures

The strain of *D. pteronyssinus* used in this study originated from a commercial culture (Dermauw *et al.*, 2009) and was kindly provided by Dr A.C. Mailleux (Catholic University of Louvain, Louvain-la-Neuve, Belgium). The standard culture conditions have been previously described (Vidal-Quist *et al.*, 2015). Briefly, mites were grown in 50-mL wide-mouth sterile Erlenmeyer flasks with 2 g of medium, into which air exchange was allowed through a hydrophilic cotton cap. Flasks were kept at 24 °C and 76% RH in permanently dark conditions. Subcultures (20-fold dilution of the culture) were performed every 6 weeks of culture growth. The standard medium used as the control material in the experiments was selected as being optimal for *D. pteronyssinus* growth (Ree *et al.*, 1997), and consisted of a 1 : 1 mixture of fish food flakes (Sera Vipar; Sera GmbH, Heinsberg, Germany) and brewer's yeast flakes (Santiveri SI, Barcelona, Spain). This medium was supplemented with electrophoresis-grade gelatin from porcine skin (G-8150; Sigma-Aldrich Corp., St Louis, MO, U.S.A.), refined cod liver oil (1100471; Acofarma SA, Terrassa, Spain) or analytical-grade potato starch (1.01252.0250; Merck Group, Darmstadt, Germany) to obtain a protein-, lipid- or carbohydrate-enriched culture medium, respectively. These supplements were selected based on their presence in the standard medium (fish food flakes contain cod liver oil and starch) and/or their common use in rearing media. As a result, quantitative differences in the nutritional values of the media were expected. Relative proportions of each supplement in the three culture media and approximate percentages of macronutrients are shown in Table 1. All components were thoroughly mixed in approximately 1 mL of sterile distilled water per gram to produce a homogeneous paste. In the case of gelatin, warm water was used to allow dissolution. The mixtures were spread on flat glass plates in a thin layer that was dried in an oven at 60 °C for approximately 1 h. Once dried, the layers were scraped off the glass and gently crushed to obtain a flake-like texture similar to that of the standard medium. In order to avoid bias derived from the procedure, the standard medium was mixed with water and processed in the same manner as the other media. The medium containing beard shavings consisted of a 1 : 1 : 2 mixture of fish food flakes, brewer's yeast flakes and beard shavings, respectively. Beard shavings were obtained by dry shaving using an electric razor without the use of pre-shaving lotion. This medium was not processed as the other supplemented media. In order to minimize the interference of undesired biological contaminants, all media were treated at 60 °C for 6 h prior to storage or assays. Mites were allowed to adapt to the supplemented media for 12 weeks (one subculture, approximately three generations) prior to any

Table 1. Composition of the mite culture media used in this study.

		Culture medium			
		Standard medium	Protein-enriched	Lipid-enriched	Carbohydrate-enriched
Ingredients*	Fish food	10	8	9	9
	Brewer's yeast	10	8	9	9
	Gelatin	—	4	—	—
	Cod liver oil	—	—	2	—
	Starch	—	—	—	2
Macronutrients†	Proteins	48.1%	58.5%	43.3%	43.3%
	Lipids	6.0%	4.8%	15.4%	5.4%
	Carbohydrates	29.9%	23.9%	26.9%	36.9%
	Others	16.1%	12.9%	14.5%	14.5%

*Relative weights for each ingredient are indicated.

†Nutrition data are estimated based on the information labels on fish food (Vipan Sera; Sera GmbH) and brewer's yeast flakes (Santiveri SI). Gelatin (G-8150; Sigma-Aldrich Corp.), cod liver oil (1100471; Acofarma SA) and starch (1.01252.0250; Merck Group) were considered as 100% pure protein, lipid and carbohydrate, respectively.

analysis. In the case of the medium containing beard shavings, the adaptation process was performed in two steps. Firstly, to demonstrate that mites were able to feed on beard shavings, a culture in which beard shavings were used as the only food source was started from isolated mixed-instar crawling mites and maintained for 12 weeks. Then, mites were adapted to the supplemented medium (1 : 1 : 2 mixture) as described for the other media. The concentration of the supplement corresponding to each treatment was established after preliminary tests at different concentrations, and the highest concentration that maintained a good level of culture growth in relation to the control [$> 90\,000$ mites per gram of culture at 6 weeks after initiating the culture (Vidal-Quist *et al.*, 2015)] was selected.

