

Population growth and allergen accumulation of *Dermatophagoides farinae* cultured at 20 and 25 °C

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Received: 22 June 2012 / Accepted: 5 October 2012
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Abstract House dust mites are cultured to obtain mite allergen material to produce allergen extracts (vaccines) for diagnostic tests, immunotherapy, and research purposes. Research laboratories and manufacturers have their own culturing protocols to grow these mites and these may vary between manufacturers and between research laboratories. The temperature at which mites are cultured may influence the allergen composition, allergen ratio of Der 1: Der 2 and endotoxin levels in the extracts produced from these cultured mites. In order to produce standardized and uniform extracts, across the industry and in various research laboratories, the influence of culture conditions must be understood. Here we determined how temperature affects mite population growth rates, dynamics of allergen production, Der f 1: Der f 2 ratio and endotoxin levels in extracts made from *Dermatophagoides farinae* mites cultured at 20 and 25 °C. We found that Der f 1 and Der f 2 accumulated exponentially in the cultures with Der f 1 accumulating faster than Der f 2. When the live mite populations peaked, the ratios for Der f 1: Der f 2 were 4.1 and 4.7 for cultures reared at 20 and 25 °C, respectively. Most of the Der f 1 and Der f 2 allergen in whole cultures is not in mite bodies and is lost when the mite material is washed. Thus, if the ratio of Der f 1 and Der f 2 is an important consideration for commercial and research extracts, then the temperature at which the mites are cultured and the collection procedure are important considerations.

Keywords *Dermatophagoides farinae* · Growth rate · Allergen · Endotoxin

Introduction

The two most common species of allergy-causing house dust mites found in homes in humid geographical areas of the United States and worldwide are *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*. Allergic disease induced by house dust

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mites (asthma, allergic rhinitis and atopic dermatitis) is a major health problem in the United States and worldwide. The latest National Health and Nutrition Examination Survey (NHANES III) showed that 27.7 % of 18–59 year olds in the United States were sensitive to these house dust mites (Arbes et al. 2005). This survey reported that house dust mite sensitivity is more prevalent than sensitivity to ragweed, grass pollen, molds, dogs and cats. House dust mite allergy is a perennial (year round) disease. Thus, it requires continuous diagnosis and treatment compared to pollen and mold allergies that are seasonal and absent most of the year.

House dust mite allergy is diagnosed by skin tests and patients may be treated by immunotherapy using extracts (vaccines) produced from cultured house dust mites. Diagnosis, treatment of mite-induced allergy, and research to characterize mite allergens are dependent on the production of extracts that are well defined and that properly represent the allergen profile and characteristics of these disease-causing mites in nature (Brunetto et al. 2010; Fernandez-Caldas 2013). Each pharmaceutical company and research laboratory uses different environmental isolates of inbred mite cultures to produce their extracts. A recent study clearly showed that commercially-available *D. pteronyssinus* extracts from eight different European manufacturers varied in allergen composition and allergenic activity (Casset et al. 2012). Similarly, immunochemical analysis and in vivo testing of extracts from eight companies showed heterogeneity among extracts of both *D. farinae* and *D. pteronyssinus* (Brunetto et al. 2010). Burazer et al. (2011) observed differences in IgE reactivity of three *D. pteronyssinus* extracts from manufacturers in Europe, South America and Australia. Likewise, three commercially-available whole crushed mite body extracts of *D. pteronyssinus* varied biochemically and induced divergent responses by bronchial epithelial cells (Post et al. 2012). Clearly, these studies illustrate that the composition and biological activity of mite allergen extracts vary among sources.

Culture climatic conditions and diet influence the population growth and allergen accumulation rates, allergen concentration, ratio of the various allergens, and endotoxins in the cultures and thus the quality of extracts produced from them (Avula-Poola et al. 2012; Yella et al. 2011). In particular, our previous study showed that *D. pteronyssinus* cultured at 20 °C had different population growth rates and allergen and endotoxin dynamics compared to mites cultured at 25 °C (Yella et al. 2011). The current parallel study reports data for *D. farinae* cultured at the same temperatures and illustrates the differences between the two species.

