

Effects of air pollution and other environmental factors on birch pollen allergens

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To determine the effects of anthropogenic pollution on water-soluble proteins and specifically allergens in birch (*Betula pendula* and *B. pubescens*) pollen, we analyzed extracts of pollen from the pollution gradient around a factory complex (emitting sulfur oxides and heavy metals) by sodium dodecyl sulfate (SDS) – polyacrylamide gel electrophoresis (PAGE) and IgE immunoblotting. In addition, tree density-associated shading of the tree habitat, and quantity and quality of proteins and allergens in pollen of the two birch species were studied. The two studied birch species gave identical allergen profiles even though their protein profiles differed. Distance from the factory did not affect the amount of birch pollen major allergen, Bet v 1 (17 kDa), or of two other strong allergens (23 and 36 kDa). Trees growing in shaded places had significantly stronger responses to Bet v 1 and to the 23-kDa allergen than trees growing in open or half-open environments. Thus, we propose that combined heavy metal and sulfur dioxide pollution does not have an important effect on birch pollen allergens. Instead, other factors, e.g., shading and soil properties of the tree habitat, as well as the genetic background of the tree, may have a stronger influence on the quantity and relative composition of allergens.

Over the centuries, air pollution has increased from a local nuisance to a global problem, and at the same time the incidence of allergies has been observed to increase in industrial countries (1–3). Air pollutants, such as exhaust gases from cars, ozone (O₃), nitrogen oxides (NO_x), sulfur dioxide (SO₂), and other inorganic airborne particles, have been shown to correlate positively with allergies and respiratory diseases (4–9). However, the mechanisms of the effects of air pollution on the human immune system are still poorly understood.

Air pollution can also affect biologic allergens; e.g., pollen grains. Airborne pollen grains can be attacked directly by air pollutants. Indirect effects of air pollution on pollen grains are possible via the soil. If a plant grows in polluted soil, its physiologic functions may change and affect properties of the developing pollen grains. Laboratory experiments have shown that NO₂, SO₂, and CO treatments can cause changes in the soluble protein composition of pollen grains (10, 11). Studies on pollen allergens collected from polluted and unpolluted areas have shown contradictory results. Jilek et al. (12) claimed

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differences in amounts of the birch pollen major allergen, Bet v 1, between polluted and control areas, although they could not show this difference to be statistically significant. Instead, Hjelmroos et al. (13) have observed that birch pollen collected near highways or other heavy traffic is low in allergens compared to samples from control areas.

The present paper aimed to determine whether

- 1) air pollutants affect birch pollen allergens around a copper-nickel smelter
- 2) other environmental factors, e.g., tree density-associated shading, affect birch pollen allergens
- 3) two birch species, *Betula pubescens* Ehrh. and *B. pendula* Roth., differ in the quantity or quality of proteins, specifically allergens, in their pollen grains.

Material and methods

Study area

The sampling area was around the industrial town of Harjavalta (61°19'N, 22°9'E) in southwestern

Finland. The factory complex, established in the early 1940s, produces copper, nickel, sulfuric acid, and fertilizers. The emissions contain large amounts of sulfuric oxides and heavy metals. The yearly copper emission is 60 000 kg, the nickel emission 10 000 kg, and the SO₂ emission 4800 t (14). This is an ideal place to study the effects of air pollution on the environment, because of the long history of pollution and clearly observable pollution gradient around the factory complex. Heavy metal concentration in the vegetation decreases with distance from the factories and reaches background values approximately 10 km from the emission source (15, 16).

Birch pollen samples were collected from 10 sites in different directions and at varying distances from the factories in May 1995. The closest two sampling sites were next to the factory, and the farthest site was 12 km from the emission source. Sites similar in their soil properties and vegetation type were chosen.

Study trees and sampling

At each site, from four to five birch trees were randomly chosen as study trees (45 trees together). Thirty-two of the trees were silver birch (*B. pubescens*) and 13 white birch (*B. pendula* = *B. verrucosa*). *B. pubescens* was represented at each study site, but *B. pendula* was absent from four sites.

The study trees were assigned to three classes according to tree density-associated shading:

- 1) open sites, over 15 m to the closest trees and bushes ($n = 25$)
- 2) half-open sites, on the edge of forest, less than 10 m to the closest trees and bushes ($n = 15$)
- 3) trees growing in shade inside a forest ($n = 5$).

