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Differential butterfly performance on host plant variants from populations under intense vs. low mammalian herbivory

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Abstract. Evolutionary interactions between a pair of species can be modified by the presence of another species that interacts with either or both species. However, only a few studies have demonstrated such complex interactions. Here, we report a case where a population of the red admiral butterfly (*Vanessa indica*) is assumed to change fitness traits in response to an evolutionary change in its major host plant, the Japanese stinging nettle (*Urtica thunbergiana*), caused by intense browsing pressure from sika deer (*Cervus nippon*). Nara Park (NP) in Japan has been home to several hundred protected sika deer for approximately 1200 yr, and their intense browsing pressure is assumed to have selected for heavily haired nettles that are more resistant to deer browsing compared to normal lightly haired nettles occurring in areas with low densities of sika deer. We found that the water and nitrogen contents of leaves are lower and the specific leaf mass is higher in heavily haired nettles at NP compared to the lightly haired nettles at the Takatori Castle Site (TCS) 30 km south of NP. Feeding experiments showed that *V. indica* larvae from NP reared on heavily haired nettles reach a greater adult body mass and relative abdomen mass than those from TCS, suggesting the possibility that the NP *V. indica* population has adapted to the heavily haired, nutritionally poorer variant. Analysis of the nucleotide sequence of the mitochondrial cytochrome c oxidase subunit I gene of *V. indica* from NP and nearby locations including TCS found one haplotype exclusive to NP, suggesting that movement of this butterfly is somewhat restricted. However, analysis using amplified fragment length polymorphism markers showed no genetic differentiation between the seven *V. indica* populations including the NP and TCS populations. These results suggest that adaptation of the NP population to the heavily haired nettle occurred in a relatively short period. Our study demonstrates a rare example of an indirect evolutionary impact of one herbivore (sika deer) on another herbivore (red admiral butterfly) through an evolutionary change in their shared food plant (Japanese nettle).

Key words: body mass; diffuse evolution; genetic differentiation; local adaptation; reciprocal feeding experiments; relative abdomen mass; sika deer; *Urtica thunbergiana*; *Vanessa indica*.

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INTRODUCTION

Most species are subjected to selection pressures both from directly interacting species and from indirectly interacting species (Strauss et al. 2005, Walsh 2013, Wise and Rausher 2013). Strong direct interaction can lead to pairwise evolution

of species involved, whereas indirect interaction can lead to diffuse evolution where selection by one species on another is influenced by the presence or absence of another species (Gould 1988, Strauss et al. 2005, Wise and Rausher 2013). There is extensive literature on pairwise evolution, but only limited reports on diffuse evolution.

In plant–animal interactions, diffuse evolution has been investigated mainly from the viewpoint of whether the magnitude of selection for tolerance or resistance of plants to particular herbivore species changes with the presence or absence of other herbivores (Strauss et al. 2005, Núñez-Farfán et al. 2007, Wise and Rausher 2013). For example, Stinchcombe and Rausher (2001, 2002) showed that the strength of selection for resistance of the ivyleaf morning glory *Ipomoea hederacea* to the white-tailed deer *Odocoileus virginianus* depended on the presence or absence of other insect herbivores. It is also possible that evolutionary change in a plant species exerted by one herbivore can have an impact on a second herbivore on the same plant, but there are few studies on this. A rare example is a study of interactions between pine squirrels, limber pines, and nutcrackers (Benkman et al. 2012, and references therein). Selection exerted by granivorous pine squirrels (*Tamiasciurus* spp.) favors evolution of increased seed defenses (e.g., thicker and larger scales) in the limber pine (*Pinus flexilis*), which in turn selects for an increase in the bill size of Clark’s nutcracker (*Nucifraga columbiana*), a mutualistic seed disperser. When pine squirrels are absent, cones with thinner scales evolve, so large bills are not necessary to harvest or disperse seeds, leading to decreased bill size in the nutcracker.

This study focuses on evolutionary interactions between the Japanese stinging nettle *Urtica thunbergiana* Siebold et Zucc. (Urticaceae), the sika deer *Cervus nippon* Temminck (Artiodactyla, Cervidae), and the Indian red admiral butterfly *Vanessa indica* (Herbst) (Lepidoptera, Nymphalidae).

Nettle leaves and stems bear stinging hairs containing toxic liquid. Nettles in Nara Park (NP) (660 ha), central Japan, where several hundred sika deer have been protected for about 1200 yr, bear many more stinging hairs than nettles in surrounding areas, where the sika deer density is low (Kato 2001, Shikata et al. 2013). Our previous studies revealed that sika deer avoid browsing on heavily haired nettles and that variation in the hair density has a genetic basis, suggesting that the high hair density of nettles in NP has evolved in response to heavy browsing by sika deer (Kato et al. 2008, Shikata et al. 2013). Such change in the hair density could impact the preference or

performance of other animals feeding on the same nettles. Our previous study has shown that the density of stinging hairs has little effect on the oviposition preference and the larval feeding preference of *V. indica* butterflies feeding on nettles in NP (Iwamoto et al. 2014), but it is unclear whether the nettle hair density affects larval performance or not. This study focuses on this topic and on whether *V. indica* in NP are locally adapted to heavily haired nettles.

