Elucidation of Triadic Relationship Among Light Environment, Host Plant Quality, and Feeding Behavior of *Zizeeria maha* (Kollar) (Lepidoptera: Lycaenidae) IN *Oxalis corniculata* L. (Oxalidales: Oxalidaceae)

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Abstract

In the dynamics of light-plant-insect interaction, the light affects plant metabolisms which may directly influence the production of defensive secondary metabolites and may consequently alter the feeding behavior of herbivores. The present study aimed to investigate the triadic interactions by using *Oxalis corniculata* L. (Oxalidales: Oxalidaceae) and its specialist herbivore, *Zizeeria maha* (Kollar) (Lepidoptera: Lycaenidae), in relation to the light intensity of plant habitats and physicochemical properties of the plants which would affect the larval feeding behavior of *Z. maha*. Firstly, leaves of *O. corniculata* in the field with seven different light conditions were collected. A part of which was subjected to chemical analyses, and the rest was fed to *Z. maha* larvae to evaluate growth and feeding activity; larval period, death rate, weight, amount of consumption, and amount of frass were measured to calculate the relative growth rate, approximate digestion rate, and relative consumption rate. Secondly, light/shade mock environment test tests were conducted with laboratory-grown *O. corniculata*. The results under both field and laboratory conditions showed positive effects of light intensity on the production of the defensive compound, oxalic acid, in the plants. Furthermore, the larval feeding activity was higher when fed with leaves in higher light intensities. These results relate to our previous study that demonstrated oxalic acid stimulates the feeding of *Z. maha* larvae. Thus, the triadic interaction among light, *O. corniculata*, and *Z. maha* larvae could be explained by the light-driven up-regulated production of oxalic acid positively influenced the larval feeding.

Introduction

Environmental conditions, biotic and/or abiotic factors, greatly influence plant physiology (Takabayashi et al. 1994). Biotic factors include the effects of microorganisms (virulent or nonvirulent), insect herbivory, and animal grazing. Abiotic factors, such as light, water, soil, and temperature, are important for plant growth and reproduction. These environmental factors can alter the plant physiology and may affect the activities of herbivore insects (Scriber and Slansky 1981). Among all the environmental factors, light is known to have the greatest influence on plant existence and affect all aspects of growth and development (Herms and Mattson 1992; Kareiva et al. 1993; Schoonhoven et al. 2005).

The previous studies have shown that differences in light intensity of the plant environment would strongly affect insect behaviors. Bentz (2003) found that the shading level altered the *Rhododendron mucronatum* (Ericales: Ericaceae) nutrient and carbon: nitrogen (C: N) ratio. These changes in plant quality influenced the feeding, and oviposition preference of azalea lace bug, *Stephanitis pyriodes* (Heteroptera: Tingidae). Jansen and Stamp (1997) determined that the leaves of tomato (*Lycopersicon esculentum*: Solanaceae) grown in full sunlight had higher concentrations of allelochemicals, such as chlorogenic acid, rutin, and tomatine. These differences in qualities also greatly impacted the behavior and growth of a Solanaceae specialist, a tobacco hornworm (*Manduca sexta*: Sphingidae). A number of studies explain the triadic interaction among plants, light, and herbivores, based on particular chemicals (Crone and Jones 1999; Hemming and Lindroth 1999); however, chemo ecological evaluation of host selection in triadic relations is yet to be unknown..

Light affects plants in two different ways; quantitatively and qualitatively. The quantitative effect can be explained as the amount of the light quanta applied to the plants, equals light intensity and is often considered in terms of resource-based theory. The sunlight provides energy for all plant species to survive by converting solar energy into organic matter, such as carbohydrates, through the process of photosynthesis (Taiz et al. 2015). Since secondary metabolites are produced from primary metabolites, a reduction in light intensity is known to impair photosynthesis, with a consequent decline in primary carbohydrate production, and also leads to lower levels of secondary metabolites such as carbon-based metabolites, including phenolics and organic acids (Schoonhoven et al. 2005). On the other hand, the qualitative effect is due to the type of light, such as wavelength, and/or wave range, and has been extensively studied in connection with shade avoidance syndrome (SAS). SAS is a set of plant responses when the plants detect far-red radiation (FR), reflected by green tissues of neighbor's leaves. The plants modulate the defense response against consumers and emit plant hormones, such as ethylene salicylic acid and jasmonic acid (Franklin 2008; Fraser et al. 2016; Pierik and de Wit 2014).

