Utilization of learned plant chemicals in host searching behavior by the egg-larval parasitoid *Ascogaster reticulata* Watanabe (Hymenoptera: Braconidae)

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Abstract

The learning systems of the parasitoid *Ascogaster reticulata* were investigated using 5 authentic chemicals from tea leaf. The learning efficiency for geraniol and linalool was higher than for the other 3 components, (Z)-3-hexenol, methyl salicylate and benzyl alcohol. Discrimination between geraniol and linalool became clear when the number of conditionings increased. Furthermore, *A. reticulata* females preferred the chemical information after successive oviposition experience and the newly learned information. A 2-choice bioassay using a mixture of these tea leaf components suggested that geraniol and linalool play a key role in the discrimination of mixtures. However, *A. reticulata* females could also discriminate minor components in the mixture. They use several key components in the discrimination of plant species as well as other hymenopteran species. Increased preference for a certain plant species after successive oviposition experience of the same plant is adaptive for female's host-searching behavior in the field.

Key words: Host-searching behavior; plant chemicals; switching; memory processing

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INTRODUCTION

Parasitic wasps exploit plant chemicals as host-searching cues. Female wasps respond to plant chemicals mainly by three factors; innate behavior, sensitization and learning (Vinson, 1998). Learning is especially efficient in generalist parasitoid host-searching because plant exploitation by multiple species of hosts is spatially and temporally variable and associative learning of plant chemical cues provides flexibility for adaption to changes in plants (Vet, 2001).

Ascogaster reticulata Watanabe is an egg-larval parasitoid of *Adoxophyes honmai* Yasuda and seven other tortricid species (Watanabe, 1967; Kamijo, 1973; Takagi, 1974; Kawakami and Kainoh, 1986) and its host, *A. honmai*, is a polyphagous insect that feeds on more than 90 species of plants (Minamikawa and Osakabe, 1979). Since it is difficult for *A. reticulata* to innately respond to many species of plants, the associative learning of plant chemicals is adaptive to this species. Previous stud-

ies have shown that *A. reticulata* females can learn to associate plant contact chemicals with the host egg mass as host-searching cues (Kainoh, 1997; Honda and Kainoh, 1998; Honda et al., 1998). Moreover, females are able to distinguish learned plant species and other plants (Seino and Kainoh, 2008).

In general, when insects learn plant chemicals, they establish responses to several key compounds from the total mixture of plant chemicals. For example, most honeybees (*Apis mellifera* L.) conditioned with oilseed rape (*Brassica napus*) flower extract showed proboscis extension responses to a mixture of eight compounds from oilseed rape flower volatiles (Blight et al., 1997). Laloi et al. (2000) also reported that honeybees conditioned with nine oilseed rape volatiles established a hierarchy among the components. In a parasitic wasp, Meiners et al. (2003) reported that *Microplitis croceipes* (Cresson) conditioned with 3 components (methyl jasmonate, β -caryophyllene and 2-octanone) could not discriminate a mixture of these 3

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components and a binary mixture (methyl jasmonate and β -caryophyllene).

In this study, we used 5 authentic plant chemicals identified from tea leaf, i.e. geraniol (G), linalool (L), (Z)-3-hexenol (H), methyl salicylate (M) and benzyl alcohol (B) (Yamanishi et al., 1963), to investigate the ability of *A. reticulata* to learn to discriminate. First, we tested the learned responses of *A. reticulata* to each of the 5 chemicals, followed by a two-choice bioassay to examine the effect of 1) the number of conditionings, 2) conditioning order and 3) length of interval between conditioning for two different chemicals. Furthermore, we prepared 3- or 4-compound mixtures of these 5 chemicals and investigated how *A. reticulata* females discriminate these mixtures from the same or different compounds.