Generally, *D. farinae* and *D. pteronyssinus* are grown under the same climatic conditions. However, the two species have very different biology and ecology. The two species are not equally prevalent among homes in a single geographical area and also between homes in different geographical areas (Arlian et al. 1978, 1982, 1992; Arlian and Morgan 2003; Moyer et al. 1985; Thomas 2010). The same species can exhibit tremendous variability in prevalence between homes within and between geographical areas presumably partly because the mite population is subject to different climatic conditions. It is clear that some house dust mite allergens from mites from different environmental isolates have multiple amino acid sequences (Chapman et al. 2007; Park et al. 2002; Piboonpocanun et al. 2006; Thomas 2010). This may reflect that there are strain differences in the various geographical locations where these mites occur.

In addition to the varied species prevalence in their natural microenvironments in homes where climatic conditions fluctuate, laboratory studies at comparable relative humidity and temperature conditions, show differences in feeding, fecundity, reproduction, population growth rates, and survival among the two species that again demonstrates that these mite species are biologically different (Arlian 1977; Arlian et al. 1990, 1998a, b; Arlian and

Dippold 1996; Arlian and Morgan 2003; Colloff 2009; Hart et al. 2007). It is unknown to what extent strains of a given species from different geographical areas of the USA and world also exhibits different biology.

The biological differences in the two species that are responsible for the varied mite species prevalence are not understood but clearly each species is unique. Likewise, it is not clear why some allergens from a species may have amino acid substitutions (Thomas 2010). Environmental stressors such as temperature and relative humidity differences may have functioned as long-term selection factors that account for strain differences or they may influence short-term gene expression thereby serving as determinants of varied species prevalence and/or allergen polymorphisms.

Our previous study of house dust mite extracts produced from cultured mites showed that culture temperature affected the population growth rate, allergen production and endotoxin levels of cultured *D. pteronyssinus* (Yella et al. 2011). As a follow-up to that study, here we report how temperature influences mite population growth rate, allergen production and endotoxin levels of cultured *D. farinae* and compared these to *D. pteronyssinus*.

Materials and methods

Dermatophagoides farinae mites for this study were taken from stock cultures maintained at 75 % relative humidity and reared on a diet of laboratory rodent chow (Teklad Rodent Diet 8640, Harlan Laboratories, Indianapolis, IN, USA) and baker's yeast (9:1, vol:vol). All other materials and methods were identical to those reported previously (Yella et al. 2011) and described briefly following.

Mite cultures

Test cultures were set up in sets of 5 so that there would be 5 replicates for each temperature. Test cultures were established by adding 6 g of thriving stock culture (inoculum), that was about depleted of food, to 60 g of previously-equilibrated diet. Ventilated culture jars containing the test cultures in ventilated humidity chambers maintained at 75 % relative humidity were kept in an incubator at 20.0 ± 0.2 or 25.0 ± 0.2 °C. The experiments at the two temperatures were run in sequence so the inoculum was not identical for the two sets of test cultures.

Samples were removed from each of the five test cultures after thorough mixing at 2-week intervals until the cultures matured. Sample aliquots were collected from each individual culture as follows: two 10–50 mg samples for immediate live mite density determination and two 100 mg samples were frozen for later extraction to determine allergen concentrations and endotoxin levels.

Live mite counts

At each sample time, two 10–50 mg samples (based on anticipated mite density) were weighed into gridded Petri dishes and the numbers of live mites were counted using a dissecting microscope. Mites of all life stages (larvae, protonymphs, tritonymphs and adults) were considered live if they were moving.

Allergen analyses

Whole culture

At the conclusion of each temperature experiment, one 100 mg aliquot of each replicate whole culture that had been collected at each sample time was prepared for allergen analysis by overnight extraction in 10.0 ml of BPBST extracting solution (1 % bovine serum albumin in Dulbecco's phosphate buffered saline with 0.05 % Tween 20 and 0.05 % sodium azide). The next day, samples were sonicated on ice for 10 min, centrifuged and supernatants were collected. Der f 1 and Der f 2 allergen content were determined using enzyme-linked immunosorbent assay (ELISA) kits obtained from Indoor Biotechnologies (Charlottesville, VA, USA) according to the manufacturer's instructions.

