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Oxalic acid as a larval feeding stimulant for the pale grass blue butterfly *Zizeeria maha* (Lepidoptera: Lycaenidae)

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Abstract

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. Larvae were stimulated to feed on artificial diets containing a crude methanolic extract from host plant leaves. Fractionations and bioassays revealed that the strong feeding activity was found in the water layer, from which oxalic acid was detected as a major compound. Removal of oxalic acid as calcium oxalate precipitates by addition of calcium chloride into the water layer resulted in a significant decrease in feeding activity on the filtrate. Re-addition of oxalic acid to the filtrate recovered the feeding activity. The addition of 260 μmol oxalic acid, corresponding to 1 g fresh leaves of *Oxalis*, to 1 g of artificial diet significantly stimulated feeding compared with the intact artificial diet. Therefore, oxalic acid was concluded to be a major feeding stimulant for *Zizeeria maha* larvae.

Keywords Oxalic acid, Feeding stimulant, *Zizeeria maha*, *Oxalis corniculata*, Lycaenidae

Introduction

The pale grass blue, *Zizeeria maha* (Kollar) (Lepidoptera: Lycaenidae), is a small butterfly distributed widely throughout Asia, from Japan (except Hokkaido island in the north) to southern Iran. *Z. maha* larvae feed on a limited number of plant species of the genus *Oxalis* in the family *Oxalidaceae*. Out of eight wild and

naturalized species of *Oxalis* in Japan (Shimizu 1982), two species including three formae, namely *Oxalis corniculata* L., *O. corniculata* L. f*. atropurpurea* (Planch.) Van Houtte ex Hegi, *O. corniculata* f. *erecta* Makino, *O. corniculata* L. f*. rubrifolia* (Makino) H. Hara, and *O. dillenii* Jacq., are described as host plants for *Z. maha* (Shirouzu 2006).

Oxalis species are common perennial plants that can be found as roadside weeds and on the margin of cultivated fields. The species are characterized by the biosynthesis and accumulation of oxalic acid in leaves (Shimizu 1982). Owing to its acidity and ability to bind dibasic cations such as calcium and magnesium, oxalic acid is toxic to mammals and causes hypocalcemia (Lynn 1972), kidney stone formation (Gershoff and Prien 1967), and chronic food poisoning of sheep (Seddon and Ross 1929). In insects, oxalic acid is an antibiotic factor that inhibits larval growth of *Helicoverpa armigera* (Lepidoptera: Noctuidae) (Yoshida et al. 1995) and is a sucking inhibitor of the brown planthopper, *Nilaparvata lugens* (Homoptera: Delphacidae) (Yoshihara et al. 1980). Therefore, oxalic acid can be regarded as a defensive chemical for both mammal and insect grazing. However, *Z. maha* evidently evolved a mechanism to overcome this defensive barrier.

In this study, we examined the effects of host plant extracts on feeding responses of *Z. maha* larvae, in order to gain insights into the mechanism by which oxalic acid toxicity is avoided.

Materials and methods

Insects

Adults and larvae of *Z. maha* were collected on the campus of University of Tsukuba, Tsukuba, Ibaraki, Japan. The adult butterflies were fed with 7% sucrose solution once per day and kept under laboratory conditions with the host plant and allowed to lay eggs. New-born and field-collected larvae were reared on fresh host plants (*Oxalis corniculata*) until the molt for the 3rd instar in an incubator (BioTRON, model LH120S, NK system, Osaka, Japan) under a 16L:8D photoperiod at 25 °C and 60–70% relative humidity.

Plant material, extraction and fractionation

The leaves of *O. corniculata* were collected from full sun and partial sun area at the campus of University of Tsukuba, Tsukuba, Ibaraki, Japan in August 2014. Larvae of *Z. maha* do not usually feed petioles and peduncles, thus the fresh trefoil leaves of *O. corniculata* (247.5 g) were extracted twice with 1.6 L of 70% ethanol for 2 weeks at 25 °C. Combined extracts were evaporated to dryness under an aspirator vacuum by a rotary evaporator (water bath temperature less than 40 °C). The residue (13.32 g) was dissolved in 350 ml of 70% methanol and stored as a stock solution at −25 °C. For fractionation, one-tenth (35 mL) of the stock methanolic solution was dried up and resuspended in 100 mL distilled water, then successively extracted with diethyl ether (100 mL, three times) and ethyl acetate (100 mL, three times) as shown in Fig. 1. Organic layers were dried over sodium sulfate and solvents were evaporated, weighed and stored in a freezer (−25 °C). The water layer was evaporated, weighed and stored as an aqueous solution in a refrigerator (4 °C).