MATERIALS AND METHODS

Insect rearing. Colonies of *A. reticulata* and its host *A. honmai* were obtained from a stock culture at the University of Tsukuba. *A. reticulata* were reared based on the methods described by Kainoh (1988), and that of *A. honmai* by Tamaki (1966). Parasitized and unparasitized *A. honmai* larvae were reared on an artificial diet containing tea leaf powder. *A. reticulata* cocoons and *A. honmai* pupae were taken out of the rearing containers and each *A. reticulata* cocoon was separately placed into a 5 ml sample tube until emergence. Emerged adult wasps were sexed and 10–20 of each were kept in a plastic container (15 cm diam., 9 cm high) with wet cotton on the bottom and honey on the walls. Rearing and experiments were conducted in the laboratory at $25 \pm 1^\circ\text{C}$ and $60 \pm 10\%$ relative humidity (RH) under a 16L8D photoperiod. Three- to four-day-old unmated female wasps with no oviposition experience and 1-day-old host egg masses were used in all experiments.

Plant chemicals. Five authentic plant chemicals from the tea leaf, geraniol (G), linalool (L), (Z)-3-hexenol (H), methyl salicylate (M) and benzyl alcohol (B) (Yamanishi et al., 1963), were used in this study. Each chemical was dissolved in hexane and stored at -20°C until use.

Bioassay responses to 5 plant chemicals. The $5 \mu\text{l}$ of the 5 plant chemicals from tea leaves (G: geraniol, L: linalool, H: (Z)-3-hexenol, M: methyl salicylate, B: benzyl alcohol) at different concen-

trations (1.0 , 10^{-1} , 10^{-2} and $10^{-3} \mu\text{g}/\mu\text{l}$) were applied to a 9 cm line across the center of a 9 cm glass Petri dish. After the solvent had evaporated by air drying, a host egg mass was placed in the center and 3- to 4-day-old female wasps were released into the dish. The female was allowed to oviposit in the host egg mass for 2 min, and was then gently removed with an insect aspirator and placed in a container (15 cm in diam., 9 cm high) with wet cotton on the bottom and honey on the walls until used in the next conditioning or the bioassay of learned responses. Conditioning was repeated three times with the same chemicals at 30–60 min intervals and the females were tested in a bioassay 30–60 min after the third conditioning.

The bioassay to measure the response of females to plant chemicals was similar to the conditioning method described by Kainoh (1997). After the solution of a sample had been air-dried, a female was released into a 9 cm Petri dish and her behavior was observed for 3 min. The approximate total distance of walking zigzag along the chemically treated line was measured by sight based on scale marks on the underside of the dish. This zigzag walking distance was used as a measure of the strength of a learned response. Ten to 15 females were used for each conditioning group.

Effect of the number of conditionings. Three- to 4-day-old females were allowed to oviposit on a line treated with $1.0 \mu\text{g}/\mu\text{l}$ geraniol or linalool in a Petri dish at different times, i.e., once (G and L), twice (GG and LL) or three times (GGG and LLL) at 30–60 min intervals. Subsequently, females were tested in a two-choice bioassay 30–60 min after the last conditioning. Geraniol and linalool ($1.0 \mu\text{g}/\mu\text{l}$) were applied crosswise on the bottom of a 9 cm Petri dish. The chemicals were not applied in the center (1 cm in diam.) to prevent the mixing of lines. After the solution had been air dried, a female was released into the dish and her behavior was observed for 3 min. The total distance of zigzag walking along each component-treated line was measured using the scale on the bottom of the Petri dish. Ten to 18 females were used for each conditioning group.

Effect of conditioning order. Females were allowed to oviposit on G and L. Oviposition occurred in different patterns of conditioning order (GGL and LLG, GLG and LGL, GGLL and LLGG) at 30-min intervals. Females were tested in a two-

choice bioassay 30–60 min after the last conditioning. The G-L choice bioassay was conducted by the same methods as described above. Ten females were used for each conditioning group.

Discrimination of new and old information.

Females were conditioned in the order of GGLL or LLGG as in the previous conditionings; however, the intervals between conditioned G and L or conditioned L and G treatment were 30, 120 and 240 min. G-L choice bioassays were conducted by the same methods as in the above bioassays. Ten to 15 females were used for each conditioning group.