Washed mite bodies

Wash columns were prepared by replacing the frits of 1.5 ml spin columns (Pierce Thermo Scientific, Rockford, IL, USA) with pieces of 400 mesh (38 μ m) stainless steel screen (Small Parts, Miami, FL, USA). A 20–30 mg aliquot of whole culture from each replicate culture at each test time was weighed into a column and was washed with 4.5 ml of PBST (Dulbecco's phosphate buffered saline with 0.05 % Tween 20). After washing, the columns containing the washed mite samples were placed in 15 ml tubes and were extracted into 10.0 ml of BPBST and subjected to allergen ELISA as described above.

Endotoxin analysis

Aliquots (20–30 mg) from each replicate culture at each sample time were also extracted overnight into 1.0 ml endotoxin-free water (Lonza, Walkersville, MD, USA), sonicated and centrifuged as above. Supernatants were collected and the endotoxin content in endotoxin units (EU) in each of these extracts was measured using the QCL-1000 endotoxin test kit (Lonza).

Data analysis

All data from measurements of live mite counts and allergen, and endotoxin content in individual aliquots were normalized to a standard aliquot size of 100 mg whole culture (wet weight). For a given sample time/temperature, means were calculated from the five replicate cultures and data are presented $\pm$ standard error of the mean (SEM). Rate constants for population growth and allergen and endotoxin accumulation were calculated using an exponential model as previously described (Arlian et al. 1998a).

Results

Population growth

The population growth of *D. farinae* was exponential at both 20 and 25 °C until the culture media became limiting (Fig. 1). Peak population densities were reached at 6 weeks ($3,745 \pm 125$ live mites per 100 mg whole culture material) and 10 weeks ($3,507 \pm 246$ live mites per 100 mg) at 25 and 20 °C, respectively. During the exponential growth phase,

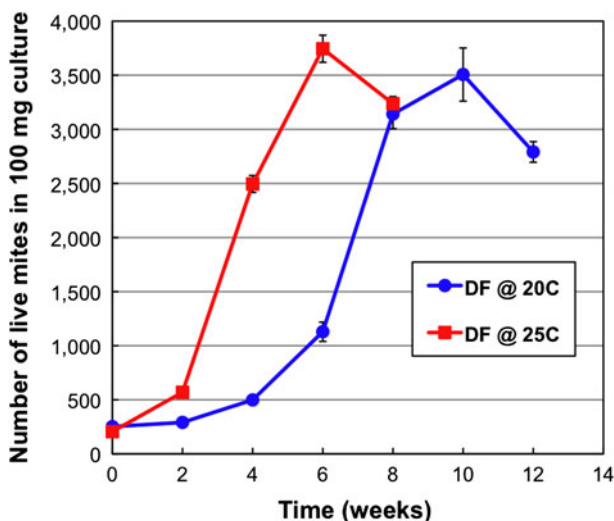


Fig. 1 Population growth profiles for *Dermatophagoides farinae* cultures grown at 20 and 25 °C

the population densities increased at 30.2 % per week ($r^2 = 0.95$) and 50.9 % per week ($r^2 = 0.96$) for cultures grown at 20 and 25 °C, respectively.

Der f 1 and Der f 2 in whole culture

Der f 1 and Der f 2 allergen concentrations also increased exponentially in parallel with the increases in live mite densities in the cultures (Fig. 2). Although the sizes of the live mite populations peaked at 6 and 10 weeks at 25 and 20 °C, respectively, Der f 1 and Der f 2 allergen concentrations continued to increase as the live mite density declined.

Der f 1 accumulated more rapidly in whole culture material at 25 °C (35.3 %/week, $r^2 = 0.96$) than at 20 °C (21.2 %/week, $r^2 = 0.99$) and greater quantities of Der f 1 were present at comparable times in cultures grown at 25 °C. Likewise, Der f 2 also accumulated more rapidly at 25 °C (29.6 %/week, $r^2 = 0.85$) than at 20 °C (17.6 %/week, $r^2 = 0.91$) and the amount present after 4 weeks was greater at 25 °C than 20 °C.

