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How do two specialist butterflies determine growth and biomass of a shared host plant?

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Abstract Although insect herbivory can modify subsequent quantity and quality of their host plants, change in plant quantity following herbivory has received less attention than plant quality. In particular, little is known about how previous herbivore damage determines plant growth and biomass in an insect species-specific manner. We explored whether herbivore species-specific food demand influences plant growth and biomass. To do this, we conducted a series of experiments and field survey using two specialist butterflies, *Sericinus montela* and *Atrophaneura alcinous*, and their host plant, *Aristolochia debilis*. It is known that *A. alcinous* larva requires four times more food resources to fulfill its development than *S. montela* larva. Despite that *A. alcinous* larvae imposed greater damage on plants than *S. montela* larvae, plant growth did not differ due to herbivory by these species both in single and multiple herbivory events. On the other hand, total aboveground biomass of the plants was reduced more by *A. alcinous* than *S. montela* feeding regardless of the number of herbivory events. Feeding on plants with a history of previous herbivory neither decreased nor increased larval growth. Our results suggest that food demand of the two butterfly species determined subsequent plant biomass, although the plant response may depend on tolerance of the host plant (i.e., ability to compensate for herbivore damage). Such difference in the effects of different herbivore species on host plant biomass is more likely to occur than previously thought, because food demand differs in most herbivore species sharing a host plant.

Keywords *Aristolochia* · Food demand · Herbivory history · Plant growth response

Introduction

A number of studies have focused on how insect herbivores decrease plant growth and reproduction, because it was hypothesized that herbivory decreases plant fitness (Hendrix 1988). However, recent studies have documented that plants can respond to herbivory in ways that enhance their defense levels or compensatory growth (Karban and Baldwin 1997; Fornoni 2011; Karban 2011). In previous studies, the effects of herbivory on plant responses have been mainly investigated following a single herbivory event (e.g., Agrawal 2000; Viswanathan et al. 2005; Ali and Agrawal 2014). However, multivoltine insect herbivores repeatedly attack host plants throughout the growing season, and plant responses to herbivory often differ between single and multiple attacks (Poelman et al. 2008; Underwood 2012). Therefore, to evaluate how previous herbivory affects subsequent plant characteristics, we should consider a plant's history of herbivory in terms of the number of herbivory events and the order in which herbivore species fed on the plant (Viswanathan et al. 2007; Erb et al. 2011; Miller-Pierce and Preisser 2012).

Recent studies examining how plants respond to previous herbivory have mainly focused on changes in plant

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quality (e.g., defensive chemicals, nutritional status, and physical traits such as leaf toughness and trichomes) rather than on changes in plant quantity, and they have explored which herbivore traits (e.g., type and extent of feeding damage and salivary constituent) are responsible for changes in plant quality (Van Zandt and Agrawal 2004; Viswanathan et al. 2007; Mooney et al. 2009; Ali and Agrawal 2012; Erb et al. 2012). Several reviews have emphasized that plant quality can limit herbivore populations and mediate interactions among herbivores (Denno et al. 1995; Ohgushi 2005; Kaplan and Denno 2007). On the other hand, changes in the biomass of host plants have received less attention, except for cases of compensatory growth where growth in response to herbivory reduces fitness loss due to tissue damage (Belsky 1986). The compensatory growth response indicates that herbivory does not necessarily decrease plant growth, but can enhance it depending on intrinsic (e.g., relative growth rate, reallocation of resources, and photosynthetic enhancement) and extrinsic factors (e.g., resource availability, timing of defoliation, and intensity of herbivory) (reviewed by Tiffin 2000; Del-Val and Crawley 2005). However, previous studies have evaluated plant growth by total standing biomass instead of directly measuring plant growth (Hawkes and Sullivan 2001; Massad 2013), which makes it difficult to separate the effects of consumption and plant growth on plant biomass. For example, an increase in the amount of tissue removal and the number of attacks decreased subsequent aboveground biomass of grassland weeds in Britain, but this may have resulted from not only an increase in consumption but also from changes in subsequent growth (Del-Val and Crawley 2005). Also, the effects of previous herbivory on plant growth have often been evaluated by artificial clippings (Tiffin 2000; Fornoni 2011), and few studies have used natural herbivory by different herbivore species (but see Ritchie and Tilman 1992; Gavloski and Lamb 2000). Thus, we know little about which traits of herbivores are critical in generating herbivore species-specific effects on subsequent plant growth, when focusing on effects of consumption by herbivores on plant growth.

Food demand of herbivorous insects to fulfill a larval development differs across herbivore species, and it may influence subsequent plant growth and biomass. Increasing food demand may increase consumption, thereby enhancing the degree of damage by a focal herbivore, and decreasing subsequent plant growth (Guillet and Bergström 2006; Mundim et al. 2012) and total biomass (Del-Val and Crawley 2005). Herbivory can also modify plant quality by changing the secondary compounds and/or nutrition in host plants, depending on herbivory intensity (Baldwin and Schmelz 1994; Underwood 2000). In addition, plant growth produces young tissues with high nutrient status (Raupp and Denno 1983), which in turn improves plant quality (Damman 1989; Utsumi et al. 2009). Thus, herbivore species-specific food demand may affect subsequent quantity and quality of plants. However, we are unaware of studies investigating whether herbivore-specific food demand changes plant growth after single and multiple herbivory events.