Local adaptation is diagnosed by two criteria: (1) Individuals of a local population exhibit higher fitness in their own environment compared to individuals originating from other populations in the same environment (“local vs. foreign” criterion), and (2) individuals of a population exhibit higher fitness in their own environment compared to other environments (“home vs. away” criterion; Kawecki and Ebert 2004). Ideally, fitness difference between populations should be deemed local adaptation when both criteria are satisfied. However, there are occasions where only one criterion is satisfied. In such cases, the “local vs. foreign” criterion should be used as a diagnosis of local adaptation, because the “local vs. foreign” criterion is directly relevant to divergent selection, whereas the “home vs. away” criterion confounds the effects of divergent selection associated with intrinsic differences in habitat quality (Kawecki and Ebert 2004). Furthermore, Kawecki and Ebert (2004) suggested that local adaptation should be examined by reciprocally designed experiments in which fitness of local and foreign populations is compared in their local and foreign environments. A limited number of studies have revealed the occurrence of local adaptation by fulfilling both criteria and using reciprocally designed experiments (Greischar and Koskella 2007, Hoeksema and Forde 2008, Leimu and Fischer 2008, Ågren and Schemske 2012).

This study investigates whether *V. indica* in NP is locally adapted to the sympatric, heavily haired variant of nettles that has evolved under selection imposed by intense deer browsing. First, we compare the physical and chemical properties of leaves between heavily haired nettles from NP and lightly haired nettles from another location at Takatori Castle Site (TCS). Next, we examine *V. indica* larval survival, development time from hatching to pupation, and adult mass using

reciprocal feeding experiments with both butterfly larvae and nettle variants from NP and TCS. Last, we evaluate genetic differentiation between several *V. indica* populations including NP and TCS using nucleotide sequences of the mitochondrial cytochrome c oxidase subunit I (COI) gene and amplified fragment length polymorphism data.

MATERIALS AND METHODS

Study species and populations

The Japanese stinging nettle *Urtica thunbergiana* is a perennial plant distributed in central and southern Japan, Taiwan, Korea, and China (Kitamura and Murata 1961). Stinging hairs on the nettles are composed of a multicellular pedestal surmounted by an elongate, tapering, silicified stinging cell (Thurston 1974) and contain toxic fluid that causes sharp pain to humans upon injection (Pollard and Briggs 1984; H. Sato, *personal communication*). Nettles prefer a nutrient-rich soil and are often found at forest edges (Pullin and Gilbert 1989, Kato 2001).

The Indian red admiral butterfly *Vanessa indica* occurs from India to East Asia. In Japan, its major host plants are *Boehmeria nivea*, *B. sylvestris*, *B. spicata*, *B. japonica*, *U. angustifolia*, and *U. thunbergiana* of the family Urticaceae (Teshirogi 1990). In Nara City, central Japan, *V. indica* overwinters as adults and produces several generations each year.

In reciprocal feeding experiments, we used butterflies and nettles collected from near the Great Buddha Hall of Todaiji Temple in NP (34°69' N, 135°84' E, 110 m a.s.l.) and from TCS (34°43' N, 135°82' E, 550–580 m a.s.l.) located approximately 30 km south of NP (for location, see Shikata et al. 2013). NP is located close to the town and has an area of 660 ha with temples, shrines, evergreen woods, and lawns. Hundreds of sika deer have been protected as a sacred animal in NP for about 1200 yr. A census in July 2013 counted 1094 deer inhabiting NP (Foundation for the Protection of Deer in Nara Park 2013). Nettles in NP have several dozen times more stinging hairs on leaves than on nettles from surrounding or nearby locations (e.g., TCS), where there are no or few sika deer. The high hair density on nettles in NP is assumed to have evolved as a mechanism to resist deer browsing (Kato et al. 2008, Shikata et al. 2013, Iwamoto et al. 2014).

Properties of *U. thunbergiana* leaves

Twenty nettle plants were selected arbitrarily for the study at NP and TCS. Two leaves on the second node of the main shoot(s) were sampled from each plant on 25 or 26 April 2007 and placed in a plastic case with a sheet of moistened filter paper. The cases were kept in a cooler box with refrigerant and taken to the laboratory. Leaves were weighed, and the upper surfaces were scanned using an image scanner (EPSON GT-X770; Seiko Epson Co., Suwa, Japan) at a resolution of 300 dpi. Using the digital image, stinging hairs on the whole upper leaf surface were counted and the leaf area was measured using the free software LIA for Win32 ver. 0.376β1 (Yamamoto 2003) to determine the density of stinging hairs. After scanning, leaves were dried at 70°C for 48 h and measured for dry weight to determine specific leaf mass (mg/cm²) and water content (%). Then, two leaves from the same plant were pulverized together and analyzed for nitrogen and carbon contents using a CN analyzer (Yanako CN coder MT-700; Yanako Co, Kyoto, Japan).

Vanessa indica survival and growth performance

To investigate whether *V. indica* butterflies are locally adapted to the sympatric nettle populations, we designed reciprocal feeding experiments using butterfly larvae and nettle leaves collected from NP and TCS. In mid-April 2007, 110 and 137 butterfly eggs were collected haphazardly from NP and TCS, respectively. Eggs from each location were divided into two groups, one for rearing on nettles from NP and the other for rearing on nettles from TCS. Eggs were placed individually on a leaf in a transparent plastic case (10 × 10 × 2 cm) with a sheet of moistened filter paper on the bottom and incubated at 24°C under a 14-h light/10-h dark (14L:10D) conditions. A total of 80 and 96 neonates hatched from the NP and TCS samples, respectively. At 2 or 3 days after hatching, old leaves were replaced by new undamaged leaves. At 4 days after hatching, larvae were transferred to 20°C and 14L:10D. They were provided with fresh undamaged leaves every 1 or 2 days and monitored for survival each day. Nettle leaves were collected from NP and TCS every 2 days, stored in plastic bags at 4°C, and provided to larvae within 2 days after collection. The position of rearing cases in the incubator was changed daily to avoid temperature position effects.

