TITLE:

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CITATION:

Moriyama, Minoru ...[et al]. Dead-twig discrimination for oviposition in a cicada, *Cryptotympana facialis* (Hemiptera: Cicadidae). *Applied Entomology and Zoology* 2016, 51(4): 615-621

ISSUE DATE:

2016-11

URL:

<http://hdl.handle.net/2433/218683>

RIGHT:

The final publication is available at Springer via <http://dx.doi.org/10.1007/s13355-016-0438-z>; The full-text file will be made open to the public on 01 November 2017 in accordance with publisher's 'Terms and Conditions for Self-Archiving'; この論文は出版社版ではありません。引用の際には出版社版をご確認ご利用ください。; This is not the published version. Please cite only the published version.

Dead twig-discrimination for oviposition in a cicada, *Cryptotympana*
facialis (Hemiptera: Cicadidae)

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Abstract In phytophagous insects, in spite of some general advantages of oviposition on a vital part of their host food plants, certain species prefer dead tissues for oviposition. In the present study, we examined oviposition-related behaviors of a cicada, *Cryptotympana facialis* (Walker), which lays eggs exclusively into dead twigs. From behavioral observation of females experimentally assigned to live or dead plant material, we found that egg laying into freshly cut live twigs is abandoned in two phases, i.e. before and after initiation of egg nest-creating behavior with the ovipositor. Behavioral sequence analyses revealed that oviposition was generally preceded by rubbing with the rostrum tip and brief stylet-penetration behavior, suggesting that oral assessment may play a primary role in decision-making of oviposition in the earlier discriminating phase. From the similarity in behavioral flows of this assessment to vital tissue-seeking behaviors, cicada females are presumed to judge a twig as dead by sensing the absence of vital cues. These findings contribute to understanding a behavioral basis of dead plant recognition for oviposition, potentially giving an insight into ecological and evolutionary aspects of diverse oviposition preferences.

Keywords Cicada · Dead twig · Kinematic diagram · Oviposition behavior · Oviposition site selection

Introduction

Due to the immobility of eggs and limited dispersal ability of neonates, insect performance at the early life stages heavily depends on oviposition site selection by their mothers (Refsnider and Janzen 2010; Thompson 1988). The mothers receive a variety of chemical and physical cues that predict future circumstances that would optimize offspring's performance (Afify and Galizia 2015; Nottingham 1988). Suitable oviposition sites, however, vary among species depending on the species' own feeding habits, life cycle strategies, and inter- and intra-specific interactions (Martínez et al. 2013; Resetarits 1996; Thompson and Pellmyr 1991).

In phytophagous insects, eggs are typically located on their host plants to facilitate access to food resources (Martínez et al. 2013; Thompson and Pellmyr 1991). Even within a host plant, mothers generally prefer a particular part possessing higher nutritional quality and greater vitality (Awmack and Leather 2002). In spite of the intuitive virtue of such proximity to vital tissues, some phytophagous species deposit their eggs preferably on dead parts of a host plant (Desurmont et al. 2009). In contrast to the accumulation of knowledge about oviposition site selection among vital plant tissues, little has been discovered about the processes of discriminating dead tissues from live tissues of a plant.

Cicadas (Hemiptera: Cicadidae) are xylem fluid feeders, and they suck this fluid from aboveground parts of plants in the adult stage, but from roots in the underground nymphal stages (Cheung and Marshall 1973; White and Strehl 1978). Although there are species-specific host plant preferences for feeding, cicadas generally

exploit a wide variety of plant species (Beamer 1928; Hayashi and Saisho 2011). They lay eggs into the inside of plant tissues. Females bore a hole, an egg nest, first by inserting their arrow-shaped ovipositor with a twisting motion (see Online resource 1), and then deposit eggs into the hole while gradually withdrawing the ovipositor (see Online resource 2). This boring–egg laying process is usually repeated sequentially, consequently creating a series of egg nests on the plant. At the time of hatching, first-instar nymphs escape from the egg nests, fall on the ground and burrow into the soil to reach a suitable rootlet (Ito and Nagamine 1981; Karban 1997; Moriyama and Numata 2006). Although a single cicada species usually uses a broad range of plant species as oviposition substrates, females, in principle, select either live or dead tissues of a plant depending on the cicada species (Beamer 1928; Hayashi and Saisho 2011). For example, periodical cicadas in the genus *Magicicada* lay eggs into the live twigs of trees (Mattingly and Flory 2011; White 1980; Yang et al. 2006), and twig viability is essential for their embryonic development (White 1981, White and Lloyd 1981). Oviposition of the sugar cane cicada, *Mogannia minuta* Matsumura, is made in the mid-rib of a green leaf (Azuma 1976; Hayashi 1976). On the other hand, oviposition of *Quesada gigas* (Oliver), a coffee plant pest in Brazil, is made exclusively on dead twigs remaining on a live tree (Decaro Júnior et al. 2012), as is the oviposition of five cicada species common in Japan (Hayashi and Saisho 2011; Moriyama and Numata 2011, 2015).