Removal and re-addition of oxalic acid in the water layer

Oxalic acid in solution is known to precipitate as calcium oxalate upon addition of calcium ion. Given the low solubility of oxalic acid in water (0.67 mg/100 mL, 20 °C; Hagler and Herman 1973), addition of calcium chloride solution to a solution containing oxalic acid followed by filtration and weighing the calcium oxalate precipitate is one of the methods used historically to quantify the oxalic acid content in plant materials (Baker 1952). We used this method to remove the oxalic acid that was expected to be present in the water layer. In advance of the addition of calcium chloride (Kanto Chemical Co., Inc., Tokyo, Japan), the concentration of oxalic acid in the water layer was estimated by gas chromatography after derivatization of an aliquot. To a 100 mL aliquot of the water layer containing 24.75 grams leaf equivalent (g.l.e.) of extract, calcium chloride (0.69 g, 6.2 mmol) was added portion-wise while swirling manually. The solution was left overnight and filtered through 1 μm filter paper (No. 4, Kiriyama Glass Works Co., Tokyo, Japan). Thus obtained filtrate, with oxalic acid removed from the water layer, was divided into two portions for use in the feeding tests: one was subjected to the feeding test unmodified (OA removed); to the other portion 0.28 g (3.1 mmol) oxalic acid (anhydrous, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was dissolved in the solution before the feeding test (OA re-added). Larval feeding activity with the OA removed and OA re-added water layers was compared with the artificial diets prepared as described below. In addition, pH values of the water layer before and after addition of calcium chloride were measured and the effect of hydrogen chloride liberated by OA removal was

tested against *Z. maha* larvae with an artificial diet containing hydrochloric acid (6.2 mmol) in the control diet as described below.

Artificial diet for feeding tests

A control diet was prepared by mixing 5.0 g of commercially available artificial diet (Insecta F-II, Nosan Corporation Life-Tech Department, Yokohama, Japan), 2.5 g agar powder (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 17.25 ml distilled water (total weight: 24.75 g). The mixture was heated to near boiling with a microwave, then placed in a refrigerator and used as a control diet.

Test diets were prepared by addition of crude methanolic extract or fractions from *O. corniculata* as aqueous solutions (or suspensions) to the artificial diets. For example, 24.75 g.l.e. crude methanolic extract (35 mL of the stock solution), after being dried up, was resuspended in 17.25 mL water and mixed with 5.0 g Insecta F-II and 2.5 g agar powder to prepare a test diet of the crude methanolic extract at a concentration of 1.0 g.l.e./g of diet.

In order to test feeding responses of *Z. maha* larvae to artificial diets containing various amounts of oxalic acid, oxalic acid was dissolved in 17.25 mL water and mixed as above to prepare the test diets in the range of 65 to 1040 μmol/g (corresponding to 0.25 to 4.0 g.l.e/g) of diet.

Feeding test

Bioassays for feeding activity of chewing insects can be set up by combinations of substrates (filter paper, artificial diet, plant tissues, etc.) and types of data

collection (weight or area consumed, number of chewing marks, weight gain of the test insect, number or weight of feces pellets produced, direct and/or indirect observation, electrophysiological responses, etc.) (Hare JD 1998; Lewis AC and Van Emden HF 1986). Depending on the nature of feeding habit of test insect, not all of these experimental components can be applied. Preliminary tests revealed that *Z. maha* larvae were unable to chew neutral substrates such as filter papers or other cellulose based thin discs. Therefore, direct measurements of amount ingested by measuring area consumed or by counting chewing marks were not applicable for our feeding assay. Trials with artificial diets containing crude host plant extract showed that neonate to 2nd instar larvae were unable to feed on the artificial diets presumably because their mandibles were too small. On the other hand, the 3rd instar larvae immediately after molting, when the dorsal nectary organ was observed, were able to continue feeding on the artificial diet. With this success, we set up our feeding test to identify stimulants responsible for continuous feeding by using a combination of artificial diet as substrate and frass measurement as data collection in no choice situation. One group of three 3rd instar larvae was placed in a plastic Petri dish (90 mm diameter, 15 mm height) lined with moistened filter paper. Five groups were used as replicates for a sample. Each group was offered one to four pieces of diet (30 mg per piece) and was kept in an incubator as described above. Frass pellets were collected daily from each group, dried at 60 °C until constant weights were obtained and weighed to evaluate the feeding activities. In the test with OA removed and OA re-added water layer, ten 3rd instar larvae were tested individually. All larval feeding tests were continued until pupation (20–25 days).

157

158 Statistics

159 Statistical analyses were performed with the statistical software EZR (Easy R,
160 version 1.29; Kanda 2013).

161

162 Chemical analyses

163 Aliquots of the crude methanolic extract and fractions obtained by liquid–liquid
164 partitioning were analyzed by gas chromatography–flame ionization detection
165 (GC-FID) and by gas chromatography–mass spectrometry (GC-MS) as *tert*-
166 butyldimethylsilyl (TBDMS) or trimethylsilyl (TMS) derivatives (Knapp DR 1979).
167 Samples were placed in glass conical vials (GL Sciences, Inc., Tokyo, Japan)
168 and dried, reacted with a mixture of 50 μL *N*-methyl-*N*-*tert*-
169 butyldimethylsilyltrifluoroacetamide (MTBSTFA; Sigma-Aldrich Co. LLC., St
170 Louis, MO, U.S.A.) and 50 μL acetonitrile at 60 °C for 1 h. For TMS derivatives,
171 *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Sigma-Aldrich) was used
172 instead of MTBSTFA in the above reaction.

