

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

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Abstract The house dust mites, *Dermatophagoides pteronyssinus* and *D. farinae* are cultured commercially and in research laboratories and material is harvested from these cultures to make extracts that are used for diagnosis, immunotherapy and research. Temperature and other climatic conditions can influence population growth rates, dynamics of allergen production, and the associated endotoxin, enzyme and protein levels of the mite material harvested from these cultures. Here we determined how temperature affected these parameters. *Dermatophagoides pteronyssinus* was cultured at 20 and 25°C at 75% relative humidity, and at 2-week intervals the concentrations of mites, Der p 1 and Der p 2 allergens, endotoxin, and selected enzymes were determined. Mite density increased exponentially but growth rate and final population density were greater at 25°C compared to 20°C. The combined allergen (Der p 1 + Der p 2) concentrations accumulated in the cultures at about the same rate at both temperatures. However, individual Der p 1 and Der p 2 accumulation rates varied independently at the two temperatures. Der p 1 accumulated faster at 20°C whereas Der p 2 accumulated faster at 25°C. The amount of Der p 1 in whole cultures was greater than the amount of Der p 2. The concentration of allergen for washed mites harvested from the cultures was much less than for the whole cultures. Our study demonstrated that temperature is an important factor in population growth and the dynamics of allergen production in cultured mites.

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

Introduction

Allergy to house dust mites continues to be an important disease in the general population in the United States and worldwide. A cross-sectional study of 18–59 year olds in the US population (NHANES III study) revealed that 27.5% were skin prick test (SPT) sensitive to

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dust mite (Arbes et al. 2005). Likewise, Arshad (2003) reports that about 20% of the adult population in Europe is sensitized to *Dermatophagoides pteronyssinus*. Among atopic individuals, a high prevalence of sensitivities to dust mites is also generally reported worldwide. However, there are geographical differences in the prevalence of dust mite sensitization in various general and atopic populations in many regions including the United States, Europe, South America, Australia, and Asia.

Generally, the house dust mites *D. farinae* and *D. pteronyssinus* are prevalent in humid geographical areas worldwide if sufficient food is available in the microhabitat where they are found. These mites obtain sufficient water for survival by absorbing water from unsaturated air that is above 60–70% relative humidity (RH) depending on temperature conditions. They are active and reproduce well in the 18–30°C temperature range. Although the two species can co-exist in nature (homes) at some climatic conditions the ranges of optimum temperature and relative humidity conditions for each species do not completely overlap. Laboratory studies have identified differences in the biology of the two species including differences in fecundity and reproductive potential at different temperatures (Arlian et al. 1990; Arlian and Dippold 1996). In addition, extract made from cultures of *D. pteronyssinus* contain much less endotoxin than extracts of *D. farinae* (Trivedi et al. 2003; Valerio et al. 2005). The enzymatic activities of extracts for the two species are different (Morgan and Arlian 2006). In spite of these differences, these mites are generally cultured for commercial and research purposes under the same climatic conditions, 75% RH and 22–25°C.

In nature, in temperate climates, populations of house dust mites grow exponentially under optimal climatic conditions in homes and populations crash when conditions are no longer optimal (Arlian et al. 1982, 1983, 1992, 2001; Lang and Mulla 1978). This generally explains the seasonal fluctuations in mite levels in homes in temperate climates. Likewise, populations of live house dust mites in culture grow exponentially until food becomes limiting even when climatic conditions are optimal (Arlian et al. 1998). Therefore, mite population growth curves exhibit a lag growth phase (population growth is slow), a log growth phase (population growth is exponential or logarithmic), a stationary phase (food becomes limiting and the population ceases to increase), and the death phase (population decreases due to depletion of food and bacterial and fungal growth) (Andersen 1988; Arlian et al. 1998; Eraso et al. 1997, 1998; Martinez et al. 2000). It has been reported that the highest allergenic potency and antigenic diversity in cultures grown at 23–25°C and 75–80% RH occurred during the exponential growth phase (Eraso et al. 1997, 1998; Martinez et al. 2000) and the cross-reactivity between extracts of *D. farinae* and *D. pteronyssinus* was greatest and most evident in extracts made from mite material harvested from cultures during the maximum exponential growth phase (Martinez et al. 2000).

House dust mites are cultured commercially and mite material is harvested to make extracts that are used for diagnostic tests and immunotherapy. In addition, many researchers maintain mite cultures as a source of material to use for research purposes. The biological differences between the two species may influence the quality and quantity of extract material and allergen they produce in culture and in nature, the mite material added to their microenvironment. Like most terrestrial arthropods, if food and relative humidity are adequate, small temperature changes within their normal biological range have large effects on the reproduction and life cycle of dust mites. The influence of temperature on the culture of dust mites and the quality and composition of material harvested to make allergen extracts has never been reported. As concluded by the CREATE project (van Ree et al. 2008) and reviewed by van Ree (2007), improving and standardizing extracts made from cultured mites and used for research, diagnosis and immunotherapy is important. The

purpose of this study was to determine the mite population growth rates and allergen concentrations, endotoxin levels, and enzyme activity of *D. pteronyssinus* material harvested from cultures grown at two temperatures.

Materials and methods

Mite cultures

A stock culture of *D. pteronyssinus* for this research was started from thriving mite cultures maintained at Wright State University. This stock culture of *D. pteronyssinus* was reared on a 9:1 (vol:vol) mixture of laboratory rodent chow (Teklad Rodent Diet, Harlan Laboratories, Indianapolis, IN, USA) and bakers yeast at 75% RH and room temperature conditions (19–22°C) (Arlian et al. 1984). The inoculum for the test cultures was taken from these thriving *D. pteronyssinus* stock cultures. The culture medium for the test cultures was the same diet as that used for the stock culture.