Branches growing in different directions and at different heights of the study tree were collected a few days before the birch pollen season started in southern Finland (17) and taken to the greenhouse (22°C) at Turku University. The branches were placed in water containers, and small paper bags were placed over each catkin. Pollen released to bags was collected in test tubes regularly every few hours. Pollen from one tree was combined to form one sample and frozen at -70°C. The purity of the pollen was estimated with a transmission microscope. In 20 randomly chosen samples, the purity varied between 95.2 and 98.2%.

Pollen extracts

The antigenic components of birch pollen are readily extractable (18) and stable to proteolysis

(19). Pollen was extracted in 0.125 M ammonium carbonate (1/10 w/v) with 0.015 M sodium azide by rotating at 4°C for 24 h. The samples were then centrifuged at 13 000 rpm for 20 min, and the clear supernatant was removed and frozen at -70°C.

Sera

Sera from 30 birch-allergic patients were individually tested for IgE reaction to birch pollen allergens. From these analyses, a pool comprising 12 samples giving responses to allergens located at 6, 14, 17, 23, 29, 36, 70, and 90 kDa was chosen. Sera from five patients not allergic to birch pollen were tested, and three of them were chosen for the control sera pool. In sera tests, the international standard for birch pollen extract was used (ALK, Copenhagen, Denmark).

SDS-PAGE and IgE immunoblotting

Proteins from the samples and molecular weight markers (Rainbow, Amersham, UK) were separated by SDS-PAGE, by the method of Laemmli (20), in a 5–17.5% gradient gel under nonreducing conditions. The gels for soluble protein detection were stained with Coomassie brilliant blue (CBB). In another set of samples, SDS-PAGE-separated antigens were transferred to nitrocellulose sheets (Schleicher & Schuell, Kassel, Germany, 0.2 µm) in a Transphor devise (LKB, Bromma, Sweden), according to Towbin et al. (21). The specific IgE binding of allergens on nitrocellulose was studied according to Bengtson et al. (22), but instead of radiographic film, a sensitive phosphor image (Bio-Rad Laboratories, Canada) was used. To confirm that different exposures were comparable, we used ¹²⁵I-conjugated anti-IgE antibodies reference disks (23). To block unoccupied binding sites, we incubated the sheets in phosphate-buffered saline (PBS) containing 0.5% Tween 20 (PBS-Tw). The sheets were incubated in pooled sera and thereafter rinsed three times in NaCl-Tween. ¹²⁵I-labeled anti-IgE antibodies (RAST-RIA, Pharmacia, Uppsala, Sweden) diluted 1:10 in PBS-Tw were used to probe bound IgE. Sheets were washed three times in NaCl-Tween and rinsed in distilled water. ¹²⁵I-labeled bands were visualized with the Molecular Imaging Screen (Bio-Rad Laboratories), and band intensities were analyzed with Phospho Analysis 1.1. software.

Statistical analyses

The data fulfilled requirements for analysis of variance (24). Differences between the sites at