When larvae were about to spin for pupation, they were transferred to transparent plastic cups (10 cm in lower diameter, 13 cm in upper diameter, and 10 cm in height) with a sheet of moistened filter paper. They usually pupated within 2 or 3 days after transfer. Adults emerged about 10 days after pupation. After meconium excretion (4 to 6 h after emergence), they were killed with ammonia gas and dried at 70°C for 48 h. Each adult was sexed and weighed for whole body mass and abdomen mass.

Mitochondrial DNA analysis

Eggs of 23 or 24 *V. indica* were collected from nettle leaves from each of five locations: NP, TCS, the site of Nara Women's University (34°69' 135°83' E; located 1.2 km west of the Great Buddha Hall in NP), Sakurai (34°54' N, 135°86' E; 10.5 km south of NP), and Kochidani (34°57' N, 136°11' E; 28.5 km east-southeast of NP) in late March to mid-April 2008 (for location, see Shikata et al. 2013). Nettles in these locations have only few stinging hairs except in NP (Shikata et al. 2013). At 2 days after hatching, larvae were preserved in 100% ethanol until examination.

Genomic DNA was extracted from the whole body of each specimen by the Chelex method described by Ito et al. (2008). A 302-bp region of the mitochondrial *COI* gene was amplified using the following primers: LCO11490-J-1514 (5' GGT CAA CAA ATC ATA AAG ATA TTG G) and HCO2198-N-2175 (5' TAA ACT TCA GGG TGA CCA AAA AAT CA) (Folmer et al. 1994). Amplifications were performed with standard PCR techniques. The reaction mixture and thermal cycling conditions followed the protocol of Ito et al. (2008). The PCR product was purified using a QIAquick PCR Purification Kit (Qiagen Inc., Valencia, California, USA). Sequencing was outsourced to MacroGen Inc., Seoul, South Korea.

Amplified fragment length polymorphism (AFLP) analysis

Eggs of 20 to 27 *V. indica* were collected from nettle leaves from seven locations, NP, TCS, Noto (34°68' 135°85' E; 2.0 km southeast of the Great Buddha Hall in NP), Kohbohji (34°69' 135°84' E; 0.57 km north of the Great Buddha Hall), the site of Nara Women's University, Sakurai, and Kochidani in late March to mid-April 2012 (for location, see Shikata et al. 2013). At 2 days after

hatching, larvae were preserved in 100% ethanol until examination.

An AFLP analysis was performed using the AFLP Plant Mapping Kit (Applied Biosystems, Foster, California, USA) according to the manufacturer's recommended protocol. Six primer combinations were used for selective amplifications (*EcoRI*+/ *MseI*+: AC/CAA, TG/CTG, AA/CTT, AG/CAT, AT/CAG, and TT/CAC). Amplification products were analyzed with an ABI 3130xl DNA Analyzer (Applied Biosystems) and a GeneScan ROX-500 size standard (Applied Biosystems). Twenty-one randomly selected samples (12.4% of the total of 169 samples) were subjected to AFLP analysis twice to estimate genotyping error. To detect and calculate the size of AFLP bands, raw electropherograms were analyzed using Peak Scanner 1.0 (Applied Biosystems) with default parameters. A binary matrix of AFLP band presence (1) or absence (0) was built using the automated scoring RawGeno package ver. 2.0-1 (Arrigo et al. 2012) in R ver. 2.15.1 (R Core Team 2012) with the following parameters: scoring range, 100–500 bps; minimum intensity, 100 rfu; reproducibility limit, 90%; minimum bin width, 1 bp; and maximum bin width, 1.5 bps.

Data analysis

Six indicators were determined for leaf properties of nettles (the number of stinging hairs per leaf, the density of stinging hairs (per cm²), water and nitrogen contents, the nitrogen/carbon (N/C) ratio, and specific leaf mass), and five indicators were determined for the growth performance of *V. indica* butterflies (larval survival from hatching to adult emergence, development time from hatching to pupation, adult body mass, relative abdomen mass (abdomen mass/body mass), and relative growth rate). Relative growth rate (mg⁻¹·mg⁻¹·d⁻¹) was calculated using Radford's (1967) formula, [ln(adult body mass) – ln(neonate mass)]/development time. The mean weight of five neonates, 0.2 mg, was used as neonate mass. For statistical analyses, proportional data (i.e., water and nitrogen contents and relative abdomen mass) were transformed using arcsine-square-root to improve normality of residuals (Krebs 1999).

Differences in the number of hairs per leaf, the density of hairs, water content, and specific leaf mass between nettles from NP and TCS were tested with a mixed-model nested ANOVA (Sokal

and Rohlf 2012), where nettle population was a fixed factor and individual was a random factor nested within population. Differences in nitrogen content and the N/C ratio between the two populations were tested with t tests. The effects of butterfly origin and nettle population on growth performance of *V. indica* were examined separately for females and males by two-way factorial ANOVA, where butterfly origin and nettle population were fixed factors. When the interaction effect of the two factors was statistically significant, contrasts between home (or sympatric) and away (or allopatric) nettle populations and between local and foreign butterfly origins were constructed, and differences were tested by the Scheffé method for multiple comparisons (Sokal and Rohlf 2012). The effects of butterfly origin and nettle population on survival were examined with the Mantel-Haenszel procedure (Sokal and Rohlf 2012). Statistical analyses were performed

with SPSS ver. 15.0J (SPSS Inc. 2006). The statistically significant level was set at 0.05.