Discrimination of major or minor component in mixtures. The discrimination of major components (G, L) in 4-component mixtures was tested. Females were conditioned 3 times with mixtures of GHMB or LHMB and then differences between responses to GHMB and LHMB were tested in a two-choice assay. The total amount of chemicals in each mixture was $0.25 \mu\text{g}/\mu\text{l}$. Choice bioassays for GHMB and LHMB were conducted by the same methods as in the G-L choice bioassay. Fifteen females were used for each conditioning group.

Discrimination of minor components (H, M, B) in 3-component mixtures was tested. Females were conditioned 3 times with mixtures of GLH, GLM or GLB and then differences between responses to conditioned and non-conditioned mixtures were tested by a two-choice bioassay. The amount of each component was $0.33 \mu\text{g}/\mu\text{l}$. A two-choice bioassay of female responses was tested between the three conditioned mixtures and two other non-conditioned mixtures, e.g. GLH-conditioned females used in bioassays of GLH-GLM and GLH-GLB. Twenty females were used for each conditioning group.

RESULTS

Responses to 5 plant chemicals

Responses of *A. reticulata* females to each of the 5 plant chemicals increased in proportion to the concentration (Fig. 1). There was no significant difference among the 5 chemicals at the lowest dose ($0.001 \mu\text{g}/\mu\text{l}$), but linalool was higher than the other 4 chemicals at $0.01 \mu\text{g}/\mu\text{l}$. Linalool was also highest at $0.1 \mu\text{g}/\mu\text{l}$, but was not significantly different from geraniol. At this dose, geraniol was significantly higher than (*Z*)-3-hexenol and benzyl alcohol, but was not significantly higher than

Fig. 1. Responses of *A. reticulata* females conditioned with 5 plant chemicals from tea leaf (geraniol, linalool, (*Z*)-3-hexenol, methyl salicylate and benzyl alcohol) ($n=10-15$). Different letters in the same concentration indicate significant differences among the chemicals tested by Tukey-Kramer test ($p<0.05$).

methyl salicylate. At the highest dose ($1 \mu\text{g}/\mu\text{l}$), geraniol and linalool were significantly higher than the other 3 compounds, (*Z*)-3-hexenol, benzyl alcohol and methyl salicylate. Females showed stronger learning responses to both geraniol and linalool at higher doses.

Effect of the number of conditionings

Females conditioned with G showed a significant preference for geraniol ($p<0.05$), but L-conditioned females did not show a significant preference for linalool (Fig. 2A). GG-conditioned and LL-conditioned females significantly preferred ($p<0.01$) the components on which they were conditioned (Fig. 2B). Females conditioned 3 times (GGG- and LLL-conditioned) also showed a significant preference ($p<0.01$) for the components on which they were conditioned (Fig. 2C).

Effect of conditioning order

GGL-conditioned females significantly preferred G ($p<0.01$), and LLG-conditioned females significantly preferred L ($p<0.01$; Fig. 3A). Females preferred a twice-conditioned component rather than a newly conditioned component. GLG-conditioned females responded significantly longer to G, but LGL-conditioned females did not significantly prefer L ($p>0.05$; Fig. 3B). GGLL- and LLGG-conditioned females did not show a significant preference for G and L ($p>0.05$; Fig. 3C).

Fig. 2. Responses of *A. reticulata* females conditioned with G or L (A), GG or LL (B) and GGG or LLL (C) ($n=10-18$) (mean $\pm$ SE). 'ns' indicates no significant difference in each test group (paired t -test, $p>0.05$). Asterisks indicate a significant difference in each test group (paired t -test, *: $p<0.05$, **: $p<0.01$).

Discrimination of new and old information

GG-30min-LL conditioned females (Fig. 4A) and LL-30min-GG conditioned females (Fig. 4B) did not show a significant preference for G or L ($p>0.05$). In the 120-min interval, GG-120min-LL conditioned females did not discriminate G and L ($p>0.05$; Fig. 4A), but LL-120min-GG conditioned females clearly discriminated between G and L and chose G ($p<0.01$; Fig. 4B). In 240-min intervals, females significantly preferred the chemical that they had learned after the interval ($p<0.01$; Fig. 4A, B).

Discrimination of major or minor components in mixtures

GHMB-conditioned and LHMB-conditioned females significantly preferred the mixture on which they had been conditioned ($p<0.01$; Fig. 5).