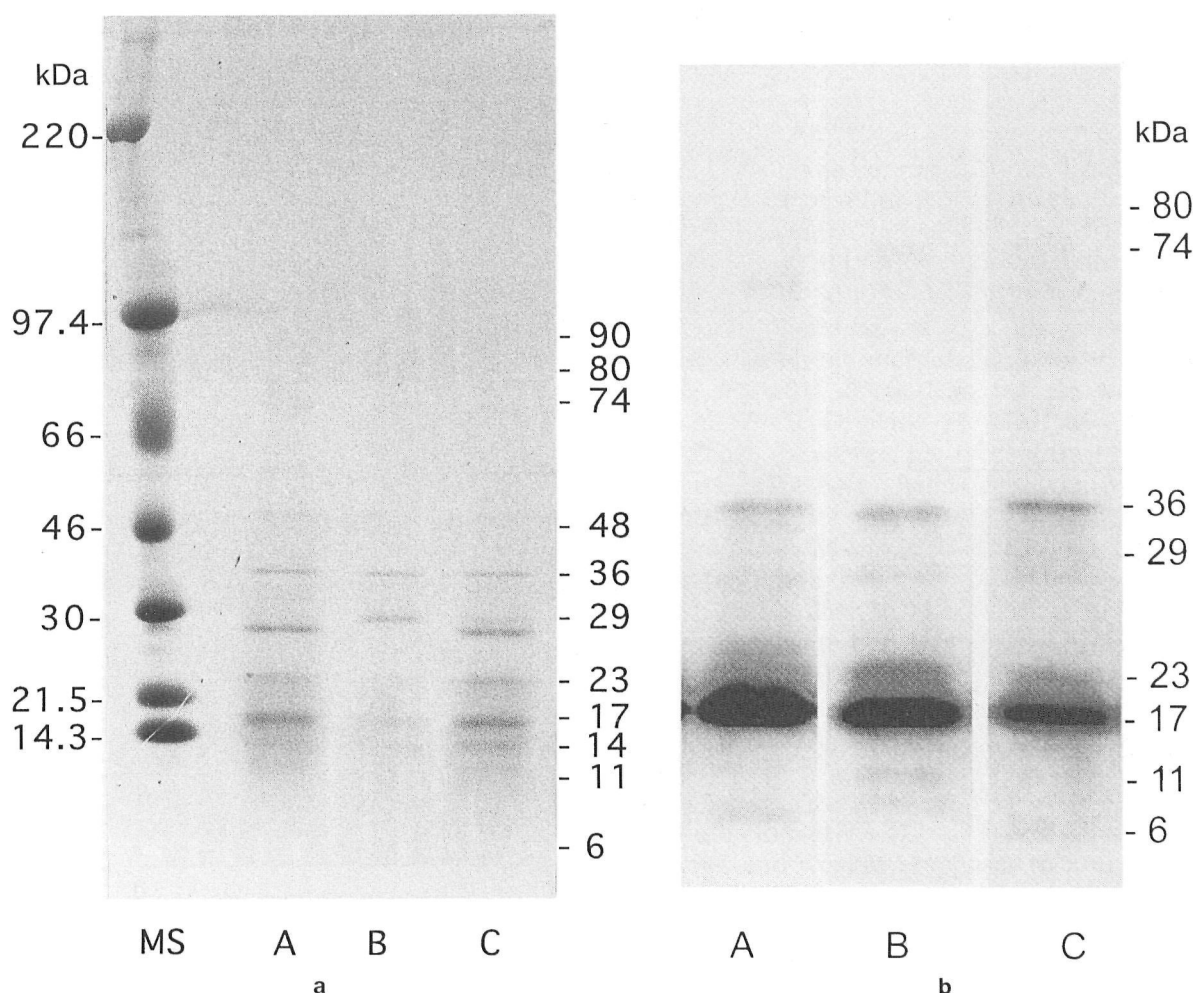


Fig. 1. Soluble proteins (a) and IgE responses (b) of pollen grains from two birch species and their possible hybrid. *Betula pubescens* (A) with 28-kDa band, *B. pendula* (B) with 29-kDa band, and their hybrid with both 28- and 29-kDa bands (C). MS: molecular mark standard.

different distances from the factory complex, the two birch species, and shade classes and their interactions were used in the model (25). The strongest allergens, 17- (Bet v 1), 23-, and 36-kDa proteins, were used as response variables. In pairwise testing, Tukey's test was used.

Results

Sera

All tested sera from birch-allergen patients (30) showed strong binding to a 17-kDa protein (Bet v 1). One-third (10/30) of the tested patient sera had a strong response to the 36-kDa protein and one-fifth (6/30) to the 23-kDa protein. Therefore, these three proteins were considered the most important allergens in this study population of randomly chosen birch-allergic individuals in southwestern Finland.

B. pendula and *B. pubescens* pollen extracts and allergens

When *B. pendula* and *B. pubescens* pollen extracts were compared by CBB staining, 12 different protein bands at 6, 11, 14, 17, 23, 28, 29, 36, 48, 74, 80, and 90 kDa were achieved. The *B. pendula* and *B. pubescens* pollen bands differed clearly: *B. pendula* had a 29-kDa protein which was absent from *B. pubescens*, while *B. pubescens* had a 28-kDa band absent from *B. pendula* samples (Fig. 1a). However, two *B. pubescens* samples had both 28- and 29-kDa bands, indicating that these trees were probably hybrids of *B. pendula* and *B. pubescens* (Fig. 1).