The percentage of polymorphic loci and expected heterozygosity in butterfly populations were computed as indices of the genetic diversity on the basis of AFLP data with AFLP-SURV ver. 1.0 (Vekemans 2002). Pairwise F_{ST} values were also obtained to assess genetic differentiation between populations with AFLP-SURV ver. 1.0 (Vekemans 2002). The significance of the F_{ST} values was evaluated on the basis of 1000 permutations.

RESULTS

Urtica thunbergiana leaf properties

Nettles from TP and TCS showed significant differences in the number of stinging hairs per leaf, density of stinging hairs, water and nitrogen contents, N/C ratio, and specific leaf mass. The number and density of stinging hairs were

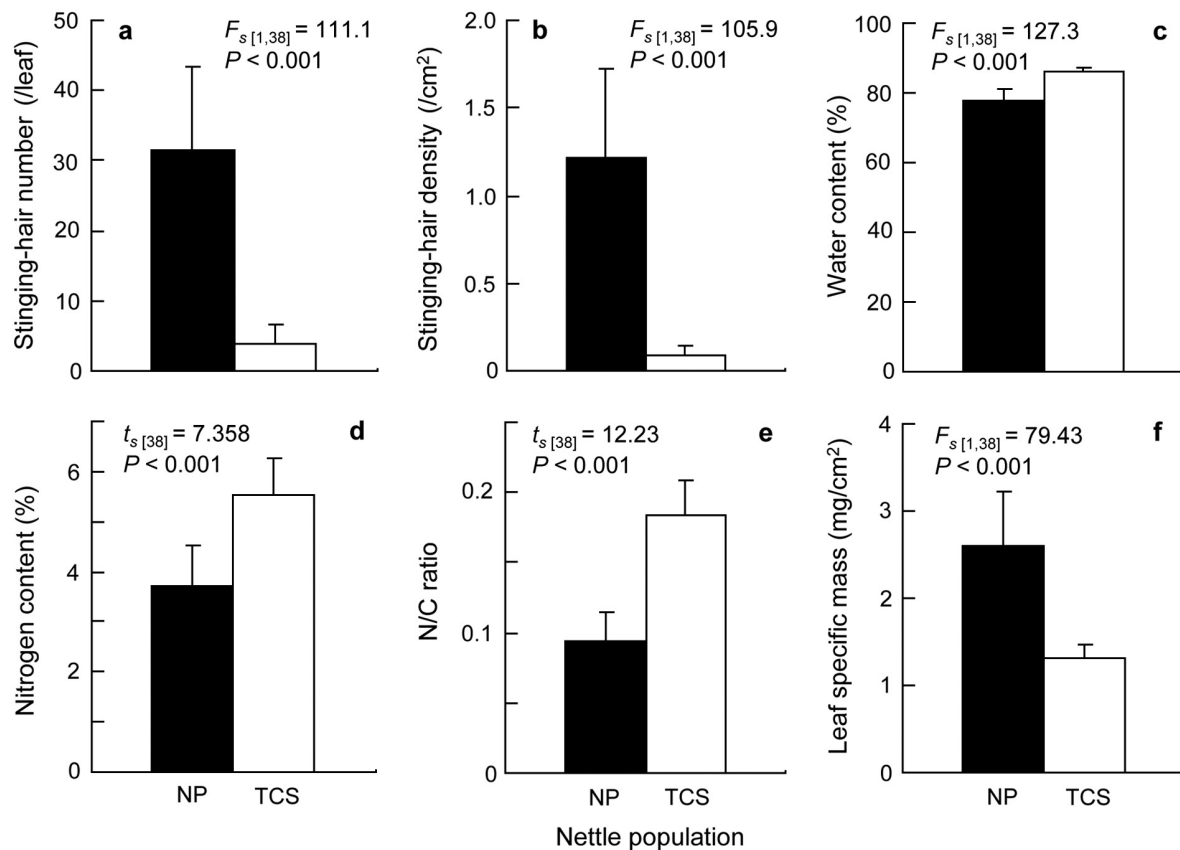


Fig. 1. Comparison of leaf properties of two *Urtica thunbergiana* populations, that is, Nara Park (NP) and Takatori Castle Site (TCS). $n = 20$ for each population.

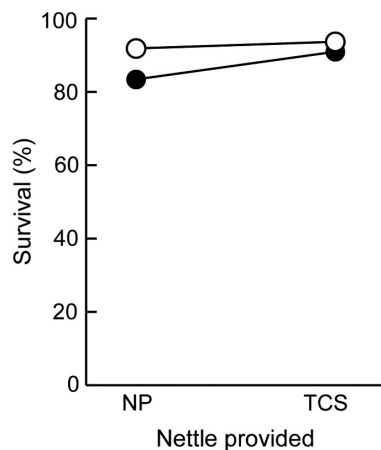


Fig. 2. Larval survival of *Vanessa indica* from Nara Park (solid circles) and Takatori Castle Site (open circles) when reared on nettles from Nara Park (NP) and Takatori Castle Site (TCS). For results of statistical analysis, see section *Vanessa indica larval performance*. $n = 36$ for NP butterfly on NP nettle, 44 for NP on TCS, 49 for TCS on NP, and 47 for TCS on TCS.

approximately 10 and 14 times greater in nettles from NP compared to nettles from TCS, respectively (Fig. 1a, b). Water content, nitrogen content, and the N/C ratio were approximately 10%, 30%, and 50% lower in leaves of nettles from NP, respectively (Fig. 1c–e), and specific leaf mass was approximately twice as large in nettles from NP (Fig. 1f).

