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Among-population variation in resistance traits of a nettle and its relationship with deer habitat use frequency

Abstract Because leaf hairs serve as resistance against herbivores, among-population variation in hair production may arise from adaptation to local herbivore communities. It is possible that Japanese nettle (*Urtica thunbergiana*) shows among-population variation in stinging hair abundance that is associated with the frequency of habitat use by sika deer (*Cervus nippon*). We examined 31–32 individuals of each of 19 populations for leaf area, stinging hair number (/leaf) and stinging hair density (per square centimeter) in and away from Nara Park (6.6 km^2), where many deer have been protected for 1,200 years. At each site we also measured deer habitat use frequency, light intensity and soil fertility as environmental factors potentially affecting leaf traits. We analyzed our hierarchical data at the levels of individuals and populations using multilevel structural equation modeling. Leaf area had a positive direct effect on stinging hair number at the individual level but no significant effect at the population level. At the population level, deer habitat use frequency had a negative direct effect on leaf area and positive direct effects on stinging hair number and density, generating a negative indirect correlation between leaf area and stinging hair number. Light intensity had a negative direct effect on leaf area, while soil fertility had no significant effect on any trait. These results suggest that the relationships between leaf area and stinging hair number at the two levels do not align. We discussed what processes were involved in the effects of environmental factors on leaf traits.

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Introduction

Understanding the forces that influence genetic and phenotypic variation within and among wild populations has been a major object of evolutionary ecology (Mazer and Damuth 2001). Leaf hairs provide a good study system to achieve this object, because their morphology and production often vary within and among populations and because they should have evolved to deter herbivores from attacking the plant (Jeffree 1986). Thus many studies have attempted to explain the maintenance of variation in leaf hair morphology and production within and among populations of a variety of plants in the context of adaptive evolution through natural selection (Agrawal et al. 2002; Hare et al. 2003; Fornoni et al. 2004; Kärkkäinen et al. 2004; Løe et al. 2007; Holeski 2007; Holeski et al. 2010). For example, the perennial herb *Arabidopsis lyrata* has genetically controlled phenotypes for leaf trichome production, i.e., glabrous form and trichome-producing form, and the frequency of the forms varies considerably among populations in northern Europe (Løe et al. 2007). Glabrous plants are damaged more severely by herbivorous insects than trichome-producing plants in polymorphic populations. The intensity of herbivory tends to be higher in polymorphic populations than in monomorphic glabrous populations. Therefore, it is likely that trichomes serve as resistance against herbivorous insects and that herbivore-mediated selection contributes to the maintenance of the polymorphism in trichome production.

However, evolutionary processes of divergence in leaf hair production should be more complex than are supposed because, besides resistance against herbivores, (1) leaf hairs may serve several functions such as temperature and water regulation and UV-reflectance (Ehleringer 1984; Hare and Elle 2001; Wagner et al. 2004), (2) they

may not deter all herbivores (Hare and Elle 2002; Løe et al. 2007), (3) their production entails costs in terms of individual fitness (Mutikainen and Walls 1995; Hare et al. 2003), and (4) plants often change their hair production plastically in response to herbivory and contact stimuli (Jaffe 1973; Karban and Baldwin 1997; Pigliucci 2005; Dalin et al. 2008).

Nettles such as *Urtica dioica* L. and *U. thunbergiana* Siebold et Zucc. bear stinging hairs on stems, petioles and laminae. Stinging hairs cause a sharp pain to humans and animals (Aston 1923), and deter mammalian herbivores from browsing (Pollard and Briggs 1984b; Kato et al. 2008). However, they do not influence the behavior of insect herbivores (Davis 1983; Pollard and Briggs 1984b). Unlike ordinary hairs such as trichomes and pubescence, physiological functions of stinging hairs such as light reflection and reduction of leaf transpiration are yet to be reported.

The abundance of stinging hairs on leaves often varies among nettle populations. For example, the population of *U. dioica* at Wicken Fen, Cambridgeshire, England, where large mammalian herbivores have been historically absent, almost lack stinging hairs on leaves (Pollard and Briggs 1982, 1984a). This population is surrounded by ordinary nettle populations with stinging hairs. In another example, *U. thunbergiana* with an extremely high number of stinging hairs on leaves are found in Nara Park, Nara City, central Japan, where many sika deer (*Cervus nippon* Temminck) have been protected for 1,200 years, while plants with few or no stinging hairs occur in four locations with few or no sika deer, 18–45 km away from the park (Kato et al. 2008). Such among-population variation is known to be genetically based (Pollard and Briggs 1982; Kato et al. 2008). Combining this with the resistant function of stinging hairs, it seems likely that mammal browsing pressure has acted as a selective force for the evolution of hairy nettles (Pollard and Briggs 1982, 1984b; Kato et al. 2008).