173 GC-FID was conducted on a HP6890 gas chromatograph (Hewlett-Packard,
174 Palo Alto, CA, U.S.A.) equipped with a fused silica capillary column (HP-5MS, 30
175 m × 0.25 mm, 0.25 μm film thickness). Samples were injected in the splitless
176 mode (sampling time: 1 min.) at an injection port temperature of 280 °C. Helium
177 was used as the carrier gas at 1 mL/min in the constant flow mode. The oven
178 temperature was set at 50 °C for 1 min, then raised to 280 °C at 10 °C/min, and
179 held at the final temperature for 10 min. A flame-ionization detector (FID) was

operated at 280 °C and the chromatograms were analyzed with HP ChemStation software.

Mass spectra were obtained by GC-MS. Samples were injected into a HP6890N gas chromatograph operated in the same condition as GC-FID, except that the column (DB-5MS, 25 m × 0.25 mm, 0.25 μm film thickness) outlet was introduced at 280 °C into a MS-600H mass spectrometer (JEOL, Tokyo, Japan). The temperature of the ionization chamber was 190 °C and the ionization was performed in the electron impact mode at 70 eV. The data were acquired in scan mode (scan range 40–600 amu, scan speed 0.29 s) and analyzed with TSS2000 software (version 2.00, Shrader Analytical and Consulting Laboratories Inc., Detroit, MI, U.S.A).

Results

Feeding responses of *Z. maha* larvae to plant extracts and fractions

Larval feeding was significantly stimulated by addition of the crude methanolic extract to the artificial diet, indicating the presence of feeding stimulant(s) in the extract. Mean dry weight of the frass obtained from larvae fed with a diet containing the crude methanolic extract was 2.2 times higher (mean ± SE: 36.8 ± 7.70 mg; $N = 5$, $p < 0.05$, *t*-test) than that obtained from the control treatment (16.7 ± 2.80 mg; $N = 5$). After fractionation of the methanolic extract into three layers, significantly enhanced feeding activity was restricted to the water layer only. Namely, a significantly larger amount of frass was produced by the larvae fed with an artificial diet containing water layer (78.7 ± 6.41 mg; $N = 5$, $p < 0.05$, Steel test against the control, two-tailed; Fig. 2). In contrast, larvae fed with the

204 artificial diet containing the ether layer produced much less frass (5.2 ± 1.59 mg;
205 *N* = 3), although the difference was marginally significant (*p* = 0.07, Steel test
206 against control, two-tailed).
207
208 Chemical analyses
209 After TBDMS derivatization, GC-MS analysis of the active water layer yielded a
210 major peak (63.2% of the total peak area) at a retention time of 13.46 min (Fig.
211 3). The mass spectrum of this compound showed characteristic ions of bis(*tert*-
212 butyldimethylsilyl) oxalate at m/z 303 (relative intensity: 2.0%, M^+-15), 261
213 (53.6%, M^+-57), 189 (15.1%), 147 (67.1%), and 73 (100%). By comparisons of
214 retention times and mass spectra using both TBDMS and TMS derivatives of an
215 authentic compound, the major compound in the water layer was identified as
216 oxalic acid.
217 Oxalic acid in the methanolic extract was quantified as the TBDMS derivative
218 by using GC-FID against a known concentration of bis-TBDMS oxalate prepared
219 with authentic oxalic acid. Fifteen samples of the methanolic extract yielded oxalic
220 acid ranging from 2.9% to 9.9% (mean ± SE: 7.2 ± 2.7) of the dry weight of *O.*
221 *corniculata* leaves.
222
223 Effects of removal and re-addition of oxalic acid in the water layer on larval
224 feeding
225 A significantly lower mean frass weight (15.0 mg/larva) was obtained from larvae
226 fed with the OA removed artificial diet, whereas 1.75 times higher weight of frass
227 (26.3 mg/larva) was produced by larvae fed with the OA re-added artificial diet (*p*

228 < 0.01, *t*-test, two-tailed; Fig. 4). Upon addition of calcium chloride to water layer,
229 pH value slightly decreased from 1.4 to 1.3. Mean dry frass weight obtained from
230 the larvae fed with artificial diet containing hydrochloric acid was 12.4 mg per
231 larva which was not significantly different from that of the control.
232
233 Feeding responses of *Z. maha* larvae to artificial diets containing various
234 amounts of oxalic acid
235 Dry frass weights were increased with increasing concentration of oxalic acid in
236 the artificial diets and reached the maximum (59.5 mg, $p < 0.05$, Steel test against
237 the control, one-tailed) at 1.0 g.l.e (260 μmol) of oxalic acid per gram of artificial
238 diet. However, addition of oxalic acid at 520 μmmol/g and higher resulted in less
239 frass production compared with that of the control (520 μmol/g: 9.60 mg, 780
240 μmol/g: 0.17 mg, 1040 μmol/g: 0.08 mg; Fig. 5).
241
242 **Discussion**
243 In this study, we characterized oxalic acid for the first time as a lepidopteran larval
244 feeding stimulant for *Z. maha* from its major host, *O. corniculata*.
245 Oxalic acid was one of the first acids identified, and was discovered in 1796
246 from wood sorrel (*Oxalis acetosella* L.) by Johann Christian Wiegleb (Vaneker
247 2015). The free acid and its salts have been found from a variety of organisms,
248 such as plants, animals including humans, fungi and microorganisms (Vaneker
249 2015). More than 215 plant families are known to accumulate calcium oxalate in
250 their tissues (McNair 1932), indicating that oxalic acid is ubiquitously distributed
251 in the plant kingdom. In *Oxalis*, oxalic acid comprising some 16% of the dry weight