Mite extracts

Purified mite body (PMB) fractions were obtained by separating mixed-instar crawling mites using the paper disc method (Vidal-Quist *et al.*, 2015). For enzyme-linked immunosorbent assays (ELISA) and enzymatic assays, 15–30 mg of mites were transferred to microtubes, immediately frozen in liquid nitrogen (N_2) and stored at -20°C until extraction. Mite extracts were obtained by homogenization in ice-cold 0.15 M NaCl solution using a microtube pellet pestle, centrifugation (10 000g, 15 min, 4°C) and collection of supernatants. Samples were then adjusted to 5 mg protein/mL, and aliquots were stored at -80°C . For gene expression analysis, approximately 5 mg of mites were immersed in ice-cold TRIzol Reagent (Life Technologies, Inc., Carlsbad, CA, U.S.A.) in 1.5-mL microtubes and immediately submerged in liquid N_2 , thoroughly homogenized and stored at -80°C until further processing. All samples were collected 6 weeks after the first subculture in the supplemented medium or the processed standard medium. Four biological replicates were performed per treatment, except in the lipid-enriched medium for which three replicates were analysed in ELISA and enzymatic assays. A biological replicate consisted of a PMB extract obtained from an independent (separate) culture flask. Extracts from a 5-week-old whole mite culture (WMC) on the standard

medium were obtained by homogenization in ice-cold 0.15 M NaCl and processed as described above.

ELISA and enzymatic assays

Total soluble protein concentration was estimated by the Lowry method using the DC Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.) and a bovine serum albumin (BSA) standard curve. Der p 1 and Der p 2 allergens were quantified by sandwich ELISA using the Der p 1 ELISA kit (EL-DP1A) and Mite Group 2 ELISA kit (EL-D2) (Indoor Biotechnologies, Inc., Charlottesville, VA, U.S.A.). Nine enzymatic activities related to known allergens were estimated on mite extracts following previously described methods specifically designed for *D. pteronyssinus* (Vidal-Quist *et al.*, 2017a). Briefly, activities related to allergens Der p 1 (cysteine protease), Der p 3 (trypsin), Der p 6 (chymotrypsin), Der p 9 (elastase-like) and Der p 15 (chitinase; both exo- and endo-chitinase activities) were analysed using fluorometric assays, whereas Der p 4 (α -amylase), Der p 8 (GST) and Der p 20 (arginine kinase) were estimated by colorimetric assays. Reaction conditions, including substrates, additives (such as protease inhibitors or activators), buffer pH and mite extract concentration are summarized in Table 2. More detailed protocols are available in Vidal-Quist *et al.* (2017a).

Testing the presence of protease inhibitors in culture media

The presence of protease inhibitors in beard shavings or in the standard medium was tested. Extracts from 40–50 mg of beard shavings and standard medium (not exposed to mites) were obtained by homogenization in ice-cold 0.15 M NaCl solution using a microtube pellet pestle, centrifugation (10 000g, 15 min, 4°C) and collection of supernatants. Samples were adjusted to 2 mg of soluble protein per mL. The fluorogenic substrates for each reaction were pre-incubated for 15 min with 10 μg of soluble proteins from the extracts (beard shavings or standard medium), or with BSA. After pre-incubation, mite extracts

Table 2. Summary of analytical methods to estimate enzymatic activity in *Dermatophagoides pteronyssinus* extracts.

Enzymatic activity*	Related allergen†	Substrate	pH	Additional components (reaction molarity)	Protein extract‡ (reaction volume)
Der p 1-like protease	Der p 1	Boc-QAR-AMC	7	DTT (0.80 mM), STI (1 µM)	15 µg (100 µL)
Trypsin	Der p 3	Boc-QGR-AMC	8	—	2 µg (100 µL)
Chymotrypsin	Der p 6	Suc-AAPF-AMC	8	—	50 µg (100 µL)
Elastase	Der p 9	Suc-AAA-AMC	7	—	50 µg (100 µL)
α-Amylase	Der p 4	Soluble starch	6.5	CaCl ₂ (16 mM)	3 µg (200 µL)
Exo-chitinase	Der p 15	4MU-NAG	5	—	5 µg (100 µL)
Endo-chitinase	Der p 15	4MU-(NAG) ₃	5	—	5 µg (100 µL)
Glutathione S-transferase	Der p 8	CDNB	7	GSH (4.6 mM)	15 µg (250 µL)
Arginine kinase	Der p 20	Coupled assay§	8.6	—	2.5 µg (300 µL)

*Procedures summarized and adapted from Vidal-Quist *et al.* (2017a).