Test cultures were set up in sets of five so that there would be five replicates for each experiment. Test cultures were established by adding 6 g of thriving stock culture (inoculum), that was about depleted of food, to 60 g of diet. The food was equilibrated at 75% RH (48 h) in the test culture jars before the mite inoculum was added. The test cultures were grown in half-pint Ball® jars with ventilated plastic lids inside of ventilated humidity chambers. The humidity chambers containing the test cultures were kept in an incubator at 20.0 ± 0.2 or $25.0 \pm 0.2^\circ\text{C}$. The temperature inside the incubator was monitored daily. The experiments at the two temperatures were run in sequence so the inoculum was not identical for the two sets of test cultures.

Once the test cultures were inoculated, samples were removed from them after thorough mixing at 2-week intervals for up to 12 weeks when the cultures had matured. Sample aliquots were collected from each individual culture as follows: two 10–50 mg samples for immediate live mite density determination; two 50 mg samples in 95% ethanol for life stage composition examination; and two 100 mg samples were frozen for later extraction to determine allergen concentrations, enzyme activities, endotoxin levels, and protein concentrations.

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.

Life stage composition

At each sample time, two 50 mg samples were removed from each culture and stored in 95% ethanol for later life stage determination. Mites were cleared in lactic acid on microscope slides then using a compound microscope, the life stage of 250 specimens for each of the 5 replicate test cultures determined. The numbers of eggs, larvae, protonymphs, tritonymphs, males and females were counted and recorded. The life stage counts for each sample time were normalized to 1000 specimens.

Allergen analyses

Whole culture

At the conclusion of each experiment, one 100 mg aliquot of each replicate whole culture that had been collected at each sample time was prepared for allergen analysis. Each aliquot was extracted overnight at room temperature with shaking 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 and 1.0 ml of each was centrifuged for 10 min at $14,000\times g$ in a microcentrifuge. Supernatants were collected and Der p 1 and Der p 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 μm) 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.

Biochemical analyses

Aliquots (20–30 mg) from each replicate culture at each sample time were also extracted into 1.0 ml endotoxin-free water (Lonza, Walkersville, MD, USA) for biochemical analyses. These samples were also extracted overnight, sonicated and centrifuged. Supernatants were collected and protein concentrations were determined using the A280 settings of a NanoDrop 1000 spectrophotometer (Thermo Scientific). The endotoxin content in each of these extracts was also measured using the QCL-1000 endotoxin test kit (Lonza).

Semi-quantitative enzyme analyses were performed on the water-extracted samples using ApiZym strips (bioMérieux, Hazelwood, MO, USA) as previously described (Morgan and Arlian 2006). For this analysis, equal volumes of extracts from each of the five replicate cultures from a given sample time/temperature were pooled.

Data analysis

All data from measurements of live mite counts and allergen, protein 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 and data are presented $\pm$ standard error of the mean (SEM). The growth patterns of the mite populations and accumulation of allergen at the two temperatures (20 and 25°C) were exponential. Therefore, rate constants for population growth, allergen accumulation rate and doubling times were calculated using an exponential model as previously described (Arlian et al. 1998).

Results

Population growth

Population growth of *D. pteronyssinus* was exponential at both 20 and 25°C (Fig. 1). During the first two weeks of culture (lag phase), the mean population densities for the populations at the two temperatures were about the same. After the second week, the population growth in the cultures at 25°C was more rapid than it was for the population in cultures at 20°C. The mite population growth rate until week 8 (when populations leveled or declined) was 26.5%/week at 20°C and 45.2%/week at 25°C (Fig. 1; Table 1). Therefore, the doubling times for the populations were 2.62 and 1.53 weeks at 20 and 25°C, respectively (Table 1). At 8 weeks, the mite population density was 2.1-fold greater at 25°C compared to 20°C (Fig. 1; Table 1). For the cultures grown at 20°C, the population peaked during week 10 at $6,331 \pm 284$ mites per 100 mg whole culture whereas at 25°C the population reached a peak of $11,614 \pm 415$ mites per 100 mg at week 8 (Table 1; Fig. 1).

Life stage composition

The life stage compositions of the cultures are shown in Fig. 2. Eggs were the most numerous life stage during the first 4–6 weeks of cultivation and their concentrations declined as the cultures matured. Larvae were generally the most numerous active life stage. Protonymphs were the least numerous life stage present in the cultures during population growth. The proportion of adult mites in the cultures remained relatively constant over the course of the experiment with roughly equal numbers of males and females present at all times.

Total allergen

The combined Der p 1 + Der p 2 allergen concentration profiles during mite population growth in cultures at 20 and 25°C are shown in Fig. 3. Allergen concentrations also increased exponentially during the population growth phase and this increase continued for 2 more weeks at 25°C and for 4 weeks at 20°C even though food was depleted and the live mite population declined (Figs. 1, 3). Combined Der p 1 + Der p 2 allergen accumulation rates were similar over the course of the experiment at both temperatures (Fig. 3; Table 2). At the conclusion of the experiment, the spent culture material contained 47.68 ± 1.99 µg (12 weeks) and 60.90 ± 3.66 µg (10 weeks) Der p 1 + Der p 2 allergen/100 mg for the cultures at 20 and 25°C, respectively.