252 appears to be present in vacuoles largely as the free acid (Ranson et al. 1965).
253 Levels of oxalates in *O. corniculata* are reported to be 4.1% soluble and 7.0%
254 total oxalate (Libert and Franceschi 1987). The present data on oxalic acid
255 content of *O. corniculata* are consistent with the above descriptions both
256 qualitatively and quantitatively. Oxalic acid was present in the biologically active
257 water layer mainly as the free acid, hence it was easily derivatized to its bis-
258 TBDMS ester and detected in the range of 2.9% to 9.9% (dry weight of leaves).
259 Furthermore, upon addition of $CaCl_2$ to the water layer, which is one of the well-
260 established procedures for quantitative analysis of oxalic acid (Hodgkinson 1970),
261 the water layer became turbid and fine precipitates of calcium oxalate were
262 obtained. After removal of oxalic acid as calcium oxalate from the active water
263 layer, the filtrate showed reduced stimulation of feeding activity (mean dry frass
264 weight; 15.0 mg/larva), which was comparable to the control. Re-addition of
265 oxalic acid to the filtrate significantly enhanced the feeding activity (26.3 mg/larva;
266 Fig. 4). These results supported our hypothesis that oxalic acid elicits a feeding
267 response by *Z. maha* larvae. Finally, the results of dose-response feeding
268 bioassays with authentic oxalic acid (Fig. 5) led us to the conclusion that oxalic
269 acid is a feeding stimulant of *Z. maha* larvae.

270 Although the outcome of feeding experiments is dependent upon their design
271 (e.g., not all experiments offer a wide range of doses of a single stimulant),
272 feeding responses in dose-response experiments may be classified into two
273 types. The first type represents a typical sigmoidal dose-response curve, as in
274 the case of the olive weevil to (-)-olivil (Kadowaki et al. 2003). The second type
275 shows a bell-shaped dose-response curve with a peak followed by a decrease

with increasing dose of a stimulant (David and Gardiner 1966; Hicks 1974; Yamamoto and Fraenkel 1960). *Z. maha* larvae showed the latter type of feeding response toward the increasing dose of oxalic acid in the artificial diet (Fig. 5). A declining response at a higher dose may be simply explained as a toxic effect of excessive amounts of the stimulant because the larvae stopped feeding and eventually died. On the other hand, such decrease in feeding response may be the result of aversive post-ingestive feedback to excess stimuli (Provenza 1995). 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. The chemical composition of plants often changes with age, exposure to sunlight, or other environmental factors (Flück 1963; Hemming and Lindroth 1999). Thus, oxalic acid may vary in concentration among individual host plants or among leaves of an individual plant. Plants grown under a high-light environment tend to invest surplus carbon (excess for growth) in secondary metabolites. If the light condition is positively correlated with the concentration of oxalic acid in *Oxalis* leaves, the observation that larvae occur more frequently on host plants growing in light shade (personal observation by MY) may reflect a preference of *Z. maha* larvae for a moderate concentration of oxalic acid in the leaves. Investigation of the triadic relationships among light condition, oxalic acid concentration in *Oxalis* leaves, and feeding response of *Z. maha* larvae is ongoing in our laboratory.

One of the primary functions of oxalic acid in plants is considered to be chemical defense against herbivores. Oxalic acid has been reported as a sucking inhibitor of brown planthopper, *Nilaparvata lugens* (Homoptera: Delphacidae) (Yoshihara et al. 1980). Yoshida et al. (1995) tested oxalic acid as a part of

300 resistant mechanism of chick pea, *Cicer arietinum* L., to *Helicoverpa armigera*
301 (Lepidoptera: Noctuidae) and found that larval weight after 10 days was reduced
302 to 53% of control when the larvae were fed with semi-artificial diet containing 250
303 µmol/g oxalic acid. Likewise, a number of oxalate-induced impacts on animals,
304 including humans, have been reported (Duncan et al. 1997; Lynn 1972). These
305 include chronic poisoning of sheep (Bull 1929), death of human due to ingestion
306 of oxalate containing plant, *Rumex crispus* (Reig et al. 1990). In mammalian
307 species, LD_{50} values of oxalic acid for rats were determined as 475 mg/kg for
308 males and 375 mg/kg for females by single oral administration (Vernot et al. 1977).
309 By the same method, they reported LD_{50} values of allyl isothiocyanate (mustard
310 oil, released by enzymatic action on Sinigrin) and nicotine sulfate as 490 mg/kg
311 and 75 mg/kg respectively. These data supports that oxalic acid serves as a
312 defense against herbivores. In the present feeding experiment, *Z. maha* larva
313 ingested about 75 µmol (6.8 mg) oxalic acid during the test period (250–300 mg
314 of artificial diet containing 0.25 mmol/g oxalic acid was consumed). Given that
315 the mean body weight of 3rd instar larvae was 5.6 mg at the beginning of the
316 feeding test, it is surprising that a *Z. maha* larva was able to ingest a quantity of
317 oxalic acid that exceeded its own body weight, which indicates that this insect is
318 highly adapted to *Oxalis* as a specialist herbivore.

