

Feeding Stimulant in *Cinnamomum camphora* for the Common Bluebottle, *Graphium sarpedon nipponum* (Lepidoptera: Papilionidae)

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The acceptance of camphor tree (*Cinnamomum camphora*) as a host plant for the larvae of common bluebottle (*Graphium sarpedon nipponum*) was explained by the presence of feeding stimulants in the leaves. When the active methanol extract of *C. camphora* leaves was separated into hexane and water layers, both layers showed high feeding activities for the larvae of *G. sarpedon nipponum*. Bioassay-guided fractionation of the hexane layer resulted in the isolation of a highly active compound, which was identified as α -linolenic acid by nuclear magnetic resonance spectrometry and gas chromatography-mass spectrometry.

Key words: Feeding Stimulants, *Graphium sarpedon nipponum*, *Cinnamomum camphora*, α -Linolenic Acid

Introduction

It is now known that plants contain numerous substances biologically active to insects; such as hormone-like active substances, attractants, feeding stimulants, antifeedants, and repellents (Koul, 2008). Among the broad range of chemical plant metabolites, some compounds serve as important host recognition cues for herbivorous insects. Such cue compounds can occur as volatile attractants or as contact stimulants that insects only perceive after biting the tissue. The identification of these kairomones, and also of repelling and deterring compounds, provides a mechanistic basis for explaining insects' preferences for certain plants as food source and ovipositional site (Nielsen, 1978a, b; Endo *et al.*, 2004; Müller and Renwick, 2001; Honda *et al.*, 1997; Reifenrath and Müller, 2008). Of these compounds, the chemicals of the Papilionidae family have been widely studied. Especially many secondary metabolites (Renwick *et al.*, 1994; Honda, 1995, 2005; Honda and Nishida, 1999; Chachin *et al.*, 2007), which often play an important role in the assessment of a plant as oviposition stimulant (Nishida, 2005; Ono *et al.*, 2004) and feeding stimulant (Ômura and Honda, 2003) including amino acid derivatives, sugar-related acids, alkaloids, flavonoids, and hydroxycinnamic acid derivatives (Nakayama *et al.*, 2003), were reported.

Graphium sarpedon is a species of swallowtail butterfly distributed in South and Southeast Asia. There are approximately 15 subspecies with differing geographical distributions. The common bluebottle, *Graphium sarpedon nipponum*, just one of the subspecies, inhabits mainly southern Japan. It appears from May to October each year. This butterfly is a Lauraceae-feeding butterfly, especially specialized on camphor tree, *Cinnamomum camphora*. Because of mating, ovipositing, and feeding on the nectar, many males and females are commonly observed around this tree.

This insect genus is known to locate intermediate between the Troidini and Papilionini genera in the evolutionary tree. Thus the chemicals relating to the host selection of the Graphiini genus should be studied, in order to elucidate the evolutionary process in the Papilionidae family; their study have been rarely done. Consequently, the feeding stimulant for *G. sarpedon nipponum* in *C. camphora* leaves was investigated.

Material and Methods

Insect and plant

The eggs and larvae of *G. sarpedon nipponum* were collected from fresh shoots of *C. camphora* at Monobe Campus, Kochi University, Japan.

Both the eggs and larvae of the butterfly were reared at (25 ± 3) °C and 70% relative humidity in Petri dishes (9 cm i.d. \times 1 cm) with a photocycle of 15 h light/9 h dark. The first/second instar larvae were fed on fresh shoots whose cut-ends were covered with a water-dipped kimwiper for keeping moisture, and the larvae from the third instars were fed on mature leaves or fresh shoots. The animate fifth instar larvae were used for the bioassay. Fresh shoots for extraction and bioassay were also collected at Monobe Campus, Kochi University, Japan.