Der p 1 in whole culture

Der p 1 increased exponentially over the entire life of the cultures (Fig. 4). Der p 1 accumulated at a faster rate at 20°C than at 25°C (Table 2). The rates of increase in Der p 1 during the mite population growth phase were 37.0%/week and 26.9%/week for 20 and 25°C, respectively. The rates of increase for the entire duration of the culture (even after mite population declined due to depletion of food) were 34.0%/week and 29.3%/week at 20 and 25°C, respectively. The Der p 1 allergen concentrations at the end of the 8 week exponential mite population growth time were 10.92 ± 0.80 µg/100 mg of culture and 21.11 ± 0.59 µg/100 mg of culture at 20 and 25°C, respectively. However, the highest concentrations of Der p 1 allergen in the cultures were 33.70 ± 1.30 µg/100 mg spent culture and 41.95 ± 1.83 µg/100 mg spent culture and these were observed at 10 weeks for

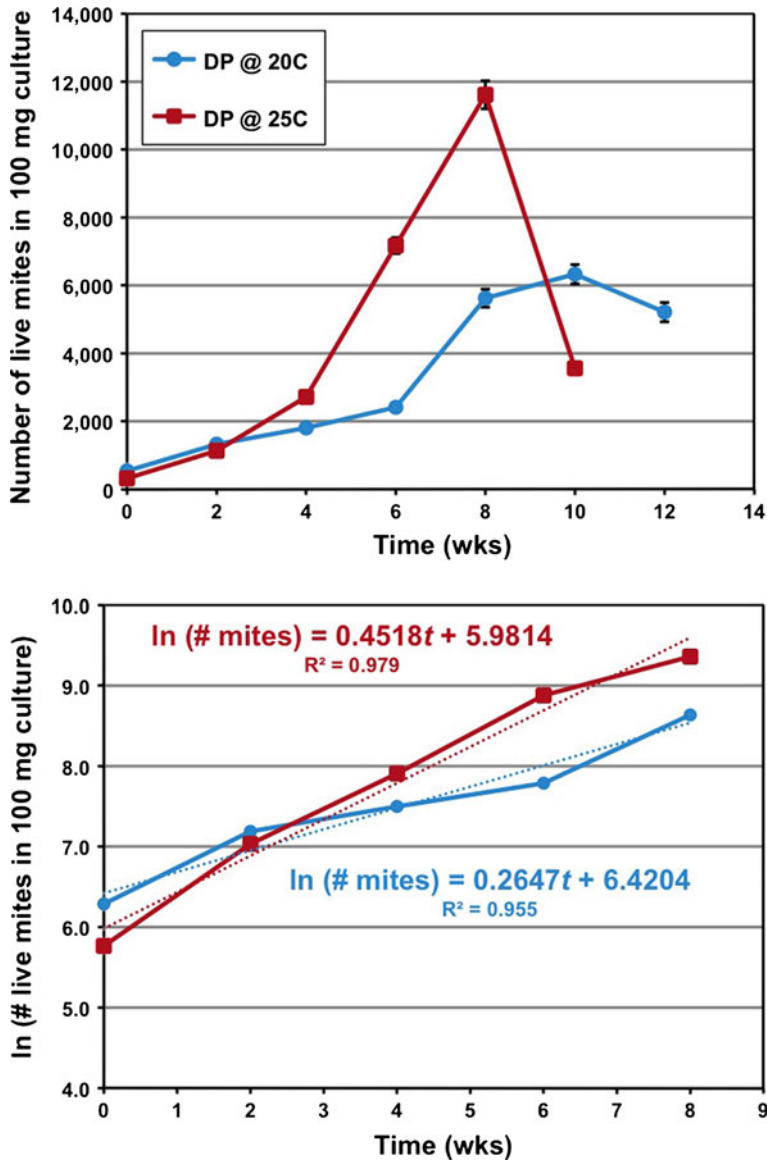


Fig. 1 Population profiles and growth rates for *Dermatophagoides pteronyssinus* cultures grown at 20 and 25°C

25°C and 12 weeks for 20°C, respectively or 2 and 4 weeks after the population had peaked when mites were dying and the populations were declining (Figs. 1, 4; Table 2).

Der p 1 in washed mite bodies harvested from cultures

The rate of accumulation of Der p 1 in washed mite bodies harvested from cultures grown at 25°C (34.9%/week) was 2.2 times greater than for washed mite bodies from cultures grown at 20°C (16.2%/week) (Table 2; Fig. 4). Washed mites harvested from thriving cultures

Table 1 Population growth and total allergen (Der p 1 + Der p 2) accumulation for *Dermatophagoides pteronyssinus* cultures grown at 20 and 25°C

In 100 mg whole culture	Culture temperature (°C)	
	20	25
<i>Live mites</i>		
Number at T = 0	538	319
Peak time (weeks)	10	8
Number at peak	6,331	11,614
Weeks used in <i>k</i> calculation	8	8
Number at <i>k</i> calc. time	5,625	11,614
Growth rate <i>k</i> (% per week)	26.5	45.2
Doubling time (weeks)	2.62	1.53
<i>Allergen (Der p 1 + Der p 2)</i>		
µg at T = 0	1.96	3.98
µg at 8 weeks	16.04	29.17
Allergen accumulation rate <i>k</i> (% per week)	25.4	25.0
Doubling time (weeks)	2.73	2.77
Time to culture end (weeks)	12	10
µg at culture end	47.68	60.90
Overall allergen accumulation rate <i>k</i> (% per week)	27.1	28.2
Doubling time to end (weeks)	2.56	2.46

contained much less allergen than the whole culture material. At 20°C, washed mites collected from 100 mg of whole culture contained 2.12 ± 0.26 µg of Der p 1 at week 8 whereas the whole culture material contained 10.92 ± 0.80 µg/100 mg (Table 2; Fig. 4). Likewise, for cultures at 25°C, washed mites from 100 mg of culture contained 9.24 ± 2.18 µg at 8 weeks whereas whole culture contained 21.12 ± 0.59 µg/100 mg of material (Table 2; Fig. 4). Two to 4 weeks later while mites were dying due to depletion of food, the concentrations of Der p 1 in washed bodies were 17.39 ± 1.74 and 16.84 ± 1.28 µg/100 mg whereas in whole culture they were 33.70 ± 1.30 and 41.95 ± 1.83 µg/100 mg of material for cultures grown at 20 and 25°C, respectively. Throughout the course of the experiment, the Der p 1 concentration in washed mite bodies collected from cultures grown at 25°C was more than double that in mite bodies collected from cultures maintained at 20°C (Fig. 4).