This study explored whether the species-specific food demand of an herbivore individual to complete its development influences subsequent plant growth and biomass. We carried out a field experiment using two specialist multivoltine butterflies, *Sericinus montela* Gray and *Atrophaneura alcinous* (Klug), and a shared host plant, *Aristolochia debilis* Sieb. et Zucc. *Aristolochia debilis* has aristolochic acids, which are toxic defensive chemicals (Kumar et al. 2003) that protect the plant from attacks by generalist herbivores. Only these specialist butterfly larvae can feed on *A. debilis*, and they repeatedly attack them throughout a growing season. Since *A. alcinous* larva is four times greater in dry weight than *S. montela* larva, it requires four times more food resources for its development than *S. montela* larva (Suzuki 1998). Therefore, we examined whether the difference in the food demand between the two butterfly species alters subsequent plant growth in cases of single and multiple herbivory, and examined how these changes in plant growth modify subsequent host plant biomass. Also, we conducted a rearing experiment to explore the possibility that previous herbivory affects plant quality, which may influence larval performance. Specifically, we addressed the following questions. (1) Does the food demand of the butterflies account for the impact of previous herbivory on plant growth and biomass? (2) Does the growth of butterfly larvae depend on leaf age and herbivory history of plants?

Materials and methods

Study area and organisms

This study was conducted on banks of the Kizu River (34°49′0″–34°52′20″N, 135°44′40″–135°48′0″E), in Kyoto prefecture, central Japan in 2013 (Fig. 1). Vegetation on the riverbanks consists primarily of common grassland weeds such as *Lolium multiflorum*, *Viola mandshurica*, *Taraxacum japonicum* and *Sophora flavescens*. Regular mowing occurs in summer and autumn in every year.

Aristolochia debilis (Aristolochiaceae) is a perennial, herbaceous vine. Aboveground parts die in winter, and new shoots sprout from overwintering roots in early spring.

Sericinus montela and *Atrophaneura alcinous* are papilionid swallowtail butterflies, and their larvae exclusively feed on *A. debilis* in our study area.

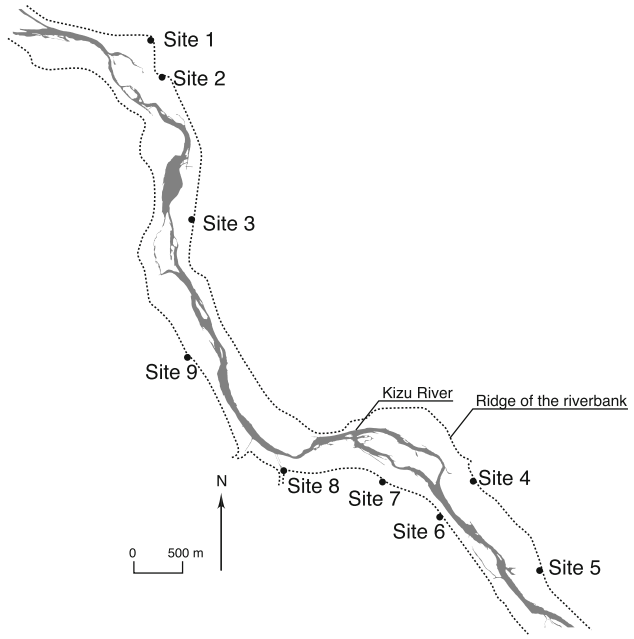

Fig. 1 Location of study sites. In the study area we conducted a field experiment (site 3), sampling for analyzing leaf quality (site 7), and field survey (all sites). Modified by Digital Japan portal web site (http://maps.gsi.go.jp)

Sericinus montela is originally distributed across Korea, China and Maritime Province in Russia, and it was introduced to Japan in the late 1970s, and has expanded its distributed region (Matsuka and Ohno 1981; Sakuratani and Kanno 2003). In our study area, it was first recorded in 1993 (Shoji 1997). *Atrophaneura alcinous* is a native species distributed all over Japan except in Hokkaido. These butterflies sequester aristolochic acids from *A. debilis* and use them in their own defense (Nishida and Fukami 1989; Nishida 1994). The first generation of *S. montela* emerges in early spring, followed by the first generation of *A. alcinous*. *Sericinus montela* and *A. alcinous* produce five and four generations a year, respectively (K. Hashimoto, personal observations), and overwinter at the pupal stage (Sakuratani et al. 2003).

Effects of single and multiple herbivory on plant growth and biomass

To assess how single and multiple herbivory affects subsequent plant growth and biomass, we conducted a field experiment that manipulated herbivore species and the number of herbivory events (Table 1a). We measured subsequent plant growth by summing the biomass of young leaves and stems, which were newly emerged following herbivory. Herbivory trials were performed twice: first herbivory trial was in the first week (August 7–14) and second herbivory trial was in the fourth week (August 30 to September 6) in a 6-week experimental period. This experimental design was based on the alternating occurrences of *S. montela* and *A. alcinous* larvae observed in the field. The time between two consecutive generations of *S. montela* and *A. alcinous* is approximately 2–3 weeks (K. Hashimoto, personal observation). We assigned plants to seven treatments: SC, SS, and SA plants received *S. montela* herbivory in the first trial, followed by no herbivory (SC), *S. montela* herbivory (SS), and *A. alcinous* herbivory (SA) in the second trial. AC, AS, and AA plants received *A. alcinous* herbivory in the first trial, followed by no herbivory (AC), *S. montela* herbivory (AS), and *A. alcinous* herbivory (AA) in the second trial. CC plants received no herbivory throughout the experiment. Each treatment had 11–14 plant individuals (see Table 1a).