GLH-conditioned females showed a significant

Fig. 3. Responses of *A. reticulata* females conditioned with GGL or LLG (A), GLG or LGL (B) and GGLL or LLGG (C) ($n=10-15$) (mean $\pm$ SE). 'ns' indicates no significant difference in each test group (paired t -test, $p>0.05$). Asterisks indicate a significant difference in each test group (paired t -test, *: $p<0.05$, **: $p<0.01$).

preference for the GLH mixture in GLH-GLB and GLH-GLM choice assays ($p<0.01$; Fig. 6A, C). GLM-conditioned females also showed a significant preference for the GLM mixture in GLM-GLB ($p<0.01$; Fig. 6B) and GLH-GLM ($p<0.05$; Fig. 6C) choice assays. GLB-conditioned females showed a significant preference for the GLB mixture in GLM-GLB choice assays ($p<0.05$; Fig. 6B); however, in the GLH-GLB choice assay, GLB-conditioned females did not significantly prefer the GLB mixture ($p>0.05$; Fig. 6A). In these three mixtures, the learned response to GLH was rather high and to GLB rather low.

DISCUSSION

Generalist parasitoids learn and discriminate plant chemicals as host-searching cues. Parasitoids of multiple species of hosts are required to search

Fig. 4. Responses of *A. reticulata* females conditioned with GG-interval-LL (A), LL-interval-GG (B) ($n=10-15$) (mean $\pm$ SE). 'ns' indicates no significant difference in each test group (paired t -test, $p>0.05$). Asterisks indicate a significant difference in each test group (paired t -test, **: $p<0.01$).

on variable plant species. We therefore conducted a bioassay to investigate in a generalist parasitoid, *A. reticulata*, how they establish a priority sequence in chemicals when they encounter several plant species.

Ascogaster reticulata females showed a longer walking distance in G and L than H, B and M (Fig. 1); therefore, the five chemicals from tea leaf were categorized into two groups, preferred (G, L) and non-preferred (H, B, M) components.

GG-conditioned females significantly preferred G, whereas LL-conditioned females significantly preferred L. The ability to discriminate G and L was maximized in females that experienced 3 conditionings (Fig. 2). Honda et al. (1998) reported that learned responses for tea leaf extracts are maximized when *A. reticulata* females experience more than 3 conditionings. Discrimination of geraniol and linalool was also higher after more than 3 times conditioning. Another parasitoid, *M. croceipes*, significantly preferred learned odors when they experienced more than 3 conditionings (Takasu and Lewis, 1996).

GGL and LLG choice assays were conducted to compare the effect of the number of conditionings and newness of information to discriminate G and

Fig. 5. Responses of *A. reticulata* females conditioned with GHMB or LHMB ($n=15-20$) (mean $\pm$ SE). Asterisks indicate a significant difference in each test group (paired t -test, **: $p<0.01$).

Fig. 6. Responses of *A. reticulata* females conditioned with GLH or GLB (A), GLM or GLB (B) and GLH or GLM (C) ($n=15-20$) (mean $\pm$ SE). 'ns' indicates no significant difference in each test group (paired t -test, $p>0.05$). Asterisks indicate a significant difference in each test group (paired t -test, *: $p<0.05$, **: $p<0.01$).

L. The results of this assay suggest that the number of conditionings is more important for discrimination than the newness of the information (Fig. 3A); however, in the GLG and LGL choice assays, LGL-conditioned females did not significantly prefer L (Fig. 3B). Responses of LLG- and LGL-con-

ditioned females suggest that successive conditioning is one key factor in *A. reticulata* females' discrimination of chemicals. GGLL- and LLGG-conditioned females did not prefer G or L (Fig. 3C). Two successive conditionings for each chemical had the same value in the utilization of G and L. When the cabbage butterfly, *Pieris rapae*, learned two types of information, the first information interrupted the learning of the second information (Lewis, 1986). Moreover, when the parasitoid *Lepidopilina boulardi* experienced the same number of conditionings, they exhibited a priority for new information (De Jong and Kaiser, 1992; Kaiser and De Jong, 1993).