Hence, plants have different response systems to the light intensity (quantitative) and light wavelength, and/or, wave range (qualitative). And in this study, we aimed to evaluate the quantitative light effect on the plant and its defense compound to the herbivore.

Larvae of the pale grass blue butterfly, *Zizeeria maha* (Kollar) (Lepidoptera: Lycaenidae), feed exclusively on *Oxalis corniculata* L. (Oxalidales: Oxalidaceae), which accumulates oxalic acid as with other Oxalidaceae species. In the course of our study on feeding stimulants of the *Z. maha* larvae showed a bell-shaped dose-response curve with a peak followed by a decrease with increasing doses of oxalic acid, a feeding stimulant, in the artificial diet (Yamaguchi et al. 2016). Such a decrease in feeding response was suggested as a result of aversive post-ingestive feedback to excess stimulants (David and Gardiner 1966). By such post-ingestive feedback, *Z. maha* larvae may be able to select leaves that contain a moderate, adequate concentration of oxalic acid in the field. In other words, larvae may detect the concentration of oxalic acid at the first bite of a leaf and may move to another for a preferable stimulus level. Variation in the intensity of light conditions where *O. corniculata* grows might cause changes in the concentration of oxalic acid in plant material, and that difference could then account for the feeding preferences of *Z. maha* larvae. If the light condition is positively correlated with the concentration of oxalic acid in *Oxalis* leaves. In that case, the observation that larvae occur more frequently on host plants growing in light shade may be a result of a larval preference of *Z. maha* for a moderate concentration of oxalic acid in the leaves. In this study, our objectives were to determine: (1) the correlation between oxalic acid and light intensity, both in the field and in artificial environments, and (2) the correlation between *Z. maha* larval feeding preferences and light intensity in *O. corniculata* populations. An investigation into the relationship between light conditions, oxalic acid concentration in *Oxalis* leaves, and feeding responses of *Z. maha* larvae would possibly be able to conduct and evaluate the chemical ecology of plant-environment and plant-insect interaction.

Methods And Materials

Field Selection and Measurement of Relative Photon Density

The field selection was done the University of Tsukuba, Tsukuba, Ibaraki, Japan (GPS: 36.10678911411827, 140.10188638634745). First, we investigated *O. corniculata* patches on the University of Tsukuba campus from April to June 2011. The study area of the *O. corniculata* patch was selected to have dimensions of 3 m × 3.5 m, and more than 500 g of fresh leaves in this patch were expected. The expected weight of leaves was calculated based on the measurement of the fresh weight of six randomly selected individuals of *O. corniculata* in the patch.

Quantum meters (LI-250 Light Meter, Li-Cor, USA) were used to determine the relative photon density (%) in the photosynthetically active wavelength range. Measurements were performed from 9:00 to 11:00 AM on a cloudy day, May 14, 2011. The relative photon density was measured at two different points at the same time with two quantum meters to compare the study area and the control area where there was nothing to block the light and was measured at six points in each study area. Relative photon density (%) was calculated from the following equation.

Relative photon density in the study area (%) = photon density of the study area ($\mu\text{mol m}^{-2} \text{s}^{-1}$) / photon density of the control area ($\mu\text{mol m}^{-2} \text{s}^{-1}$) × 100

There were differences in relative photon density among the seven study areas, and the study areas are designated as A to G in descending order of relative photon densities recorded. The average photon density among the control areas was $353 \mu\text{mol m}^{-2} \text{s}^{-1}$. The highest relative photon density in the area was 85.4%, and the lowest area was 9.5% (Fig. 1).

Growth of O. corniculata under two different artificial light conditions at laboratory

Oxalis corniculata plants were grown from the seeds in artificial light conditions inside an incubator.