On August 6, 2013, we randomly selected 89 plants without damaged leaves at study site 3 (see Fig. 1). We counted leaf number of each plant (mean ± SD 22.6 ± 10.3) and randomly assigned the plants to the seven treatments mentioned above. There was no difference in leaf number of plants among the seven treatments (one-way ANOVA, $F_{6,82} = 0.4$, $P = 0.9$). Each plant was covered with a mesh bag to prevent natural herbivory throughout the experiment.

Table 1 Experimental design (a) and statistical procedure (b) to examine the effects of single and multiple herbivory on leaf and stem consumption, and subsequent plant growth (i.e., the biomass of young tissues) and total biomass

(a)

First herbivory	No	S			A		
Second herbivory	No	No	S	A	No	S	A
Treatment	CC	SC	SS	SA	AC	AS	AA
Sample size	14	14	13	13	13	11	11

(b)

Main effect	Combination	Analysis	Fixed effect of the models
Effects of herbivore species with single herbivory	CC, SC, AC	One-way ANCOVA	First herbivory, initial leaf number (covariate), interaction
Effects of herbivore species with multiple herbivory	SC, SS, SA, AC, AS, AA	Two-way ANCOVA	First herbivory, second herbivory, initial leaf number (covariate), their second order interactions

C, control (no herbivory); S, *S. montela* herbivory; A, *A. alcinous* herbivory

On August 7 and 8, we inoculated one fourth or fifth instar larva either of the two butterfly species to each plant. The larvae were allowed to feed until August 14 (i.e., first herbivory trial). We checked the plants every day, and when the larva died or disappeared, another larva was added. On August 14, we removed all larvae, and then estimated leaf consumption rate (consumed leaf area/the initial leaf number) and stem consumption rate (consumed number of stem nodes/the initial number of stem nodes) of the plants. Consumed leaf area of a plant was obtained from total sum of visual estimates of a ratio of consumed area of individual leaves, which was classified into 0–100% with 10% interval. The visual estimates per leaf were significantly correlated with the measured leaf consumption (linear regression with intercept of zero, slope = 0.97, $R^2_{adj} = 0.95$, $n = 20$). After removing the larvae, we allowed the plants to grow until August 30, when they were then inoculated with one fourth or fifth instar larva to each of SS, SA, AS, AA plants until September 6 (i.e., second herbivory trial). On September 6, we removed all larvae and recorded leaf and stem consumption rate of the plants. Then, we allowed the plants to grow until September 19, when aboveground parts of the plants were harvested. At the end of the experiment, dry masses of aboveground parts were measured.

We determined tissue age by the timing of their emergence during the herbivory trials (i.e., old: emerged before the first trial, intermediate: emerged between the first trial and the second trial, and young: emerged after the second trial). Therefore, the age of old, intermediate, and young tissues was >6, 6–2, and <2 weeks, respectively. The samples were dried in a laboratory at room temperature for 60 days (25 °C; 32% RH), and weighed, except for leaves that were used in a rearing experiment (see next section). We also examined feeding behavior of the larvae to determine whether they feed on stems.

Effects of leaf age and previous herbivory on larval growth

To examine the effects of leaf age on larval growth, we carried out a rearing experiment using *S. montela* and *A. alcinous* larvae. We also examined whether previous herbivory affects larval growth via herbivore-induced qualitative changes in plants. From two to eight replicates of each species were reared on both young and old leaves of each herbivory treatment.

We used young and old leaves for each herbivory treatment in the rearing experiment. Six to eight plants were randomly selected from each treatment, and 1–23 old and young leaves per plant were used in the rearing experiment. We estimated dry weight of these leaves by multiplying leaf area by mean leaf mass area (LMA) of each leaf age in each treatment, and we added these values to the biomass values of the other part of the plants. A single third instar larva each of *S. montela* and *A. alcinous* was reared with young or old leaves from each plant treatment. An ample amount of leaves was placed on wet paper, and the larva was placed on the leaves in a plastic case (75 × 90 × 40 mm). The cases were kept in an environmental chamber (23 °C, 14L10D) for 24 h. Before and after the experiment, leaf area and larval weight were measured. Leaf area was determined by a software ImageJ (http://imagej.nih.gov/ij/). LMA was also measured using other leaves that were kept in an oven for 72 h (60 °C), and weighed after dried. LMA was calculated as (dry weight/leaf area). Consumed leaf mass was estimated by multiplying consumed leaf area of each larva by mean LMA of each leaf age in each treatment. Relative growth rate (RGR) and relative consumption rate (RCR) of larvae were calculated as [(final larval mass − initial larval mass)/initial larval mass] and (consumed leaf mass/initial larval mass), respectively.

Plant quality related to leaf age

To determine whether *A. debilis* plant quality depends on leaf age, we examined aristolochic acid contents, C/N ratio, and water content of old and young leaves. On August 6, 2012, we randomly selected nine plants without damaged leaves at study site 7 (Fig. 1). On August 24, 26, 28, and 30, we recorded the position of all individual leaves when each leaf emerged. We defined leaves emerged before 6 August as old (>3 weeks) and emerged from August 25 to 30 as young (<1 week), respectively. On August 30, we harvested aboveground parts of all experimental plants, brought them to a laboratory, and took 1 or 2 leaves of each leaf age from all individual plants for analysis of aristolochic acids and water content. The remaining part of leaves was dried in an oven at 60 °C for 72 h for analysis of C/N ratio.