As for *U. thunbergiana* in Nara Park, however, among-population variation in the abundance of stinging hairs might arise in a more complex way than is expected for several reasons. First, stinging hair production may be affected by light intensity and soil fertility as *U. dioica* shows (Pollard and Briggs 1982; Pullin and Gilbert 1989). Second, the number of stinging hairs on a leaf may be correlated with leaf area. Third, nettles may show plastic responses such as a damage-induced response (i.e., an increase in stinging hairs on regrowth shoots after plants suffer damage by herbivores) (Pullin and Gilbert 1989; Mutikainen et al. 1994; Mutikainen and Walls 1995) and also a thigmomorphogenic response (i.e., the production of smaller leaves in response to contact stimuli such as touching and trampling) (Jaffe 1973; Pigliucci 2005). Finally, although nettles are distributed patchily in Nara Park (Kato 2001) and sika deer use the habitat unevenly there (Torii and Tatsuzawa 2009), nettles have been examined for leaf traits at only one site that is one of those exposed to deer's grazing most heavily in the park (Kato et al. 2008). In fact, our preliminary study shows that nettles vary in the number of stinging hairs on leaves considerably among populations in the park.

The present study expanded the previous study (Kato et al. 2008) and examined 19 nettle populations in and away from Nara Park to analyze the contribution of environmental factors such as deer habitat use frequency, light intensity and soil fertility to among-population variation in stinging hair abundance. More specifically, applying multilevel structural equation modeling to our data with a hierarchical structure, we examined (1) whether leaf area directly affects stinging hair number at the individual level and the population level, and (2) the degree to which each environmental factor affects leaf area, stinging hair number and stinging hair density at the population level.

Materials and methods

Study sites and species

Nara Park is adjacent to the town of Nara City, with an area of 6.6 km², containing grassy areas and woody areas (Fig. 1). In the park, more than several hundred sika deer (*Cervus nippon*) have been protected for 1,200 years for religious reasons (Torii and Tatsuzawa 2009). As of 2008, there were 1,128 sika deer (Foundation for the Protection of Deer in Nara Park 2008).

The Japanese stinging nettle *Urtica thunbergiana* is a perennial plant that grows at the edge of woods (Kitamura and Murata 1961; Kato 2001). Plants continue to elongate their shoots from April through November in Nara. The main shoots wither in December, while short lateral shoots from the basal part of the plant overwinter and resume elongating in the following April. The longevity of plants is unknown.

We examined nettles at 13 sites in or nearby Nara Park (sites 1–13), 0.1–2.5 km apart from each other, and six sites 18–45 km away from the park (sites 14–19) (Fig. 1; Table 1). Sites 7, 14, 16, 17 and 19 were the same as Kato et al. (2008) examined. Sites 15–19 are inhabited by few or no sika deer. In this paper we refer to a group of nettles at each site as a population irrespective of the location, although subpopulations may be more preferable for groups at sites 1–13 in an ecological context. This treatment is practically expedient.

Measurement of leaf traits

We chose 31 or 32 well-grown plants randomly from each population in late May 2008. For each plant, two intact fully grown leaves were sampled on the second or third node from the tip of one or two major shoots. For each leaf, the upper surface was scanned using an image scanner (EPSON GT-X770, Seiko Epson, Suwa, Japan) with a resolution of 300 dpi. On the basis of the digital
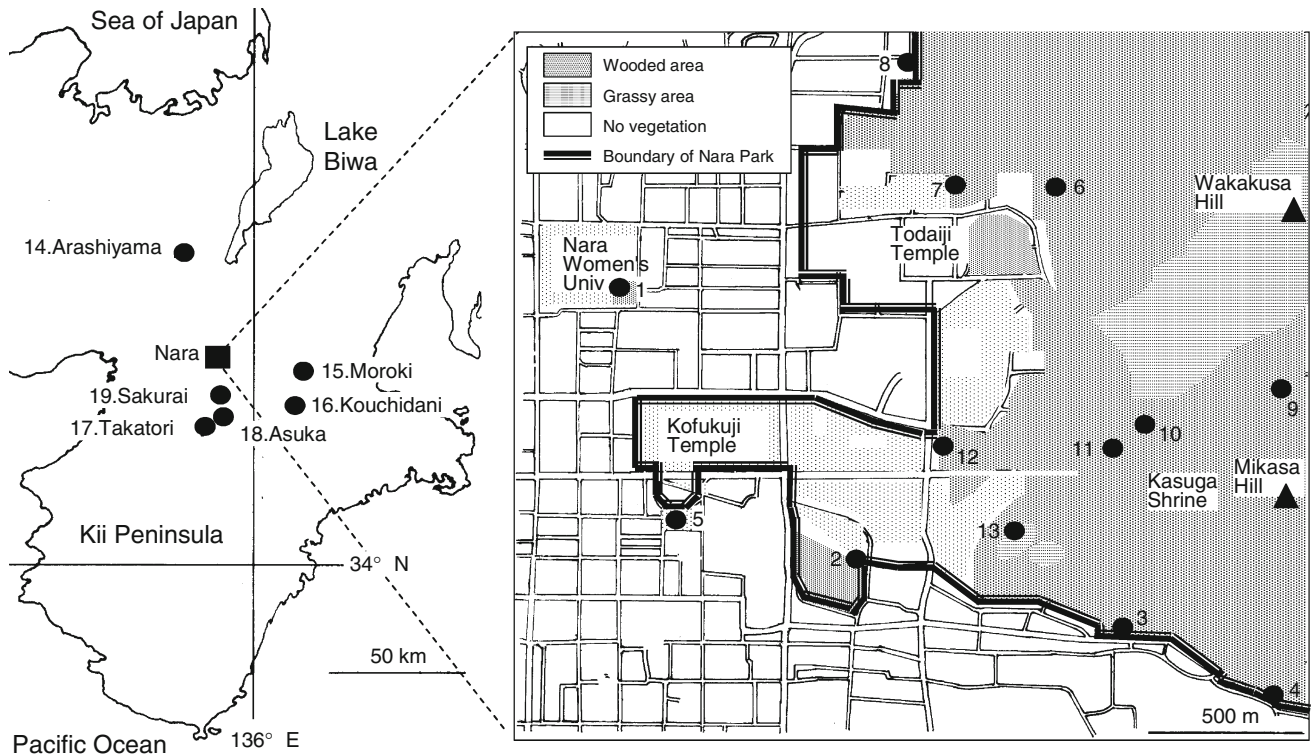
Fig. 1 Location of 19 sampling sites for Japanese stinging nettle, *Urtica thunbergiana*. Sites *1–13* are located in or nearby Nara Park. For topographical description, see Table 1