The seeds of *O. corniculata* were collected from study area A on the campus of the University of Tsukuba, Tsukuba, Ibaraki, Japan. The soil was also collected from study area A and placed in four commercially available plastic planters (39.2 × 11.5 × 9.1 cm, Baby leaf planter: type 40, 4973655 81634-9, Richell Corporation, Toyama, Japan). Approximately 100 seeds were placed in one planter and cultivated under 100% relative photon density light conditions in an incubator (BioTRON, model LH120S, NK System, Osaka, Japan) under a 16-h light: 8-h dark photoperiod at 25°C and 60–70% relative humidity. When the stalks reached 1 cm, two of the four planters [designated as Light (L) 1 and Light 2] were further cultivated under light with 100% relative photon density. The other two planters [designated as Dark (D) 1 and Dark 2] were covered with a shading net (Dionet 1010, Dio Chemicals, Ltd., Tokyo, Japan) so that the relative photon density was reduced to 9% and were cultivated as dark conditions.

Chemical Analysis: Oxalic Acid

The leaves of *O. corniculata* were collected from August to September of 2011 at the University of Tsukuba (Tsukuba, Ibaraki, Japan).

Fresh trifold leaves of *O. corniculata* were cut into small pieces with the scissors and soaked in 5 mL of 80% ethanol, which is generally used for the extraction of several organic acids, for two days at room temperature. Then extracts were centrifuged (H-36, Kokusan Co. Ltd., Osaka) for 15 minutes at 3000 rpm, and the supernatant was carefully transferred into the new glass tube. The samples were evaporated with the centrifugal evaporator (CVE-2000, Eyela, Tokyo) at 55°C, for a day. A dried sample after trimethylsilylation by MSTFA was analyzed to identify oxalic acid *bis*-TMS ester. Quantification of oxalic acid was done by HPLC/UV-Vis.

Mass spectra were obtained by GC-MS. Samples were injected into a Shimadzu GC (Shimadzu Co., Ltd., Kyoto, Japan) equipped with a fused silica capillary column (Rtx-5MS, 0.25 mm × 30 m, 0.25µm film thickness). Samples were injected in the splitless mode (sampling time: 1 min) at an injection port temperature of 280°C. Helium was used as the carrier gas at one mL/min in the constant flow mode. The oven temperature was set at 80°C for 2 min, then raised to 250°C at 5°C/min, and held at the final temperature for 10 min. The column outlet was introduced at 250°C into an MS-QP2010 (Shimadzu Co., Ltd., Kyoto, Japan). The temperature of the ionization chamber was 200°C, and the ionization was performed in the electron impact mode at 70eV. The data were analyzed with Wiley RegistryTM of Mass spectra library, 9th edition (Wiley, Hoboken, U.S.A.) software.

HPLC was obtained by LC- 10Avp Series equipped with a UV-Vis detector (Shimadzu, Kyoto, Japan) and was analyzed at 210 nm. Samples were injected into an ODS column (Inert Sustain C18, particle size five µm, 4.6 × 250 mm, GL Science, Tokyo, Japan). The oven temperature was at 40°C. The samples were analyzed with the isocratic mode, and the mobile phase was 10 mM NH₄H₂PO₄ in pH 2.6 at 1.0 mL/min flow.

Plant Extraction and Chemical Analysis for laboratory grown O. corniculata

As a representative chemical affected by different light conditions, oxalic acid in the leaves of *O. corniculata* was analyzed chronologically by GC-FID and GC-MS. Specifically, three trifoliate leaves were randomly selected from each of L1 and L2 (light condition replicates), and D1 and D2 (dark condition replicates), and collected on each of the following days; day 0 (at the start of the experiment, when the stalks were equally 1 cm), day 3, day 14, day 28, and after incubation. The leaves were weighed and cut into small pieces with scissors and immersed in 5 mL of 70% methanol, which is generally used for the extraction of general phenolics as well as several organic acids. The samples were extracted for two days at room temperature, around 25°C.