Aristolochic acids (AAs) were analyzed by the method described in Nishida and Fukami (1989). We extracted AAs from leaves twice, first in 5 mL of 99% ethanol and then in 2 mL of acetone. We purified the crude extract using an acid–base extraction procedure. Then, we obtained a yellow solid mass of acidic components after removing the solvent. We dissolved the mass in 1 mL of 2-butanone, thereby yielding a 2-butanone solution. We quantified AA contents using a Shimadzu Corporation HPLC system and a reverse-phase column (Shiseido Capcell Pak C18 S-5 μm, 4.6 mm ID × 250 mm), eluting isocratically with a mixture of methanol, distilled water, and acetic acid (66:33:1). The chromatograms were monitored by UV detection at 252 nm. Five analogs of AAs (AA-C, B, II, E, and I) were identified based on their

retention times (Rt: AA-C = 4.9, B = 6.0, II = 10.1, E = 11.1, I = 13.5 min). We determined AA contents by comparing areas to a standard curve generated with AA-I standards (LKT Laboratories, Inc.). We assumed that absorbance per unit mass of AA-I was equal to those of other AAs. Total AA contents were represented as (absolute mass of aristolochic acids/fresh mass of the leaf).

To estimate C/N ratio, we ground and homogenized dried leaves of each treatment, and determined nitrogen and carbon contents using an elemental analyzer (NC-analyzer Sumigraph nc-900, Sumika Chemical Analysis Service Ltd., Osaka, Japan). C/N ratio was calculated as (carbon/nitrogen contents).

To measure water content, we weighed the fresh mass of leaf samples and the dry mass after drying at 60 °C for 72 h. Water content was calculated as [(fresh mass − dry mass)/fresh mass].

Effects of previous larval density on growth and current quantity of plants

To confirm whether effects of previous herbivory on plant growth, which were examined by the manipulative experiment, can work in the field condition, we examined whether previous larval density affects plant growth (i.e., new leaf number) and quantity (i.e., total leaf number) in the field. In April 2013, we set nine study sites along the riverbank (see Fig. 1). We placed a single 5 × 5 m quadrat at each site, and all plants in the quadrats were protected from the regular mowing.

We monitored young and total leaf numbers of 10 randomly selected plants (90 plants in total), in each quadrat. From July 1 to September 13, we counted all leaves of the selected plants every 2 weeks. To determine young leaves (younger than 2 weeks), we used the following method; on each census date, we painted a mark in black ink on the stem adjacent to the apical meristem of each shoot. On the next census, we determined the leaves growing above the marks as young leaves.

To estimate larval densities, from May 25 to October 4, we counted the number of *S. montela* and *A. alcinous* larvae on 10–21 randomly selected plants once a week. The larval density in each quadrat on each census date was obtained by dividing the total number of larvae by the number of plants surveyed. In each quadrat, we found several peaks of *S. montela* and *A. alcinous* densities from May 25 to October 4, except for the quadrat in site 2 in which no larvae appeared.

Statistical analyses

We used ANCOVA to examine whether leaf and stem consumption rate differed among the treatments in the first herbivory trial (i.e., CC, SC, and AC plants, Table 1b). First herbivory treatment (main factor), initial leaf number (covariate), and their interaction were as explanatory variables. We also used ANCOVA to examine whether leaf and stem consumption rate differed among the treatments in the second herbivory trial (i.e., SC, SS, SA, AC, AS, and AA plants, Table 1b). We excluded CC plants because our focus is on the effects of second herbivory when first herbivory occurred (i.e., multiple herbivory, see Table 1b). First and second herbivory treatments (main factors), initial leaf number (covariate), and their second order interactions were as explanatory variables. We also examined whether the final biomass of young and total tissues depended on the treatments using ANCOVA in the same way as for leaf and stem consumption rate; first, we examined the effect of the first herbivory trial with single herbivory event, and second, we examined the effects of the first and second herbivory trial with multiple (in this case, twice) herbivory events (Table 1b). The leaf and stem consumption rate were arcsine square-root transformed and the biomass was $\log(x + 0.1)$-transformed to meet the assumption of normality and homoscedasticity. In addition, a Tukey–Kramer post hoc test was performed among treatments, when these variables were significant.

We used linear mixed models to examine the relationships between RGR, and RCR, leaf age, and herbivory treatments. The models for *S. montela* and *A. alcinous* were constructed separately. The response variable was RGR, and the explanatory variables were RCR, leaf age, treatment, and their second order interactions. Plant individual was included as a random factor.

To examine whether leaf quality (i.e., aristolochic acid contents, C/N ratio, and water content) was dependent on leaf age, we constructed linear mixed models. The models included the leaf traits as dependent variables, with leaf age (old vs. young) as an explanatory variable, and plant individual as a random factor. Aristolochic acid contents were $\log(x)$-transformed to meet the assumption of normality and homoscedasticity.

We examined the effects of the previous larval densities of *S. montela* and *A. alcinous* on young and total leaf number using linear mixed models. Leaf number was $\log(x + 1)$-transformed to improve normality and homoscedasticity. Explanatory variables were the latest peak density of *S. montela* and *A. alcinous* and census week (treated as a categorical variable). Plant individual was included as a random factor nested within site. Peak density was $\log(x + 1)$-transformed because of highly right-skewed distribution.

