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The evolution of nettle resistance to heavy deer browsing

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Abstract We examined whether heavy browsing by sika deer, *Cervus nippon* Temminck, changed morphological characteristics of a Japanese nettle, *Urtica thunbergiana* Sieb. et Zucc., in Nara Park, where a large population of sika deer has been maintained for more than 1,200 years. Wild nettles of Nara Park exhibited smaller leaf area, 11–223 times more stinging hairs per leaf, and 58–630-times higher stinging hair densities than those of other areas where there was no evidence of sika deer browsing. There were no significant differences in stinging hair length between the areas. Nettles from Nara Park that were cultivated from seeds in a greenhouse retained a larger number and higher density of stinging hairs. In the field, nettles of Nara Park were less frequently browsed by sika deer and showed higher survivorship than nettles that were transplanted from an unbrowsed area into Nara Park. These results indicate that: (1) the *U. thunbergiana* population of Nara Park has an extremely high stinging hair density compared with those of unbrowsed areas; (2) this characteristic has a genetic basis, and (3) stinging hairs serve as a defensive structure against sika deer, contributing to an increase in survivorship. Thus, we conclude that a *U. thunbergiana* population in Nara Park, with extremely high stinging hair densities, has evolved through natural selection due to heavy browsing by sika deer.

Keywords Genetic variation · Plant defense · Plant–herbivore interactions · Regional variation · *Urtica thunbergiana*

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Introduction

Plants exposed to heavy herbivory by mammals often have more or sturdier spinescence, which includes thorns, spines, prickles and stinging hairs, than plants not exposed to heavy herbivory. For instance, brambles (*Rubus hispidus*), angelica trees (*Aralia spinosa*) and spiny shrubs (*Dammacanthus indicus*) bear longer, sharper and larger thorns in areas with high activities of mammalian herbivores than in areas with low activities (Abrahamson 1975; White 1988; Takada et al. 2001). Plants with spinescence are less damaged by mammalian herbivores than plants whose spinescence is experimentally removed, and, therefore, spinescence has been considered as a defensive structure against herbivores (Cooper and Owen-Smith 1986; Obenso 1997; Takada et al. 2003).

If spinescence characteristics are influenced by heavy herbivory, changes may result from ecological or evolutionary responses by the plants to the herbivory. The former is exemplified by a damage-induced response (Karban and Baldwin 1997); that is, plants regrow with more or sturdier spinescence on shoots newly produced following damage by herbivores. The latter involves selection for plants with more or sturdier spinescence; as a result, such characteristics are genetically fixed within plant populations. Many studies have demonstrated that real or simulated herbivory induces spinescence in various plants, including *Glochidion* (Okuda 1987), acacia (Young 1987), nettle (Pullin and Gilbert 1989; Mutikainen and Walls 1995), cactus (Myers and Bazely 1991), alder (Bauer et al. 1991), bramble (Bazely et al. 1991) and mustard (Agrawal 1999; Traw and Dawson 2002). However, few studies have established the evolutionary basis of spinescence as a response to herbivory. Danell and Bergström (2002) give two reasons for this: it is difficult to separate genotypic and phenotypic variation, and some environmental processes can cause physical damage and the removal of biomass in ways similar to herbivory. As far as we know, only Pollard

and Briggs (1982, 1984) and Mutikainen and Walls (1995) examined the adaptive significance and genetic basis of spinescence using the stinging nettle *Urtica dioica*.

The stinging nettle (*U. dioica*) bears many stinging hairs on stems and leaves. These hairs contain a toxic liquid containing histamine, acetylcholine and serotonin (Emmelin and Feldberg 1949; Collier and Chesher 1956). The stinging nettle is known to have higher stinging hair densities in grazed areas than in ungrazed areas (Pullin and Gilbert 1989). Rabbits and sheep prefer nettles with few or no hairs to nettles with many stinging hairs (Pollard and Briggs 1984). These facts indicate that stinging hairs provide a defense against mammalian herbivores. Intraspecific variation of stinging hair density has a genetic basis, with a heritability of 0.3–0.4 (Pollard and Briggs 1982). Stinging hair density is also influenced to some degree by shading (Pollard and Briggs 1982). Furthermore, *U. dioica* increases its stinging hair density on leaves newly produced following simulated herbivore damage, which suggests that a damage-induced increase in stinging hairs occurs in the field. It is uncertain whether production of stinging hairs is costly for the plant in terms of fitness; Mutikainen and Walls (1995) found that there is a trade-off between reproduction and stinging hair density, whereas Puustinen et al. (2004) found no such trade-off. However, because *U. dioica* is a highly nitrophilous and phosphophilous species (Olsen 1921; Rorison 1968), stinging hairs may compromise plant fitness under nutrient-poor conditions (Pullin and Gilbert 1989). Therefore, in *U. dioica*, both ecological and evolutionary responses to herbivory are influenced by the availability of soil nutrients and combine to determine the stinging hair density.