Part of the methanol extract (100 µL) was placed in a conical glass vial (GL Sciences, Inc., Tokyo, Japan), dried in a glass tube oven (GTO-250RN, Shibata, Saitama, Japan) at 40°C and then analyzed by gas chromatography-flame ionization detection (GC-FID) as *tert*-butyldimethylsilyl (TBDMS) derivatives (Knapp 1979). Samples were reacted with a mixture of

50 µL *N*-methyl-*N*-*tert*-butyldimethylsilyl-trifluoroacetamide (MTBSTFA; Sigma-Aldrich Co. LLC., St Louis, MO, U.S.A.) and 50 µL acetonitrile at 60°C for one hour. Oxalic acid in the methanolic extract was quantified by GC-FID against a known concentration of *bis*-TBDMS oxalate prepared with authentic oxalic acid. Both the GC quantification method and MS procedure are described in Chemical Analysis of oxalic acid.

Insect

Adults and larvae of *Z. maha* were collected at the University of Tsukuba (Tsukuba, Ibaraki, Japan).

The adult butterflies were fed with 7% sucrose solution once per day, kept under laboratory conditions (16-h light: 8-h dark photoperiod at 25°C and 60–70% relative humidity) with the host plant, and allowed to lay eggs. Newborn and field-collected larvae were reared on fresh host plants (*O. corniculata*) until the molt for the third instar in an incubator (BioTRON, model LH120S, NK Systems, Osaka, Japan) under a 16-h light: 8-h dark photoperiod at 25°C and 60–70% relative humidity.

A feeding experiment with *Oxalis corniculata* growing under different light conditions among study areas

Larvae were fed with *O. corniculata* leaves collected from each study area to evaluate the effects on growth and feeding activity.

Feeding experiments were performed from August to September of 2011. One group of three third instar larvae was placed in a plastic petri dish (90 mm diameter, 15 mm height) lined with moistened filter paper. Three groups were used as replicates for a sample. Each group was offered one to four leaves and kept in an incubator as described above. The leaf samples fed to the larvae were collected from each study area every day or every other day. The leaf samples were weighed before they were fed. Frass pellets were collected daily from each group, dried at 60°C until constant weights were obtained, and weighed to evaluate the feeding activities. The larval period (day), the death rate (%), weight (F.W. g), amount of consumption (D.W. g), and amount of frass pellets (D.W. g) were measured. Relative growth rate, approximate digestion rate, and relative consumption rate were calculated using Scriber's method (Scriber 1978).

$$RelativeGrothRate = \frac{LarvalWeightGain (FWg)}{AverageofLarvalWeight (FWg) \times LarvalPreriod (day)} \times 100$$

$$ApproximateDigestionRate = \frac{IngestedAmountofLeaves (DWg) - MassFrassWeight (DWg)}{IngestedAmountofLeaves (DWg)} \times 100$$

$$RelativeFoodConsumption = \frac{IngestedAmountofLeaves (DWg)}{AverageofLarvalWeight (FWg) \times LarvalPeriod (day)} \times 100$$

Larval food consumption was measured as the difference in dry weight of the leaves before and after feeding.

A feeding experiment with Oxalis corniculata growing under two different artificial light conditions at the laboratory

The larvae were fed with leaves grown under two different artificial light environments, Light (L1 and L2) and Dark (D1 and D2), to investigate the feeding response of *Z. maha* larvae effect by host plant quality grown under different light environments.

The larval feeding activities were evaluated by five parameters (larval period, larval weight, mean ingested amounts of leaves, mean frass weight, larval death rate), and correlations with light intensities of the seven study areas were calculated. Two parameters that showed positive correlations between light intensity mean frass weight and mean ingested amount of leaves were selected to represent larval feeding activity.

A group of three third-instar larvae was placed in a plastic Petri dish (90 mm diameter, 15 mm height) lined with moistened filter paper. Five groups with a total of 15 larvae were used as a sample. Each group was offered one to two leaves from light (L1 and L2) and dark (D1 and D2), which were cultivated after 28 days. Frass pellets were collected daily from each group, dried at 60 °C until constant weights were obtained, and weighed to evaluate feeding activities. One gram of fresh leaves (light- or dark-treated) was placed in the Petri dish and fed to the larvae for 24 h. The leaves were then weighed to determine the amounts eaten by the larvae. All larval feeding tests were continued until pupation (20–25 days).