The same allergens were detected in both *B. pendula* and *B. pubescens* samples (Fig. 1b). The components which bound IgE most strongly were those at 17 (Bet v 1), 23, and 36 kDa, and these were used in statistical analyses. Weaker

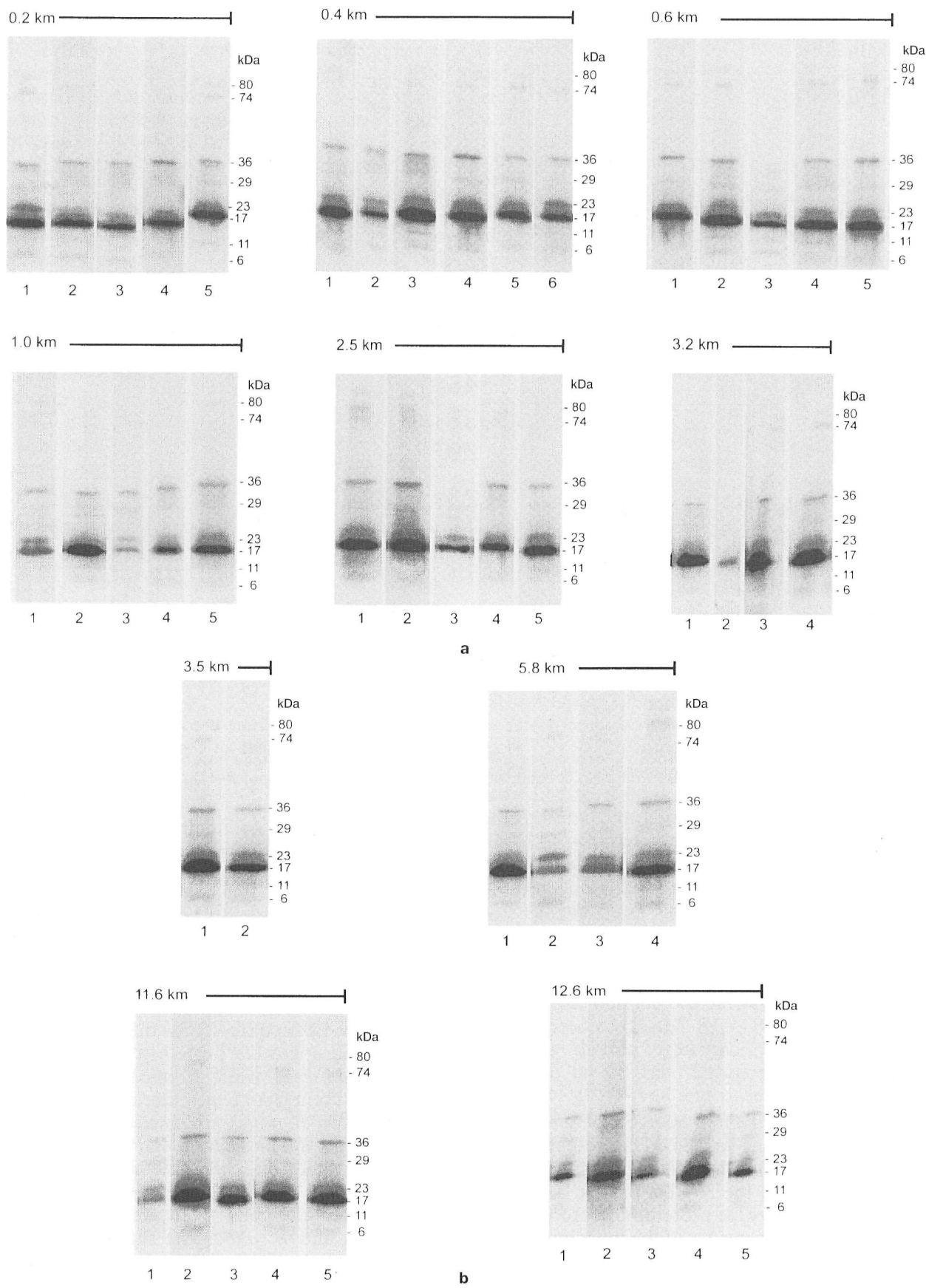
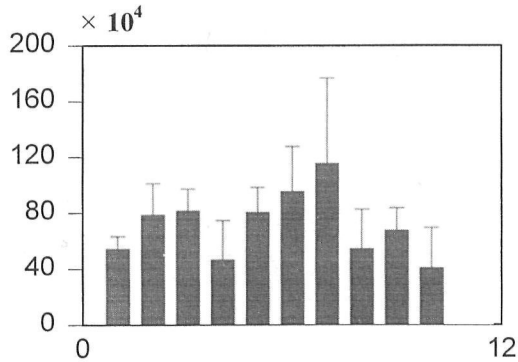
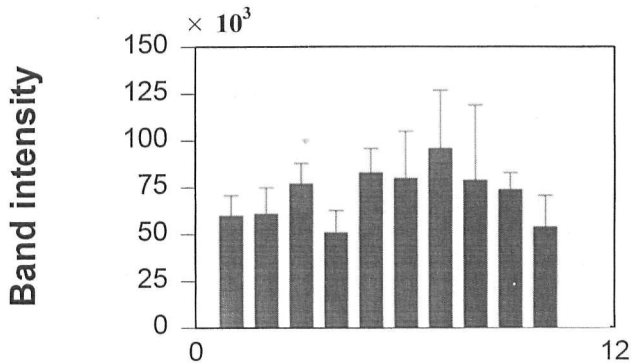