At both temperatures, much greater quantities of Der f 1 were produced than Der f 2. The Der f 1: Der f 2 ratios were 3.9 and 5.0 for cultures at 20 and 25 °C, respectively, when allergen levels peaked at 12 and 8 weeks, respectively. However, at the live mite population peak at 10 weeks (20 °C) and 6 weeks (25 °C), these ratios were 4.1 and 4.7, respectively.

Washed mite bodies

Commercial and research extracts may be prepared from whole spent culture of harvested mites that have been washed. The washing process removes much of the mite allergen in the whole culture that is not inside the mite body (e.g. fecal material, secretions such as saliva and molting fluids that contain allergens). Therefore, washed mite culture material contains mostly mite and little external mite allergen. All soluble allergen outside of the mite body should have been washed away.

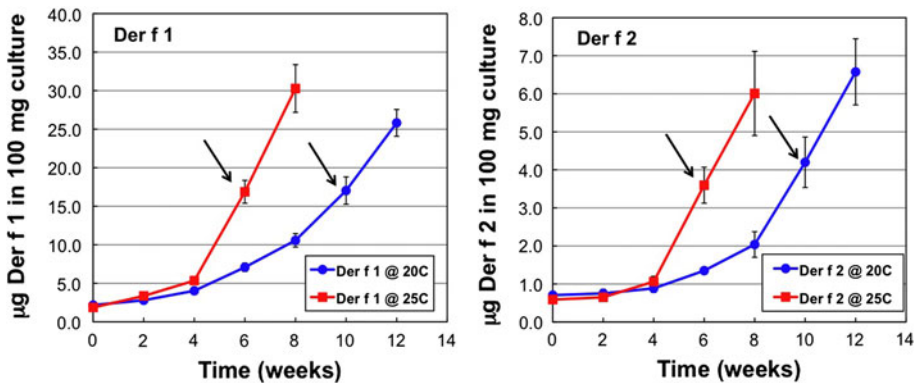


Fig. 2 Der f 1 and Der f 2 allergen accumulation profiles for *Dermatophagoides farinae* cultures grown at 20 and 25 °C. Arrows indicate the allergen concentration at the time of peak population density

On a weight basis (whole culture), extracts prepared from washed mite bodies had much less Der f 1 and Der f 2 allergen compared to that present in whole spent culture material during the population growth phase. When the population peaked at 6 weeks of growth for culturing at 20 °C, only 37.6 % of the total Der f 1 allergen in the whole culture was present in the mite bodies (6.4 vs. 17.0 µg per 100 mg whole culture). Likewise, only 21.7 % (0.91 vs. 4.20 µg per 100 mg whole culture) of the total Der f 2 in the whole culture was in the mite bodies. For mites cultured at 25 °C, only 27.8 % (4.69 vs. 16.88 µg) and 26.9 % (0.97 vs. 3.60 µg) of the total Der f 1 and Der f 2, respectively, in the whole culture was in the mite bodies when the mite population peaked.

Endotoxin

Endotoxin content in the whole cultures was also monitored over time as the mite populations grew to their peaks. Endotoxin concentration in the extracts prepared from cultures grown at both temperatures increased exponentially (Fig. 3). Although endotoxin accumulated more rapidly at 25 °C (22.4 %/week, $r^2 = 0.61$) than 20 °C (16.1 %/week, $r^2 = 0.80$), the total endotoxin concentrations at the times when the mite populations peaked were essentially the same (821 ± 188 kEU per 100 mg at 20 °C vs. 921 ± 99 kEU per 100 mg at 25 °C).

Discussion

Nineteen allergens from *D. farinae* and *D. pteronyssinus* have been identified and partially characterized (Thomas et al. 2007). Mite allergic patients have differential sensitivities (IgE recognition patterns) to the multiple allergens of each species (Arlian et al. 1987a, b, 1993; Taketomi et al. 2006; Thomas et al. 2002; Thomas 2010). Diagnosis and treatment of mite-induced allergy are dependent on the production of extracts that represent the allergen profiles of these disease-causing mites in nature. Understanding the influence of temperature and other culture factors such as diet and relative humidity on the dynamics of allergen production in cultured mites has the potential to provide higher quality house dust mite extracts with the full array of natural allergens.