Der p 2 in whole cultures

Initially (T = 0) the concentrations of Group 2 allergens in cultures at 20 and 25°C were essentially identical (Fig. 4; Table 2). During the first 4 weeks of population growth, the Der p 2 concentrations were not appreciably different for cultures grown at the two test temperatures (Fig. 4). From week 4 until the food supply in the cultures became limiting at 8 weeks, the cultures contained increasingly more Der p 2 allergen at 25°C than at 20°C (Fig. 4). The rates of accumulation of Der p 2 in cultures where the populations were exponentially increasing were 14.8 and 20.9% per week at 20 and 25°C, respectively (Table 2). During the exponential growth phase of the cultures, the doubling time for Der p 2 concentration for cultures grown at 20°C was 1.4 times longer than for those grown at 25°C (Table 2). At 8 weeks, the cultures contained 5.12 ± 0.45 µg and 8.06 ± 0.64 µg of Der p 2/100 mg of culture material for cultures grown at 20 and 25°C, respectively. After

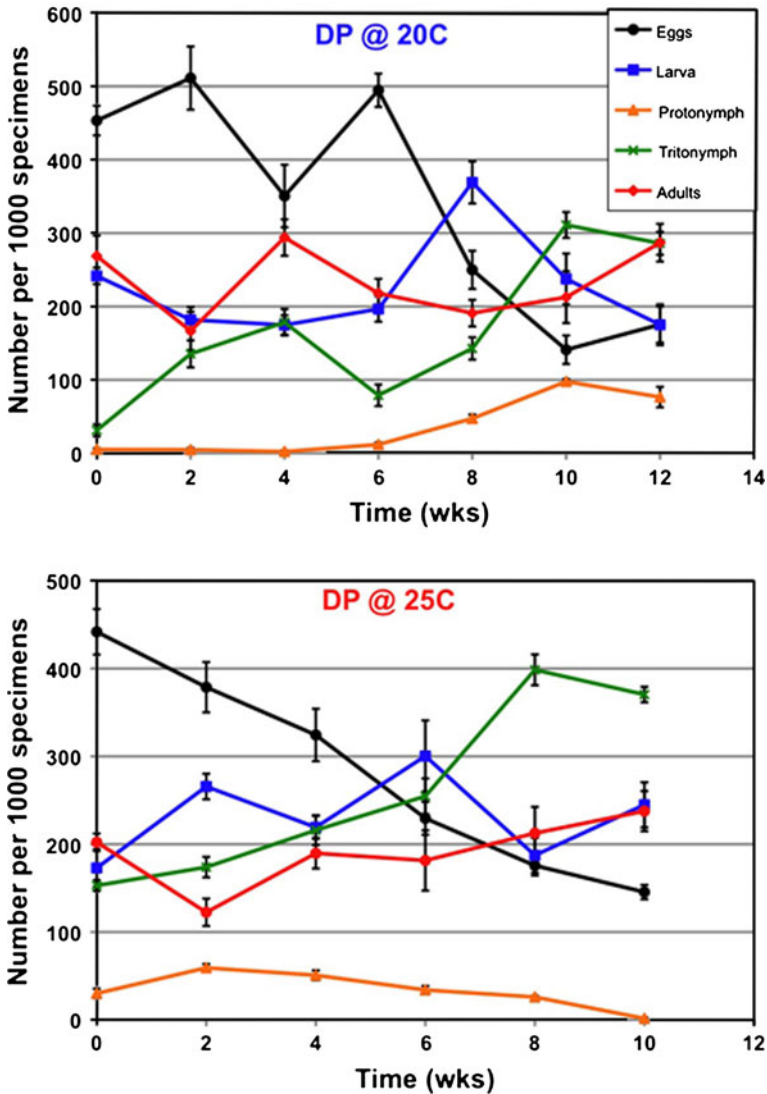


Fig. 2 Mite life stage composition of *Dermatophagoides pteronyssinus* cultures grown at 20 and 25°C

the mite population peaked and then declined, the concentration of Der p 2 in cultures at both temperatures continued to increase for the next 2–4 weeks. At the termination of the experiment, the cultures contained $13.98 \pm 0.98 \mu\text{g}$ (12 weeks at 20°C) and $18.96 \pm 1.90 \mu\text{g}$ (10 weeks at 25°C) of Der p 2/100 mg of material (Table 2).