Vanessa indica larval performance

Larvae from TCS showed higher survival (92.7%) than from NP (87.5%) as a whole, but the larval survival was not significantly different between the two populations (Breslow–Day test, $\chi^2_H = 0.167$, $df = 1$, $P = 0.683$; Fig. 2). The joint odds, or the Mantel–Haenszel estimate, were 0.601, suggesting that the odds for larval survival on NP nettles were, on average, approximately 0.6 times as high as those on TCS nettles, but this estimate was not significantly different from 1 (Mantel–Haenszel test, $\chi^2_{HM} = 0.531$, $df = 1$, $P = 0.466$). These results indicate that there was no significant difference in survival between larvae reared on NP nettles (88.2%) and those on TCS nettles (92.3%) irrespective of their origin.

The interaction effect of butterfly origin and nettle population on development time from hatching to pupation was not significant in either sex

(Fig. 3a, Table 1). Female development time was significantly longer when reared on NP nettles than on TCS nettles, but was not significantly different between butterflies of different origins. No significant effect of butterfly origin or nettle population was observed on male development time.

The interaction effect of butterfly origin and nettle population was significant on adult body mass and relative abdomen mass in both sexes (Fig. 3b, c, Table 1). The Scheffé test for multiple comparisons showed that NP larvae reached significantly greater adult body mass when reared on NP nettles than TCS larvae, regardless of sex. When reared on TCS nettles, no significant difference was observed between butterflies of different origins. Furthermore, NP butterflies did not show significant differences when reared on NP or TCS nettles, whereas TCS butterflies had significantly larger body mass when reared on TCS nettles than when reared on NP nettles.

NP males reared on NP nettles showed significantly greater relative abdomen mass compared to NP males reared on TCS nettles and also to TCS males reared on NP nettles. Both NP and TCS females had significantly greater relative abdomen mass when reared on sympatric nettle populations than when reared on allopatric populations. In addition, when reared on either NP or TCS nettles, females of sympatric origin had significantly greater relative abdomen mass than those of allopatric origin.

The interaction effect of butterfly origin and nettle population on relative growth rate was not significant in either sex (Fig. 3d, Table 1). No significant effect of butterfly origin or nettle population was observed on male relative growth rate, whereas female relative growth rate was significantly larger when reared on TCS nettles than when reared on NP nettles.

Mitochondrial COI gene and AFLP analyses

Only two haplotypes (α and β) were found in the partial nucleotide sequence (302 bps) of the COI gene of 118 individuals from five *V. indica* populations (Fig. 4), but haplotype- α was only found in the NP population (in six out of 24 individuals). Haplotype- α was not found in specimens from Nara Women's University located only 1.2 km from NP.

A total of 466 polymorphic AFLP loci were obtained in 169 individuals collected from seven