Table 1 Description of 19 sampling sites for Japanese stinging nettle, *Urtica thunbergiana*. Sites are numbered as in Fig. 1

1. The campus of Nara Women's University, close to Nara Park, sparsely planted with evergreen and deciduous trees, sometimes entered by sika deer. 80 m a.s.l.
2. On the edge of Nara Park, by Sagi Pond, enclosed by a fence 1 m in height, at the edge of a glove of broad-leaved trees. 100 m a.s.l.
3. On the edge of Nara Park, on a roadside along the Noto Stream, at the edge of a broad-leaved forest. 130 m a.s.l.
4. On the edge of Nara Park, on a shallow slope along the Noto Stream, at the edge of a broad-leaved forest. 150 m a.s.l.
5. On the edge of Nara Park, by Sarusawa Pond, surrounded by a building and an artificial stream. 70 m a.s.l.
6. Within Nara Park, on a gentle slope along a brook, at the edge of a broad-leaved forest. 150 m a.s.l.
7. Within Nara Park, in a glove of Japanese cedars (*Cryptomeria japonica*) and cypresses (*Chamaecyparis obtusa*), behind the Todaiji Temple Hall, heavily grazed by sika deer. 110 m a.s.l.
8. On the edge of Nara Park, on a vacant lot enclosed by a fence 1 m in height, at the edge of a broad-leaved forest. 100 m a.s.l.
9. Within Nara Park, on a shallow slope along the Yoshiki Stream, at the edge of a primary forest. 180 m a.s.l.
10. Within Nara Park, on a roadside along the Yoshiki Stream, at the edge of a Japanese cedar plantation. 140 m a.s.l.
11. Within Nara Park, in a glove of Japanese cedars, in the vicinity of the parking lot for Kasuga Great Shrine, heavily grazed by sika deer. 135 m a.s.l.
12. Within Nara Park, in a glove of Japanese cedars and evergreen oaks, heavily grazed by sika deer. 110 m a.s.l.
13. Within Nara Park, in a glove of evergreen oaks, heavily grazed by sika deer. 120 m a.s.l.
14. Arashiyama, 45 km north from Nara Park, on steep slopes, at the edge of a broad-leaved forest along the Katsura River. Sika deer have rapidly increased for some years, so that the forest floor has been bared except steep slopes. 60 m a.s.l.
15. Moroki, 35 km east from Nara Park, on the right bank of the Moroki Stream, at the edge of a Japanese cedar plantation, with no sign of sika deer. 420 m a.s.l.
16. Kouchidani, 30 km south-east from Nara Park, on a roadside, at the edge of a Japanese cedar plantation, along the Shorenji River. Foot prints of sika deer are observed. 280 m a.s.l.
17. Takatori, 28 km south from Nara Park, in a Japanese cedar plantation, with no sign of sika deer. 550 m a.s.l.
18. Asuka, 25 km south from Nara Park, on a roadside, at the edge of a Japanese cedar plantation, with no sign of sika deer. 320 m a.s.l.
19. Sakurai, 18 km south from Nara Park, on a roadside, at the edge of a Japanese cedar plantation, along the Makimuku River, with no sign of sika deer. 140 m a.s.l.

image, the number of stinging hairs on the whole leaf surface was counted and the leaf area was measured using free software LIA for Windows95 ver. 0.373 (Yamamoto 1997). The stinging hair density ($/cm^2$) of a leaf was calculated by dividing the stinging hair number of the leaf by the leaf area.

