Stylet penetration and survival of three psyllid species on adult leaves and ‘waxy’ and ‘de-waxed’ juvenile leaves of *Eucalyptus globulus*

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Accepted: July 26, 2001

Key words: Ctenarytaina eucalypti, Ctenarytaina spatulata, Glycaspis brimblecombei, salivary sheath, stylet tracks, probing, oil glands, heteroblasty, epicuticular wax, herbivory

Abstract

The stylet probing behavior and survival of *Ctenarytaina eucalypti* Maskell, *C. spatulata* Taylor and *Glycaspis brimblecombei* Moore (Homoptera, Psyllidae) on adult leaves and ‘waxy’ (untreated) and ‘de-waxed’ (rubbed) juvenile leaves of the heteroblastic plant *Eucalyptus globulus* Labillardiere were compared. Psyllids were confined within clip-on cages and after 15 days the leaf tissue under each cage was sectioned and stained to characterize and quantify stylet tracks. Across all leaf treatments and psyllid species 1438 stylet tracks were observed in 7239 leaf sections and 571 of these stylet tracks reached the vascular tissue. Survival and the number of stylet tracks of *C. eucalypti* on the different leaf types did not differ. In contrast, *C. spatulata* survived significantly longer and produced significantly more stylet tracks on adult leaves and ‘de-waxed’ juvenile leaves than on ‘waxy’ juvenile leaves. Although *G. brimblecombei* survived equally on all treatments, it produced more stylet tracks on adult leaves and ‘de-waxed’ juvenile leaves than on ‘waxy’ juvenile leaves. For all three psyllid species, there was a positive correlation between survival and number of stylet tracks reaching the vascular tissue. Stylet tracks of all species were generally intercellular from the leaf epidermis to the vascular tissue. Oil glands were avoided by all species, as indicated by the repeated branching and formation of stylet tracks at the periphery of the glands. In ‘de-waxed’ juvenile leaves, *C. spatulata* and *G. brimblecombei* produced stylet tracks that entered the leaf at the adaxial surface and exited at the abaxial surface. This pattern was rarely observed on adult leaves, and never occurred with *C. eucalypti* in any leaf type. We conclude that (1) the epicuticular wax on juvenile leaves reduced stylet probing by *C. spatulata* and *G. brimblecombei*, (2) there were apparently no internal physical barriers in either juvenile or adult leaves to prevent the stylets of any of the psyllid species from reaching the vascular tissue, (3) the psyllids avoided oil glands in both leaf types, and (4) *C. spatulata* and *G. brimblecombei* may lack cues to orient their stylets towards the vascular tissue in the juvenile leaves.

Introduction

During the past decade, several exotic *Eucalyptus* psyllids were reported in California, including *Ctenarytaina spatulata* Taylor and two economically important pest species (*C. eucalypti* Maskell and *Glycaspis brimblecombei* Moore) (Brennan et al., 1999). In their native range (Australia) and as exotics, *C. spatulata* and *G. brimblecombei* are generally found on glossy leaved species, whereas *C. eucalypti* is found primarily on glaucous leaved *Eucalyptus* spp. (Morgan, 1984; Taylor, 1997; Brennan et al., 1999). In studies with these three species on the heteroblastic tree *Eucalyptus globulus* Labillardiere, Brennan (2000) found that the glossy, sickle-shaped adult leaves exhibited ovipositional antixenosis to *C. eucalypti*, whereas the glaucous, simple-shaped juvenile leaves exhibited ovipositional antixenosis to *C. spatulata* and *G. brimblecombei*. This was one of the first examples where heteroblasty [which describes
plants whose juvenile leaves and shoots differ morphologically from the adult leaves and shoots (Johnson, 1926; Gould, 1993; Day, 1998a) was shown to affect herbivory (see also Edwards, 1982; Edwards & Wanjura, 1990). Epicuticular wax on juvenile leaves of *E. globulus* plays a primary role in resistance to *C. spatulata* and *G. brimblecombei*, because these species survived longer and settled more often on ‘de-waxed’ than on ‘waxy’ juvenile leaves (Brennan & Weinbaum, 2001a).