All statistical analyses were conducted using R version 3.0.3 (R Development Core Team 2014). Computing least square means and performing multiple comparisons were made by lsmeans function in the 'lsmeans' package version 2.15 (Lenth 2015). All linear mixed models were

constructed by lmer function of 'lme4' package version 1.1-5 (Bates et al. 2014) and significance of their explanatory variables was tested by type II F test using Kenward-Roger approximate denominator degree of freedom computed by 'pbkrtest' package version 0.3-8 (Halekoh and Højsgaard 2013).

Results

Effects of single and multiple herbivory on plant growth

Leaf consumption rate by *A. alcinous* in the first trial was 83% greater than that by *S. montela* (SC vs. AC in Table 2; Tukey–Kramer test, $t = -6.6$, $P < 0.001$). On the other hand, in the second trial the leaf consumption rate did not significantly differ between *S. montela* and *A. alcinous* (back-transformed least square means, 81 vs. 89%; Tukey–Kramer test, $t = -1.2$, $P = 0.5$). Stem feeding by *A. alcinous* larvae was more frequently observed than that by *S. montela* larvae. Moreover, *A. alcinous* occasionally consumed whole stems. As a result, stem consumption by *A. alcinous* in the first trial was eight times greater than that by *S. montela* (SC vs. AC in Table 2; Tukey–Kramer test, $t = -10.9$, $P < 0.001$). Also, stem consumption by *A. alcinous* in the second trial was 2.5 times greater than that by *S. montela* (back-transformed least square means, 87 vs. 35%; Tukey–Kramer test, $t = -8.9$, $P < 0.0001$).

Despite the obvious difference in damage intensity between *S. montela* and *A. alcinous*, previous herbivory did not influence subsequent growth of *A. debilis* regardless of the number of herbivory events. When herbivory occurred once, young tissue biomass, which emerged after the first herbivory had finished, was not significantly affected by the first herbivory event (Table 3). This was also true when herbivory events occurred twice; young tissue biomass was not affected by the first and second herbivory of the two butterfly species [Fig. 2; Table S1a in Electronic Supplementary Materials (ESM)]. The interaction between the first and second herbivory events was not significant ($F_{2,65} = 0.075$, $P = 0.9$), suggesting that the first herbivory did not change the effects of the second herbivory event on plant growth.

Effects of single and multiple herbivory on plant biomass

When herbivory occurred once, effects of herbivory on plant biomass differed depending on herbivore species (Table 4); *A. alcinous* herbivory reduced total biomass by 52% (CC vs. AC in Table 2; Tukey–Kramer test, $t = 2.6$, $P = 0.03$) but *S. montela* herbivory reduced total biomass only by 5% and this was not significant (CC vs. SC in Table 2; Tukey–Kramer test, $t = 0.4$, $P = 0.9$). As a result, AC plants had 49% less total biomass than SC plants, although this difference was insignificant ($t = 2.3$, $P = 0.07$). When herbivory occurred twice, the total biomass of plants with *A. alcinous* herbivory in the first trial was 30% less than plants with *S. montela* herbivory (Fig. 3; Table S1b in ESM). In the second trial, *A. alcinous* herbivory caused a 65% reduction in total biomass while *S. montela* herbivory reduced total biomass by 49%. As a result, plants with *A. alcinous* in the second herbivory trial had 43% less in total biomass than plants with *S. montela* herbivory. This difference (0.16 g in back-transformed least square means) was similar to the difference in first herbivory trial between the plants with *S. montela* and *A. alcinous* (0.18 g in back-transformed least square means, Fig. 3).

Effects of leaf age and previous herbivory on larval growth

We found significant positive relationships between RGR and RCR in both *S. montela* ($F_{1,40.1} = 13.90$, $P < 0.001$) and *A. alcinous* ($F_{1,48.6} = 55.83$, $P < 0.001$), indicating

Table 2 Leaf and stem consumption rate, and biomass of plants (mean ± SE) at the end of the experiment

Treatment	Leaf consumption (%)		Stem consumption (%)		Biomass of plant tissues (leaves and stems) (g)			
	First	Second	First	Second	Total	Old	Intermediate	Young[a]
CC	0	0	0	0	1.45 ± 0.30	0.86 ± 0.14	0.34 ± 0.12	0.26 ± 0.07
SC	47.85 ± 7.56	0	9.72 ± 1.83	0	1.38 ± 0.30	0.67 ± 0.14	0.43 ± 0.14	0.29 ± 0.09
SS	49.47 ± 7.24	63.24 ± 9.55	7.93 ± 0.92	10.24 ± 4.25	0.71 ± 0.19	0.44 ± 0.15	0.06 ± 0.05	0.20 ± 0.05
SA	61.28 ± 7.21	87.83 ± 6.37	10.70 ± 1.69	78.51 ± 6.14	0.30 ± 0.10	0.09 ± 0.06	0.04 ± 0.03	0.17 ± 0.03
AC	88.15 ± 4.83	0	76.68 ± 7.19	0	0.70 ± 0.15	0.16 ± 0.06	0.26 ± 0.05	0.28 ± 0.06
AS	84.68 ± 7.62	74.85 ± 11.10	71.87 ± 8.72	27.85 ± 8.29	0.65 ± 0.27	0.27 ± 0.15	0.18 ± 0.11	0.20 ± 0.05
AA	84.55 ± 7.63	75.82 ± 10.86	73.33 ± 7.65	70.79 ± 8.92	0.27 ± 0.07	0.01 ± 0.004	0.04 ± 0.03	0.21 ± 0.05