Measurement of environmental factors

At each study site, we measured light intensity, soil fertility and deer habitat use frequency as environmental factors that potentially affected leaf area, stinging hair number and stinging hair density. Light intensity was estimated as relative photosynthetically active photon-flux density (PPFD). PPFD was measured above ten randomly selected nettles at a height of 1 m from the ground and at an open area adjacent to the site on a cloudless day using a LI-1400 data logger equipped with a LI-190SA quantum sensor (LI-COR, Lincoln, NE). Relative PPFD was calculated as the ratio of PPFD above each nettle to PPFD at the adjacent open area. The mean value was used as the representative of relative PPFD at the site. Soil fertility was estimated as soil nitrate concentration (mg/L), because nettles are nitrophilous (Olsen 1921; Molisch and Dobat 1979; Kato 2001). Soil nitrate concentrations were measured in the soil about 2 cm in depth beside ten randomly selected nettles using a PRN-41 portable soil nitrate ion measuring device (Fujiwara Scientific, Tokyo, Japan), and the mean value was calculated. Since deer move throughout in Nara Park, there seemed to be little sense in estimating deer density at each site. Instead, deer habitat use frequency was estimated as the number of deer dung pellets per unit area (Loft and Kie 1988; Sato et al. 2005). Dung pellets were counted within 20 quadrats of 50 cm × 50 cm located randomly at each site, and the sum of pellets was calculated. We assumed that deer habitat use frequency reflected the intensity of browsing and the frequency of touching and trampling.

It was not possible to include other mammalian herbivores, serrows (*Capricornis crispus*) and hares (*Lepus brachyurus*). Serrows are mountainous ungulates, and do not inhabit around study sites. Hares are certainly distributed in Nara Park and its vicinity (Maeda 1994). We found, however, no signs of their presence at any site. In addition, there were no reports that they considerably damage crops or planted trees around any site (Y. Yoneda, personal communication). Hence, we focused only on sika deer as the potent mammalian herbivore of *U. thunbergiana*.

Data analysis

Before performing statistical analysis, we transformed all variables except leaf area for normality as follows: arcsin-square root for relative PPFD; $\ln (x + 1)$ for dung pellet number, stinging hair number and stinging hair density; and cube root for soil nitrate concentration (Quinn and Keough 2002). As for each leaf trait, the arithmetic mean of the transformed measurements of the two leaves sampled from a plant was used as the measurement for that plant in all subsequent analyses.

Our data on leaf traits were obtained by two-level sampling: primary sampling units were nettle populations and secondary sampling units were nettle individuals. Accordingly, our data had a hierarchical structure in which individuals were nested within populations. This required us to analyze our data at the level of individuals and at the level of populations. Probably, the best means for the analysis was multilevel structural equation modeling (SEM) (Shipley 2000). However, multilevel SEM was not implemented in available standard SEM software such as EQS (Bentler and Wu 2005) and Amos (Arbuckle 2006). This made us adopt Muthén's (1994) approach, which is a method of tricking such SEM software into fitting a multilevel SEM by treating it like a simultaneous multigroup SEM with particular cross-group constraints (Shipley 2000). This method consists of two procedures. First, the covariance of sample data is decomposed into a pooled within-group covariance matrix obtained from group-centered variables and a between-group covariance matrix obtained from the group means of each variable centered around the overall mean of each variable. This procedure is similar to the decomposition of the total variance into a within-group variance and a between-group variance in one-way ANOVA (Shipley 2000). Second, simultaneous multigroup SEM is conducted as if the within-group covariance matrix and the between-group covariance matrix were obtained from different samples.

Following Muthén (1994), we constructed structural equation models that included a within-group part (or individual-level part) and a between-group part (or population-level part) (Fig. 2a, d). We separated stinging hair density (Fig. 2d) from leaf area and stinging hair number (Fig. 2a), because stinging hair density was calculated by dividing the number of stinging hairs on a leaf by the leaf area, naturally resulting in correlation of hair density with hair number and leaf area. In the models, it is assumed that all nettles within a given population experience the same environmental conditions for light intensity, soil fertility and deer habitat use frequency, which are expressed by the mean relative PPFD, the mean soil nitrate concentration and the sum of dung pellets, respectively. Each observed variable is a linear function of a within latent variable at the individual level and a between latent variable at the population level. The models hypothesized that (1) each leaf trait was affected by the properties of individuals but not by environmental factors at the individual level; (2) each leaf trait was affected directly by each environmental factor at the population level; (3) leaf area affected stinging hair number directly at both levels; and (4) deer habitat use frequency affected soil fertility, because ungulates might increase soil nitrate concentration through nitrification of amino acids and ammonium in their dung and urine (Ritchie et al. 1998).

We computed the within-group covariance matrix and the between-group covariance matrix of our data using free software HAD ver. 8.4 (Shimizu et al. 2006; Shimizu 2011). To conduct simultaneous multigroup SEM implemented in Amos 7.0J (Arbuckle 2006), we specified within-group models for individual-level analysis (Fig. 2b, e) and between-group models for

population-level analysis (Fig. 2c, f). The within- and between-group models have the same structure but differ in the numerical strengths of the parameters. In the within-group models, all path coefficients from within latent variables to observed variables were fixed at one, while all variances of and all path coefficients from between latent variables were fixed at zero. In the between-group models, all path coefficients from between latent variables to observed variables were fixed at the scaling constant C (Muthén 1994):

$$C = \left(N^2 - \sum_{i=1}^{G} N_i^2\right) / N(G-1)$$

where G, N_i and N denote the number of populations (groups in statistical sense), the number of individuals for population i, and the total number of individuals, respectively. All free parameters in the between-group model, except path coefficients from between latent variables of environmental factors to between latent variables of leaf traits, were constrained to be equal to the equivalent parameters in the within-group model. Amos 7.0J provided standardized solutions (i.e., path coefficients) by maximum-likelihood method and their P values on the assumption that the data followed a multivariate normal distribution.