319 For specialist herbivores, so-called secondary plant metabolites, such as
320 cyanide, cyanogenic glycosides, alkaloids, glucosinolates, terpenoids, coumarins
321 and cardenolides, serve as positive cues (Rosenthal and Berenbaum 1991).
322 Oxalic acid is no exception to this. Verschaffelt (1910) observed that the
323 Polygonaceae-feeding leaf beetle *Gastroidea viridula* Deg. (Coleoptera:

Chrysomelidae) gnawed the leaves of *Lathryrus sylvestris* (non-host) when the leaves had been immersed for some time in a solution of oxalic acid. Subsequently, Renner (1970) confirmed that the feeding of *G. viridula* was stimulated by oxalic acid. Matsuda and Matsumoto (1975) tested a variety of organic acids, including oxalic acid, against four species of Polygonaceae-feeding leaf beetles to analyze host plant specificity. The authors observed that the feeding of *Gallerucida bifasciata* Motschulsky (Coleoptera: Chrysomelidae) was stimulated by oxalic acid, as well as by malic, tartaric and citric acids. Studies of interactions between herbivorous insects and plant secondary metabolites have been conducted and have made noteworthy progress, especially in three families of butterflies, namely Papilionidae (Feeny 1991; Honda et al. 2011; Li et al. 2010; Murata et al. 2011; Nakayama et al. 2003; Nishida et al. 1987; Nishida and Fukami 1989a; Nishida and Fukami 1989b; Ono et al. 2000), Pieridae (Honda et al. 1997; Huang et al. 1993; Renwick and Lopez 1999; Renwick and Radke 1983), and Nymphalidae (Ackery 1988). On the other hand, the chemical ecology of lycaenid–plant interaction remains almost unexplored (Fiedler 1996) with the remarkable exception of the cycasin-sequestering aposematic lycaenid *Eumaeus atala florida* (Röber) (Rothschild et al. 1986). Considering that lycaenid larvae show phytophagy and entomophagy, the family Lycaenidae, which is the second-largest butterfly taxon with over 4500 species (Shields 1989) and possibly 6000 species (Robbins 1988), is a complex yet fascinating group of butterflies on which to study the chemical basis of host selection.

The fate of oxalic acid ingested by *Z. maha* larvae is not known. Specialist herbivores are demonstrated to metabolize, catabolize (detoxify), and/or

348 sequester plant defensive secondary metabolites (Ali and Agrawal 2012; Malcolm
349 and Brower 1989). Preliminary analysis of *Z. maha* fecal extracts for presence of
350 free oxalic acid resulted in detection of much less amounts of the acid than those
351 ingested (unpublished data). Sequestration of intact oxalic acid in the body
352 seems negative. These findings imply that oxalic acid may be catabolized
353 (degraded) and excreted in feces or metabolized (transformed) as a carbon
354 source in *Z. maha* larvae. The synthesis of oxalate-degrading enzymes, such as
355 oxalate oxidase (EC 1.2.3.4; Datta et al. 1955) and oxalate decarboxylase (EC
356 4.1.1.2; Jakoby et al. 1956), is known from plants and bacteria, but, to the best
357 of our knowledge, no information is available on the production of these enzymes
358 in insects. Given that oxalotrophic bacteria (capable of utilizing oxalates as their
359 sole or major carbon and energy source) have been identified from *Oxalis* (Sahin
360 2003, 2005), the involvement of these bacteria in the digestive tract of *Z. maha*
361 larvae is possible. Ongoing investigations of the fate of oxalic acid in *Z. maha*
362 larvae may provide insights into oxalate toxicosis.
363
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571 Figure Captions

572 **Fig. 1** Procedure for separation of feeding stimulants for *Z. maha* larvae from
573 the crude methanolic extract of *O. corniculata* leaves. Extracts and fractions in
574 boxes were subjected to bioassays

575

576 **Fig. 2** Feeding responses of *Z. maha* larvae to a control artificial diet and to
577 artificial diets containing *O. corniculata* leaf extract. Each bar represents dry
578 frass weight (mean ± SE, mg per larva, $N = 3–5$). * $p < 0.05$ (Steel test against
579 the control, two-tailed)

580

581 **Fig. 3** Typical total ion current chromatograms obtained by TBDMS
582 derivatization of blank control (MTBSTFA in acetonitrile; A), standard oxalic acid
583 (B), and water layer (C).
584 Chromatograms were obtained with a HP6890N gas chromatograph (Hewlett-
585 Packard, Palo Alto, CA, U.S.A.) equipped with a fused silica capillary column
586 (DB-5MS, 25 m × 0.25 mm, 0.25 μm film thickness), programed from 50 °C for
587 1 min, then raised to 320 °C at 10 °C/min, and held at the final temperature for
588 12 min. The arrows indicate the peaks of oxalic acid bis-TBDMS ester.