Der p 2 in washed mites

Interestingly, the amount of Der p 2 allergen in washed mites harvested from cultures grown for 8 weeks at 25°C was about the same as it was in the whole culture material from which the mites were harvested. Likewise for cultures grown at 20°C, the amount of Der

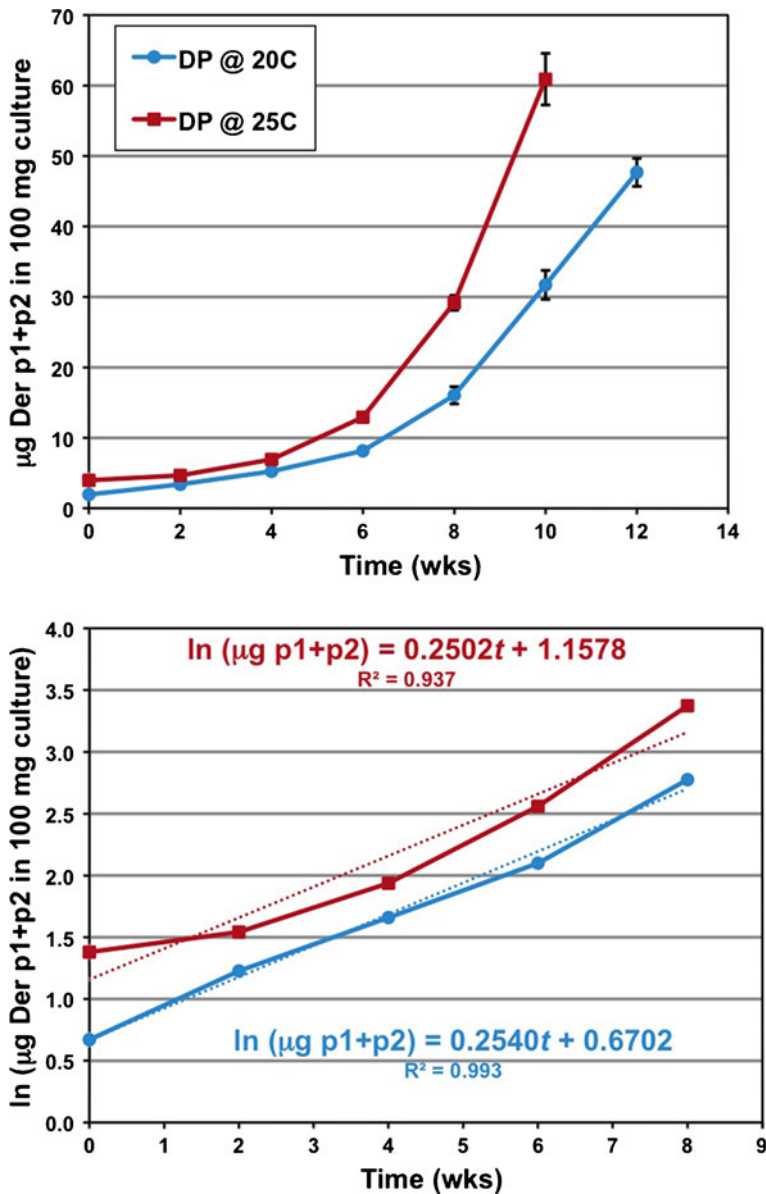
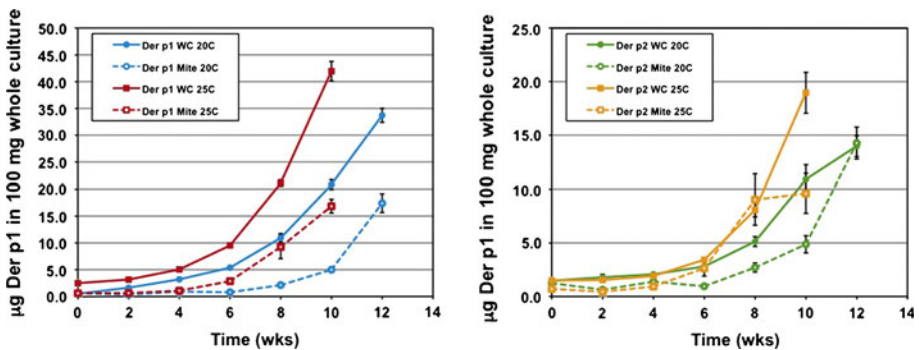


Fig. 3 Total allergen (Der p 1 + Der p 2) profiles and allergen accumulation rates for *Dermatophagoides pteronyssinus* cultures grown at 20 and 25°C

p 2 in washed mite bodies and spent culture was about the same after 12 weeks (4 weeks after the mites were dying and the population was declining) (Fig. 4). In contrast, the level of Der p 2 from washed mite bodies was about 1/2 that in whole culture at 8 weeks at 20°C and 10 weeks at 25°C. Notably, the rate of accumulation of Der p 2 in washed mite bodies from cultures grown at 25°C (24.5%/week) was 2.5 times faster than at 20°C (9.8%/week) during exponential population growth.

Table 2 Der p 1 and Der p 2 levels and accumulation rates in whole culture and washed mites collected from *Dermatophagoides pteronyssinus* cultures grown at 20 and 25°C

In 100 mg whole culture	Cultures at 20°C		Cultures at 25°C	
	Der p 1	Der p 2	Der p 1	Der p 2
<i>Whole culture</i>				
µg at T = 0	0.49	1.47	2.48	1.49
µg at 8 weeks	10.92	5.12	21.11	8.06
Allergen accumulation rate k (% per week)	37.0	14.8	26.9	20.9
Doubling time (weeks)	1.87	4.70	2.57	3.32
Time to culture end (weeks)	12	12	10	10
µg at culture end	33.70	13.98	41.95	18.96
Overall allergen accumulation rate k (% per week)	34.0	20.2	29.3	26.1
Doubling time to end (weeks)	2.04	3.43	2.37	2.66
<i>Washed mites</i>				
µg at T = 0	0.63	1.24	0.60	0.70
µg at 8 weeks	2.12	2.69	9.24	9.03
Allergen Accumulation Rate k (% per week)	16.2	9.8	34.9	24.5
Doubling time (weeks)	4.27	7.04	1.98	2.01
Time to culture end (weeks)	12	12	10	10
µg at culture end	17.39	14.26	16.84	9.60
Overall allergen accumulation rate k (% per week)	28.9	21.6	36.8	33.2
Doubling time to end (weeks)	2.40	3.21	1.88	2.09