†*D. pteronyssinus* allergen putatively exhibiting a related enzymatic activity (www.allergen.org).

‡Amount of purified mite body extract needed for optimal reaction, expressed as weight of soluble protein estimated by the Lowry method using bovine serum albumin as standard.

§Three connected reactions using L-arginine, adenosine triphosphate (ATP), phosphoenolpyruvate, pyruvate kinase, NADH and lactic dehydrogenase.

(from WMC or PMB reared on standard medium) were added to complete the reaction. Double blanks consisting of substrate without/with extracts (or BSA) in the absence of mite extract were used as controls of residual activity.

Gene expression analysis

RNA was extracted from TRIzol homogenates using the manufacturer's standard protocol and stored in 75% ethanol at –80 °C until further use. Gene expression of three reference genes (*RPL13a*, *RPS4*, *EF1a*) and 17 allergen genes was analysed by real-time quantitative polymerase chain reaction (RT-qPCR), as previously described (Vidal-Quist *et al.*, 2015, 2017b). Raw gene expression data were efficiency-corrected and allergen transcription was transformed to normalized relative quantities (NRQ) (Hellemans *et al.*, 2007).

Statistical analysis

Specific enzymatic activity, allergen concentration and allergen gene transcription data (Cq' values, obtained by $\log_2$ transformation of NRQs) were compared with those in controls using parametric tests, unpaired *t*-test or one-way analysis of variance (ANOVA) coupled with Dunnett's post-test, as indicated for each assay. The analysis of correlation was performed using Pearson's correlation coefficient (*r*). Statistical analyses were computed using GraphPad Prism Version 6.00 (GraphPad Software, Inc., San Diego, CA, U.S.A.).

Results

Der p 1 and Der p 2 contents

Concentrations of major allergens estimated by ELISA in PMB extracts are summarized in Table 3. Rearing on the protein-enriched medium significantly increased the Der p 1 concentration (122% relative content in comparison with the control), but not that of Der p 2, and as a result the Der p 1 to

Der p 2 ratio significantly increased. Der p 1 content was not affected in the lipid- and carbohydrate-enriched media, but both produced significant reductions in Der p 2 (59 and 57%, respectively, in comparison with the control), resulting in a significant increase in the Der p 1 to Der p 2 ratio. The medium containing beard shavings induced significant reductions in both Der p 1 and Der p 2 (41 and 40%, respectively, in comparison with the control), but did not alter their ratio.

Allergen transcription

Rearing on the medium supplemented with beard shavings induced significant downregulations of eight allergen genes, including the major allergen genes *Der p 1* (2.46-fold) and *Der p 2* (5.00-fold), and the upregulation of one gene (*Der p 10*), as estimated by RT-qPCR (Table 4). Mites growing on the lipid-enriched medium showed significantly increased transcription in *Der p 14*. Rearing on protein- or carbohydrate-enriched medium did not significantly affect allergen transcription.

Allergen-related enzymatic activities

Figure 1 shows the results of nine allergen-related enzymatic activity assays comparing PMB from mites feeding on the four supplemented media with PMB from mites feeding on the standard medium. Mites growing on the protein-enriched medium showed significant increases in four enzymatic activities, including Der p 1-like cysteine protease (116% relative activity in relation to control) (Fig. 1A), chymotrypsin (115%) (Fig. 1C), elastase (163%) (Fig. 1D) and α-amylase (109%) (Fig. 1E). Mites growing on the lipid-enriched medium showed significant increases in trypsin (191%) (Fig. 1B) and chymotrypsin (137%) (Fig. 1C) activities, and reductions in elastase (69%) (Fig. 1D), GST (77%) (Fig. 1F) and endo-chitinase (66%) (Fig. 1H) activities. Growth on the carbohydrate-enriched medium significantly increased chymotrypsin (116%) (Fig. 1C) and GST (109%) (Fig. 1F) activities, but reduced α-amylase (79%) (Fig. 1E), exo-chitinase (73%) (Fig. 1G) and endo-chitinase

Table 3. Mean $\pm$ standard error major allergen content in purified mite body fractions from *Dermatophagoides pteronyssinus* cultures using different growth media, estimated by enzyme-linked immunosorbent assay.