Two nettles, *U. thunbergiana* and *U. platyphylla*, are found in Japan. They often occur in areas with high activities of sika deer (*Cervus nippon*) (Takatsuki 1980; Kaji et al. 1991). Of these nettles, *U. thunbergiana* occurs in Nara Park, Nara, central Japan. This park has an area of 660 ha in which there are Buddhist temples, Shinto shrines, open grasslands and evergreen woods, and it serves as a habitat for about 1,200 sika deer, despite being in the vicinity of an urban area. According to the Man'yōshū—the earliest anthology of Japanese verse—sika deer had already inhabited the park by about AD 750. Because sika deer were regarded as sacred animals, they have been protected for more than 1,200 years in the park (Ohigashi et al. 2003). If stinging hairs reduce damage from sika deer, and if stinging hair variation has a genetic basis, the nettles in Nara Park might have higher stinging hair densities as an adaptive (i.e., evolutionary) response to heavy browsing.

In this study, we investigated whether length and density of stinging hairs differ among *U. thunbergiana* populations in the absence and presence of sika deer browsing, whether these characteristics have a genetic basis, and whether stinging hairs serve as a defensive structure against sika deer. We first examined leaf area

and the number, density and length of stinging hairs of nettles in Nara Park and in other areas where there was no evidence of sika deer browsing. Second, we cultivated nettles from these areas from seeds in a greenhouse to compare leaf and stinging hair characteristics. Third, we transplanted nettles from an unbrowsed area into Nara Park and compared their vulnerability to deer browsing with that of nettles from Nara Park.

Materials and methods

Study organism

Urtica thunbergiana is distributed in central and southern Japan. It is a perennial plant that grows in nutrient-rich habitats mainly at the edges of woods (Kitamura and Murata 1961; Kato 2001). Like *U. dioica*, it bears stinging hairs on its stems and the upper and lower surfaces of its leaves. These stinging hairs contain a toxic liquid with chemical compounds that have not been identified but that probably include histamine, acetylcholine and serotonin, as found in *U. dioica* (Emmelin and Feldberg 1949; Collier and Chesher 1956).

In Japan, the mammals that can browse nettles are hares, Japanese serows and sika deer. However, we did not observe any nettles browsed by hares in this study. Because Japanese serows inhabit mountainous regions, their browsing would not affect our study area. Sika deer are widely distributed from the lowlands to highlands and from northern to southern Japan. Thus, only sika deer browsing was taken into consideration in this study.

Study sites

Nara Park (34°41' N, 135°51' E) is situated at the center of the Kansai District. It has an area of 660 ha, with open grasslands and evergreen woods (Fig. 1). Sika deer have been protected in the park for more than 1,200 years because they are regarded as sacred animals (Ohigashi et al. 2003). As of 2005, the population of sika deer in the park was about 1,200 (The Nara Foundation for Protection of Deer, unpublished data). The study site in the park [110 m above sea level (a.s.l.)] is sparsely planted with Japanese cedars.

Five other study sites are located at the edges of cedar forests, 20–50 km from Nara Park (Fig. 1): Kouchidani (34°34' N, 136°6' E, 280 m a.s.l.), Takatori (34°25' N, 135°49' E, 550 m), Arashiyama (35°00' N, 135°40' E, 60 m), Sakurai (34°32' N, 135°51' E, 138 m), and Yoshino (34°19' N, 135°58' E, 320 m). Sika deer have increased in number since the 1980s in the Kansai District, including these sites, and they have now spread throughout the district (Biodiversity Center of Japan 2004). However, these sites are situated near residential areas or beside pathways leading to a main road, and, thus, few sika deer may have migrated there at least before the 1980s. Indeed, local woodland officers have