Multivariate normality of the observed data for the within- and between-group models was tested using Mardia's multivariate kurtosis statistic (Mardia 1974; Shipley 2000),

$$(g_{2v} - v(v+2))/\sqrt{8v(v+2)/N}$$

where g_{2v}, v and N denote Mardia's coefficient, the number of observed variables and the total number of individuals in the within-group model (the number of populations in the between-group model), respectively. This statistic asymptotically follows a standard normal
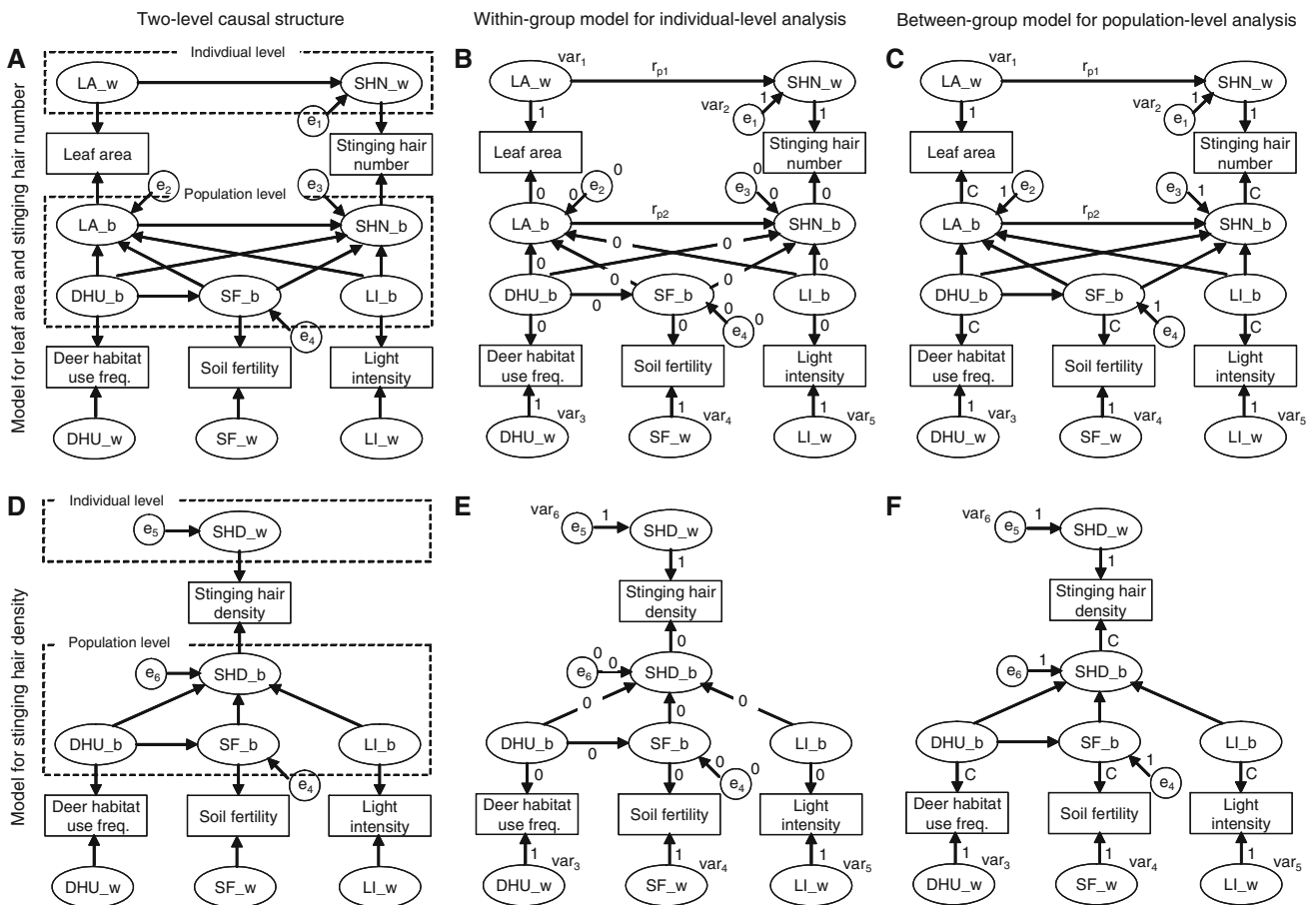