	Standard medium	Protein-enriched	Lipid-enriched	Carbohydrate-enriched	50% beard shavings
Der p 1 ($\mu\text{g}/\text{mg}$ soluble protein)	2.04 ± 0.09	$2.49 \pm 0.05^{**}$	2.23 ± 0.06 (NS)	1.92 ± 0.05 (NS)	$0.84 \pm 0.12^{***}$
Der p 2 ($\mu\text{g}/\text{mg}$ soluble protein)	7.95 ± 0.17	7.17 ± 0.39 (NS)	$4.71 \pm 0.07^{***}$	$4.55 \pm 0.35^{***}$	$3.21 \pm 0.10^{***}$
Ratio: Der p 1 to Der p 2	0.26 ± 0.01	$0.35 \pm 0.02^*$	$0.48 \pm 0.02^{**}$	$0.43 \pm 0.04^{**}$	0.26 ± 0.03 (NS)

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$.

Data refer to mean $\pm$ SE in $n = 4$ biological replicates; except lipid-enriched ($n = 3$). Comparisons refer to differences in relation to the control (standard medium) as assessed by one-way ANOVA coupled with Dunnett's post-test.

NS, not significant.

Table 4. Transcription of allergen genes in *Dermatophagoides pteronyssinus* reared on different culture media, estimated by real-time quantitative polymerase chain reaction.

	Relative transcription†			
	Protein-enriched	Lipid-enriched	Carbohydrate-enriched	50% beard shavings
Der p 1	NS	NS	NS	– 2.46****
Der p 2	NS	NS	NS	– 5.00****
Der p 3	NS	NS	NS	– 3.21****
Der p 4	NS	NS	NS	NS
Der p 5	NS	NS	NS	– 1.87**
Der p 6	NS	NS	NS	– 3.68****
Der p 7	NS	NS	NS	NS
Der p 8	NS	NS	NS	– 1.63***
Der p 9	NS	NS	NS	– 4.54****
Der p 10	NS	NS	NS	1.30*
Der p 11	NS	NS	NS	NS
Der p 14	NS	1.57*	NS	NS
Der p 15	NS	NS	NS	NS
Der p 18	NS	NS	NS	NS
Der p 20	NS	NS	NS	NS
Der p 21	NS	NS	NS	– 2.25***
Der p 23	NS	NS	NS	NS

†Relative gene transcription expressed as fold-change in relation to control (standard medium), calculated from normalized relative quantities (NRQ) ratios. Negative values indicate lower transcription than the control; only data showing statistically significant differences in relation to the control are displayed, as assessed by one-way ANOVA coupled with Dunnett's post-test. Data are means of four biological replicates.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

NS, not significant.

(85%) (Fig. 1H) activities. Mites reared on the medium containing beard shavings showed significant alterations in all enzyme activities studied except that of elastase. Increased activity was reported for trypsin (140%) (Fig. 1B), whereas decreased activities were observed for Der p 1-like cysteine protease (68%) (Fig. 1A), chymotrypsin (87%) (Fig. 1C), α -amylase (78%) (Fig. 1E), GST (78%) (Fig. 1F), exo-chitinase (71%) (Fig. 1G), endo-chitinase (27%) (Fig. 1H) and arginine kinase (91%) (Fig. 1I). All estimates of Der p 1-like activity and Der p 1 content (by ELISA), across the four supplemented media and the standard medium, showed a highly significant positive correlation ($r = 0.94$, $P < 0.0001$; $n = 19$).