Fig. 2. IgE responses of birch pollen from individual trees along pollution gradient.

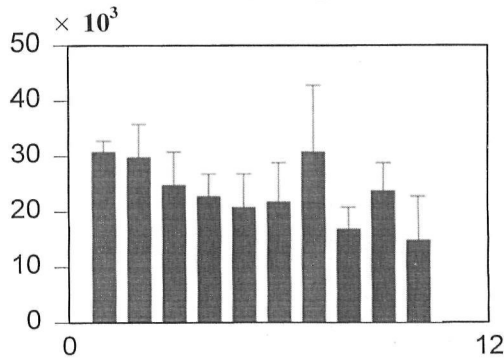
Bet v 1 (17 kDa)



23 kDa



36 kDa



Distance (km)

Fig. 3. IgE responses of birch pollen to Bet v 1 and 23- and 36-kDa proteins along pollution gradient. Note different y-axis scales on each picture.

responses were detected at 6, 11, 28, 29, 74, and 80 kDa.

On average, Bet v 1 was responsible for 70% of all allergen responses. Its response was positively correlated with other allergens in the samples (with

23 kDa, $n=45$, $r=0.69$, $P<0.0001$; with 36 kDa, $n=45$, $r=0.68$, $P<0.0001$).

Environmental factors

Distance from the factory did not affect the amount of Bet v 1 or of other major allergens (23 and 36 kDa) (Figs. 2 and 3) in samples (for Bet v 1 $df=9$, $F=1.26$, $P<0.3119$; for 23 kDa, $df=9$, $F=1.1$, $P<0.4040$; for 36 kDa $df=9$, $F=1.07$, $P<0.4183$). The birch species (*B. pendula* or *B. pubescens*) did not have different allergen responses (for Bet v 1, $df=1$, $F=0.01$, $P<0.9126$; for 23 kDa, $df=1$, $F=0.27$, $P<0.6088$; for 36 kDa, $df=1$, $F=1.31$, $P<0.2652$). However, the trees growing in shaded habitats (Fig. 4) had significantly stronger responses to Bet v 1 ($df=2$, $F=5.22$, $P<0.0024$) and to the 24-kDa protein ($df=2$, $F=4.64$, $P<0.0209$) than trees growing in open or half-open environments. For 36 kDa, responses at open or shaded sites were not significantly different ($df=2$, $F=2.42$, $P<0.1095$; Fig. 5).

Discussion

The pooled patient sera showed clear IgE responses to Bet v 1 (17 kDa) and 23- and 36-kDa proteins, and weaker responses to four other proteins, but differences in allergens between the two species of *Betula* were not detected. However, a difference was found between *B. pendula* and *B. pubescens* proteins (28 and 29 kDa), while the used sera did not show measurable responses to either of these protein bands. Jarolim et al. (26) have observed that 12% of tested sera from allergic patients responded to the *B. pendula* (= *B. verrucosa*) 29-kDa protein. Therefore, it is possible that these two *Betula* species differ in their allergens.