Fig. 2 Proposed structural equation models for our data with a hierarchical structure. **a** Leaf area and stinging hair number, **d** stinging hair density. Each model is modified into a within-group model for individual-level analysis (**b**, **e**) and a between-group model for population level analysis (**c**, **f**) by simultaneous multigroup SEM according to Muthén's (1994) method. Variables in *squares* are observed variables, and those in *ellipses* are latent variables. Latent variables include within-latent variables (indicated by _w) and between-latent variables (_b). *C* scaling constant of Muthén (1994), see text for estimation; *DHU* deer habitat use frequency (expressed by dung pellet number); e_1–e_6 error variables, variances of which are fixed to one; *LA* leaf area; *LI* light intensity [expressed by relative photosynthetically active photon-flux density (PPFD)]; r_{p1}, r_{p2} path coefficients which are constrained to be equal in the within model and the between model; *SF* soil fertility (expressed by soil nitrate concentration); *SHD* stinging hair density; *SHN* stinging hair number; var_{1-6} variances which are constrained to be equal in the within model and the between model

distribution. When its absolute value was more than 1.96, the data was considered not to follow a multivariate normal distribution at $P < 0.05$ level of significance. The fit of the simultaneous multigroup structure model to the observed data was evaluated by the maximum likelihood Chi-square statistic, χ^2. When its P value was larger than 0.05, the model was judged to be consistent with the data (Shipley 2000). In addition, adjusted goodness of fit index (AGFI), the comparative fit index (CFI) and the root mean square error of approximation (RMSEA) were calculated. When AGFI and CFI closely approximated to 1 and RMSEA was < 0.05, the model was judged to be acceptably fit (Toyoda 2007). These statistics were calculated by Amos 7.0J.

One-way ANOVA and Kendall's rank correlation were performed with SPSS 15.0J (SPSS 2006).

Results

Environmental factors

Relative PPFD varied considerably among sites, ranging from 0.016 to 0.222 (Fig. 3 left column). Soil nitrate concentration was as variable as relative PPFD, ranging from 17.3 to 391 mg/L (Fig. 3 middle column). Dung pellet number varied widely among sites from 0 to 391 (Fig. 3 right column). Differences in dung pellet number among sites in and nearby Nara Park were caused partly by vegetation and barriers to deer's free movement. Sites 7, 11, 12 and 13 in woody areas were adjacent to deer's main grazing areas, or grassy areas, showing higher pellet numbers than others. Sites 1, 2 and 8 were enclosed by a fence, and sites 4, 9 and 10 contained shallow slopes, so that they showed relatively low pellet numbers.

Leaf traits

Leaf area (cm^2), stinging hair number (/leaf) and stinging hair density (/cm^2) varied significantly among populations (in order, $F_s = 11.96$, $P < 0.001$; $F_s = 123.61$, $P < 0.001$; $F_s = 231.18$, $P < 0.001$; one-way ANOVA, $v_1 = 18$, $v_2 = 586$; Fig. 3). Stinging hair number and density were highly divergent across populations compared with leaf area. The population mean of stinging hair number ranged from 0.016 to 62.4, while that of stinging hair density ranged from nearly 0 to 2.34.
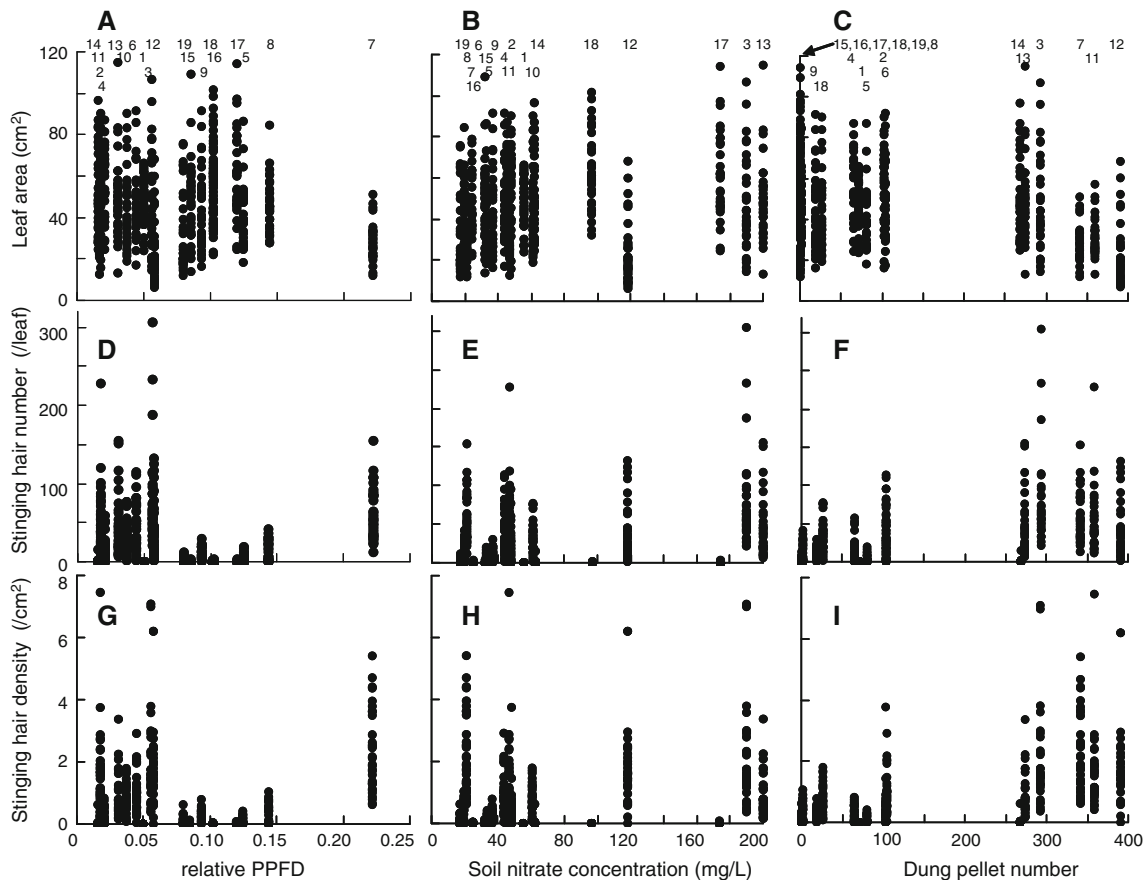