589

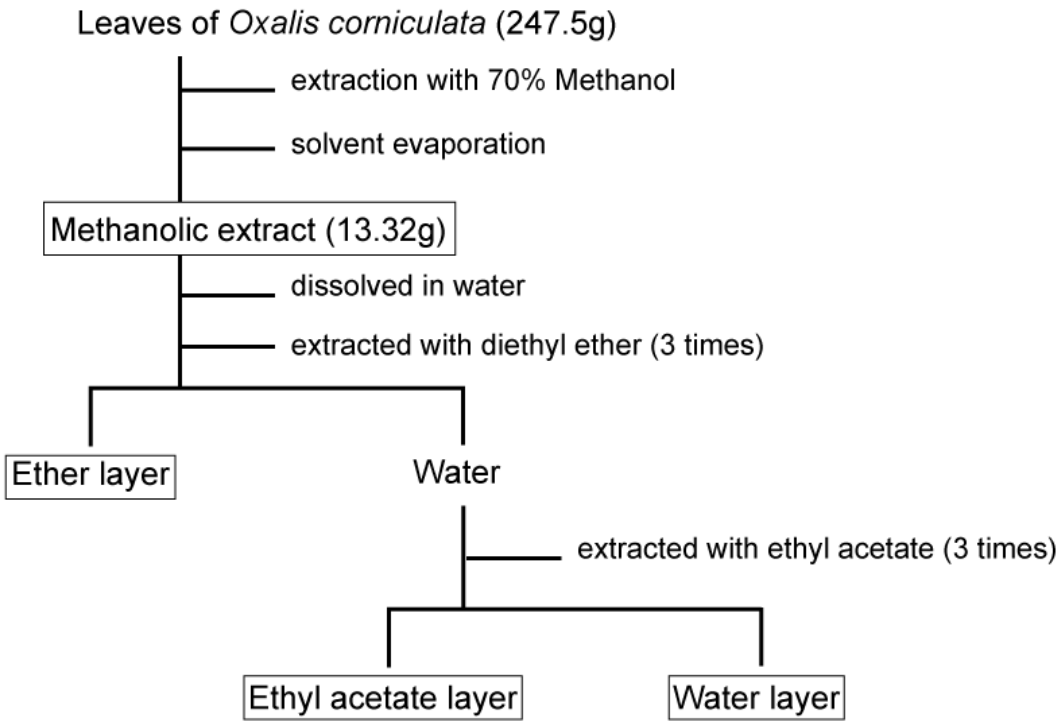
590 **Fig. 4** Feeding response of *Z. maha* larvae to removal and re-addition of oxalic
591 acid in the active water layer. Each bar represents dry frass weight (mean ± SE,
592 mg per larva, $N = 8$ for OA removed; $N = 9$ for OA re-added). * $p < 0.01$ (*t*-test)

593

Fig. 5 Feeding response of *Z. maha* larvae to artificial diets containing various amounts of oxalic acid. Each bar represents dry frass weight (mean ± SE, mg per larva, N = 2–5). * $p < 0.05$ (Steel test against the control, one-tailed)

598 Tables and Figures

599 **Fig. 1**



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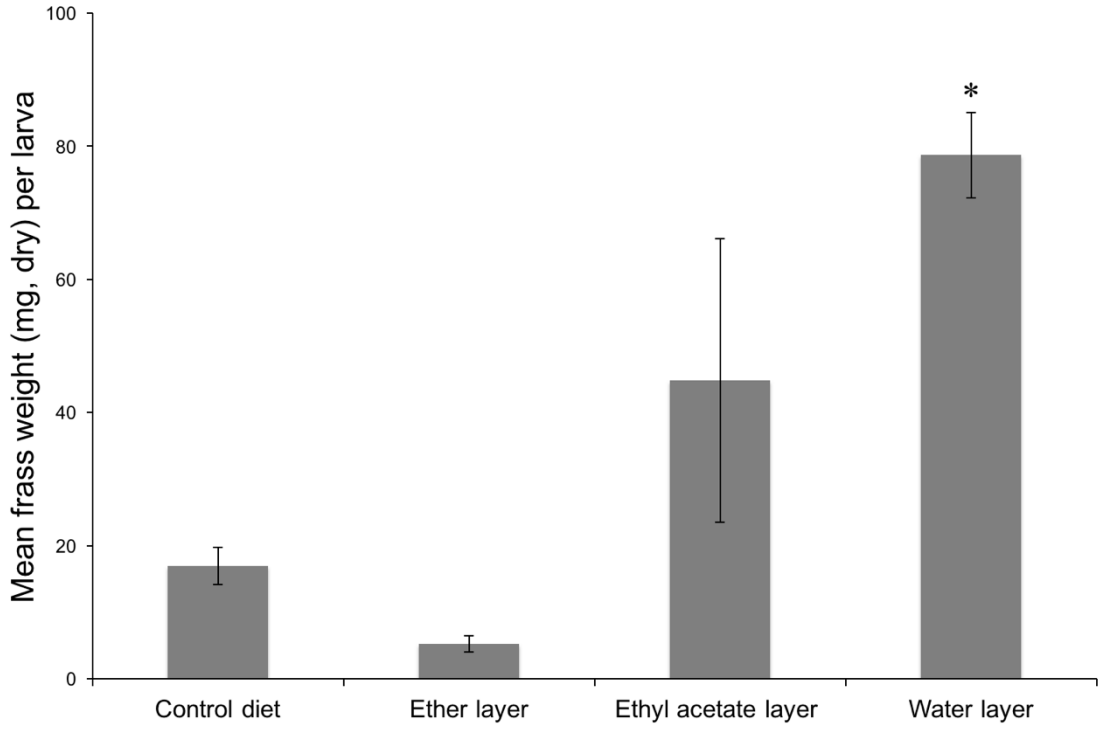