**Fig. 4** Der p 1 and Der p 2 accumulation profiles for whole culture (WC) and washed mites (Mite) collected from *Dermatophagoides pteronyssinus* cultures grown at 20 and 25°C

Ratio of Der p 1/Der p 2

At most times during population growth at both temperatures, Der p 1 allergen in the whole culture was generally twice that of Der p 2 (Fig. 4). Der p 1 accumulated faster in cultures

than Der p 2 at both temperatures (Table 2). Therefore, the ratio of Der p 1 to Der p 2 increased as the mite population grew. Interestingly, this ratio continued to increase when the mite population at 20°C was declining whereas the ratio of Der p 1 to Der p 2 declined after week 6 for cultures at 25°C (data not shown).

Protein and endotoxin concentrations

The protein concentration in the cultures increased slowly over time until the cultures were terminated and this was associated with the increases in mite population and allergen content of the cultures (Fig. 5). Endotoxin levels also increased exponentially when the cultures were thriving and about to be food limited (Fig. 5). Endotoxin levels increased more rapidly in cultures at 20°C compared to cultures at 25°C.

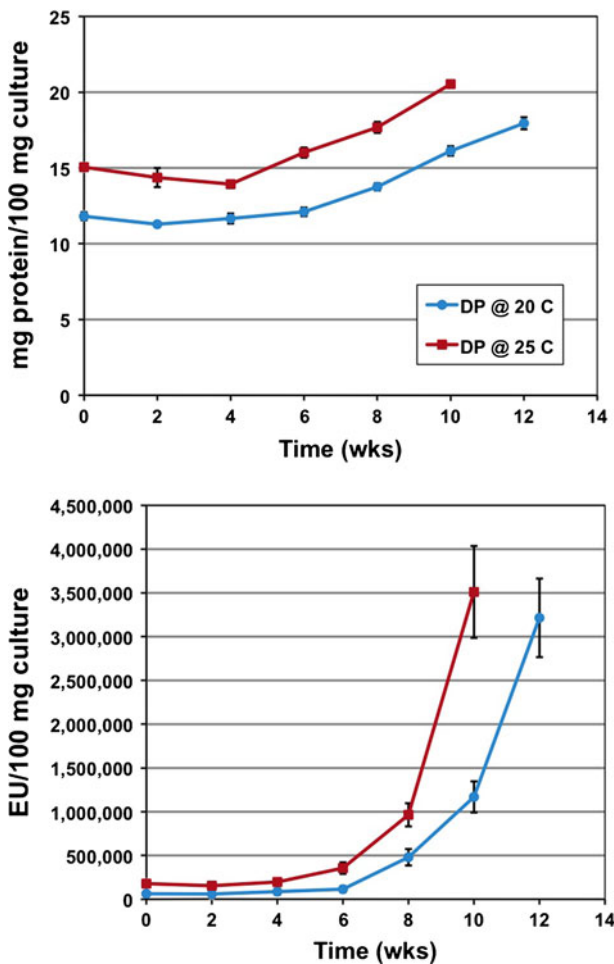


Fig. 5 Protein concentrations and endotoxin contents for *Dermatophagoides pteronyssinus* cultures grown at 20 and 25°C

Table 3 Enzymatic activity of mite extracts measured using ApiZym strips

Time in culture (weeks)	Cultures at 20°C							Cultures at 25°C						
	0	2	4	6	8	10	12	0	2	4	6	8	10	
<i>Phosphatases</i>														
Acid phosphatase	5	5	5	5	5	5	5	5	5	5	5	5	5	
Alkaline phosphatase	2	1	2	3	2	3	4	2	2	3	4	2	3	
Naphthol-AS-BI-phosphohydrolase	5	5	5	5	5	5	5	5	5	5	5	5	5	
<i>Esterases</i>														
Esterase (C4)	2	1	2	3	3	4	4	2	2	2	3	4	3	
Esterase lipase (C8)	1	1	1	2	3	4	4	1	1	1	2	3	4	
Lipase (C14)	0	0	0	0	0	1	1	0	0	0	0	1	1	
<i>Aminopeptidases</i>														
Leucine arylamidase	1	1	2	3	4	5	5	1	1	2	4	5	5	
Valine arylamidase	0	0	0	1	1	2	2	0	0	0	1	2	2	
Cystine arylamidase	0	1	1	1	1	1	1	0	0	0	1	1	1	
<i>Serine peptidases</i>														
Trypsin	0	0	0	1	1	1	1	0	0	0	0	1	1	
α -Chymotrypsin	0	0	0	1	1	1	1	1	0	0	0	1	1	
<i>Glycosidases</i>														
α -Galactosidase	1	1	1	2	2	2	2	1	1	1	2	2	3	
β -Galactosidase	3	3	3	4	3	4	4	2	2	2	3	2	4	
β -Glucuronidase	0	0	0	0	0	0	1	0	0	0	0	1	1	
α -Glucosidase	4	3	3	5	4	3	1	4	5	5	4	1	2	
β -Glucosidase	2	1	1	2	2	3	3	1	2	2	2	2	3	
N-Acetyl- β -glucosaminidase	1	1	1	1	2	3	3	1	1	1	1	2	3	
α -Mannosidase	0	0	0	0	1	2	3	1	1	1	2	3	4	
α -Fucosidase	0	0	0	0	0	1	2	0	0	0	1	2	3	

Scoring is 0 (no activity) to 5 (most activity)

Enzymes activities

The activity profiles for a collection of enzymes present in culture extracts were determined using ApiZym strips (Table 3). The activities of most enzymes remained unchanged over the course of the experiment at both temperatures. Of note were the increases in the activities of esterase lipase, leucine arylamidase, N-acetyl- β -glucosaminidase, and α -mannosidase as cultures matured at both temperatures. In contrast, levels of α -glucosidase waned as the cultures aged.