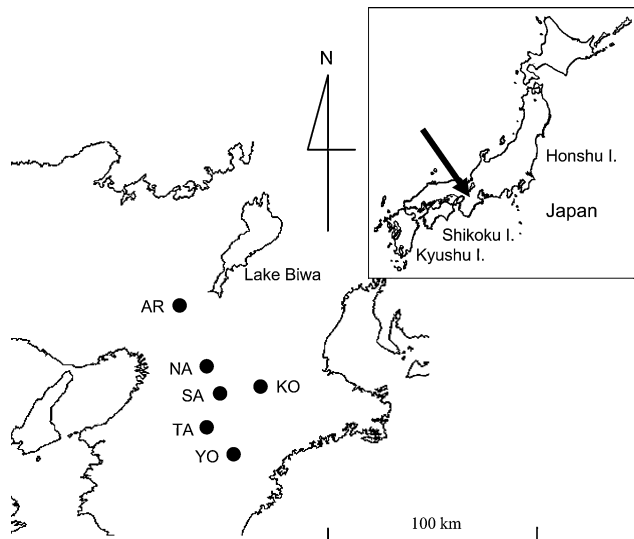


Fig. 1 Locations of study sites in the Kansai District (*AR* Arashiyama, *KO* Kouchidani, *NA* Nara Park, *SA* Sakurai, *TA* Takatori, *YO* Yoshino)

said that they had seen no sika deer around the sites. We also found neither fecal pellets of sika deer nor evidence of browsing by sika deer during the period of this study. Therefore, it can safely be assumed that densities of sika deer at these sites are much lower than that at Nara Park.

Field survey

We examined the stinging hairs of *U. thunbergiana* between 6 and 20 October 2002 in Nara Park, Kouchidani, Takatori and Yoshino. At each site, we randomly selected ten nettles and sampled one leaf from each of the 2nd, 4th, and 6th or 7th nodes. These leaves are referred to as upper, middle and lower, respectively. Leaf area was measured with an automatic area meter (Model AAM-7, Hayashi Denko Co. Ltd., Tokyo, Japan). Stinging hairs on the lower and upper surfaces of the leaves were counted, using photocopies of leaves enlarged two or four times. The density of stinging hairs on a leaf was calculated as the number of stinging hairs divided by leaf area. Five stinging hairs were randomly selected on each leaf surface, and their lengths were measured to 0.01 mm under a binocular microscope. The mean value was used to represent the stinging hair length for the leaf.

Light intensity at the study sites was expressed as the relative photosynthetically active photon-flux density (RPPFD). RPPFD is defined as the ratio of the photosynthetically active photon-flux density (PPFD) at a spot in question to the PPFD at an adjacent unshaded place without vegetation. At each study site in mid-October 2002, the RPPFD was measured ten times using the LI-190SA Quantum sensor connected to the LI-1400 data logger (LI-COR, Lincoln, USA).

Greenhouse experiment

In October 2001 we collected seeds from four nettles that were more than 3 m apart, to avoid the progeny of one maternal plant or clones at four of the study sites: Nara Park, Sakurai, Arashiyama and Kouchidani. The low number of selected plants was due to small population sizes, ranging from 50 to 200 individuals, and the small proportions of plants bearing seeds at the sites, except Nara Park. Seeds from the four plants were kept together at room temperature in a well-ventilated room until the following spring. On 20 April 2002, the seeds were put on moistened cellulose mats in petri dishes. They were kept at 4°C for 10 days in an incubator. Afterwards, they were kept at 25°C for 10 days to promote synchronous germination. Twelve seedlings with two intact cotyledons were selected to represent each location. Seedlings were planted into plastic pots (two seedlings per pot; upper caliber, 16 cm; lower caliber, 10 cm; depth, 12.5 cm) filled with 1 l of perlite, and were cultivated in a greenhouse, with each pot receiving 100 ml of a nutrient solution containing 1.0 mM NaNO_3 every 2 days (for details of the solution, see Koyama et al. 2001). The nitrate ion concentration in the solution was considerably higher than those in the soil at the study sites, which ranged from 0.3 mM to 0.8 mM (Kato et al. unpublished). Pots were randomly moved weekly within the greenhouse, to avoid position effects. On 22 November 2002, leaf area and the number and length of stinging hairs were measured as described previously.