General bioassay design

To investigate the feeding stimulation of *G. sarpedon nipponum*, 1 g of fresh leaves equivalent amounts (g.l.e.) of test leaf extracts, fractions of the extract or authentic standards solution were applied on Styrofoam semicircle discs (45 mm i.d. \times 0.7 mm), respectively. The discs were introduced into a kimwiper box (13 cm \times 12 cm \times 9 cm) which had the upper end opened. One end of each disc was sandwiched between two Styrofoam sticks (3 cm \times 3 cm \times 13 cm). All test discs stood against the Styrofoam sticks like a ceiling board. Three fifth instar larvae starved for 6 h were introduced into the box, and the open end was covered with a glass slide. During the bioassay, larvae were allowed to feed on the discs freely for 24 h at (25 ± 3) °C and 70% relative humidity with a photocycle of 15 h light/9 h dark (5–8 replicates per assay). The MeOH and crude extracts were used as negative and positive controls, respectively. At the end of each bioassay, the area of remaining Styrofoam semicircle disc fragments was calculated using a flat-bed scanner. Averaged consumed areas of Styrofoam semicircle discs as an index of feeding stimuli were recorded, and the data were statistically analysed using one-way analysis of variance (ANOVA) followed by Tukey HSD test ($P \leq 0.05$).

Bioassay by using several fresh leaves

One animate fifth instar larva and one mature leaf or one fresh shoot with nine leaves were put into a Petri dish (10 cm i.d. \times 1 cm) at (25 ± 3) °C and 70% relative humidity with a photocycle of 15 h light/9 h dark for 24 h. Averaged consumed areas of leaf as an index of feeding stimuli were recorded.

Isolations

Leaves of fresh shoots of *C. camphora* (1.9 kg) were extracted twice in 80% MeOH in H₂O for 3 d. The combined extract was evaporated under reduced pressure. The residue (118.2 g) was dissolved in H₂O (2.8 l) and partitioned with *n*-hexane (2 l) for four times. Subsequently, the *n*-hexane layer was concentrated and chromatographed on a reversed phase open column (ODS, 87 g, 100 mesh, 30 mm i.d. \times 500 mm, Fuji Silysia Chemical Ltd., Kasugai, Japan). The column was eluted with MeOH/H₂O mixtures of decreasing polarity: 40% MeOH/H₂O, 60% MeOH/H₂O, and 100% MeOH. The 60% MeOH/H₂O fraction was rechromatographed on a reversed HPLC column (CAPCELL PAK C18 UG 80 Å 5 μ m, 10 mm i.d. \times 250 mm) eluted with a 65% MeOH/H₂O mixture, and three fractions (A–C) were collected. 500 g of fresh leaves yielded 3.7 mg of compound **B**.

Methyl esterification of compound **B** by diazomethane gas

After compound **B** (10 mg) was dissolved in MeOH, diazomethane gas was introduced into the methanol solution for 30 min at room temperature, according to an ordinary method (Stransky *et al.*, 2005; Sýkora *et al.*, 2007).

Methyl ester of compound **B** (methyl α -linolenate)

m/z (%) = 41 (53), 55 (45), 67 (70), 74 (10), 95 (78), 108 (50), 121 (26), 135 (23), 149 (19), 163 (8), 173 (3), 178 (3), 191 (8), 217 (2), 223 (5), 236 (9), 249 (3), 261 (8), 292 (19).

Results and Discussion

Polarity of feeding stimulant

Almost 200 cm² of the disc applied on the crude methanol extract was consumed by the larvae. This result clearly showed that the extract contained some feeding stimulants for *G. sarpedon nipponum*.

In order to find out a feeding stimulant or antifeedant of Lepidoptera larvae from a certain plant, filter paper or agar is generally used as a medium. The biggest limitations of these techniques are that they can not be applied to larvae with a weak biting power, and also it is difficult to calculate the exact quantity fed by the

larvae. Because Styrofoam semicircle discs can be applied to any larvae with a weak biting power like *G. sarpedon nipponum* and the amount can be exactly calculated using a flat-bed scanner, it can be adapted as a common method to elucidate feeding stimulants or antifeedants associated with Lepidoptera larvae.