Discussion

In this study we found that temperature greatly affected the exponential growth rate of *D. pteronyssinus* populations and the population size that could be achieved on equal quantities of food. The population growth rate was 1.7 times greater at 25°C compared to

20°C. This is likely caused by the greater fecundity and shorter life cycle that occurs at 25°C compared to lower temperatures as previous laboratory studies have reported (Arlian et al. 1990). However, the peak live mite population density achieved was much greater for mites cultivated at 25°C compared to 20°C. It is unclear why the population size peaks at lower density for populations grown at 20°C compared to 25°C given that the cultures contained the same amount of starting food. *D. pteronyssinus* may be better adapted to thrive and reproduce at 25°C. Our study results suggest that for commercial and research purposes, greater numbers of mites are available for harvest in a shorter cultivation time from cultures grown at 25°C compared to 20°C.

In spite of the big differences in temperature-induced population growth rates, the combined allergen (Der p 1 + Der p 2) concentrations accumulated in whole culture at about the same rate regardless of the temperature during exponential mite population growth and this continued for the 2–4 weeks after the live mite population peaked and began to decline due to depletion of food in the cultures. However, individual Der p 1 and Der p 2 accumulation rates varied independently at the two temperatures. During the exponential growth phase for the cultures, Der p 1 accumulated 1.38 times faster at 20°C than at 25°C. In contrast, Der p 2 accumulated 1.41 times faster at 25°C than at 20°C. Therefore, if one wants to maximize Der p 1 accumulation in whole culture material, 20°C is the better temperature for culturing than 25°C. In contrast, 25°C is a better culture temperature to maximize Der p 2 accumulation in whole cultures. Interestingly, the greatest amounts of allergen are present in cultures 2–4 weeks after the mite population has begun to die off due to starvation. Therefore, if methods permit and group 1 and 2 allergens are the allergens of interest, then harvesting these allergens after the live mite population is dying as growth media is depleted would give the greatest yield of allergen. The dynamics for accumulation of the other 18 + allergens from these cultivated mites is unknown at this time.

A study by Eraso et al. (1997) found that at 23–25°C and 75–80% RH *D. pteronyssinus* cultures increased exponentially and reached maximum live mite density at about 16–18 weeks (grown on rat and mouse fodder and dried yeast powder). However, this is considerably longer than it took our cultures to mature. Also, in contrast to our results, their study found that Der p 1 and Der p 2 concentrations increased exponentially in parallel in whole culture material but they peaked between 14 and 16 weeks of cultivation which was before the live mite population peaked in the cultures. During exponential increase, the concentration of Der p 1 was always greater than that of Der p 2 as we observed in this study.

Batard et al. (2006) characterized house dust mite allergens in culture material for populations of *D. pteronyssinus* grown on a medium of wheat germ, yeast and amino acids (Stalmite APF®) at 75% RH and 25°C. A major difference between their study and ours and that of Eraso et al. (1997) was that culture medium was added twice during the 70-day culture period and the mite population growth was not reported. As in the other two studies, the concentration of Der p 1 (µg/ml of extract) in whole culture material during the 70 days of culture growth was always greater than that of Der p 2 and the concentration of Der p 1 increased at a faster rate than did Der p 2 over time until harvest. The ratio Der p 1 / Der p 2 in spent culture material after 70 days was 4.1.

Unlike the situation for whole cultures, the influence of temperature on the amount of Der p 1 and Der p 2 in washed mite bodies harvested from the cultures was different than it was for whole culture material. Both the Der p 1 and Der p 2 content of washed bodies accumulated more than twofold faster at 25°C than at 20°C. However, for both temperatures, Der p 1 accumulated 1.65–1.42 times faster in washed bodies than did Der p 2.

Therefore, if mites are harvested from cultures and washed to prepare extracts, culturing mites at 25°C yields more total allergen and essentially equivalent amounts of Der p1 and Der p 2 in shorter cultivation time than does culturing at 20°C.

The amount of total Der p 1 and Der p 2 was 1.65–2.49 fold greater in mature whole cultures at 10 or 12 weeks of cultivation compared to harvested mites bodies. The harvesting and washing of mites resulted in loss of significant amounts of the allergen that was present in whole culture. Much of the allergen that was lost was Der p 1 which is associated with fecal material that was washed away. Efforts should be made to develop methods to harvest more of the mite allergen that is present in mite cultures. Alternatively, culture medium should be developed that would allow whole spent culture to be used in the preparation of vaccines.

Similar to our results, Osterberg et al. (2007) found that the maximum yield of purified live *D. pteronyssinus* during culturing occurred between 40 and 60 days (5.7–8.6 weeks) of cultivation at 75% RH and 25°C and then the yield declined over the following 40 days. In parallel, the Der p 1 content of purified mite bodies increased exponentially and peaked at about 6 mg/g of purified mite bodies (60 days). In contrast, the Der p 2 content of purified dried bodies was relatively constant and ranged between 8 and 10 mg/g of dried bodies during the 100 days of cultivation. In contrast to our results at 25°C, the amount of Der p 2 in purified bodies was always greater than the amount of Der p 1. However, in that study the concentration of Der p 1 was always much greater than Der p 2 in whole culture. Also, in contrast to our results, maximum Der p 1 and Der p 2 concentrations were reportedly 5- and 20-times lower, respectively, in whole culture than in purified mite bodies.