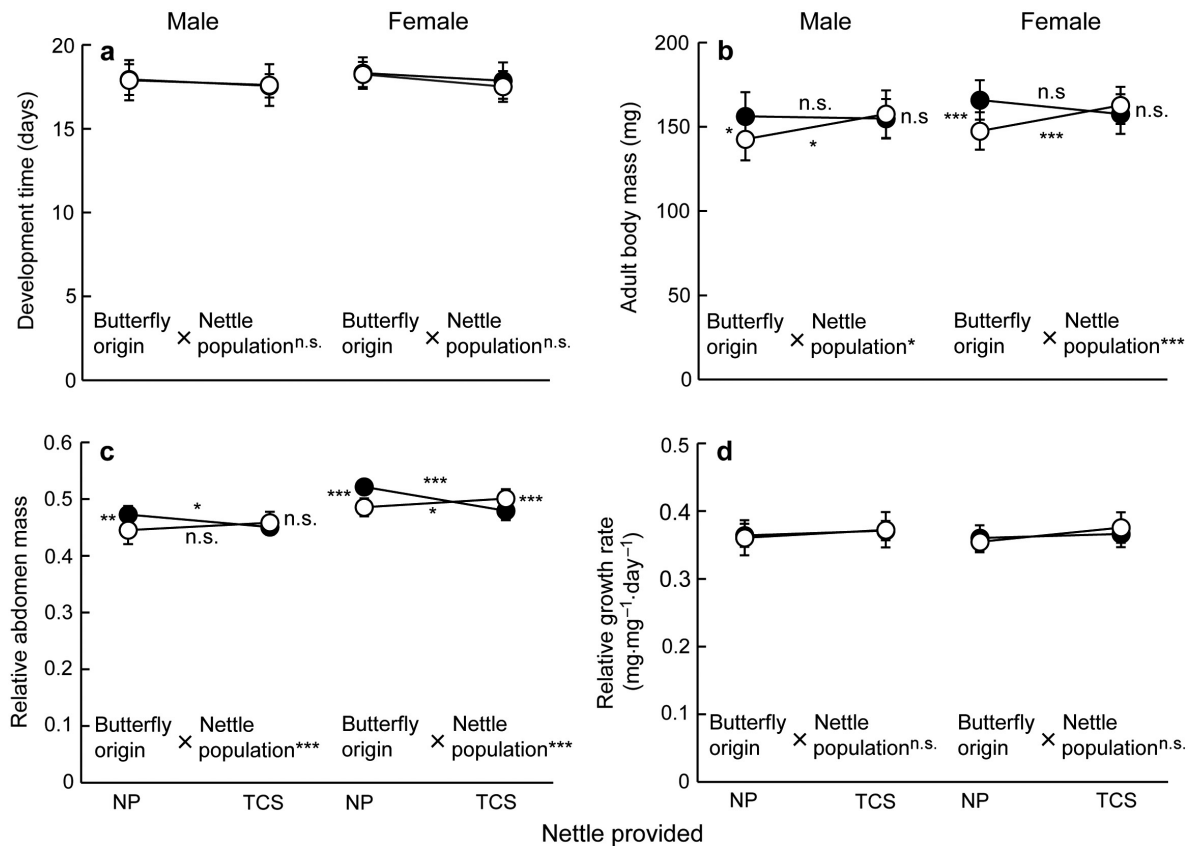


Fig. 3. Development time, adult body mass, and relative abdomen mass, and relative growth rate of *Vanessa indica* from Nara Park (solid circles) and Takatori Castle Site (open circles) in reciprocal feeding trial experiments in which larvae were reared on nettles from Nara Park (NP) or Takatori Castle Site (TCS). When two-way factorial ANOVA indicated that the butterfly origin $\times$ nettle population interaction was statistically significant (Table 1), multiple comparisons for contrasts were conducted using Scheffé method. Male: $n = 14$ for NP butterfly on NP nettle, 18 for NP on TCS, 28 for TCS on NP, and 15 for TCS on TCS; females: $n = 16$ for NP on NP, 22 for NP on TCS, 17 for TCS on NP, and 29 for TCS on TCS. n.s., not significant, $*0.01 < P \leq 0.05$, $**0.001 < P \leq 0.01$, $***P \leq 0.001$.

V. indica populations. The overall rate of genotyping error was 0.0318. Percentages of polymorphic loci (PL) and expected heterozygosity (H_j) were only slightly different between populations (PL: median = 74.7, minimum = 66.5, maximum = 78.8; H_j : median = 0.219, minimum = 0.211, maximum = 0.232; Table 2). Pairwise F_{ST} values were significant in most population pairs but very small (median = 0.013, minimum = 0.004, maximum = 0.034; Table 2).

DISCUSSION

Leaves of nettles from NP not only have a higher density of stinging hairs but also lower water and

nitrogen contents, lower N/C ratio, and larger specific leaf mass compared to leaves of nettles from TCS (Fig. 1). In general, larval growth performance is lower when leaf water and nitrogen contents are lower (Awmack and Leather 2002, Schoonhoven et al. 2005, Munguira et al. 2009). In addition, stinging hairs contain silica that might hinder digestion of leaves (Thurston 1974, Massey and Hartley 2009). Moreover, leaves with larger specific leaf mass are usually tougher and less digestible for insect herbivores (Wright and Cannon 2001, Schoonhoven et al. 2005). Therefore, it is expected that nettles from NP are nutritionally poorer at least for insect herbivores compared to TCS nettles. In fact, female larvae of *V. indica*

Table 1. Two-way factorial ANOVA table for differences in development time, adult body mass, relative growth rate, and relative abdomen mass when butterfly (*Vanessa indica*) larvae from Nara Park and Takatori Castle Site were reared on nettles from Nara Park or Takatori Castle Site.

Fitness-related trait	Sex	Source of variation	SS	df	MS	F	P
Development time	Male	Butterfly	0.0003	1	0.0003	<0.001	0.986
		Nettle	1.9331	1	1.9331	1.723	0.194
		Butterfly × Nettle	0.0280	1	0.0280	0.025	0.875
		Error	79.6515	71	1.1219		
	Female	Butterfly	0.8916	1	0.8916	1.014	0.317
		Nettle	6.7658	1	6.7658	7.696	0.007
		Butterfly × Nettle	0.3600	1	0.3600	0.410	0.524
		Error	70.3286	80	0.8791		
Adult body mass	Male	Butterfly	5.13×10^{-4}	1	5.13×10^{-4}	3.011	0.087
		Nettle	7.99×10^{-4}	1	7.99×10^{-4}	4.684	0.034
		Butterfly × Nettle	11.66×10^{-4}	1	11.66×10^{-4}	6.774	0.011
		Error	121.13×10^{-4}	71	1.71×10^{-4}		
	Female	Butterfly	8.80×10^{-4}	1	8.80×10^{-4}	6.797	0.011
		Nettle	2.34×10^{-4}	1	2.34×10^{-4}	1.909	0.182
		Butterfly × Nettle	27.39×10^{-4}	1	27.39×10^{-4}	21.147	<0.001
		Error	138.03×10^{-4}	80	1.30×10^{-4}		
Relative abdomen mass	Male	Butterfly	17.55×10^{-4}	1	17.55×10^{-4}	4.435	0.039
		Nettle	3.59×10^{-4}	1	3.59×10^{-4}	0.908	0.344
		Butterfly × Nettle	53.22×10^{-4}	1	53.22×10^{-4}	13.456	<0.001
		Error	280.82×10^{-4}	71	3.96×10^{-4}		
	Female	Butterfly	10.06×10^{-4}	1	10.06×10^{-4}	4.044	0.048
		Nettle	35.89×10^{-4}	1	35.89×10^{-4}	14.420	<0.001
		Butterfly × Nettle	168.05×10^{-4}	1	168.05×10^{-4}	67.528	<0.001
		Error	199.09×10^{-4}	80	2.49×10^{-4}		
Relative growth rate	Male	Butterfly	2.32×10^{-5}	1	2.32×10^{-5}	0.047	0.829
		Nettle	152.81×10^{-5}	1	152.81×10^{-5}	3.094	0.083
		Butterfly × Nettle	9.37×10^{-5}	1	9.37×10^{-5}	0.190	0.664
		Error	3506.28×10^{-5}	71			
	Female	Butterfly	6.44×10^{-5}	1	6.44×10^{-5}	0.163	0.687
		Nettle	368.77×10^{-5}	1	368.77×10^{-5}	9.356	0.003
		Butterfly × Nettle	105.25×10^{-5}	1	105.25×10^{-5}	2.670	0.106
		Error	3153.23×10^{-5}	80	39.42×10^{-5}		

Note: SS, sum of squares; MS, mean squares.

showed significantly longer development time and lower relative growth rate when reared on NP nettles than when reared on TCS nettles (Fig. 3a, d), although males did not show such differences.