Transplantation experiment

Before the transplantation experiment, we examined stinging hair densities of nettles in Nara Park and Sakurai. In July 2002 we collected ten nettles randomly within a quadrat with an area of 60 m² at both sites and measured stinging hair densities of those nettles in the same way as described previously. As a result, nettles from Nara Park had significantly higher stinging hair densities than nettles from Sakurai (means \pm SDs for Nara Park and Sakurai, $n = 10$: upper leaves, $12.12 \pm 7.51 \text{ cm}^{-2}$ and $0.023 \pm 0.031 \text{ cm}^{-2}$; middle leaves, $6.08 \pm 2.57 \text{ cm}^{-2}$ and $0.030 \pm 0.044 \text{ cm}^{-2}$; lower leaves $6.43 \pm 4.00 \text{ cm}^{-2}$ and $0.139 \pm 0.187 \text{ cm}^{-2}$) [analysis of variance (ANOVA) $F = 128.113$, d.f. = 1, $P < 0.001$].

In Nara Park, five plots (90 cm \times 90 cm) were placed at least 10 m apart, and each of them was divided into two subplots. On 2 April 2004, four young nettles were transplanted from the quadrat at Nara Park to one subplot and from that of Sakurai to the other subplot in each plot, for fear that tall nettles from Sakurai (6.0 ± 1.97 cm) would shade short nettles from Nara Park (3.7 ± 1.37 cm) (the difference was significant; t -test, $P = 0.008$, d.f. = 38). Nettles were arranged at least 25 cm apart. Immediately after being transplanted,

the nettles were left for exposure to sika deer browsing. Browsed nettles, which were identified by teeth marks on their shoots, were counted twice each month from April to August 2004. The surviving nettles were counted the following spring.

Statistical analysis

To analyze the differences in leaf area and the number, density and length of stinging hairs between nettles from Nara Park and from each of the other sites, we performed multiple comparisons using the Dunnett method. In the transplantation experiment, we analyzed differences in vulnerability to deer browsing between nettles from Nara Park and Sakurai by applying the log-rank test (Krebs 1998) to cumulative percentage curves of browsed nettles. All statistical analyses were carried out with SPSS version 9.01J (SPSS 1999). A probability of 0.05 was chosen as the level of statistical significance.

Results

Field survey

The RPPFDs did not differ significantly between study sites ($F = 0.192$, $d.f. = 2$, $P = 0.826$). Values for RPPFDs (means \pm SDs) were as follows: Nara Park, $10.7 \pm 3.3\%$; Kouchidani, $12.2 \pm 7.7\%$; Takatori, $12.5 \pm 1.1\%$. The site at Yoshino was submerged after the construction of a dam, and so the RPPFD could not be obtained.

Nettles in Nara Park had smaller leaf area than nettles at all other sites at any leaf position (Fig. 2a), although statistical significance of the differences depended on leaf position along shoots. Middle leaves of

Nara Park nettles exhibited significantly smaller leaf area than middle leaves collected at any other site. Upper leaves were significantly smaller only when compared with those at Takatori, and lower leaves were significantly smaller than those from both Takatori and Yoshino.

The number of stinging hairs per leaf in Nara Park was significantly higher than that at any other site at each leaf position by a factor of 11–223 (Fig. 2b). Similarly, the density of stinging hairs in Nara Park was 58–630 times as high as those at other sites (Fig. 2c).

In contrast, there were no significant differences in the length of stinging hairs between Nara Park and any other sites at any leaf position except for the middle leaves at Yoshino (Fig. 2d).

Greenhouse experiment

The leaf area of nettles grown from seeds did not differ significantly between Nara Park and any other sites at any leaf position except for the upper leaves at Sakurai (Fig. 3a). This was obviously inconsistent with the results from comparisons of wild plants.

Like wild nettles, however, nettles from Nara Park had significantly more stinging hairs per leaf and higher stinging hair densities on leaves than those from any other site at each leaf position (Fig. 3b, c). Compared with wild nettles, cultivated nettles exhibited high stinging hair densities at any site and a small variation in stinging hair density among study sites.

Stinging hair length did not differ significantly between Nara Park and any other sites at any leaf positions except for the middle and lower leaves at Sakurai (Fig. 3d). Such small variation in stinging hair length among sites was common to both cultivated and wild nettles.