Old: emerged before the first trial, intermediate: produced between the first and second trials, young: emerged after the second trial

[a] Plant growth was represented by young tissue biomass

Table 3 Effects of first herbivory on plant growth in single herbivory event

	df	*F*	*P*
First herbivory	2	0.56	0.6
Leaf number (covariate)	1	8.85	**0.005**
First herbivory × leaf number	2	1.03	0.4
Residuals	35		

Bold shows statistical significance

Table 4 Effects of first herbivory on plant total biomass in single herbivory event

	df	*F*	*P*
First herbivory	2	4.22	**0.02**
Leaf number (covariate)	1	39.82	**<0.001**
First herbivory × leaf number	2	0.69	0.5
Residuals	35		

Bold shows statistical significance

that larvae that consumed a greater amount of leaves achieved a higher growth rate (Fig. 4). The effects of leaf age were also significant in both species (*S. montela*; $F_{1,29.6} = 46.29$, $P < 0.001$, *A. alcinous*; $F_{1,25.8} = 63.61$, $P < 0.001$). Thus, larvae fed on young leaves showed greater RGR than larvae fed on old leaves when they consumed an equal amount of leaves (Fig. 4). Herbivory treatment (*S. montela*; $F_{6,30.1} = 0.79$, $P = 0.6$, *A. alcinous*; $F_{6,31.9} = 0.62$, $P = 0.7$) and all interactions did not affect larval RGR (Table S2 in ESM).

Plant quality related to leaf age

Total aristolochic acid contents did not differ between young and old leaves (Table 5). On the other hand, young leaves had significantly lower C/N ratio and higher water content than old leaves.

Effects of previous larval density on growth and total biomass of plants in the field

The number of young leaves was neither associated with previous *S. montela* density nor *A. alcinous* density in the field (Table 6). On the other hand, total leaf number was negatively associated with the previous *A. alcinous* density, but not associated with the previous *S. montela* density. These results are consistent with the results obtained from the field experiment.

Discussion

Our results showed that previous herbivory by two butterfly species did not affect subsequent plant growth, regardless of whether there were single or multiple instances of herbivory. However, host plant biomass was significantly reduced by previous *A. alcinous* herbivory relative to by *S. montela* in both single and multiple attacks.

Effects of species-specific food consumption on plant growth

It is widely accepted that herbivory can reduce plant growth (Hendrix 1988; Hawkes and Sullivan 2001; Massad 2013). In this study, however, although leaf and stem consumption rate of the plants in one herbivory event ranged from 0 to 100%, the butterfly species causing

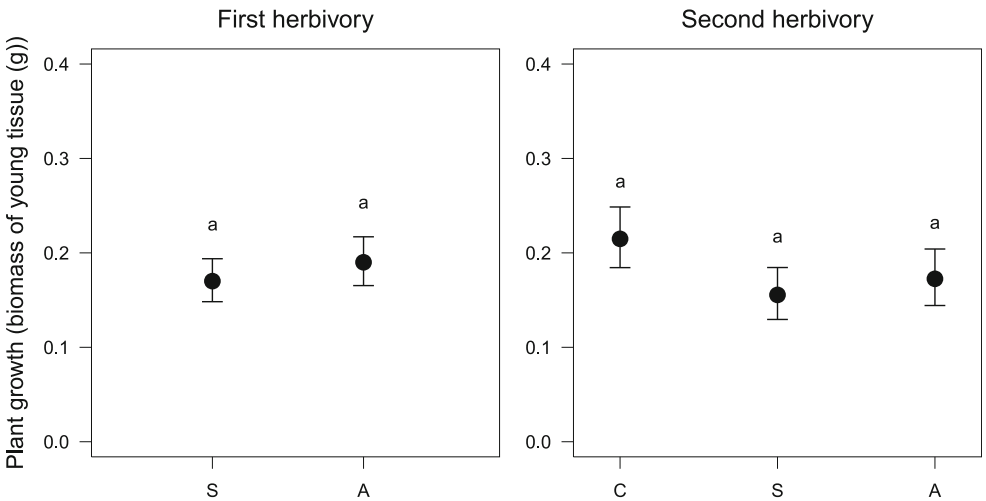

Fig. 2 Growth of plant with multiple herbivory. Back-transformed values of least square mean ± SE are presented. S, *S. montela* herbivory; A, *A. alcinous* herbivory; C, no herbivory. *Different letters* indicate significant difference