This study did not detect any differences in birch pollen allergenicity along the studied pollution gradient, indicating that combined sulfur dioxide and heavy metal pollution do not affect pollen allergens. Previous, contradictory studies have shown both positive and negative results. Jilek et al. (12) observed an increase of the major birch pollen allergen, Bet v 1, in areas where nitrogen loads are high, while Hjelmroos et al. (13) found a decrease in Bet v 1 concentration due to elevated nitrogen compounds. Because the present study showed great variation between individual trees and between shaded and unshaded trees in their allergen concentration, we propose that many unknown environmental factors other than pollution cause variation in birch pollen allergen concentrations. In future studies, more attention should be paid to adequate sampling within trees (27), between trees, and between sites

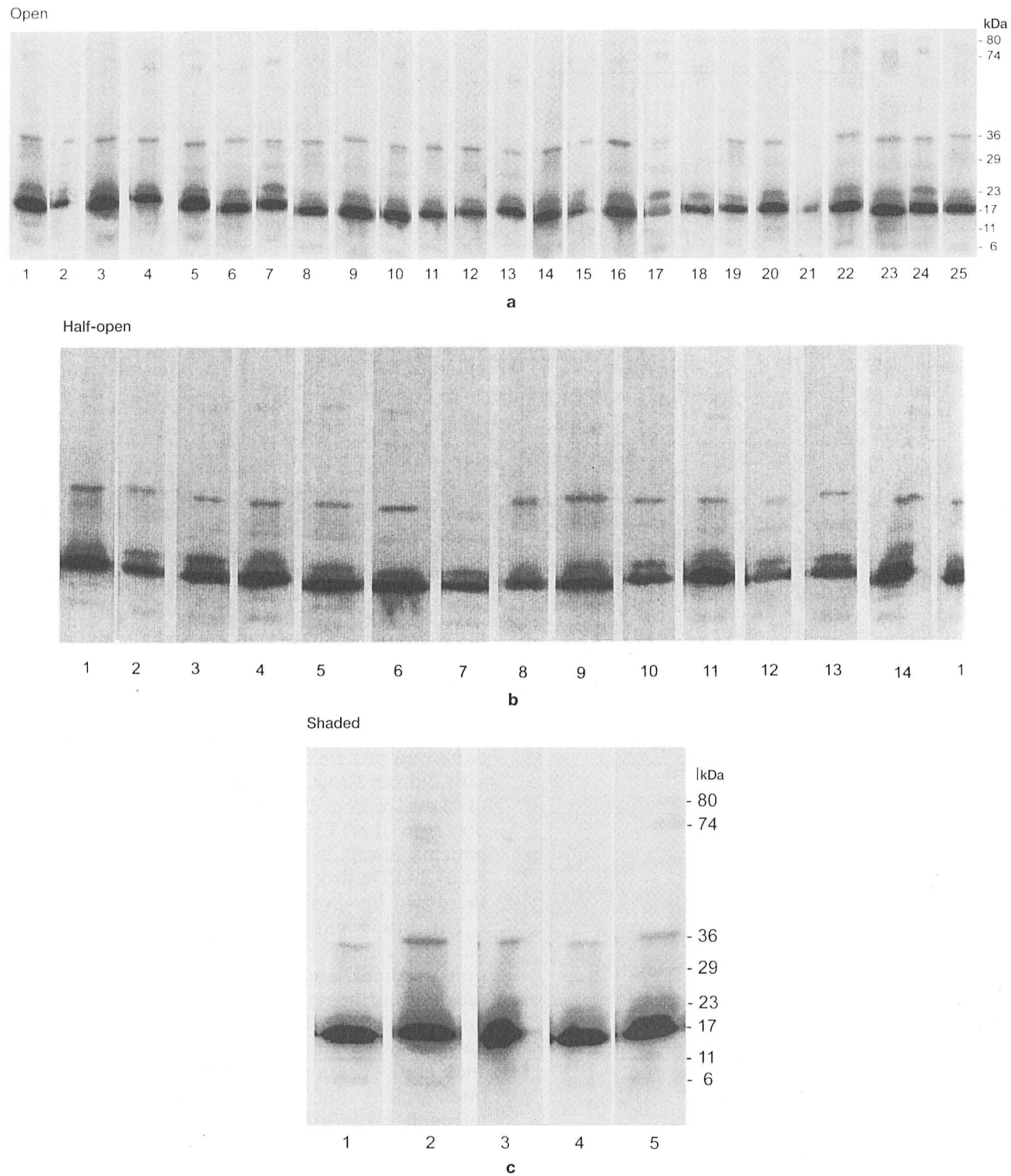


Fig. 4. IgE responses of birch pollen from individual trees grouped by shade class: open (a), half-open (b), and shaded (c) sites.

to achieve sufficient power of statistical analysis and to avoid results that are only correlated with air pollution.