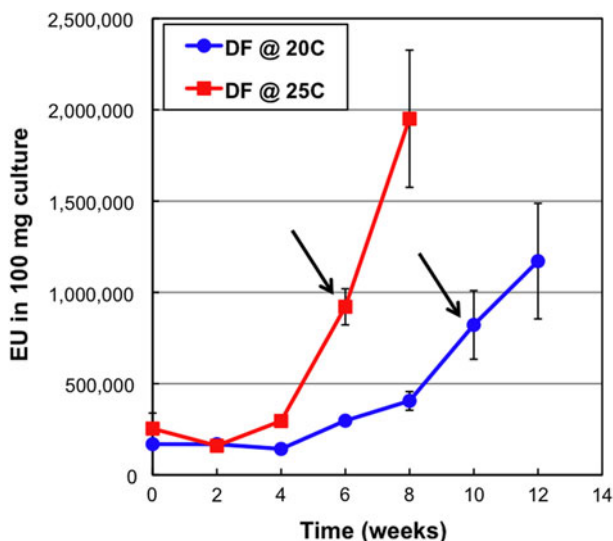


Fig. 3 Endotoxin accumulation profiles for *Dermatophagoides farinae* cultures grown at 20 and 25 °C. Arrows indicate the endotoxin concentration at the time of peak population density

Different research and commercial laboratories may culture *D. farinae* and *D. pteronyssinus* mites at different temperature conditions within the normal biological range for each species in spite of the fact that these may be different from optimal temperatures for maximum reproduction. In nature, within a home, *D. farinae* and *D. pteronyssinus* occur in a variety of microhabitats (couch, carpet, mattress) and at any time, temperature can vary among these locations. Also, temperature fluctuates within these microhabitats daily. Likewise, temperatures vary between homes in different geographical areas where dust mites are prevalent. These temperature differences influence the population growth and reproduction of dust mites and thus their allergens, profiles and accumulation. A previous study showed that for *D. pteronyssinus*, the allergen content, allergen profiles, and allergen ratios in extracts made from cultured mites were influenced by the temperature at which the mites were cultured (Yella et al. 2011). In this study we determined that culture temperature also influenced allergen content (profile) and ratios in whole spent culture and washed bodies of *D. farinae*.

Although there are at least 19 different allergens from these mites, reagents are not readily available to investigate most of these allergens in cultures, mites or in dust in homes. However, reagents are available to study the dynamics of two of these, Der 1 and Der 2. The current study shows that the total amount of these allergens produced and the ratio of these are affected by the culture temperature.

We observed, as expected, that the mite population grew 1.6 times faster at 25 °C than at 20 °C. However, at both temperatures, the populations peaked at about the same density. At the lower temperature it required more culture time to get to the peak. This was also the case for the corresponding allergen concentrations in the whole cultures.

The influence of temperature on allergen production was directly related to mite population growth rate. Der f 1 was produced faster for mites cultured at 25 °C than at 20 °C and the mite population growth rate, as expected, also was greater at 25 °C. The same pattern was observed for mites cultured at 25 °C where Der f 2 accumulated faster than at

20 °C. However, at each temperature, the Der f 1 accumulation rate was faster than the Der f 2 accumulation rate. Therefore, the ratio of these two allergens can change (increase) over time during population growth. It is not clear what metabolic processes contribute to this relationship. Der 1 is a cysteine protease and is thought to function as a digestive enzyme in the digestive tract and thus be present in high concentrations in mite feces. In contrast, Der 2 is thought to be a lipid-binding protein. These relationships that we have observed for *D. farinae* are somewhat different from the results of our similar study for *D. pteronyssinus* (Table 1). In that study, Der p 1 accumulated faster at 20 °C than at 25 °C while Der p 2 accumulated faster at 25 °C than at 20 °C (Yella et al. 2011). Therefore, for both *Dermatophagoides* species, temperature influences population growth and allergen accumulation when these mites are grown on the same diet and some of these effects are different for the two mite species. These data suggest that not only are their climatic requirements different, which influences their well-documented varied prevalence, but they also have physiological differences that influence allergen production dynamics.