The present study aimed to understand a behavioral basis of oviposition site selection in a cicada, *Cryptotympana facialis* (Walker). This is a large black cicada that lives in the western part of Japan (See Fig. 1) and uses a dead twig as an oviposition

substrate. Recently, extensive domination of this species in urban areas, which is associated with loss of cicada diversity, has become a new social problem in Japan (Moriyama and Numata 2011, 2015; Takakura and Yamazaki 2007). In fact, heavy infestation of *C. facialis* in residential areas causes breakdown of optical fiber cables due to piercing with the cicadas' ovipositor (Cyranoski 2007; Tsukamoto et al. 2008, 2010). In the first step to understand how cicada females discriminate dead twigs from live twigs, we observed behaviors of *C. facialis* females that were experimentally hung on dead or live plant material, and analyzed the behavioral sequence leading toward oviposition.

Materials and methods

Insects

Adult females of *C. facialis* were collected in Osaka City and Matsubara City, Osaka, Japan from 9 to 15 August 2010. During this time, most females were mature and devoted to laying eggs. To minimize disturbance during transport, captured females were caged in a cardboard box (approximately 250 x 300 x 300 mm) and protected from light.

Observation of oviposition behavior

A collected female was individually housed in an observation chamber (100 x 35 x 29 mm) made of plastic boards (Fig. 1). Two chambers separated by an opaque white plastic board were recorded together using a digital video camera (Panasonic,

HDC-TM300). One of the following three oviposition substrates was installed at the top of each chamber: 1) Commercially available squared wood sticks of *Albizia* (6 x 6 x 130 mm), 2) Dead twigs of *Cinnamomum camphora* (L.) (approximately 10–15 mm in diameter and 130 mm in length), and 3) freshly cut live twigs of the same tree (approximately 10–15 mm in diameter and 130 mm in length). Wood sticks were soaked in tap water for several days and dried before use, in order to remove adhesive and antiseptic agents. Cut edges of live twigs were wrapped with wet cotton to protect them from dehydration. The narrow observation chamber had enough space for females to perform normal behaviors other than flying, and a female that fell from a perch in the chamber could easily return to a hanging position. The chambers were kept in an air-conditioned room ($25 \pm 1^\circ\text{C}$) during observation.

Analyses of behaviors leading toward oviposition

From video playbacks, we recorded the start and end time of each action listed in Table. 1 (see Results for details). At the times around the end of boring and around the beginning of laying eggs, the ovipositor was completely inserted into a substrate, and the abdominal tip was in contact with the substrate, preventing us from viewing their discriminative motions. Thus, we could not know the exact time of behavioral switching from boring to laying eggs. Actions that did not end within the observation period were removed from analyses. Because our preliminary observation confirmed the existence of laid eggs after egg-laying behavior by dissecting the oviposition substrate, we judged egg laying only from video playbacks. We performed statistical analyses under a generalized linear model (GLM) framework (Crawley 2005), unless otherwise

noted. The number and duration of each action were compared with a model assuming a Poisson and Gamma error structures, respectively, while a binomial error structure was selected for oviposition success rates. When sample overdispersion was observed, we adopted a mixed model that considers individual variation as a random effect. If deviance reduction due to the treatment term was significant in a chi-square test, we compared its effect between each treatment group by Tukey-type multiple comparisons. All statistical analyses were conducted using R (ver. 3.3.0, R core team 2016).