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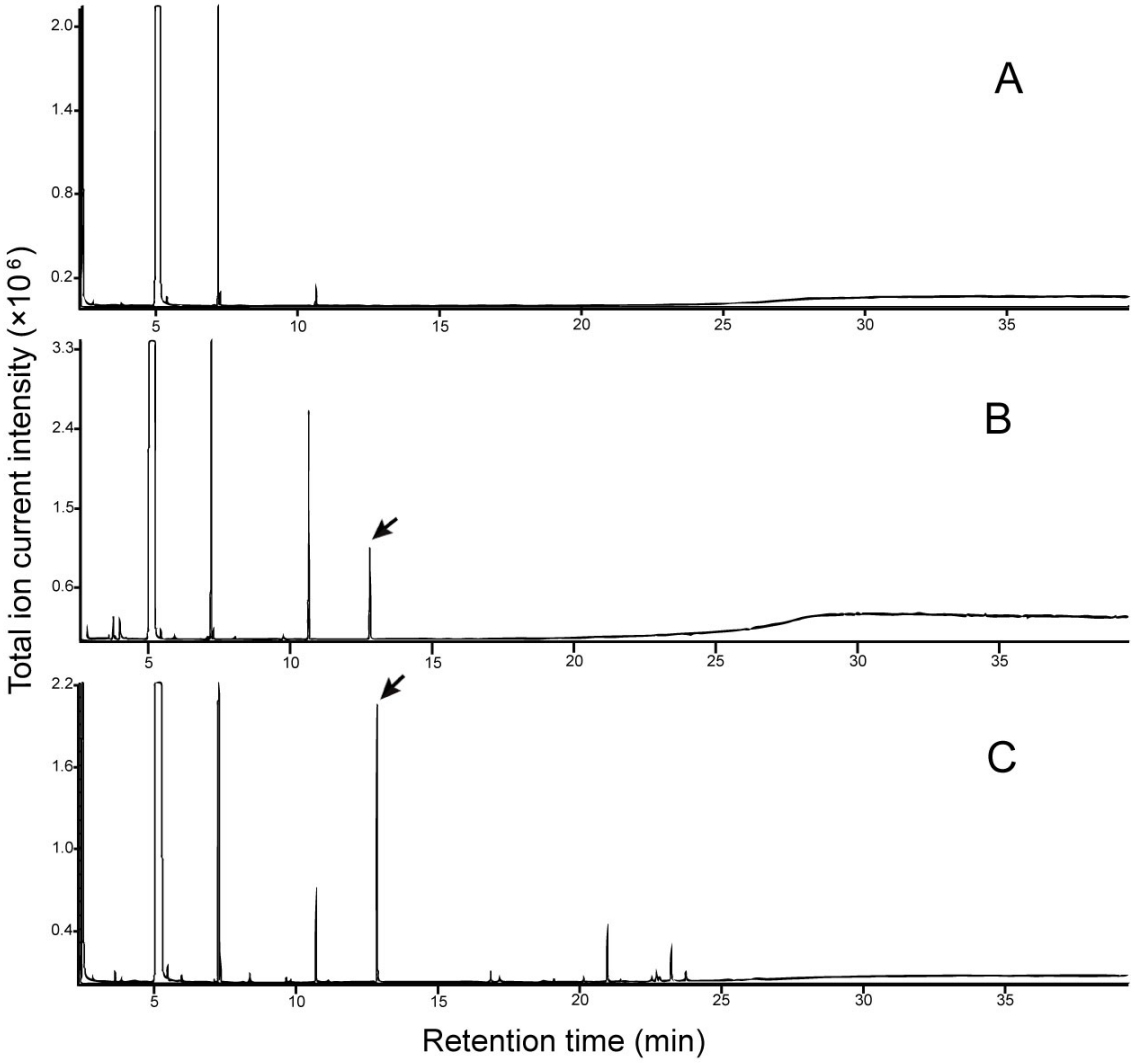