House dust mite extracts in the United States are made from washed mites (Valerio et al. 2005). Washing mites harvested from thriving cultures results in the loss of huge amounts of the mite allergen present in these cultures and also changes the ratio of specific allergens (Avula-Poola et al. 2012; Yella et al. 2011). We have previously found that washed *D. pteronyssinus* mites grown at three different temperatures have different allergen profiles (Avula-Poola et al. 2012; Yella et al. 2011). In extracts produced from whole mite spent culture, the allergens are present in both mite bodies and in the spent culture material (live

Table 1 Comparison of growth and allergen accumulation rates in *Dermatophagoides farinae* and *D. pteronyssinus* cultures grown on the same culture medium at 20 and 25 °C

In 100 mg whole culture	Cultures at 20 °C		Cultures at 25 °C	
	<i>D. farinae</i>	<i>D. pteronyssinus</i> *	<i>D. farinae</i>	<i>D. pteronyssinus</i> *
Live mites				
Time to peak population (weeks)	10	10	6	8
Growth rate <i>k</i> (% per week)	30.2	26.5**	50.9	45.2
Doubling time (weeks)	2.30	2.62**	1.36	1.53
Number live mites at peak	3,507	6,331	3,745	11,614
Der 1 allergen				
Growth rate <i>k</i> (% per week)	21.2	37.0**	35.3	26.9
Doubling time (weeks)	3.27	1.87**	1.96	2.57
µg at population peak	17.0	10.9**	16.9	21.1
Der 2 allergen				
Growth rate <i>k</i> (% per week)	17.6	14.8**	29.6	20.9
Doubling time (weeks)	3.94	4.70**	2.34	3.32
µg at population peak	4.2	5.1**	3.6	8.1
Der 1 ± Der 2 allergen				
Growth rate <i>k</i> (% per week)	20.5	25.4**	34.2	25.0
Doubling time (weeks)	3.38	2.73**	2.03	2.77
µg at population peak	21.2	16.0**	20.5	29.2
Der 1: Der 2 ratio	4.1	2.1	4.7	2.6

* Data for *D. pteronyssinus* are taken from Yella et al. (2011)

** Calculated at 8 weeks, the end of the exponential growth phase

and dead mite bodies, exoskeletons, eggs, feces, secretions of the salivary glands, molting enzymes, hemolymph proteins, etc.). By washing the spent culture material before making the extract, soluble allergens in the spent culture outside of live and dead mite bodies is removed. Thus, the allergens in extracts produced from washed spent culture are those that remain in the mite body and can include molecules in the hemocoel, salivary glands, gut, reproductive organs, and fecal material in the digestive tract, and molting enzymes in quiescent life stages preparing to molt to the next life stage. Molting enzymes, digestive enzymes in feces, and salivary secretions that contaminate culture material from the feeding process are washed away. As expected, the extracts made from washed mites contained much less Der f 1 and Der f 2 than was present in whole culture extracts. This suggested that most of the Der f 1 and Der f 2 allergen in the whole culture material was outside the mite bodies. In terms of efficiency and cost of extracts, this argues for use of whole culture to produce extracts since all of these mite products are present in the dust in homes to which patients are sensitized. Although most of the allergen was removed by washing, the percent of the total Der f 1 and Der f 2 that remained after washing mites cultured at 25 °C was about the same, leaving the Der 1: Der 2 ratio similar to that in whole culture (4.8 in washed mites vs. 4.7 in whole culture). In contrast, for mites cultured at 20 °C, there was a greater percentage of Der f 1 remaining in the mite bodies after washing than Der f 2 resulting in an altered Der 1: Der 2 ratio (7.1 in washed mites vs. 4.1 in whole culture). Thus, if the ratio of Der f 1 to Der f 2 is an important consideration for commercial and research extracts, then the temperature at which the mites are cultured is an important consideration.

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