Fig. 2 Leaf area (a), number of stinging hairs per leaf (b), density of stinging hairs (number per square centimeter) (c), and length of stinging hairs (d) of a Japanese nettle, *Urtica thunbergiana*, growing in the wild. An asterisk indicates a significant difference between Nara Park and one of the other sites ($P < 0.05$). Upper, middle and lower leaves represent the 2nd, 4th, and 6th or 7th leaves along shoots, respectively. The error bar is SD. ND, no data. For location abbreviations, see Fig. 1

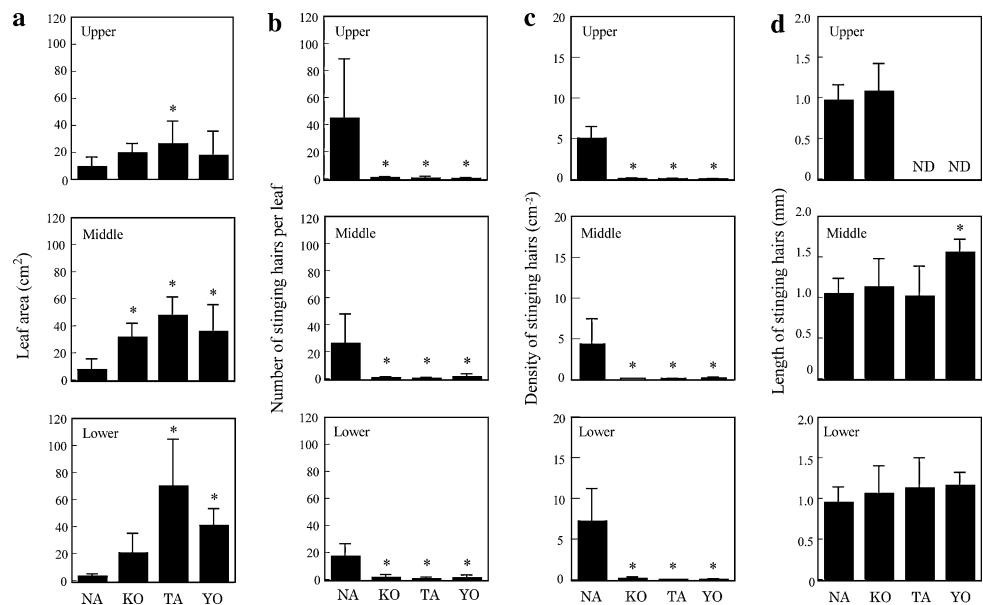


Fig. 3 Leaf area (a), number of stinging hairs per leaf (b), density of stinging hairs (number per square centimeter) (c), and length of stinging hairs (d) of a Japanese nettle, *Urtica thunbergiana*, cultivated from seed in a greenhouse. For abbreviation and symbols, see Figs. 1 and 2

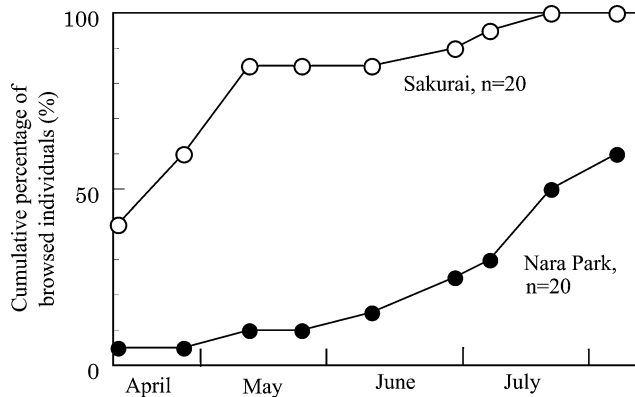
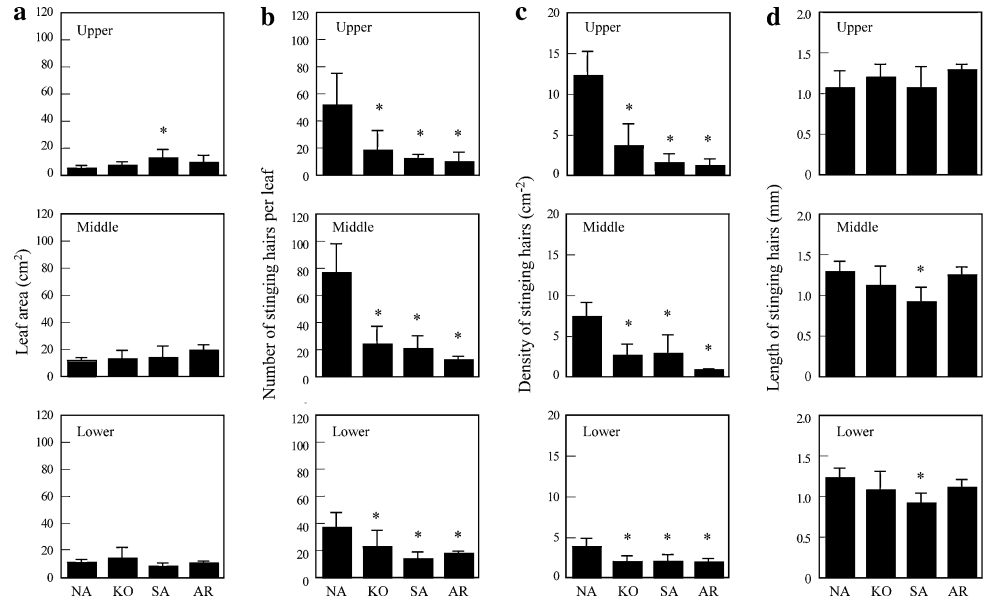