Protease inhibitory effect of extracts from beard shavings

Extracts from beard shavings significantly inhibited Der p 1-like cysteine protease activity in both PMB and WMC extracts (67 and 82% inhibition, respectively, in comparison

with untreated mite extracts), but caused no significant inhibition of mite trypsin activity (Table 5). The inhibitory effect of extracts from the standard medium was significant, but below 16% for both activities, whereas the effect of BSA (as a negative control) was not significant. Der p 1-like cysteine protease and trypsin residual activities in extracts from beard shavings were approximately six- and 30-fold lower, respectively, than the activity estimated in PMB from a standard culture, and were negligible in extracts from the standard medium.

Discussion

This study demonstrates that changes in the nutritional value of a culture medium supplemented with proteins (gelatin), lipids (cod liver oil), carbohydrates (starch) or a food source present in domestic environments (beard shavings) influence the physiology and repertoire of allergens produced by *D. pteronyssinus*. Three complementary approaches were combined to analyse

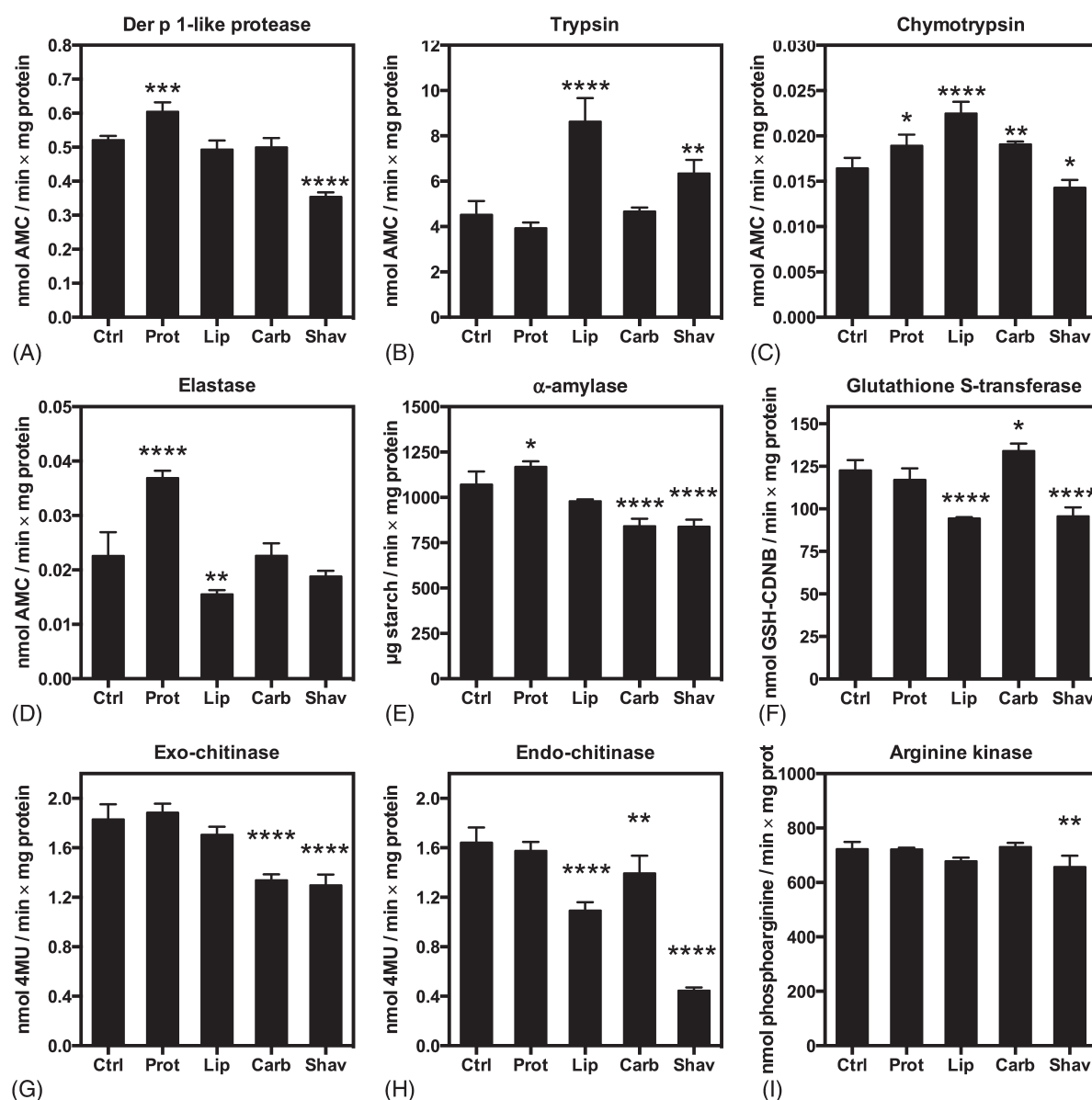


Fig. 1. Enzymatic activities estimated in purified mite body fractions of *Dermatophagoides pteronyssinus* cultures using different growth media, including standard [control (Ctrl)], protein-enriched (Prot), lipid-enriched (Lip), carbohydrate-enriched (Carb), and 50% beard shavings (Shav) media. Extracts were obtained from purified mite bodies and nine enzymatic activities were analysed (A–I). Data correspond to mean $\pm$ standard error of specific activities ($n = 4$ biological replicates, except lipid-enriched, $n = 3$). Comparisons refer to differences with the control material, as assessed by one-way ANOVA coupled with Dunnett's post-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

mite bodies extracted from rearing media: immunodetection of the two major allergens; analysis of transcription of 17 allergen genes, and quantification of allergen-related enzymatic activities associated with metabolic processes. Immunodetection allows the direct quantification of allergens and hence is the method of choice in the industry for standardization purposes (Larsen *et al.