When the active methanol extract of *C. camphora* leaves was separated into *n*-hexane and water layers, both layers showed high feeding activities for the larvae of *G. sarpedon nipponum* (Fig. 1). Differences were significant between the treatments and the negative control, but not among the Styrofoam discs treated with the lay-

ers (one-way ANOVA followed by Tukey HSD test).

These results indicate that the feeding activity is not based on a single component but on plural components.

Feeding stimulation in the *n*-hexane layer fraction

Almost no feeding stimulation was observed on the discs treated with 40% MeOH/H₂O or 100% MeOH. In contrast, the 60% MeOH/H₂O (528.6 g) fraction scored a high consumed area, which indicated that the feeding stimulants in the *n*-hexane layer were mainly from this fraction

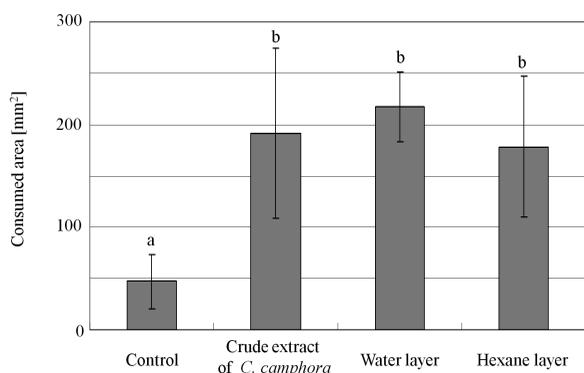


Fig. 1. Feeding response (mean ± SE) of *G. sarpedon nipponum* to negative control, crude methanol extract of *C. camphora*, and two layers. Significant difference between the control and the treated Styrofoam sticks is presented by different letters ($P \leq 0.05$, $n = 5$).

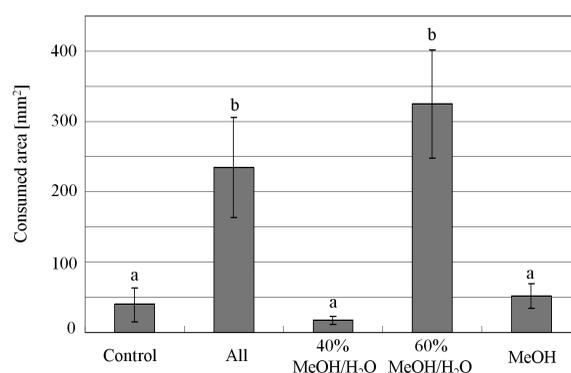


Fig. 2. Feeding response (mean ± SE) of *G. sarpedon nipponum* to negative control, 40% MeOH/H₂O fraction, 60% MeOH/H₂O fraction, MeOH fraction, and their mixture ($P \leq 0.05$, $n = 5$). For further details see Fig. 1.

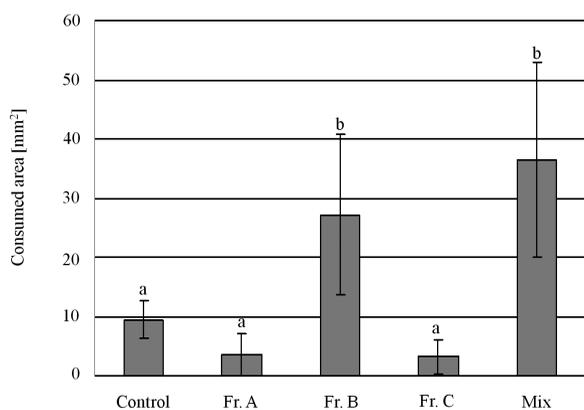


Fig. 3. Feeding response (mean ± SE) of *G. sarpedon nipponum* to negative control, three pooled HPLC fractions (A, B, C), and mixture of the fractions ($P \leq 0.05$, $n = 5$). For further details see Fig. 1.