Fig. 4 Curves showing the cumulative percentages of nettles browsed by sika deer at a study site in Nara Park. Nettles were transplanted from within Nara Park and from Sakurai into the study site

Transplantation experiment

Most of the nettles transplanted from Sakurai to the study site in Nara Park had been browsed by sika deer by mid-May, and by the end of the study all of these nettles had suffered damage (Fig. 4). Nettles transplanted from within Nara Park were rarely browsed until late May, with the percentage showing damage gradually increasing to 60% in early August. The two cumulative percentage curves differed significantly (log-rank statistic = 29.07, $P < 0.001$), indicating that nettles from Sakurai had suffered damage earlier and more frequently than those from Nara Park.

Rates of survival till the following spring for transplanted nettles were 60% for those from Nara Park and 25% for those from Sakurai. The difference in survival was only nearly significant (Fisher's exact test, $P = 0.054$).

Discussion

Three major findings resulted from this study of *U. thunbergiana* in Nara Park, where a large population of sika deer has been maintained for more than 1,200 years. First, the wild nettles of Nara Park have extremely high stinging hair densities compared with those in areas with no sika deer browsing (Fig. 2). The stinging nettle *U. dioica* also has a higher stinging hair density in grazed areas than in ungrazed areas (Pullin and Gilbert 1989). However, differences in stinging hair density of *U. thunbergiana* between Nara Park and other areas are much greater (58–620 times) than those reported for *U. dioica* between grazed and ungrazed habitats (1.4–10.3 times).

The second major finding was that the number and density of stinging hairs have a genetic basis. Both wild and cultivated nettles from Nara Park exhibited a significantly higher number and density of stinging hairs than those from other areas (Figs. 2 and 3). Pollard and Briggs (1982) reported that stinging hair density in *U. dioica* also has a genetic basis, estimating the heritability at 0.3–0.4 by parent–offspring correlation analysis. One might have some doubts about the soundness of our result because of small sample sizes, particularly in the greenhouse experiment. Cultivated seedlings from each area were descended from only four plants. Furthermore, there is a possibility that those seedlings were the progeny of one maternal plant, because they were selected from bulked seeds of the four plants. Hence, among-site variation in stinging hair traits may have merely reflected an inter-individual variation. However, the differences in the number and density of stinging hairs between Nara Park and the other sites were very distinct. This is hardly attributable to small sample size, because results from small sample sizes would exhibit a

large variation among the sites. Therefore, it is a safe assumption that the result was largely unaffected by sample sizes.

The third major finding was that nettles from Nara Park were browsed less frequently by sika deer than were nettles from Sakurai with lower stinging hair densities (Fig. 4), so that they had a trend of higher survivorship. This indicates that the stinging hairs of *U. thunbergiana* served as a defensive structure against sika deer browsing and contributed to an increase in survivorship. Possibly, the difference in plant height between nettles from Nara Park and those from Sakurai may have resulted in the difference in damage and survivorship. Because nettles from Nara Park were significantly shorter than those from Sakurai, the former might be less apparent to sika deer than those from Sakurai. However, this possibility can be ignored because nettles from the two sites were transplanted into different subplots within a plot for fear that Nara Park nettles would be shaded by Sakurai nettles.