*, 2014). However, the number of allergens that can be studied by this method is limited by the availability of specific antibodies. In addition, quantification may be influenced by polymorphism in allergen epitopes (van Ree, 2007) or may not detect partial degradation of the allergen (Vidal-Quist *et al.*, 2017a).

Real-time qPCR identifies changes in the expression of allergen genes without being subject to restriction in the number of genes (Vidal-Quist *et al.*, 2015), although estimates of gene expression may not be directly related to allergen production if the gene is regulated by post-transcriptional processes. Finally, estimations of allergen-related enzymatic activities allow the detection of changes in mite physiology that are directly or indirectly related to allergens, although this method is subject to the drawback that estimates may eventually be influenced by non-allergenic proteins belonging to the same enzymatic family of the allergen (Vidal-Quist *et al.*, 2017a).

Table 5. Protease inhibitory effect of the culture medium.

Mite extract†	Enzymatic activity	Inhibition, %, mean $\pm$ SE‡		
		Beard shavings	Standard medium	BSA
PMB	Der p 1-like cysteine protease	66.9 $\pm$ 0.1***	10.2 $\pm$ 1.4*	1.9 $\pm$ 1.8 (NS)
	Trypsin	6.8 $\pm$ 1.3 (NS)	15.7 $\pm$ 1.0**	4.5 $\pm$ 4.7 (NS)
WMC	Der p 1-like cysteine protease	82.2 $\pm$ 1.3***	NA	NA
	Trypsin	10.5 $\pm$ 2.2 (NS)	NA	NA

†Amounts of mite extract (PMB: 15 μ g and 5 μ g of soluble protein in Der p 1-like protease and trypsin assays, respectively; WMC: 5 μ g and 0.5 μ g in Der p 1-like protease and trypsin assays, respectively) were adjusted in preliminary tests to obtain an optimal increase of fluorescence signal for quantification.

‡The inhibitory effect of each extract or bovine serum albumin was tested at 10 μ g of soluble protein/assay ($n = 3$). Comparisons refer to differences in specific activity between treated mite extracts and untreated extracts, as assessed by one-way ANOVA coupled with Dunnett's post-test (PMB) or unpaired *t*-test (WMC).

* $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$.

PMB, purified mite body extracts; NA, not analysed; NS, not significant; SE, standard error; WMC, 5-week-old whole mite culture extract.