Light availability in the tree habitat is one environmental factor significantly affecting tree pollen

allergen content. The Bet v 1 and 23-kDa allergens showed greater responses in trees growing in shaded areas than in open or half-open areas. In contrast, Hjelmroos et al. (27) have found that allergens are more abundant on pollen collected

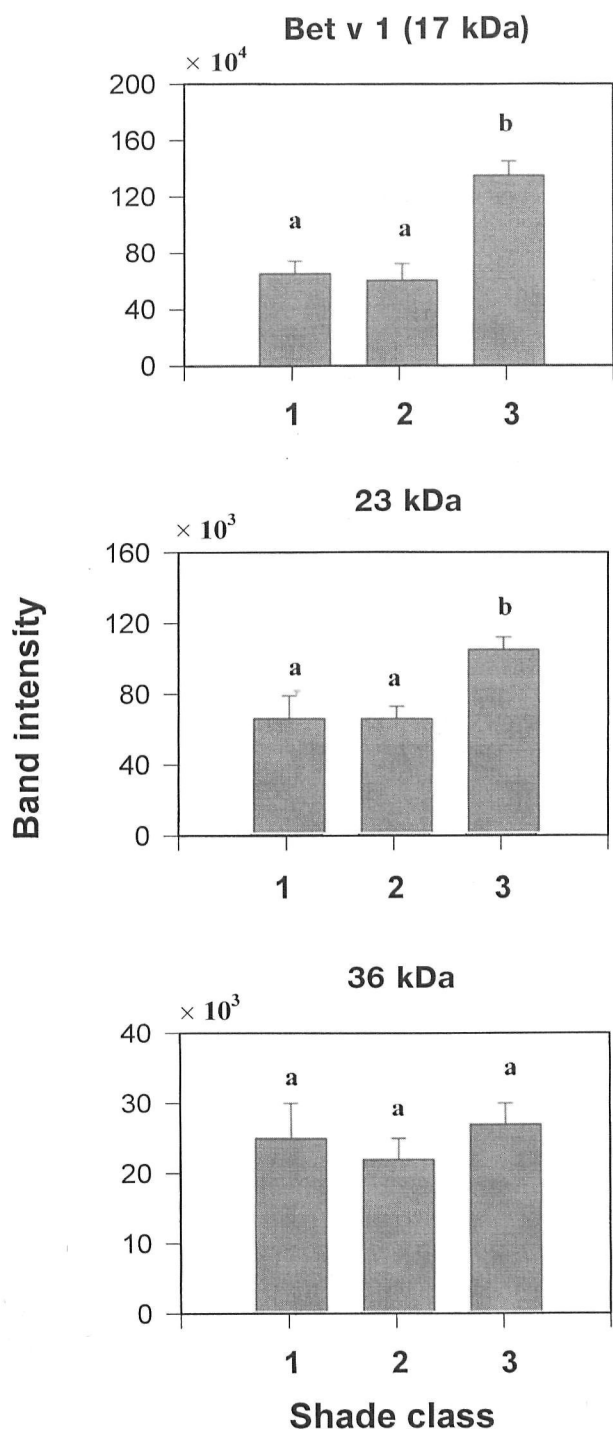


Fig. 5. IgE responses of birch pollen to Bet v 1 and 23- and 36-kDa proteins from birch trees growing in open (1), half-open (2), or shaded (3) sites. Bars with different letters were significantly different (Tukey's test, $P < 0.05$). Note different y-axis scales on each picture.

from the southern side of the tree, which usually receives more light than other sides. However, they studied only the within-tree variation of allergens, not the effects of tree habitat and light conditions on the average allergen contents of a tree.

Bet v 1 is structurally similar to some resistance proteins that are formed in response to plant pathogens (28, 29). These proteins are also synthesized after some environmental stresses (30–32). Trees that grow in shade are continuously stressed due to lack of light for photosynthesis, and may produce stress proteins in response.

According to our results, sulfur and heavy metal emissions do not have strong effects on birch pollen allergens or soluble proteins. Instead, other environmental factors, such as shading and soil properties, may be more important here. Besides environmental factors, the genetic background of the tree may also play an important role in the allergenicity of the pollen and should be taken into consideration in future studies.

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