Statistics and correlation analyses

Frass weights and ingested amounts obtained from the two groups were compared with the statistical software EZR (Easy R, version 1.29; [Kanda \(2013\)](#)). The data from the bioassay were arcsine-transformed if necessary and statistically tested by one-factor ANOVA with post-hoc *Dunnnett T3*, $P < 0.05$. The quantification of oxalic acid was statically tested with one-factor, $P < 0.05$.

To investigate the relationships among environmental factors, the feeding activity of *Z. maha* larval, and chemical properties of *O. corniculata*, the means of relative photon density among the study areas, and the mean contents of oxalic acid were analyzed by *Pearson correlation* using EZR software (Easy R, version 1.29; [Kanda \(2013\)](#)). First, the relationships between photon density and chemical contents were analyzed. Then, the relationships between larval feeding parameters and chemical contents were analyzed.

Results

Insect and Feeding Experiments

There were significant differences in the *mean ingested amount* (Fig. 2) and the *relative growth rate* (Fig. 3) among the study areas. In the *mean ingested amount*, site E and F, and site E and G showed significant differences among the study areas ($P < 0.05$, ANOVA, post-hoc: *Tukey*). Site F and G also showed a marginal difference among the study areas. In the *relative growth rate* there was a significant difference between site A and D, site A and E ($P < 0.05$, ANOVA, post-hoc: *Tukey*). However, there were no significant differences in the approximate digestion, relative food consumption, larval period, larval weight, and larval death rate.

Chemical Analysis: Organic Acids

After TMS derivatization, GC-MS analysis of the ethanol extract yielded a major peak at a retention time of 4.50 min, exactly the same as that of *bis*(trimethylsilyl) oxalate prepared from standard oxalic acid. The mass spectrum of this compound also showed characteristic ions of *bis*(trimethylsilyl) oxalate. Thus, we confirmed that the major compound in the extract was oxalic acid. Quantifying the oxalic acid in leaves of *O. corniculata* by HPLC/UV-Vis showed a significant difference in oxalic acid among the study areas. Area A showed the highest (3.7 µg/mg), and area F was the lowest (1.3 µg/mg) concentration among the study areas (Fig. 4).

Correlation Analysis

There were positive correlations between the relative photon density where *O. corniculata* grows, and the contents of oxalic acid ($r = 0.86$, $P = 0.013$) in the *O. corniculata* (Table 1, Fig. 5a). Among the eight parameters of larval feeding activities (Table 2), three parameters (i.e., ingested amount, frass weight (Fig. 5b), and relative growth rate (Fig. 5c) had positive correlations to the concentration of oxalic acid ($P < 0.05$).

Table 1
Correlation analyses between relative photon density among the study areas and oxalic acid in *O. corniculata*

Parameters	Relative photon density		
	r	P-value	sig.
Oxalic Acid	0.861	0.013	*
r: Pearson's correlation coefficient			
sig.: significance level, * $P < 0.05$			

Table 2
Correlation analysis between phytochemicals in *O. corniculata* and *Z. maha* larval feeding parameters

Larval feeding parameters	Oxalic acid		
	r	P-value	sig.
Larval Period	-0.174	0.708	
Larval Weight	-0.163	0.726	
Ingested Amount	0.950	0.001	**
Frass Weight	0.880	0.008	**
Death Rate	-0.625	0.133	
Relative Growth Rate	0.934	0.002	*
Approximate Digestion Rate	-0.074	0.875	
Relative Food Consumption	0.608	0.148	
r: Pearson's correlation coefficient			
sig.: significance level, * $P < 0.05$, ** $P < 0.01$			

Chemical Analysis: Oxalic Acid

On day 0, the oxalic acid concentrations in the leaf showed no difference between the light (L1: 3.72 µg, L2: 2.54 µg) and dark (D1: 2.42 µg, D 2: 2.78 µg) conditions. However, oxalic acid starts decreasing after 14 days of cultivation (D1: 1.51 µg, D 2: 0.85 µg).