The high hair density of NP nettles is a genetically fixed trait which evolved in response to heavy browsing by sika deer (Kato et al. 2008, Shikata et al. 2013). In addition, nettles do not show rapid or delayed damage-induced response to simulated deer browsing for stinging hair number (H. Sato, M. Ikeda, and A. Fujii, *unpublished data*). However, it is still unknown whether the lower water and nitrogen contents and higher specific leaf mass of NP nettles are genetically fixed traits or ecologically plastic traits. If these

traits are genetically fixed, there are questions about whether these traits are selected by heavy browsing or whether there are trade-offs between the density of stinging hairs and water and nitrogen contents or specific leaf mass. Future studies will focus on these issues.

Although *V. Indica* development time was longer and relative growth rate was lower when reared on nutritionally poorer NP nettles, body mass and relative abdomen mass did not show such simple trends (Fig. 3b, c). For example, NP butterflies had a significantly larger relative abdomen when reared on NP nettles than when reared on TCS nettles, although the whole body mass of NP butterflies was not significantly different when reared on NP or TCS nettles. These

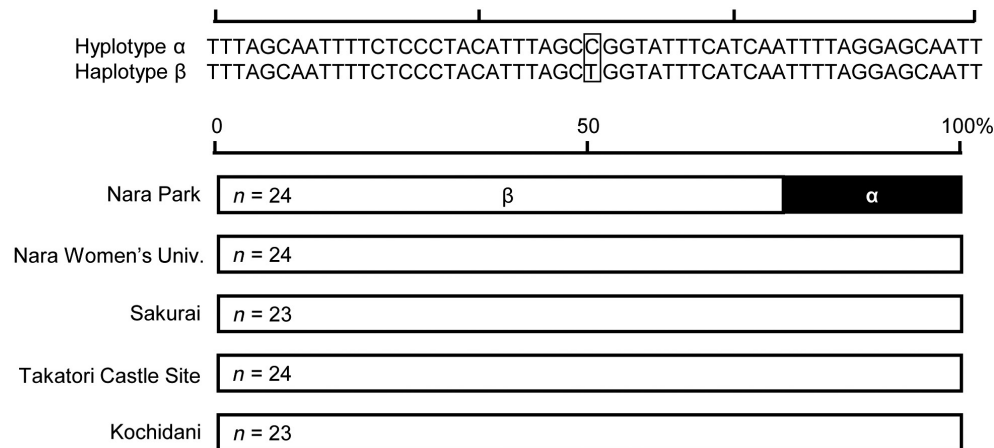


Fig. 4. Part of two cytochrome c oxidase subunit I (COI) haplotypes, α and β , obtained from 118 *Vanessa indica* individuals from five populations and percentage of each haplotype in populations.

results may be explained by local adaptation of NP butterflies.

According to Kawecki and Ebert (2004), there are two criteria for local adaptation, that is, “local vs. foreign” and “home vs. away.” If a butterfly population sympatric to a nettle variant used in experiments shows a greater fitness than an allopatric butterfly population, the “local vs. foreign” criterion is held. If a butterfly population shows a greater fitness on a sympatric variant than on an allopatric variant, the “home vs. away” criterion is fulfilled. If only one of the criteria is satisfied, the “local vs. foreign” criterion is the better criterion, because the “home vs. away” criterion confounds the effects of divergent selection associated with intrinsic

differences in habitat quality (Kawecki and Ebert 2004). In the studied *V. indica* populations, both criteria are satisfied for relative abdomen mass for both NP and TCS females and NP males, although only the “home vs. away” criterion is satisfied in TCS males. However, only the “local vs. foreign” criterion is satisfied for the whole body mass of NP females and males, and only the “home vs. away” criterion is satisfied in TCS females and males. These results suggest that the NP population of *V. indica* is locally adapted to heavily haired, nutritionally poorer nettles. In TCS butterflies, the “home vs. away” criterion is satisfied for both indices of females and males, but this may be simply because the TCS nettles are better nutritionally than NP nettles.

Table 2. Genetic diversity and differentiation indices estimated from AFLP analysis of *Vanessa indica* populations.

Population	N	PL	H_j	Pairwise F_{ST}					
				Nara Park	Noto	Kohbohji	Nara Women's Univ.	Sakurai	Takatori Castle Site
Nara Park	28	74.9	0.232						
Noto	20	74.7	0.213	0.016					
Kohbohji	20	78.8	0.220	0.013	0.005				
Nara Women's Univ.	27	73.2	0.229	0.006	0.016	0.006			
Sakurai	26	74.7	0.219	0.032	0.018	0.019	0.031		
Takatori Castle Site	25	66.5	0.211	0.008	0.013	0.012	0.007	0.033	
Kochidani	23	66.5	0.213	0.004	0.016	0.014	0.010	0.027	0.005

Notes: AFLP, amplified fragment length polymorphism; N, sample size; PL, percentage of polymorphic loci; H_j , expected heterozygosity or Nei's gene diversity. Statistically significant F_{ST} values are shown in bold ($\alpha = 0.05$).