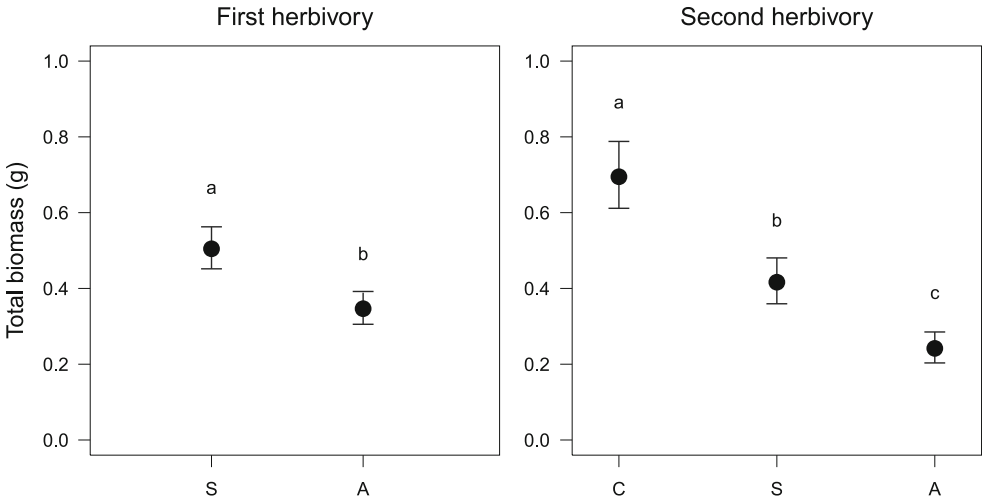

Fig. 3 Total biomass of plants with multiple herbivory at the end of the experiment. Back-transformed values (least square mean ± SE) were presented. S, *S. montela* herbivory; A, *A. alcinous* herbivory; C, no herbivory. *Different letters* indicate significant difference

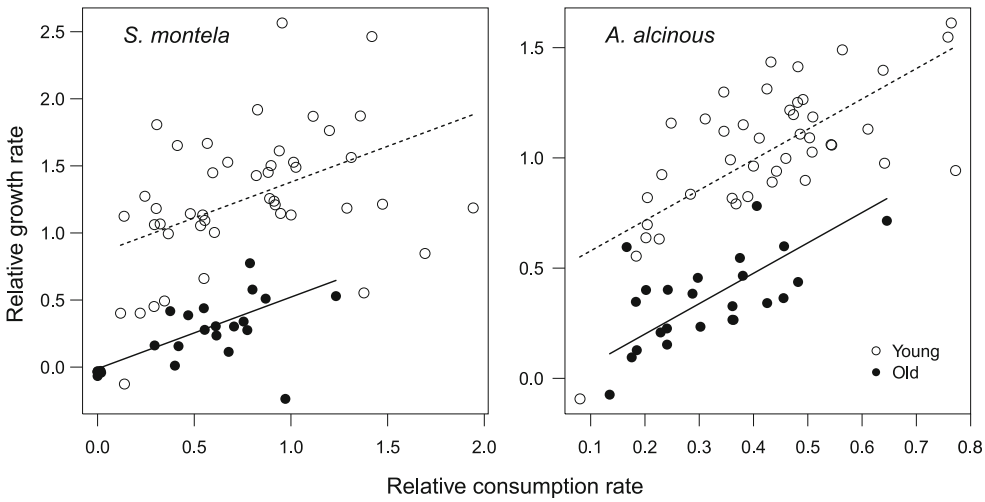

Fig. 4 Relationships between relative consumption rate and relative growth rate of *S. montela* and *A. alcinous* larvae. *Lines* represent predicted values from the most parsimonious models, in which only significant terms (relative consumption rate and leaf age) were included. *Dashed* and *solid lines* show young and old leaves, respectively

Table 5 Comparison of leaf traits in quality (mean ± SE) between young and old leaves

Leaf trait	Young	Old	*df*	*F*	*P*
Aristolochic acids (mg/g fresh weight)	0.86 ± 0.10	0.61 ± 0.09	1, 6.9	3.11	0.1
C/N	12.30 ± 0.37	15.86 ± 0.36	1, 2.4	87.72	**<0.006**
Water (%)	74.54 ± 0.54	69.45 ± 0.88	1, 6.2	17.67	**<0.005**

Bold shows statistical significance

Table 6 Effects of previous larval density on young and total leaf number

Response variable	Explanatory variable	Slope	*df*	*F*	*P*
Young leaf number	Previous *S. montela* density	−0.33	1, 87.6	1.05	0.3
	Previous *A. alcinous* density	−0.23	1, 167.7	1.15	0.3
	Census week		4, 359.3	13.01	**<0.001**
Total leaf number	Previous *S. montela* density	0.01	1, 138.9	0.09	0.8
	Previous *A. alcinous* density	−0.97	1, 364.1	31.24	**<0.001**
	Census week		4, 448.9	5.12	**<0.001**

Bold shows statistical significance

different damage did not affect subsequent plant growth irrespective of whether there were single and multiple herbivory events. This result suggests that an increase in damage intensity does not affect subsequent growth of *A. debilis*. Rausher and Feeny (1980) found a similar growth response in *Aristolochia reticulata* where leaf production by *A. reticulata* was maintained after herbivory by the specialist papilionid butterfly *Battus philenor*. This may have been achieved by allocating resource from roots to shoots, and this hypothesis was supported by the reduced root biomass of *A. reticulata* following shoot herbivory. Likewise, *A. debilis* roots may be sufficiently large, being approximately 1–2 cm diameter and typically extending vertically more than 100 cm (K. Hashimoto, personal observation), to store resources, which may enhance shoot growth following severe damage.

Plants sometimes do not decrease their growth and in other cases even increase it following herbivory, due to secondary growth to compensate for herbivory damage (Belsky 1986). However, this compensatory growth often weakens with increasing damage intensity (Belsky 1986; Del-Val and Crawley 2005). For example, shoot growth of tropical shrub *Piper arieianum* did not decrease when a small fraction of the leaf area was removed, but it decreased when leaf removal was large (Marquis 1984). Also, leaf production of palm tree *Sabal palmetto* after defoliation decreased with increasing defoliation frequency (McPherson and Williams 1998). It should be noted that *A. debilis* may have a high tolerance to intensive herbivory compared to the above examples. In fact, subsequent growth of *A. debilis* was not reduced even after severe herbivory by *A. alcinous* (leaf consumption, 76–88%, see Table 2), and it was at same level as plants without herbivory. There are several studies showing compensation even after severe defoliation (e.g., herbaceous forbs, in Harrison and Maron 1995; grasses, in Del-Val and Crawley 2005). A recent meta-analysis showed that growth of herbaceous forbs, such as *A. debilis*, were less likely to be affected by herbivory than woody plants (Massad 2013).