Many field studies have analyzed dust samples from homes for the concentration of Der p 1 and Der p 2. It is difficult to obtain an exact ratio of Der p 1/Der p 2 in dust samples because the monoclonal antibody used in the ELISA to detect Der 2 is cross-reactive and detects both Der p 2 and Der f 2 (Ovsyannikova et al. 1994). However, these studies generally report that the concentration of Der p 1 in dust from mattresses and carpets in homes is much greater than the concentration of Der 2 (Der p 2 + Der f 2 together) (Calvo et al. 2005; Carswell et al. 1999; Chen et al. 2002; Su et al. 2001; Tsay et al. 2002). The relationship (ratio) between Der p 1 and Der p 2 found in our whole cultures is consistent with what is reported for dust samples taken from homes around the world.

House dust mite extracts also contain numerous enzymes including some that are the major allergens from these mites (Morgan and Arlian 2006; Thomas et al. 2007). Some of the proteases in an extract of *D. pteronyssinus* have direct inflammatory activity in the respiratory tract (Wan et al. 1999; Winton et al. 1998). Likewise, proteases and other enzymes from mites can penetrate the skin and activate keratinocytes (Arlian et al. 2008; Mascia et al. 2002). In vitro, serine and cysteine proteases in *D. pteronyssinus* extracts induced cultured skin keratinocytes to secrete (but not synthesize) interleukin-1 α (IL-1 α) and its receptor antagonist IL-1ra (Mascia et al. 2002). *D. pteronyssinus* extract also induced mRNA expression and secretion of IL-8 and granulocyte-monocyte colony stimulating factor (GM-CSF). Both of these effects were abrogated by serine and cysteine protease inhibitors. Our data in this study and in a previous study show that *D. pteronyssinus* extract had little detectable serine and cysteine protease activity (Morgan and Arlian 2006). However this study showed increasing levels of lipase, esterase, aminopeptidase and glycosidases, particularly N-Acetyl- β -glucosaminidase (a chitinase molting enzyme) activity in extracts of culture material as the mite population grows over time. Mite chitinases have been implicated in the Th2 polarization that is characteristic of allergic asthma (Elias et al. 2005; Wills-Karp and Karp 2004). Although the focus of most studies of enzyme activity in mite extracts has been the cysteine (group 1 allergen) and

serine (groups 3, 6, & 9 allergens) proteases because they can activate protease activated receptors (PARs), this study and that of Cardona et al. (2006) using *Blomia* demonstrate that mite culture material contains a host of other enzymes that may also be important in inflammation and immune reactions and atopy.

Our study also showed that endotoxin levels in extracts of our mite cultures increased exponentially over time. Many allergen vaccines including those for house dust mite allergy contain endotoxins (Trivedi et al. 2003; Finkelman et al. 2006) but allergen vaccines in the United States are not required to report the presence of endotoxins (Trivedi et al. 2003; Valerio et al. 2005). Endotoxin is a lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria that is ubiquitous in indoor and outdoor environments (Topp et al. 2003). LPS promotes inflammatory reactions by activation of the Toll-like receptor 4 (TLR 4) (Cook et al. 2004; Kaisho and Akira 2006). Little is known about the influence of endotoxins in atopy, skin testing and immunotherapy responses. However, studies show that endotoxins in house dust mite extracts may influence inflammatory and immune reactions. In vitro, LPS stimulates cultured normal human epidermal keratinocytes and dermal fibroblasts to secrete a variety of proinflammatory cytokines (Arlan et al. 2008). Likewise, LPS stimulated dermal microvascular endothelial cells to express several cell adhesion molecules and to secrete numerous cytokines (Arlan et al. 2009). These cytokines, along with the endotoxins, can directly influence the function of other cells in the vicinity such as keratinocytes, microvascular endothelial cells, migrating monocytes, macrophages, Langerhans cells, and lymphocytes in the skin. Also, endotoxin has been shown to effect Th2 responses (IgG and IgE) and atopy (Slater et al. 1998). Thus, the presence of endotoxins in house dust mite vaccines is important but yet to be fully understood. The endotoxin in our mite extracts likely comes from intracellular endosymbiont bacteria as well as enteric bacteria. Our findings are consistent with the finding that house dust mite vaccines made from washed mites contain endotoxin and that house dust mites contain DNA from several species of Gram-negative bacteria (Trivedi et al. 2003; Valerio et al. 2005).

The results of our laboratory culturing studies coupled with data for allergen analysis of field dust samples suggest that independent of dust mite population growth phase, Der p 1 allergen concentration is usually greater than Der p 2 allergen concentration for established mite populations both in dust in homes and in laboratory cultures. Also, our study clearly demonstrates that temperature is an important factor in population growth and dynamics of allergen production in cultured mites. This is likely also the case in their natural microhabitats in homes. Global climate change may also effect dust mite populations in nature and the potential impact of this on mite allergen prevalence and mite-induced sensitization and allergic disease is unknown. Studies using only *D. pteronyssinus* and one food are reported here. Similar data for *D. farinae* and cultivation of dust mites using different foods have yet to be reported. These studies are important in order to produce standardized mite material for use in making extracts for research, diagnostic and immunotherapy purposes and to understand the relationship between the properties of cultured mites and natural mite populations.

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