Plant resistance mechanisms to Homoptera involve external (i.e. trichomes, hairs) and internal anatomical characteristics (i.e. sclerenchyma tissue) (Johnson, 1953; Jayaraj, 1967; Quiros et al., 1977; Brewer et al., 1986; Hoffman & McEvoy, 1986), and external and internal chemical and physiological features (Pollard, 1973; Montllor, 1991). Studies of homopteran stylet tracks began over 100 years ago (Prillieux, 1878; Bügen, 1891) and have provided extensive information on feeding sites and in some cases have indicated the location of plant anatomical resistance factors. Although studies of plant resistance to aphids often include histological examination of stylet tracks (Pollard, 1973; Spiller et al., 1985; Bing et al., 1991; Ni & Quirosberry, 1997) and electronic monitoring of feeding behavior (Montllor & Tjallingii, 1989; Tjallingii, 1995; Klingler et al., 1998), to our knowledge these approaches have seldom been used to study plant resistance to psyllids (however see Ullman & McLean, 1988a, b). Studies on psyllid resistance have focused primarily on nymphal development in resistant versus susceptible plants (Moran, 1968; Butt et al., 1988; Mensah & Madden, 1991; Lapis & Borden, 1993; Berrada et al., 1995) and leaf-hardness (Moran & Buchan, 1975; Mensah & Madden, 1992).

The present study addresses the following questions: (1) Does stylet probing behavior of the three psyllid species differ on adult leaves, ‘waxy’ (untreated) juvenile leaves and ‘de-waxed’ juvenile leaves of *E. globulus*? (2) Does stylet probing behavior correlate with psyllid survival? (3) Is stylet probing behavior suggestive of psyllid resistance mechanisms in the juvenile and adult leaves?

**Materials and methods**

The experiment was conducted from 19 July to 3 August, 1999 in El Cerrito, California on an *E. globulus* tree with juvenile and adult leaves 1–2 m high in the canopy. Sexually mature adult female psyllids from wild populations of the three species were used. Juvenile leaves were ‘de-waxed’ by gently rubbing a cotton swab on the adaxial surfaces of normally ‘waxy’ juvenile leaves to remove the glaucous bloom. Our use of the terms ‘waxy’ and ‘de-waxed’ refer only to epicuticular wax. Five females were placed in clip-on cages for each species in the three different leaf treatments to increase the number of stylet tracks in the leaf area under each cage. Each cage only contained one species. Each cage covered approximately 113 mm² of leaf surface.

The experiment was replicated three times and in each replicate, cages with the three psyllid species were randomly distributed to one of three positions on a single leaf of each treatment. We carefully selected leaves and shoots that were free of psyllids. The three cages on each leaf were positioned on the adaxial leaf surface over the mid rib with approximately 5 mm between cages. Adults of these psyllids naturally feed on both adaxial and abaxial surfaces and previous experiments found equal survival on both leaf surfaces (Brennan & Weinbaum, 2001a). Survival was recorded three times per day (approximately 6 AM, 12 PM, 6 PM) during days one and two, twice (6 AM, 6 PM) on day three, and once (6 AM) on days 4 to 8, 10, 12 and 14. To assess psyllid survival without a food source, we caged five individuals of each species in three cages on plastic transparency sheets within the tree canopy. On day 15, we harvested all of the leaves. A hand microtome was used to section the disk of leaf tissue under each cage, perpendicular to the mid rib, into transverse sections that were approximately 25 µm thick. The sections were cleared and stained with acid fuchsin and aniline blue as described by (Brennan et al., 2001). Under a compound light microscope, stylet tracks were counted and described. Because the sequential order of the sections was not maintained, different parts of the same stylet track may have been included in separate sections. To quantify survival we calculated the cumulative number of psyllid-days per cage. We used the PROC GLM procedure in SAS (Version 6.12, SAS Institute Inc., Cary, NC) to conduct analyses of variance comparing survival (i.e. psyllid-days), the total number of stylet tracks, and the number of stylet tracks ending in the vascular tissue of the three leaf treatments of each psyllid species separately. The analyses were conducted separately for each psyllid species due to inequality of variances between the species. The sequential Bonferroni method (Rice, 1989) which controls for the type-I error rate was used for mean
Results

Psyllid survival was generally shortest on the plastic sheets (Figure 1). Across the three leaf treatments, *G. brimblecombei* died the fastest, followed by *C. spatulata* and *C. eucalypti* (Figure 1). *Ctenarytaina eucalypti* survived equally well on all leaf treatments ($F_{2,6} = 0.2; P = 0.82$) (Figure 2). In contrast, *C. spatulata* survived significantly longer on adult leaves than on ‘waxy’ juvenile leaves, and for an intermediate period on ‘de-waxed’ juvenile leaves ($F_{2,6} = 10.1; P < 0.05$) (Figure 2). Although *G. brimblecombei* survived longer on adult and ‘de-waxed’ juvenile leaves than on ‘waxy’ juvenile leaves, these differences were not significant ($F_{2,6} = 1.97; P = 0.2$) (Figure 2).