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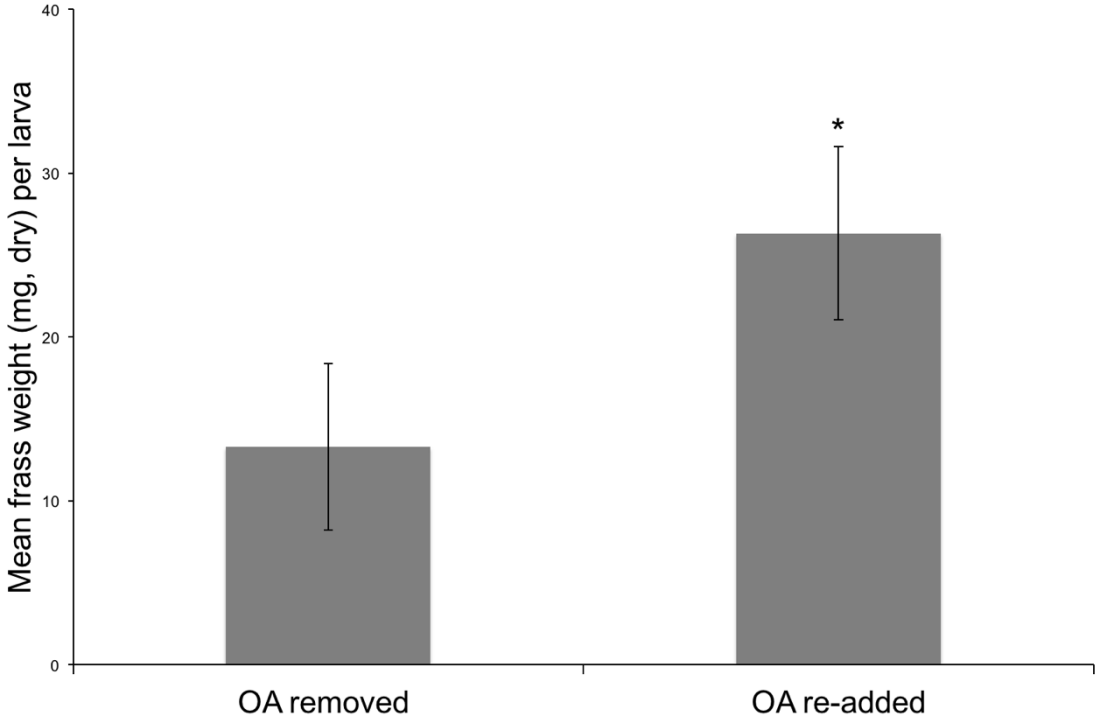

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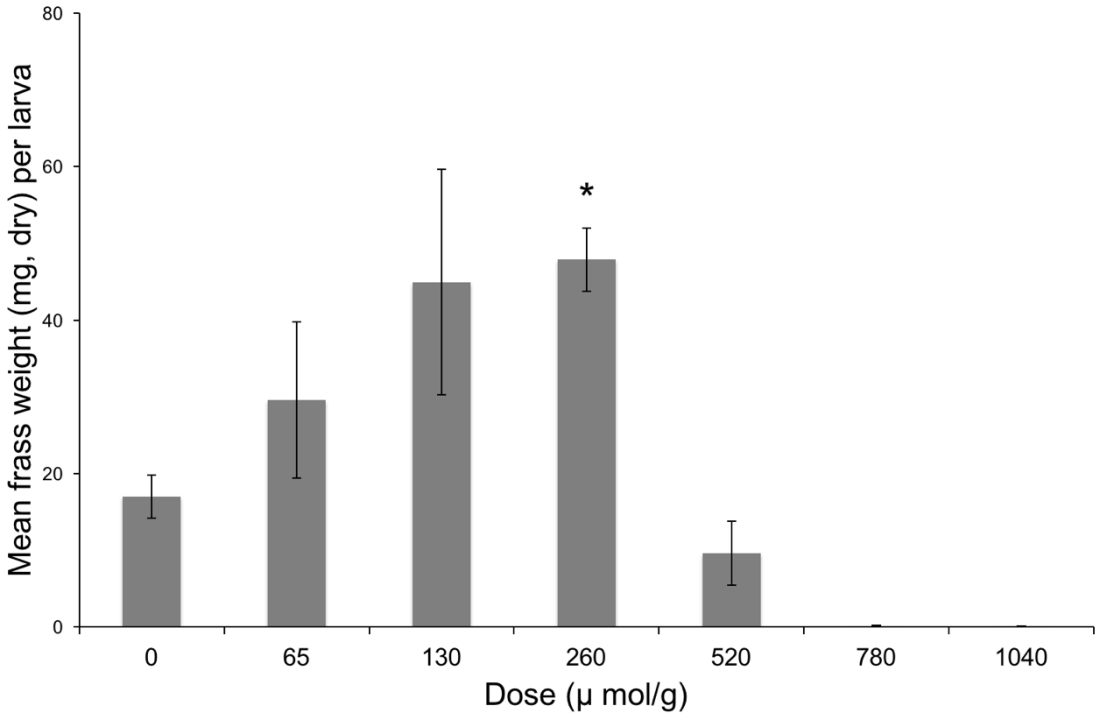
611 **Fig. 5**



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