The interval between different types of conditioning affected the females' preference (Fig. 4). LLGG-conditioned females exhibited a preference for G, new information, when they were allowed to rest for more than 120 min. GGLL-conditioned females required a 240-min interval to show a preference for new information. The responses of females conditioned 3 times to tea leaf extracts gradually decreased with increasing days between conditioning and the next bioassay (Honda et al., 1999). In this study, *A. reticulata* females experienced each chemical twice, so we speculated that the decline of the learned response occurred earlier. *M. croceipes* females that experienced two different odors while feeding at 10-min and 24-h intervals did not show a preference for a new odor (Takasu and Lewis, 1996); however, the number of conditionings of *M. croceipes* was 5 times. Long persistence of learned memory may require a longer series of conditionings. *L. boulardi*, which experienced associative conditionings 5 times, had not lost the learned response 24 h after the last conditioning (Kaiser et al., 2003).

The number of conditionings, the conditioning order and comparison of the newness of information predict female host-searching behavior; that is, *A. reticulata* females rely on the number of conditionings, especially successive oviposition experiences, as more valuable. The females may regard successive oviposition experiences as an indication of a relatively high density of host egg masses in the same plant species. Female *A. reticulata* normally show walking and short range flight while searching for hosts, and seldom fly long distances (Takagi, 1974). Therefore, they tend to search on nearby individual plants and recognize successive

oviposition experiences on the same plant species as "many host egg masses exist on this plant". In contrast, the time elapsed without oviposition (unrewarding experience, Honda et al., 1999; Seino and Kainoh, 2008) causes decreased response to the plant species that has no host. This enhancement and decreased response contributes to the efficient location of hosts in *A. reticulata* females.

Ascogaster reticulata females discriminate the mixtures of 4 components that differ in preferred components (GHMB and LHMB) (Fig. 5). G and L exhibit the highest responses in *A. reticulata* female learning. These components may play a key role in the discrimination of plant species by females. Similar results were reported in the honeybee, *A. mellifera* (Blight et al., 1997; Laloi et al., 2000) and *M. croceipes* (Meiners et al., 2003). Geraniol and linalool appear to be key tea leaf compounds for learning by *A. reticulata*.

Females also discriminated mixtures of 3 components that differed in non-preferred components (GLH, GLM and GLB); however, GLB-conditioned females did not show a significant preference for the GLB mixture in a GLH-GLB choice assay. Discrimination between 3-component mixtures may be more difficult for *A. reticulata* females than 4-component mixtures because these three components (H, M, B) are non-preferred minor components. Seino and Kainoh (2008) reported that *A. reticulata* females were unable to discriminate tea and camellia or tea and sasanqua extracts. Tea, camellia and sasanqua are categorized into the same genus, *Camellia*. These plant extracts likely contain the same or similar key compounds but may differ in minor components.

Our results suggest that *A. reticulata* females learned to respond to key components (geraniol and linalool) of 5 chemicals from tea leaf. When *A. reticulata* females learned several plant species they emphasized successive oviposition experience as a high density of hosts in the same plant species.