Species-specific effects of herbivores on plant biomass

Atrophaneura alcinous had a higher food demand than *S. montela*, resulting in differences in the reduction in total plant biomass. Although leaf consumption in the second trial was not different between herbivore species, stem consumption by *A. alcinous* was significantly higher than that by *S. montela*. When considering total consumption of leaves and stems, *A. alcinous* larvae would have fed on more plant tissues than *S. montela*. There are a few studies showing that the effects of herbivory on plant biomass differ depending on herbivore species (Ritchie and Tilman 1992; Gavloski and Lamb 2000). For example, Gavloski and Lamb (2000) reported that biomass of canola and yellow mustard following herbivory differed between lepidopteran herbivore species. This was probably due to a change in growth following herbivory, because defoliation was controlled at a constant level. Similarly, Utsumi et al. (2013) found that shoot regrowth of willow *Salix eriocarpa* following herbivory differed depending on herbivore species identity. If this is also the case in *A. debilis*, then there would be species-specific effects of previous herbivory on subsequent plant biomass. In this study, however, neither *S. montela* nor *A. alcinous* herbivory changed subsequent growth of *A. debilis*. Therefore, the stronger negative impact of *A. alcinous* on subsequent plant biomass than *S. montela* would be due to the greater resource consumption by *A. alcinous* individuals, not due to the decreased plant growth following *A. alcinous* feeding. Previous studies to date have used total standing biomass as an index of plant growth (Hawkes and Sullivan 2001; Massad 2013). Our results indicate that using total standing biomass may be misleading when we evaluate plant growth, because aboveground biomass of *A. debilis* was decreased without a decrease in plant growth. Note that our aim is to examine whether food demand of two herbivores that are same feeding type and taxonomy affects plant growth and biomass. Hence, we chose these butterfly species so as to avoid effects of feeding guilds or taxonomic relatedness, to highlight the difference in the food demand at an individual level between herbivore species.

From the viewpoint of these butterflies, the order of colonization of the herbivores may be important in determining food quantity available to the subsequently colonizing butterflies. The field survey suggested that the effects of previous herbivory on plant growth and biomass, which were revealed by the manipulative experiment, can work in the field condition. In the field, larval feeding did not influence plant growth as neither *S. montela* nor *A. alcinous* density affected the number of young leaves. However, *A. alcinous* was likely to have greater negative impact on plant biomass than *S. montela*, indicated by the negative effect of previous *A. alcinous* density on the number of total leaves. Hence, plants fed on by *A. alcinous* would provide less available food resource to subsequent herbivores than plants fed on by *S. montela*.

Responses of butterflies to age-dependent plant quality

Insect herbivores have higher performance when fed on young tissues. The underlying mechanisms involve physical, nutritional and secondary chemical traits of the plant tissue (Raupp and Denno 1983; Price 1991; Martinsen et al. 1998; Utsumi et al. 2009). Likewise, this study showed that

butterfly larvae had higher performance when fed on young leaves. This is probably because young leaves are nutritionally rich, and not because they are less chemically defended. In fact, C/N ratio was lower and water content was higher in young leaves, but aristolochic acid contents were not dependent on leaf age. On the other hand, we found no effect of damaged plants on RGR, suggesting that herbivore-induced qualitative changes in leaves were unlikely to occur, or may not be sufficient to influence larval growth. In another papilionid–*Aristolochia* system, previous damage induced aristolochic acid in *Aristolochia californica* leaves (Fordyce 2001), but increased aristolochic acid did not have negative impact on the larval growth of specialist butterfly *Battus philenor* (Dimarco et al. 2012). Since specialist herbivores generally have a tolerance to plant-specific defensive chemicals (Cornell and Hawkins 2003; Ali and Agrawal 2012), *S. montela* and *A. alcinous* larvae are unlikely to be affected by secondary chemicals such as aristolochic acids. Thus, even if an increase of aristolochic acids was induced by previous herbivory, this would be unlikely to have a large impact on larval growth of these butterflies.

In conclusion, our study revealed that herbivore-specific food demand of *S. montela* and *A. alcinous* determines the difference in subsequent biomass of *A. debilis*, because *A. debilis* growth was not changed by single or multiple *S. montela* and *A. alcinous* herbivory events. This may be due to a high tolerance of *A. debilis* to intensive herbivory, which often experiences high levels of defoliation. Consequently, the difference in subsequent plant biomass was determined by consumption by the different herbivores rather than the change in plant growth. Such a mechanism where plant biomass is determined by herbivore-specific food demand may be more common than previously thought because food demand differs among herbivore species sharing a host plant. Since plant growth following herbivory depends on the tolerance of the host plant, we need to separate the effects of consumption by herbivores and subsequent plant growth to understand the mechanisms by which herbivory affects plant biomass. Because both plant quantity and quality play a key role in the interactions among herbivores and in shaping the arthropod populations and communities on plants, understanding how species interactions between herbivores via plant quantity are modified by previous herbivory in a species-specific manner is a promising avenue for building a predictive framework of plant-mediated interactions.

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