U. dioica shows increased stinging hair density on leaves newly produced following damage (Pullin and Gilbert 1989). It is uncertain whether *U. thunbergiana* shows such a damage-induced increase in stinging hair density. However, it is unlikely, because cultivated nettles from Nara Park exhibited an increase in stinging hair density compared with wild ones; the result was the opposite of what was expected.

The evolution of an adaptive trait through natural selection requires three conditions: heritable variation in the trait, variation in lifetime reproductive success among individuals, and the correlation of the trait with lifetime reproductive success (Stearns and Hoekstra 2005). The evolution of extremely high stinging hair density in the *U. thunbergiana* population of Nara Park may satisfy these conditions. Variation in stinging hair density was shown to be heritable. Nettles with higher stinging hair densities were browsed less frequently by sika deer than those with lower densities, so that the former may survive and consequently reproduce more successfully than the latter. A large number of browsing sika deer have existed in Nara Park for more than 1,200 years (Ohigashi et al. 2003), thus acting as an agent in natural selection for nettles with higher stinging hair densities over this period. A population of *U. thunbergiana* with extremely high stinging hair densities might have evolved in Nara Park as a consequence of this selective pressure.

Two factors other than mammalian herbivory could influence stinging hair density of nettles: light conditions (Pollard and Briggs 1982) and soil nutrient conditions (Pullin and Gilbert 1989). Pollard and Briggs (1982) demonstrated that shading changes stinging hair densities in *U. dioica*. In our study, however, light conditions were unlikely to have caused variations in the stinging hair density of *U. thunbergiana*, because light intensity, expressed as RPPFD, did not differ significantly between study sites. On the other hand, Pullin and Gilbert (1989), in greenhouse experiments, suggested that nutrient-poor

conditions can lead to reduced stinging hair densities. This may be true for *U. thunbergiana* as well, because nettles cultivated in the greenhouse had high stinging hair densities compared with those in wild plants; nitrate ion concentration under cultivating conditions (1.0 mM) was considerably higher than those under natural conditions (0.3–0.8 mM). Even if soil conditions influence stinging hair density of *U. thunbergiana*, this alone could not explain the extremely large differences between nettles from Nara Park and those from other areas. The differences in the stinging hair traits were evident, even in the greenhouse experiment under nutrient-rich conditions.

The length of stinging hairs of nettles in Nara Park and other areas was similar, which suggests that stinging hair length is not influenced by sika deer browsing. This is somewhat inconsistent with observations involving other plants. Brambles (*R. hispidus*), angelica trees (*A. spinosa*) and spiny shrubs (*D. indicus*) under high browsing pressure bear longer, sharper, and larger thorns than those under low browsing pressure (Abrahamson 1975; White 1988; Takada et al. 2001). This difference between *U. thunbergiana* and other plants may reflect the difference between chemical and physical defensive tactics. The stinging hairs of nettles contain toxic compounds such as histamine, acetylcholine, and serotonin; in addition, they are fragile and penetrate the skin of browsing mammals, introducing toxins (Lenggenhager 1974). Therefore, nettles do not need sturdy stinging hairs. In contrast, thorns of other plants represent a physical defensive tactic, and, thus, may be more effective when sharper, longer and larger.

Herbivory may induce changes in the size and shape of plants (Danell and Bergström 2002). For instance, heavy grazing by sika deer causes the grass *Sasa nipponica* to become dwarf, with small leaves and short tillers (Yokoyama and Shibata 1998). Wild nettles in Nara Park had small leaves rather than those in unbrowsed areas, whereas cultivated nettles from the park had leaves as large as those of unbrowsed areas (Figs. 2 and 3). This suggests that *U. thunbergiana* might decrease leaf size following damage by sika deer, although there is also a possibility that leaf area varies with environmental conditions, such as water and soil nutrients, as it does in the mustard *Sinapis arvensis* (Roy et al. 1999).

In conclusion, stinging hairs of *U. thunbergiana* serve as a defensive structure against sika deer, and heavy browsing by these deer in Nara Park would have resulted in selection for plants with higher stinging hair densities over 1,200 years. As a consequence, the extremely high stinging hair density of the population of *U. thunbergiana* in Nara Park might have evolved through natural selection.

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