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REFERENCES

- Blight, M. M., M. L. Metayer, M. P. Delege, J. A. Pickett, F. Marion-Poll and L. J. Wadham (1997) Identification of floral volatiles involved in recognition of oilseed rape flowers, *Brassia napus* by honeybees, *Apis mellifera*. *J. Chem. Ecol.* 23: 1715–1727.
- De Jong, R. and L. Kaiser (1992) Odour preference of a parasitic wasp depends on order of learning. *Experientia* 48: 902–904.
- Honda, T. and Y. Kainoh (1998) Age-related fecundity and learning ability of the egg-larval parasitoid, *Ascogaster reticulatus* Watanabe (Hymenoptera: Braconidae). *Biol. Cont.* 13: 177–181.
- Honda, T., Y. Kainoh and H. Honda (1998) Enhancement of learned response to plant chemicals by the egg-larval parasitoid, *Ascogaster reticulatus* Watanabe (Hymenoptera: Braconidae). *Appl. Entomol. Zool.* 33: 271–276.
- Honda, T., Y. Kainoh and H. Honda (1999) The persistence of a learned response in the egg-larval parasitoid *Ascogaster reticulatus* Watanabe (Hymenoptera: Braconidae). *Entomol. Science* 2: 335–340.
- Kainoh, Y. (1988) Some factors influencing sex ratio in *Ascogaster reticulatus* Watanabe (Hymenoptera: Braconidae). *Appl. Entomol. Zool.* 23: 35–40.
- Kainoh, Y. (1997) Learning of plant's contact chemicals by the egg-larval parasitoid, *Ascogaster reticulatus* Watanabe (Hymenoptera: Braconidae). *Appl. Entomol. Zool.* 32: 416–418.
- Kaiser, L. and R. De Jong (1993) Multi-odour memory influenced by learning order. *Behavioural Processes* 30: 175–184.
- Kaiser, L., R. Prez-Maluf, J. C. Sandoz and M. H. Pham-Delegue (2003) Dynamics of odour learning in *Lepidopilina bouldardi*, a hymenopterous parasitoid. *Anim. Behav.* 66: 1077–1084.
- Kamijo, K. (1973) The parasite complex of *Choristoneura diversana* Hübner injurious to todo-fir, *Abies sachalinensis* Masters. *Jpn. J. Appl. Entomol. Zool.* 20: 380–386 (in Japanese).
- Kawakami, T. and Y. Kainoh (1986) Host specificity of the egg-larval parasitoid, *Ascogaster reticulatus* Watanabe (Hymenoptera: Braconidae) among five tortricid and one noctuid species. *Appl. Entomol. Zool.* 21: 8–14.
- Laloi, D., O. Bailez, M. M. Blight, B. Roger, M. Pham-Delegue and L. J. Wadham (2000) Recognition of complex odors by restrained and free-flying honeybees, *Apis mellifera*. *J. Chem. Ecol.* 26: 2307–2319.
- Lewis, A. C. (1986) Memory constraints and flower choice in *Pieris rapae*. *Science* 32: 863–865.
- Meiners, T., F. Wäckers and W. J. Lewis (2003) Associative learning of complex odours in parasitoid host location. *Chem. Senses* 28: 231–236.
- Minamikawa, J., and M. Osakabe (1979) *Insect Pest of the Tea Plant*. Japan Plant Protection Association, Tokyo. 322 pp (in Japanese).
- Seino, H. and Y. Kainoh (2008) Associative learning and discrimination of 10 plant species by the egg-larval parasitoid, *Ascogaster reticulata* Watanabe (Hymenoptera: Braconidae). *Appl. Entomol. Zool.* 43: 83–90.
- Takagi, K. (1974) Monitoring of hymenopterous parasite in the field. *Bull. Nat. Res. Inst. Tea* 10: 91–131 (in Japanese with English summary).
- Takasu, K. and W. J. Lewis (1996) The role of learning in adult food location by the larval parasitoid, *Microplitis croceipes* (Hymenoptera: Braconidae). *J. Insect Behav.* 9: 265–281.
- Tamaki, Y. (1966) Mass rearing of the smaller tea tortrix, *Adoxophyes orana* Fischer von Röslerstamm, on a simplified artificial diet for successive generations (Lepidoptera: Tortricidae). *Appl. Entomol. Zool.* 1: 120–124.
- Vet, L. E. M. (2001) Parasitoid searching efficiency links behavior to population process. *Appl. Entomol. Zool.* 36: 399–408.
- Vinson, S. B. (1998) The general host selection behavior of parasitoid hymenoptera and a comparison of initial strategies utilized by larvaphagous and oophagous species. *Biol. Cont.* 11: 79–96.
- Watanabe, C. (1967) Description of a new species of the genus *Ascogaster* Wesmael and notes on synonymy of *Apanteles* species (Hymenoptera, Braconidae). *Insecta Matsumurana* 29: 41–44.
- Yamanishi, T., T. Kiribuchi, M. Sakai, N. Fujita, Y. Ikeda and K. Sasa (1963) Studies on the flavor of green tea Part V. examination of the essential oil of the tea-leaves by gas liquid chromatography. *Agr. Biol. Chem.* 27: 193–198.