A total of 7239 leaf sections were examined for stylet tracks among the three leaf treatments for all psyllid species. The area under each cage included an average ($\pm$ SE) of 268 $\pm$ 7 sections. Across all treatments and species, 1438 stylet tracks were observed including 571 that reached the vascular tissue. All of the stylet tracks originated from the adaxial leaf surfaces where the psyllids were caged. Stylet tracks were found in the leaf tissue under cages of all three leaf treatments and psyllid species, however, the number of tracks differed with treatment and species (Figure 3). Across the three psyllid species, approximately half of the total number of stylet tracks reached the vascular tissue. The number of *C. eucalypti* stylet tracks (total, and to the vascular tissue)
did not differ significantly among the three leaf treatments. In contrast, *C. spatulata* produced significantly fewer stylet tracks (total, and to the vascular tissue) in ‘waxy’ juvenile leaves compared with the adult leaves and ‘de-waxed’ juvenile leaves. *Ctenarytaina spatulata* also produced about twice as many stylet tracks as the other species in the adult leaves and ‘de-waxed’ juvenile leaves. Although *G. brimblecombei* produced fewer stylet tracks to the vascular bundle in the waxy juvenile leaves than in the ‘de-waxed’ juvenile leaves and adult leaves, these differences were only marginally significant (*P* = 0.07 and 0.08, respectively). Stylet track numbers per cage varied greatly in all species as indicated by the large standard error bars (Figure 3). There was a positive correlation between survival (i.e., cumulative psyllid days) and the number of stylet tracks to the vascular tissue for *C. eucalypti* (*r* = 0.84), *C. spatulata* (*r* = 0.95), and *G. brimblecombei* (*r* = 0.91).

Qualitatively, the stylet tracks of the three psyllid species appeared similar. Generally the stylet tracks entered the leaf intercellularly including entries through the stomata. Within the mesophyll, most stylet tracks were intercellular. The oil glands were avoided by all species, as indicated by the repeated branching of the stylet track at the oil glands and subsequent formation along the edge of the oil gland (Figure 4). The stylets of all species were clearly capable of reaching the vascular tissue at the leaf mid rib and at minor veins of both the juvenile and adult leaves. A notable difference in the stylet tracks of the three species was the frequency of tracks that went from the adaxial epidermis to the abaxial epidermis and occasionally exited the leaf at the abaxial surface (Figure 5). *Ctenarytaina eucalypti* never exhibited this character in 352 stylet tracks found across all three leaf treatments and *G. brimblecombei* exhibited it rarely (i.e., in three of 126 stylet tracks in the ‘de-waxed’ juvenile leaves, and one of 119 stylet tracks in adult leaves). However, *C. spatulata* exhibited it quite frequently (i.e., in 48 of 357 stylet tracks in the ‘de-waxed’ juvenile leaves and in three of 451 stylet tracks in the adult leaves).

**Discussion**

This study demonstrated that (1) heteroblasty in *E. globulus* affected the stylet probing behavior of *C. spatulata* and *G. brimblecombei*, (2) stylet probing by these species on juvenile leaves was reduced by the presence of epicuticular wax, and (3) survival of all three species was correlated positively with the number of stylet tracks that reached the vascular tissue. To our knowledge, this is the first study on plant resistance to psyllids that investigated the relationship between survival and stylet probing behavior, and the first to study stylet probing of herbivores of heteroblastic plants.

The survival of psyllids on the three leaf treatments in the present study is consistent with a previous study (Brennan & Weinbaum, 2001a) that measured psyllid survival, leaf-settling and honeydew production. Brennan and Weinbaum (2001a) speculated that the prolonged survival of *C. spatulata* and *G. brimblecombei* on adult leaves than on ‘waxy’ juvenile leaves, and on ‘dewaxed’ juvenile leaves than on ‘waxy’ juvenile leaves was due to increased feeding. The present study supports this reasoning because *G. brimblecombei* and *C. spatulata* produced several times
more stylet tracks to the vascular tissue in adult leaves and ‘de-waxed’ juvenile leaves than in ‘waxy’ juvenile leaves. Although the presence of stylet tracks is not necessarily indicative of feeding (because psyllids may have probed without ingesting), the absence of stylet tracks precludes feeding. Therefore, the scarcity of *G. brimblecombei* and *C. spatulata* stylet tracks in ‘waxy’ juvenile leaves suggests that their relatively poor survival may have been due to starvation. Electrical penetration graph (EPG) techniques (McLean & Kinsey, 1964; Tjallingii, 1978) should be used in future studies of the feeding behavior and resistance mechanisms of the juvenile and adult leaves to these psyllid species. EPG techniques have shown that the number of stylet tracks that a homopteran produces in a plant is not always a good indicator of plant resistance. For example, some aphids and whiteflies produce a greater number of probes (stylet tracks) in resistant than susceptible cultivars, but probe duration was longer in susceptible cultivars (Montllor & Tjallingii, 1989; Lei et al., 1998).