Results

To investigate dead-twig discriminating behavior involved in oviposition, an adult female of *C. facialis* was introduced into an observation chamber with one of the following three oviposition substrates. The first one was a commercially available *Albizia* squared wood stick. This soft, homogeneous material has been used in previous studies dealing with several cicada species laying eggs into dead twigs, including *C. facialis* (Moriyama and Numata 2011, 2015). The second one was a dead twig of *Cinnamomum camphora*, which is most frequently utilized by *C. facialis* for oviposition in the collection site, while the third one was a freshly cut live twig of the same tree species. During a 90-min observation period, there was a clear difference in the number of ovipositions among the three substrates (Fig. 2a). In the groups provided with a wood stick or a dead twig, a majority of females (22/26 and 17/27, respectively) at least created an egg nest, whereas only 3 out of 31 females that were hung on a fresh twig performed oviposition. This low egg load on fresh twigs was attributed in part to the

fact that fewer females attempted to bore (Fig. 2b), and in part to the fact that fewer females achieved oviposition even after initiation of boring (Fig. 2c). Females on fresh twigs often ceased to create a hole and abandoned oviposition without laying eggs. These results suggest that *C. facialis* females can discriminate dead twigs from live twigs in at least two phases, i.e. before and after initiation of boring.

Next, we focused on how cicada females discriminate live and dead tissues before initiation of boring by analyzing behavioral sequences leading to oviposition. Besides trivial behaviors, including standing still, walking, and sweeping the antennae, we observed five discrete actions into which oviposition behavior was subdivided depending on its success (Table 1). Their behavioral relationships were analyzed by creating kinematic diagrams (Fig. 3). In these diagrams, actions that successively occurred within 60 s were regarded as a single sequential behavior. Once females were motivated to perform oviposition, they tended to carry out repeated boring–oviposition behaviors to create a series of egg nests. Therefore, we focused on behavioral sequences leading to the first oviposition in a series of repetitive ovipositions. Regardless of the substrate material, most females performed “rubbing” first, an action in which a female moved the rostrum tip along a substrate’s surface (Online resource 3). Mean duration of rubbing behavior (ranging from 12.0–14.0 s) did not significantly differ among substrates (a likelihood ratio test of GLM, $p > 0.05$). Rubbing was frequently followed by “probing”, a plant-sap feeding-like behavior in which a female pressed the rostrum perpendicularly against the substrate and seemed to penetrate the stylet into it (Online resource 4). In the group provided with a wood stick (Fig. 3a), 86.8% of cases (33/38) of boring–oviposition occurred subsequent to rubbing, either directly thereafter or after

a subsequent brief probing, whereas the other 13.2% (5/38) of boring–ovipositions occurred abruptly without rubbing. “Scuffing”, an action in which a female scuffs the abdominal tip against a substrate (Online resource 5), was exclusively observed just after oviposition. Similarly, most cases of boring (36/38) followed rubbing with or without probing in females hung on a dead twig (Fig. 3b). Boring by females, however, went through probing more frequently on dead twigs than on wood sticks (27/36 vs 14/33, χ^2 test, $p < 0.05$). In contrast to the behaviors on these two dead substrates, females hung on fresh twigs showed different behavioral flows (Fig. 3c). A higher proportion of rubbing individuals proceeded to probing (75.4%, 175/232), and a higher proportion of probing individuals terminated an action sequence without boring–oviposition behavior (52.6%, 92/175) than those of the other two groups (χ^2 test with a Bonferroni correction, $p < 0.05$). It was notable that both the frequency and duration of probing behavior were significantly increased in females hung on a fresh twig compared to females in the other two groups (Fig. 4), suggesting that females may have ingested xylem sap from the fresh twig in this probing period. Taken together, our findings show that initiation of boring for oviposition appears to be preceded by rubbing and probing behaviors, indicating that oral assessment plays a critical role in the discrimination of plant vital status.

Discussion

The results of the present study demonstrated that females of *C. facialis* achieve discrimination of dead twigs from live twigs by two discrete behaviors:

rostrum-mediated assessment before boring and ovipositor-mediated assessment after initiation of boring.

The first oral assessment phase is presumed to consist of two check points. The rubbing behavior seems to monitor plant chemicals released from the plant surface, whereas the probing or brief sap sucking-like behavior is likely to investigate plant vital status by evaluating plant sap. In the behavioral sequence leading toward oviposition, probing was skipped more often in females on wood sticks, which are more easily recognized as a dead tissue than dead twigs (Fig. 3a, b), implying that probing may function as a backup assessment if the preceding rubbing does not provide adequate assessment. This raises the question of what cue(s) cicada females sense to recognize a twig as a dead tissue. In insects preferring a dead plant material for oviposition, however, it is unusual to sense direct evidence of dead plant status. For example, females of the viburnum leaf beetle, *Pyrrhalta viburni* (Paykull), tend to lay eggs into dead twigs (Desurmont et al. 2009). Desurmont et al. (2009) demonstrated that this is accomplished not by recognizing plant vital status, but rather by preferring twigs with oviposition remains made in the previous year. Eggs of the broad-headed bug, *Neomegalotomus parvus* (Westwood), are frequently laid on dry pods of legumes, but these pods contain matured seeds that are an adequate food source to hold females on them (Ventura and Panizzi 2003). In the Japanese pine sawyer, *Monochamus alternatus* Hope, adult females are attracted to volatiles released from weakened or dying trees, but not from already dead trees (Ikeda et al. 1980). In light of these facts, it is not expected that *C. facialis* has an ability to sense direct cues of dead twigs. Rather, dead-twig discrimination seems more likely to be accomplished by sensing the absence of