Fig. 3 Scatter plot matrix of data on relationships between environmental factors and leaf traits. *Numbers* in the upper part of **a**, **b** and **c** indicate sites or population numbers in Fig. 1 and Table 1

The goodness-of-fit of models to observed data

Observed data of stinging hair density for the within-group model followed a normal distribution, while those of leaf area and stinging hair number exhibited significant departure from normality (Table 2). Observed data of leaf area and stinging hair number and those of stinging hair density for between-group models followed a multivariate normal distribution. Each simultaneous multigroup structure model provided a non-significant χ^2_s, thus having a good fit to the observed data. AGFI, CFI and RMSEA also indicated that the models had acceptable fit.

Direct effect of leaf area on stinging hair number at the two levels

Leaf area had a significantly positive direct effect on stinging hair number at the individual level (Fig. 4a).

Table 2 Mardia's measurement of multivariate kurtosis for the test of multivariate normality of the observed data and measurements of fit of the model to the data

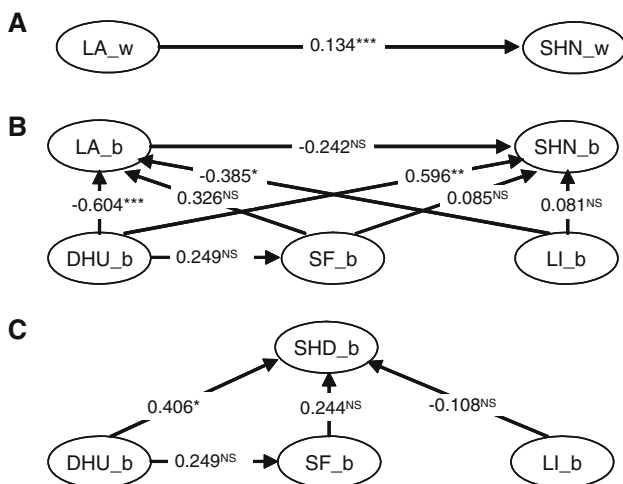|	Leaf area and stinging hair number	Stinging hair density
Mardia's kurtosis statistic		
Within-group model	5.992***	0.364 NS
Between-group model	0.365 NS	0.621 NS
χ^2_s	4.251 NS ($df = 11$)	4.251 NS ($df = 8$)
AGFI	0.993	0.992
CFI	1.000	1.000
RMSEA	0.000	0.010

*** $P \leq 0.001$, NS not significant

Fig. 4 Standardized solutions (i.e., path coefficient) between latent variables at **a** the individual level for leaf area and stinging hair number, **b** the population level for leaf area and stinging hair number, and **c** the population level for stinging hair density. For abbreviations, see legend of Fig. 2. *$0.01 < P \leq 0.05$; **$0.001 < P \leq 0.01$; ***$P \leq 0.001$; NS not significant

However, the statistical significance was not reliable, because the data did not show a multivariate normality as mentioned above. Then a non-parametric correlation coefficient, Kendall's τ, was calculated from group-centered variables, exhibiting a significant P value ($\tau = 0.114$, $n = 605$, $P < 0.001$). Therefore, the direct effect of leaf area on stinging hair number was considered significant at the individual level. In contrast, leaf area had no significant direct effect on stinging hair number at the population level (Fig. 4b).

Effects of environmental factors on leaf traits at the population level

Deer habitat use frequency, as expressed by dung pellet number, had a significantly negative direct effect on leaf area and a significantly positive direct effects on stinging hair number and density (Fig. 4b,c), generating a negative indirect correlation between the two traits. Light intensity, as expressed by relative PPFD, had a significantly negative direct effect on leaf area. Soil fertility, as expressed by soil nitrate concentration, had no significant effect on any leaf trait. Unlike the prediction of Ritchie et al. (1998), soil fertility was not significantly affected by deer habitat use frequency.

Discussion

Our findings based on multilevel SEM are summarized as follows: (1) leaf area had a positive direct effect on stinging hair number at the individual level but no significant direct effect at the population level, (2) deer habitat use frequency had a negative direct effect on leaf area and positive direct effects on stinging hair number and density at the population level, (3) light intensity had a negative direct effect on leaf area, and (4) soil fertility had no significant effect on any leaf trait.

The negative direct effects of light intensity and deer habitat use frequency on leaf area at the population level suggest that these factors contribute to among-population variation in leaf area. This variation would be mainly caused not by genetic variation according to light intensity but by phenotypic plasticity to light intensity, because leaf area generally has a high degree of plasticity in response to environmental conditions (Larcher 2003) and because genetic control of leaf area is not detected in *U. thunbergiana* (Kato et al. 2008). In many plants, leaves grown in the shade are larger and thinner than those in the sun, increasing greater photosynthetic capacity under low light intensity (Mooney and Ehleringer 1997, Larcher 2003). In fact, *U. dioica* in the shade has a higher specific leaf area (i.e., larger leaves for the weight) than that exposed to the sun (Pollard and Briggs 1982). Thus, such plasticity of leaves to light intensity would have much to do with the negative effect of light intensity on leaf area in *U. thunbergiana*.