Studies with other systems (Eigenbrode et al., 1991; Eigenbrode & Pillai, 1998; Powell et al., 1999) have shown that epicuticular wax can affect the biting and stylet probing behavior of insects. Epicuticular wax on the juvenile leaves of *E. globulus* appears to be the primary factor affecting the stylet probing behavior and survival of *C. spatulata* and *G. brimblecombei*. Due to the decreased settling of *C. spatulata* and *G. brimblecombei* on the ‘waxy’ juvenile leaves relative to the adult leaves and ‘de-waxed’ juvenile leaves, we speculated that epicuticular wax reduces adhesion of the psyllids to the leaf surface (Brennan & Weinbaum, 2001a). In a subsequent study (Brennan & Weinbaum, 2001b), we found that the epicuticular wax increased leaf slipperiness and prevented *C. spatulata* and *G. brimblecombei* from adhering to the ‘waxy’ juvenile leaves. Per unit of body weight, *C. spatulata* and *G. brimblecombei* had several times less adhesive pad area on their tarsomeres than *C. eucalypti*. Thus, it appears that *C. spatulata* and *G. brimblecombei* produced fewer stylets on the ‘waxy’ juvenile leaves because they were unable to adhere to the leaf surface. We speculate that the few stylet tracks produced by *C. spatulata* and *G. brimblecombei* on the ‘waxy’ juvenile leaves occurred in areas of the leaf where a small amount of wax was inadvertently rubbed off while placing the clip-on cages on the leaf.

Because stylet tracks of all three species were consistently oriented toward the vascular tissue and often reached the phloem, we assume that this was the preferred feeding site in both leaf types. Although stylets of *C. spatulata* and *G. brimblecombei* frequently lo-
cated the vascular tissue of the ‘de-waxed’ juvenile leaves, many of their stylet tracks also went from the adaxial to the abaxial epidermis, and occasionally exited the abaxial epidermis. To our knowledge this behavior has not been reported for other homopterans. We speculate that *C. spatulata* and *G. brimblecombei* stylets within ‘de-waxed’ juvenile leaves may have lacked cues found in the adult leaves, which orient them to the vascular tissue. McMurtry & Stanford (1960) found that stylet tracks of an aphid (*Theiosaphis maculata* Buckton) also appeared to lack orienting cues to the vascular bundles of resistant alfalfa cultivars. Although there is ample evidence that homopterans can direct the movement of their stylets to the tissue where they feed, the chemical or physical cues involved in this process are poorly understood.
(Pollard, 1973). Psyllids and other homopterans contain nerves within the stylets that are likely involved in chemoreception and selection of host plants and feeding sites within a host plant tissue (Forbes, 1972).

Foliar terpenoids (essential oils) in several plant families are thought to be involved in plant-herbivore interactions (Macedo & Langenheim, 1978; Langenheim & Stubblebine, 1983; McClure & Hare, 1984; Stone & Bacon, 1994; Goralka & Langenheim, 1996; Nehlin et al., 1996). *Eucalyptus* essential oils are compartmentalized in specialized secretory structures (oil glands) in the leaf mesophyll (Carr & Carr, 1969; 1970), and are generally thought to defend plants against insect herbivores (Morrow & Fox, 1980; Edwards et al., 1990; 1993), however this is poorly understood (Doran, 1991). In *E. globulus*, there are qualitative and quantitative differences in the essential oils in juvenile versus adult leaves (Brophy et al., 1991) that could potentially affect insect herbivores. All three psyllid species in our study apparently avoided the oil glands in both leaf types. To our knowledge, this is the first evidence that sucking insects perceive oil glands and direct their stylets around them.

We conclude that (1) the epicuticular wax on juvenile leaves reduced stylet probing by *C. spatulata* and *G. brimblecombei*, (2) there are no internal barriers in juvenile or adult leaves that prevent the stylets of any of the psyllid species from reaching the vascular tissue, (3) the oil glands in both leaf types are avoided by all three species, and (4) *C. spatulata* and *G. brimblecombei* may lack cues in the juvenile leaves to orient their stylets towards the vascular tissue. In future studies it may be useful to electronically monitor stylet penetration to understand further the psyllid resistance mechanisms of the juvenile and adult leaves of *E. globulus*.

Acknowledgements

We thank Jay Rosenheim and Rick Karban of the Department to Entomology at U.C. Davis for improving this manuscript. We appreciate the cooperation of the grounds staff of the Contra Costa Unified School District in Contra Costa County, California for allowing us to conduct this research on their grounds. We are grateful for the contributions of the late Sean Duffey at the beginning of this study. This research was funded by a Jastro Shields Research Grant.

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