evidence that twigs are alive, for the following two reasons. First, *C. facialis* females frequently attempt oviposition into various artificial substrates that lack both live and dead plant cues, such as paper poles and cables sheathed with rubber and resinous materials (Tsukamoto et al. 2008, 2010; M. Moriyama, personal observation). Even in the field, there have been more than 1000 records per year of an optical fiber cable suffering from oviposition damage by this species (Cyranoski 2007). Second, oral assessment for oviposition had the same behavioral sequence as that for food ingestion: When a female meets a live twig, probing behavior seems to be taken over directly by sap sucking instead of oviposition (see Figs. 3c and 4). Assessment using the rostrum, including both rubbing- and probing-like behaviors, is a prevalent mechanism of host plant recognition in food-seeking processes among hemipteran insects (Backus 1988; Chapman and Bernays 1989), and it serves a discriminative function also in oviposition site selection (Ferran et al. 1996; Romani et al. 2005). Some cicadas have species-specific host plant preferences for feeding (Beamer 1928; Hayashi and Saisho 2011). In addition, in cicada species that lay eggs into live twigs, plant preference for oviposition has also been demonstrated (Clay et al. 2009; Cook et al. 2001; White 1980). These facts may support the idea that cicadas possess fundamental abilities to assess plant quality for selecting suitable feeding and oviposition sites, and therefore it is more parsimonious to associate the absence of plant vital cues with the decision of oviposition in dead twig-ovipositing cicadas than to posit obtaining a novel ability to detect a dead cue. Actually, absence of vital cues is expected to be sufficient to identify dead twigs, because cicadas rarely meet stick-shaped materials other than twigs under natural circumstances.

The second discrimination, ovipositor-mediated discrimination, is conducted in the course of boring and consists of making a decision whether or not to proceed to egg laying. Most females that had initiated boring on a freshly cut twig abandoned their oviposition at this check point (Fig. 2c). Ovipositors that bear various types of sensilla responsible for chemo- and mechano-reception play an important role in oviposition site selection, especially in stimulating oviposition behavior, in various insects (Hattori 1988; Hummel et al. 2006; Justus and Mitchell 1996; Stoffolano and Yin 1987). Although the morphological details of the cicada ovipositor have not been investigated yet, it lacks clearly visible sensilla (Kato 1956). Therefore, two possible mechanisms, which are not mutually exclusive, may be involved in this discrimination phase in cicadas: 1) plant chemicals released during boring are sensed using sensillum-bearing organs adjacent to the ovipositor, and 2) physical properties of substrates are sensed through the ovipositor. Experiments using artificial cables that are coated with resins having various physical properties have revealed that elastic or hard substrates decrease the depth of boring injuries by *C. facialis* females and inhibit the subsequent egg laying process (Katsuya 2007; Tsukamoto et al. 2010). Therefore, at least the physical properties of substrates are likely to influence the decision of egg laying.

In the present study, a minor but non-negligible portion of *C. facialis* females attempted to bore into freshly cut twigs, and a few of them laid eggs into them (Fig. 2a, b). In our experimental conditions, confining a female in a narrow chamber with undesirable substrates may have forced the female to initiate oviposition into the substrates. Beamer (1928) also observed that cicadas that normally lay eggs into either dead or live substrates used the other if the preferred substrate was not available.

Although in the present study we demonstrated substantial differences in behavioral responses between fresh and dead twigs, a freshly cut twig is not the same as a living twig attached to a tree. Especially, freshly cut twigs must have lost negative pressures that are normally maintained inside the xylem vessels (Schurr 1998), and contained a tiny amount of xylem sap that would have been depleted immediately. These unnatural situations may have confused females about oral information. In the field, we have observed hundreds of *C. facialis* females boring into dead twigs, but scarcely any boring into live twigs (M. Moriyama and H. Numata, personal observation). Therefore, we expect that oral assessment more accurately discriminates plant vital status in the field than in the experimental conditions.