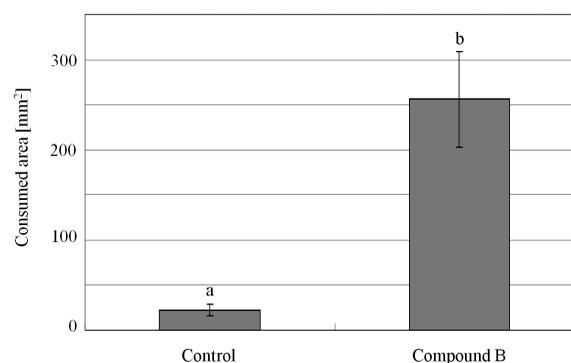


Fig. 4. Feeding response (mean ± SE) of *G. sarpedon nipponum* to negative control and compound B ($P \leq 0.05$, $n = 5$). For further details see Fig. 1.

(Fig. 2). Further fractionation of the 60% MeOH/H₂O fraction by reversed phase HPLC showed that the discs consumption was not significantly influenced by fraction A (0 ~ 39 min) or fraction C (42 ~ 60 min), but varied significantly after treatment with fraction B (39 ~ 42 min) (Fig. 3). Successive bioassay-guided fractionation of the *n*-hexane layer led to the isolation of a single oily compound **B** from fraction B, which showed significant feeding stimulation for *G. sarpedon nipponum* (Fig. 4).

Identification and feeding activity of compound **B**

The chemical structure of compound **B** was analysed by ¹H NMR, ¹³C NMR (Jeol JNM-AL400, TMS as an internal standard), and GC-MS (Hewlett-Packard 6890 coupled with Jeol MS600), and the spectra were compared with available commercial standards. The ¹H NMR spectrum in DMSO-*d*₆ (400 MHz) of compound **B** showed peaks at δ 5.31 (6H, multiplet, -CH=CH-), 2.76 (4H, multiplet, =C-CH₂-C=), 2.17 (2H, triplet, *J* = 6.8 Hz), 2.02 (4H, multiplet, -CH₂-CH=CH-CH₂-), 1.48 (2H, quintet, -CH₂), 1.24 (8H, broad), 0.91 ppm (3H, triplet, -CH₃, *J* = 7.2 Hz). In the ¹³C NMR spectrum (100 MHz, DMSO-*d*₆), the carbon signal at 174.1 ppm indicated the presence of a carboxylic acid. Other carbon signals were at 14.0, 19.9, 24.4, 25.0, 25.1, 26.5, 28.3, 28.4, 28.4,

28.9, 33.6, 126.8, 127.3, 127.7, 127.7, 129.7, and 131.3 ppm. Through arrangement of all structural elements identified so far, the compound was most likely linolenic acid (Table I).

After compound **B** (10 mg) was dissolved in MeOH, diazomethane was introduced into the methanol solution for 30 min at room temperature. Then, the methyl ester of compound **B** was analysed by GC-MS. In its GC-mass spectrum, an α -cleavage fragment ion of the ester ($M^+ - 31$, $m/z = 261$), McLafferty rearrangement fragment ion ($m/z = 74$), and M^+ ion ($m/z = 292$) were found. All data coincided with that of a methyl ester of linolenic acid.

As a normal fatty acid in plants, linolenic acid can refer to either of two fatty acids with different configuration, both of which can be found in many common vegetable oils, especially in plant seeds. α -Linolenic acid is a polyunsaturated *n*-3 (omega-3) fatty acid. γ -Linolenic acid, a polyunsaturated *n*-6 (omega-6) fatty acid, is its isomer. The configuration determination of compound **B** (α -linolenic acid) was completed by subsequent injection of authentic standards of these two fatty acids into the gas chromatograph and comparison of their ¹³C NMR data (Table I). The amount of α -linolenic acid contained in fresh shoots of *C. camphora* was 7.4 μ g/g.