The culture medium markedly influenced the production of the major and serodominant allergens Der p 1 and Der p 2, which is in agreement with a previous report (Avula-Poola *et al.*, 2012). Significant effects on the immunodetection of at least one of the two allergens were observed in mites feeding on all supplemented media. In addition, the Der p 1 to Der p 2 ratio, generally used by the industry for the quality control of API (Carreira *et al.*, 1994; Fernández-Caldas, 2013), significantly increased in mites growing on media enriched with proteins, lipids or carbohydrates. Remarkably, estimates of Der p 1 by immunodetection were confirmed by the estimation of Der p 1-like activity. The high correlation between both methods further supports previous evidence that the enzymatic assay (based on a specific substrate in combination with a protease inhibitor) preferentially targets Der p 1 over other cysteine proteases in the extract (Vidal-Quist *et al.*, 2017a). Feeding on a protein-enriched medium produced 22 and 16% increments in Der p 1 content and Der p 1-like protease activity, respectively, which is in accordance with the increased demand for proteolytic digestion derived from this diet. However, the remarkable reduction in Der p 2 content in mites growing on the lipid-, carbohydrate- and beard shavings-supplemented media will remain unclear until the specific biological function of this protein is elucidated. Changes may correspond to a general alteration of the metabolism, or may be related to the suggested antibacterial role of Group 2 allergens (Jeong *et al.*, 2002; Ichikawa *et al.*, 2009) because changes in gut-associated bacteria may eventually occur after a dietary shift. Notably, RT-qPCR could only detect significant changes in the transcription of *Der p 1* or *Der p 2* when the contents of the corresponding proteins estimated by ELISA showed the most noticeable variation: a decrease of > 50% in mites growing on the medium containing beard shavings. The lack of detection of changes by RT-qPCR may be explained by unknown post-transcriptional events regulating *Der p 1* and *Der p 2*, but may also reflect a lower resolution of the technique when dealing with moderate changes in expression, as reported at the protein level.

Feeding on beard shavings produced a drastic impact on the allergen gene expression profile (eight genes were downregulated and one was upregulated). In agreement with this, the enzymatic analysis of PMB from this culture also

showed significant changes in all allergen-related activities under study, except elastase-like activity. On this basis, it is likely that the gene regulation reported here is a consequence of the adaptation of mites to a natural food source in the culture medium. Interestingly, this adaptation was not associated with lower performance of the mite culture as population counts were in the range of those of standard cultures in this and previous studies (Vidal-Quist *et al.*, 2015). Further investigation of components in beard shavings that may alter mite metabolism demonstrated that extracts from this material contain protease inhibitors that preferentially inhibit Der p 1-like cysteine protease activity in mites. The presence of cysteine protease inhibitors in human skin is well documented (Brocklehurst & Philpott, 2013). In addition, cystatin A produced by keratinocytes and present in human sweat has been shown to specifically inhibit Der p 1 (Kato *et al.*, 2005). It can be tentatively proposed that the presence of cysteine protease inhibitors in beard shavings alters the production of mite allergens, at least those corresponding to proteolytic enzymes. Thus, a downregulation of allergen genes coding for endoproteases putatively participating in an activation cascade in the mite's digestive tract [Der p 1, 3, 6 and 9 (Dumez *et al.*, 2014)] was accompanied by a significant decrease in Der p 1-like and chymotrypsin activities, but by an increase (40%) in trypsin activity. Such a profile suggests a mechanism of compensation of the proteolytic activity, as has been reported in both herbivore and predatory insects in response to the ingestion of protease inhibitors (Bouchard *et al.*, 2003; Ortego, 2012). Interestingly, the phytophagous mite *Tetranychus urticae* Koch (Prostigmata: Tetranychidae), which possesses in its genome an outstanding proliferation of cysteine protease genes putatively participating in proteolytic digestion (Grbić *et al.*, 2011), responds similarly to protease inhibitors by regulating the transcription of protease genes (Santamaría *et al.*, 2015). It is noteworthy that an apparent discordance between RT-qPCR and enzymatic activity estimates was observed as the increment in total trypsin activity was coupled with a downregulation of the *Der p 3* trypsin-coding gene. This result may be explained by the existence of more than one gene in *D. pteronyssinus* coding for trypsins participating in digestion, and by differences in regulation imposed in response to the presence of inhibitors in the diet. As a matter of fact, a preliminary

analysis conducted by the present group on the recent draft of the partial genome of *Dermatophagoides farinae* Hughes (Chan *et al.*, 2015) using ORCAE (online resource for community annotation of eukaryotes) (Sterck *et al.*, 2012) revealed the existence of at least 28 different serine protease genes. Remarkably, 17 of these are trypsin-like genes (including *Derf3*), based on the presence of the primary specificity determinants Asp-189 and Gly-226 (trypsin numbering) (Di Cera, 2009).