The amount of oxalic acid in the leaves after 28 days of cultivation under the light condition was approximately twice as high (L1: 2.61 µg, L2: 2.38 µg) as which obtained under the dark condition (D1: 0.92 µg, D 2: 0.84 µg) (Fig. 6).

Feeding Assay

The larva which fed on the leaves cultivated with the light condition produced 22.1 mg of the mean frass weight (mean ± SE: 22.1 ± 5.1 mg; $N = 5$, $P = 0.044$, *t-test*, one-tailed). And it was significantly higher than the larva which fed on the leaf cultivated under the dark condition (16.7 ± 3.37 mg; $N = 5$) (Fig. 7).

There was a very slight trend toward significance in the mean ingested amount between the larvae which fed with the leaf cultivated with light condition (mean ± SE: 0.60 ± 0.21 g; $N = 5$, $P = 0.13$, *t-test*, one-tailed) and dark condition (0.46 ± 0.15 g; $N = 5$) (Fig. 8).

Discussion

Our study is the first example of a chemo ecological evaluation of host selection in triadic relations to the best of our knowledge. We evaluated the relationship between the light conditions, oxalic acid concentration in *Oxalis* leaves, and the feeding responses of *Z. maha* larvae by determining (1) the correlation between oxalic acid and light intensity, both in the field and in the artificial environments, and (2) the correlation between the light intensity where *O. corniculata* grows and feeding preference of *Z. maha* larva.

In the laboratory experiments, chronological analyses clearly showed that oxalic acid content decreased after shading treatment (dark condition). This is probably due to the slowdown of oxalic acid biosynthesis by low photosynthetic activity under a low light environment. It was concluded that light intensity had the highest impact on the differences in the quantity of the major secondary metabolite, oxalic acid, in *O. corniculata*. Oxalic acid in *O. corniculata* showed positive correlations with light intensity in both field and laboratory.

We strongly consider that the tangible example of light (quantitative), defense chemical, and herbivore response study would help to elucidate the whole defense (induce defense + constitutive defense) system. And we believe that our study would be one of the big pieces to elucidate this whole defense system of the plants in the future.

In the interaction among light environment, phytochemical, and phytophagous insects, the choice of a mother butterfly is the first step of host selection. Therefore, to draw a whole picture of triadic relationships, including host selection, investigating the behavior of the mother butterfly is demanded.

In our preliminary study of oviposition preference, females of *Z. maha* preferred *O. corniculata* growing under higher-light intensity in the field. This preference was also reproduced in the artificial light environment. These results suggest that the growing environmental condition of *O. corniculata* affects the oviposition choice of the *Z. maha* female butterfly. However, the chemical factor which changed the oviposition preference of the female *Z. maha* butterfly remains unknown. Evaluation of chemical stimuli of oviposition preference in *Z. maha* female butterfly would shed light on host plant selection in this species.

Our results show that larval host selection also plays a necessary part in *Z. maha* species. Our previous study found that oxalic acid is the larval feeding stimulant and larva feeding activity needs to start in the appropriate range of oxalic acid (65–260 $\mu\text{mol/g}$, corresponding to 0.25–1.0 g.l.e./g)(Yamaguchi et al. 2016). Larva immediately escapes from the artificial diets when it contains 2.0 g.l.e./g (520 $\mu\text{mol/g}$) or more of oxalic acid. We expect this larval choice to be observed in the field as a preference for leaves containing appropriate oxalic acid contents. These observations suggest that there is strict host selection by larvae as well as adults of *Z. maha*. Further experiments and investigation of preferences in both larvae and adults are desired.

From these results, we developed the following triadic relationship: the first identification of the oxalic acid in *O. corniculata* as a feeding stimulant for *Z. maha* larvae, evaluation of the positive effect of light intensity on the production of oxalic acid in *O. corniculata*, and discovery of a positive correlation between light intensity of host plant habitat and larval feeding activity. Additionally, we would like to present the ecological views and the evaluation of the oviposition behavior of the *Z. maha* butterfly in the future. We expect that this study will contribute to the understanding of biodiversity, coevolution, and applied entomology.

Declarations

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Data Availability

All data are available but not currently deposited in any public repository.

Conflicts of Interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Figures

Figure 1

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