Furthermore, it is known that leaves reduce their area on regrowth shoots after suffering damage by herbivores and on shoots which frequently receive contact stimuli. Such changes in leaf area are referred to as a damage-induced response (Karban and Baldwin 1997) and a thigmomorphogenic response (Jaffe 1973; Pigliucci 2005). Assuming that deer habitat use frequency reflects the intensity of browsing and the frequency of touching and trampling, those responses would be involved in the negative direct effect of deer habitat use frequency on leaf area in *U. thunbergiana*.

There are two possible hypotheses for the positive direct effects of deer habitat use frequency on stinging hair number and density. One is that nettles increase stinging hair production in response to deer's browsing. Pullin and Gilbert (1989) have demonstrated that the number of stinging hairs on *U. dioica* doubled after simulated herbivory. This value is far lower than the ratio of the maximum population mean of stinging hair number (62.4) or density (2.34) to the minimum (0.016 or nearly 0) in *U. thunbergiana*. Thus, it is unlikely that damage-induced response contributes greatly to among-population variation in stinging hair number and density in *U. thunbergiana*. The other hypothesis is that the constitutive production of stinging hairs is determined by a balance between costs and benefits. This hypothesis is more likely, because (1) when nettles in pots were placed in site 7, plants with few stinging hairs on leaves often had almost all the aboveground parts consumed by sika deer but those with many stinging hairs did not (unpublished data), (2) the constitutive stinging hair number and density are genetically based (Kato et al. 2008), and (3) the constitutive production of stinging hairs entails costs in terms of individual fitness (Mutikainen and Walls 1995). Natural selection would favor nettles with the optimal constitutive production of stinging hairs that is set by the balance between the risk of damage caused by deer and costs associated with the constitutive production.

Neither soil fertility nor light intensity had a significant effect on stinging hair number or density in *U. thunbergiana*. This is inconsistent with Pollard and Briggs (1982) and Pullin and Gilbert (1989) in *U. dioica*. They have shown that *U. dioica* tends to have lower stinging hair density under low nutrient conditions or in the shade than under high nutrient conditions or in the sun. This inconsistency might be attributable to differences between wild populations under natural conditions and experimental populations in common gardens.

Finally, we note the result that leaf area had a positive direct effect on stinging hair number at the individual level but had no significant direct effect at the population level. Our hierarchical models hypothesize that leaf traits are affected by the properties of individuals but not by environmental factors at the individual level. Hence, the positive correlation between leaf area and stinging hair number at the individual level might reflect the effect of genetic correlation such as pleiotropy or linkage disequilibrium (Armbruster et al. 2004). At the population level, however, there is no direct correlation between leaf area and stinging hair number. On the contrary, there is a negative indirect correlation between the two traits that is generated by deer habitat use frequency, which directly affects leaf area negatively and stinging hair number positively. Consequently, the relationships between leaf area and stinging hair number at the individual level and at the population level do not align.

Multilevel SEM is probably the best means for analyzing our hierarchical data at the levels of individuals and populations. Shipley (2000) suspected that since hierarchies were so ubiquitous, multilevel SEM would become very important in biology. However, as far as we know, no ecological studies had used multilevel SEM before our study. For example, although various statistical methods have been developed to analyze covariation between phenotypic characters at the levels of individuals, populations and species and to explain how populations and species diverge with respect to phenotypic characters, they do not include multilevel SEM (Pigliucci and Preston 2004). There appear to be two reasons why the use of multilevel SEM has not been spread even though Muthén (1994) developed a procedure through which multilevel SEM was performed in available standard SEM software (Shimizu et al. 2006). First, commercial software does not implement a program for obtaining within-group covariance matrices and between-group covariance matrices. Second, it is rather complicated to construct structural equation models with equality constraints across groups for simultaneous multigroup SEM as shown in Fig. 2. We expect our study to help popularize multilevel SEM in ecology.

In summary, at the population level, deer habitat use frequency had a negative direct effect on leaf area and positive direct effects on stinging hair number and density, while light intensity had a negative direct effect on leaf area. The effects of the two factors on leaf area are likely to involve plastic responses of leaves to light intensity and deer's browsing, touching and trampling. The effect of deer habitat use frequency on stinging hair number and density may be determined by the balance between the risk of damage caused by deer and costs associated with the constitutive production of stinging hairs. Furthermore, there was a direct correlation between leaf area and stinging hair number at the individual level, but not at the population level. At the population level, however, deer habitat use frequency generated an indirect correlation between the two traits by directly affecting leaf area negatively and stinging hair number positively. Consequently, the relationships between leaf area and stinging hair number at the individual level and the population level do not align. To corroborate our explanations for among population variation in leaf traits and their relationships with deer habitat use frequency, we are presently examining inductive and constitutive production of stinging hairs and its genetic basis.

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