Egg laying behavior was usually followed by scuffing behavior (Fig. 3). At the end of egg laying behavior, females of some cicada species close the opening of the egg nest by filling it with glue-like secretion or by pushing back the torn tissue with the ovipositor (Beamer 1928; Hayashi and Saisho 2011). Females of *C. facialis* did not secrete a glue-like material and their egg nests were left open, although the torn tissue at the opening was somewhat pressed during scuffing behavior (M. Moriyama and H. Numata, personal observation).

Until now, the ecological consequences of dead twig oviposition in cicada species have not been determined. In some beetles, concealing eggs in dead twigs is considered to be advantageous by avoiding plant defense responses (Desurmont et al. 2009; Shibata 1987). In cicadas that use a live twig as an oviposition substrate, egg nests suffer the risk of being confined by plant resin (White 1981; White et al. 1982), although the moisture supply from live twigs is essential for egg survival and

development in these species (Azuma 1976; White and Lloyd 1981). It is possible that oviposition preference for dead twigs has evolved to enable cicadas to escape from plant defense responses at the cost of acquiring desiccation tolerance. Moreover, oviposition preference for dead twigs is likely to be strongly associated with some life-cycle strategies of cicadas, such as humidity-dependent hatching (Moriyama and Numata 2006, 2011) and overwintering egg diapause (Moriyama and Numata 2008, 2011). Two congeneric species of *C. facialis*, *C. atrata* (F.) and *C. yaeyamana* Kato, perform oviposition into live twigs (Hayashi and Saisho 2011). Future investigations of the contrasting oviposition behavior and egg physiology among these three species will elucidate the ecological and evolutionary significance of oviposition site selection in cicadas.

Acknowledgements We thank Elizabeth Nakajima for linguistic corrections.

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Table 1 List of oviposition-related behaviors in *Cryptotympana facialis*

Action	Description
Rubbing	Moving the rostrum tip along a substrate
Probing	Appressing the rostrum perpendicularly to a substrate
Boring	Digging a hole into a substrate using the ovipositor
Oviposition (completed)	Inserting eggs into the hole while gradually withdrawing the ovipositor
Oviposition (abandoned)	Withdrawing the ovipositor without depositing eggs
Scuffing	Scuffing the abdominal tip against a substrate

Fig. 1 Photograph of *Cyptotympana facialis* adult females in the observation chamber

Fig. 2 Effects of oviposition substrates on induction and success of oviposition. An adult female was caged with one of the three oviposition substrates, and its behavior was observed for 90 min. (a) The number of ovipositions completed per female. (b) The number of oviposition trials, that is, initiation of boring, per female. (c) Completion rates of oviposition trials. Data are shown by Tukey box plots; they indicate the median (bold line), the 25th and 75th percentiles (box edges), the range (whiskers), and outliers, which are larger or smaller than 1.5 times the interquartile range from the box edge (open circles). The number of females in each group is indicated on the top of the graph. Groups marked with the same alphabetical character were not significantly different (a likelihood-ratio test of GLM and post-hoc multiple comparisons, $p > 0.05$)

Fig. 3 Kinematic diagrams for oviposition-related behaviors. Sequential relationships between behaviors, which occurred successively within 60 s, are linked with lines for females hung on a wood stick (a, from 25 females), a dead twig (b, from 24 females), or a live twig (c, from 25 females). The number in a box is a cumulative total number of observations of each behavior. The number associated with a line is the number of observations of a flow, and the thickness of a line indicates its proportion relative to the total number of sequences, except that flows constituting less than 10% are depicted as broken lines. T-shaped lines indicate the end of a sequence that did not lead to oviposition. For the definition of each action, see Table 1

Fig. 4 Frequency and duration of probing behavior of *Cryptotympana facialis* females on different oviposition substrates. (a) The number of probing behaviors per female during a 90-min observation period. (b) The mean duration of a single probing behavior. The number of females is indicated at the top of the graph. Groups marked with the same alphabetical letter were not significantly different (a likelihood-ratio test of GLM and post-hoc multiple comparisons, $p > 0.05$). For details of Tukey box plots, see Fig. 2

Online Resource 1 A video of boring behavior

Online Resource 2 A video of egg-laying behavior

Online Resource 3 A video of rubbing behavior

Online Resource 4 A video of probing behavior

Online Resource 5 A video of scuffing behavior