Feeding on protein-, lipid- or carbohydrate-enriched media produced minor effects on the transcription of allergen genes. Significant changes were reported only for *Der p 14*, coding for a protein showing homology to vitellogenins and apolipophorins and likely to be involved in lipid transport (Thomas, 2015). Interestingly, overexpression of this gene was observed only in mites from the lipid-enriched medium. However, the analysis of allergen-related enzymatic activities did reveal the evidence of physiological adaptation of the mite in response to the different diets. These changes may not exclusively reflect variations in the production of allergens (Vidal-Quist *et al.*, 2017a), but highlight how alterations in the environment (food) prompt mites to adapt their metabolism. Given that some allergens are metabolic enzymes, such adaptations may have a direct or an indirect impact (e.g. by compensatory effects) on their production. The mechanisms controlling digestion in arthropods are complex and highly regulated (Terra & Ferreira, 2012). In order to meet their nutritional requirements, continuous feeders, such as *D. pteronyssinus*, may compensate for variable nutrient quality and/or quantity by adjusting hydrolytic activities (Woodring *et al.*, 2009; Clissold *et al.*, 2010). The present results suggest a similar mechanism in *D. pteronyssinus*, for which little is known in terms of specific nutritional requirements or food consumption efficiency (Colloff, 2009). Significant increases in activity were observed in all mite proteases, except trypsin, when the macronutrient balance was shifted in favour of proteins. This may imply that the standard medium did not meet the intrinsic demand for protein by *D. pteronyssinus*, which agrees with recommendations for rearing using high-protein media (Fernández-Caldas, 2013). However, it may also be explained by a reduction in the quality of the supplemented diet because of the known low nutritional value of gelatin, which is deficient in mite essential amino acids such as methionine and isoleucine (Rodríguez & Hampton, 1966). By contrast, the decrease in α -amylase activity reported after supplementation with starch (23% relative increase in carbohydrates), together with the increase in this activity when carbohydrates were reduced (in the protein-enriched medium), suggests that the proportion of this macronutrient in the standard medium would be close to the optimum for *D. pteronyssinus* as any eventual imbalance was enzymatically compensated. The mechanism behind the increase in activity of the serine proteases chymotrypsin and, particularly, trypsin in response to an enrichment in dietary lipid is unknown, although it may have practical implications. Certain lipids are able to induce HDM growth, including cod liver oil (Matsumoto, 1975), and skin scales are generally rich in lipids (Colloff, 2009). Thus, appropriate lipid-rich sources are generally included in laboratory culture media. The present results suggest that lipid content should be considered with attention in the manufacture of HDM products as high lipid levels may promote trypsin activity, which, in turn, may have

negative effects on extract stability, given the prominent role of trypsin in the degradation of major allergens (Vidal-Quist *et al.*, 2017a). Interestingly, the reference standard extract WHO/IUIS 82/518, obtained from mites cultivated on a medium based on human dander (rich in lipids, and probably containing protease inhibitors), also showed proportionally higher trypsin activity compared with other reference standards and with the present group's own extracts (Vidal-Quist *et al.*, 2017a).

The substantial effects reported here caused by mild alterations in dietary macronutrients indicate that quantitative and qualitative divergences between commercial HDM products manufactured by companies that use distinct culture media can be expected. In addition, special attention should be applied when, as recommended for pharmaceutical production, mite cultures are shifted to animal/allergen-free media (Fernández-Caldas, 2013). Likewise, given the remarkable impact of beard shavings used as a dietary supplement, the possibility that the repertoire of allergens produced by mites adapted to the laboratory (or industry) may differ from that of mites breeding in homes, where skin-derived products and other food sources are available, cannot be discarded. Unravelling such eventual divergence will require further research analysing laboratory-adapted and wild populations (Hart *et al.*, 2007), the interaction of mites with natural microflora, and the impact of the microhabitat and alternative geographical and socioeconomic environments (e.g. rural vs. urban environments).

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