In the bioassays, the standard of α -linolenic acid displayed significant stronger feeding activity than the control and the standard of γ -linolenic acid (Fig. 5).

Table I. ¹³C NMR data (400 MHz, DMSO-*d*₆, δ in ppm) of compound **B** and authentic standards (α -linolenic acid and γ -linolenic acid).

Compound B	α -Linolenic acid	γ -Linolenic acid
14.0	14.1	13.9
19.9	20.0	21.9
24.4	24.5	24.1
25.0	25.1	25.2
25.1	25.2	25.2
26.5	26.6	26.4
28.3	28.5	26.6
28.4	28.5	28.6
28.4	28.6	28.7
28.9	28.9	30.9
33.6	33.7	33.5
126.8	126.9	127.4
127.3	127.4	127.7
127.7	127.8	127.8
127.7	127.8	127.8
129.7	129.8	129.4
131.3	131.4	129.8
174.1	174.3	174.2

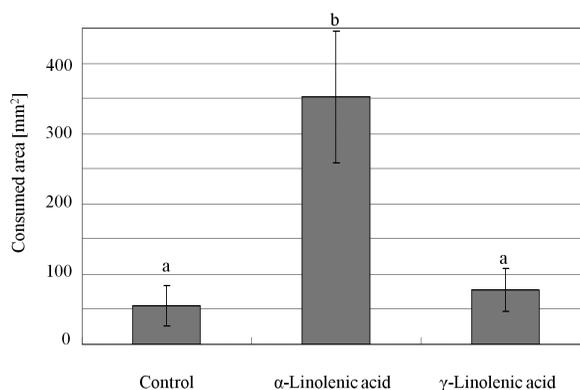


Fig. 5. Feeding response (mean \pm SE) of *G. sarpedon nipponum* to negative control and authentic standards (α -linolenic acid and γ -linolenic acid) ($P \leq 0.05$, $n = 5$). For further details see Fig. 1.

Table II. The presence of α -linolenic acid and the results on feeding stimulation to test plants.

Plant	N	α -Linolenic acid	Feeding action
<i>Machilus thunbergii</i>	5	○	+
<i>Citrus limon</i>	5	○	+
<i>Citrus grandis</i> (fresh shoots)	5	○	+
<i>Citrus grandis</i> (mature leaves)	5	○	–
<i>Citrus tamurana</i>	5	○	+
<i>Orixa japonica</i>	5	×	–
<i>Ternstroemia gymnanthera</i>	5	○	+
<i>Camellia japonica</i>	5	○	+

○, contained α -linolenic acid; ×, did not contain α -linolenic acid; +, showed feeding behaviour; –, did not show feeding behaviour; N, replicate times.

Feeding stimulation of fresh leaves from different plants

One of the feeding stimulants in *C. camphora* for *G. sarpedon nipponum* has been determined as α -linolenic acid, which is a common compound widespread in the plant kingdom. Therefore, it might be advantageous for *G. sarpedon nipponum* to utilize this compound as food cue to exploit more food resources. Here seven kinds of plants (host plants for *Papilio xuthus*, *Papilio protenor*, and *Papilio polytes*, all of which belong to the Papilionini tribe) were selected to test their feeding activity for *G. sarpedon nipponum* (Table II). Of these plants, only *Orixa japonica* contained no α -linolenic acid.

Graphium sarpedon nipponum could feed on all plant species except *Citrus grandis* (mature leaves) and *O. japonica*. As *O. japonica* contains no α -linolenic acid, this result is reasonable. Mature leaves of *C. grandis* were not fed, but fresh shoots of it were, though they contain α -linolenic acid. These results may indicate that the mature leaves contain some antifeedant(s) or are too hard for these larvae. The other plant species are not a host plant. This insect species, however, could feed on them. These results may indicate that this insect species easily can change the host plant if females laid eggs on these plants. To confirm this idea, the feeding stimulants of the H₂O layer and ovipositional stimulants for this species must be investigated.

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