This argument assumes that body mass and relative abdomen mass are positively correlated with reproductive success. It is believed that females with a larger abdomen produce more eggs, because the ovaries are in the abdomen (Karlsson and Van Dyck 2009). In fact, in Lepidoptera, larger females often have greater fecundity than smaller females (Tammaru et al. 2002, Karlsson and Van Dyck 2009). In males, larger bodies may be favored selectively by winning territorial conflicts (Rosenberg and Enquist 1991, Peixoto and Benson 2008). In addition, larger abdomen mass may be favored in producing larger ejaculates (Wedell and Cook 1999), which may increase a female's fecundity (Wiklund and Kaitala 1995, Karlsson 1998), have an advantage in sperm-competitive environments (LaMunyon and Eisner 1994, Bissoondath and Wiklund 1997), and suppress females from mating with other males (Wiklund and Kaitala 1995, McNamara et al. 2009). It is also reported that larger females and males of a butterfly species have higher longevity (Boggs and Freeman 2005). However, there is no literature on these topics for *V. indica*. Further study is needed.

Although *V. indica* larval performance differs between heavily haired NP nettles and lightly haired TCS nettles, *V. indica* females and larvae do not discriminate between them when allowed to choose at oviposition or feeding (Iwamoto et al. 2014), indicating a lack of preference-performance linkage. There is also some literature reporting the lack of this linkage in insects (Gripenberg et al. 2010), and there are only a few studies reporting strict linkage. It is not fully understood why insects sometimes preferentially oviposit on and are attracted to plants that are unsuitable for their offspring or their own development.

This study suggests that *V. indica* butterflies from NP are differentiated to some extent in some life-history traits from butterflies from TCS. In the AFLP analysis, a very weak genetic differentiation was observed between the butterfly population from NP and populations from the other localities (median of pairwise F_{ST} = 0.01, minimum = 0.004, maximum = 0.032), but such differentiation levels were also observed between most of the studied butterfly populations. On the other hand, one COI haplotype was exclusive (six out of 24 individuals) to the NP butterfly

population. These results suggest that *V. indica* butterflies in NP are sedentary to some extent and their local adaptation to the heavily haired nettle variant in NP has occurred comparatively recently. Considering the history of protected sika deer in NP, this adaptive change would not date back 1200 yr.

The response of relative abdomen mass to nettle variants showed sexual difference; both NP and TCS females showed significantly larger relative abdomen mass when reared on the sympatric variants than when reared on the allopatric variants, whereas only NP males showed this pattern. Females may be adapted to local variants better than males. Berthiaume et al. (2009) suggested that females of the hemlock looper moth *Lambdina fiscellaria* are more likely to reproduce at the birthplace and are adapted better to local environments than males. Larger relative abdomen mass in females may hinder their dispersal by flight and allow them to adapt to local environments.

Another factor that may affect local adaptation of *V. indica* to nettles is the presence of other host plants such as *Boehmeria nivea*, *B. sylvestris*, *B. spicata*, and *B. japonica*. These plants are found in areas surrounding NP. However, the effect of these plants is thought to be small even if present, because (1) the NP population of *V. indica* is thought to be sedentary to some extent, and (2) local adaptation to the heavily haired nettle variant has occurred although lightly haired nettles are found at Nara Women's University only 1.2 km from NP.

Local adaptation of *V. indica* in NP, if it is the case, is regarded as an outcome of diffuse evolution rather than pairwise evolution. In diffuse evolution, interaction between a pair of species is affected by the presence of other species, while in pairwise evolution, interaction between a pair is independent of the presence or absence of other species (Gould 1988, Strauss et al. 2005, Wise and Rausher 2013). Local adaptation of *V. indica* to heavily haired nettles would not occur unless heavy browsing by deer exerts selection for the heavily haired variant; that is, the evolutionary interaction between *V. indica* and *U. thunbergiana* has been modified by the presence or absence of sika deer.

There are also several studies suggesting that heavy browsing by large mammals causes the

evolution of particular defense traits in plants (Stinchcombe and Rausher 2001, 2002, Vourc'h et al. 2001, Didiano et al. 2014, Martin et al. 2015). For example, Martin et al. (2015) experimentally documented that populations of orange jewelweed (*Impatiens capensis*) exposed to heavy browsing by white-tailed deer for a long time produce more fruits than those historically protected from deer when plants suffered deer browsing, suggesting that deer browsing imposed selection on tolerance traits in orange jewelweed. Furthermore, Stinchcombe and Rausher (2001, 2002) indicated that the intensity of selection for tolerance or resistance of ivyleaf morning glory (*Ipomoea hederacea*) to white-tailed deer depended on the presence or absence of insect herbivores, suggesting that the community composition of herbivores can alter the evolutionary trajectory of defensive traits against deer herbivory. However, most of these studies do not investigate whether plant resistance or tolerance traits that have evolved in response to a herbivore species exert evolutionary changes in other herbivore species exploiting those plants. To our knowledge, one exception is the series of studies by Benkman and colleagues on pine squirrels, limber pines, and Clark's nutcrackers (Benkman et al. 2012 and references therein). This study is another example.

In conclusion, we suggest that, due to local adaptation, the NP population of *V. indica* has evolved some physiological processes to overcome the nutritionally poor quality of the sympatric, heavily haired variant of the nettle *U. thunbergiana*, which could have evolved under intense browsing pressure by sika deer *C. nippon*. Although there is no clear evidence of genetic differentiation between the NP and surrounding populations, *V. indica* in NP seems sedentary to some extent, because one haplotype of *COI* was found exclusively in the NP population. This study could be a rare example of diffuse evolution in which the evolutionary impact of a mammalian herbivore (sika deer) on a plant (Japanese nettle) causes an evolutionary change in an insect herbivore (red admiral butterfly). To further understand this multispecies system, we are now studying the genetic bases of differences in the nutritional contents of heavily and lightly haired nettles.

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