Variation in Eastern Cottonwood (*Populus deltoides* Bartr.) Phloem Sap Content Caused by Leaf Development May Affect Feeding Site Selection Behavior of the Aphid, *Chaitophorous populicola* Thomas (Homoptera: Aphididae)

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ABSTRACT Apterous populations of *Chaitophorous populicola* Thomas (Homoptera: Aphididae) appear to track Eastern cottonwood (*Populus deltoides* Bartr.) leaf development. Few aphids occur on mature leaves. Marked individual aphids on leaves of different developmental stages were observed through a period of new leaf initiation. Nymph and adult *C. populicola* frequently track leaf development by moving up to younger leaves. A comparison of phloem sap constituents and leaf toughness among leaf developmental stages revealed some differences that could be used by *C. populicola* to determine leaf age. Phloem sap exudates, collected from *P. deltoides* leaves of different developmental stages, were analyzed by high-performance liquid chromatography for free amino acids and the phenolic glycoside salicin. Sucrose concentration in exudates, indicative of phloem sap exudation rate, was uniform among leaf stages. Of 20 amino acids examined, only aspartic acid and γ-amino-n-butyric acid (GABA) concentrations differed significantly between leaf stages. Forward stepwise discriminant function analysis showed that seven of the amino acids analyzed are useful for classifying leaf maturity groupings. Aphid-infested cottonwoods had lower cystine concentrations in phloem sap than aphid-free plants. Salicin concentration was significantly higher in new leaves. Leaf toughness was assessed by lignin density and distance measurements in petiole cross-sections. Rapidly expanding leaves had significantly less lignification and new leaves had shorter distances to the vascular bundles than senescent leaves. These physiological and phytochemical differences among *P. deltoides* leaf developmental stages may contribute to the leaf stage selection patterns exhibited by the aphid, *C. populicola*.

KEY WORDS aphid feeding behavior, leaf development, phloem sap, amino acid, salicin

The settling and feeding location of ambulatory aphid populations within a given host plant seems to be nonrandom and based, in part, on a preference for palatable leaves of a certain developmental stage or physiological age. The aphid *Chaitophorous populicola* Thomas (Homoptera: Aphididae), prefers rapidly expanding, metabolite importing "sink" and senescing, resource exporting "source" leaves of Eastern cottonwood (*Populus deltoids* Bartr) (Coleman and Jones 1988). *C. populicola* is host specific and aggregates on *Populus* spp. shoot tips throughout its North American range (Solomon 1985, 1986, Blackman and Eastop 1994). We hypothesized that *C. populicola* might relocate feeding sites, or track leaf development, to feed from preferred leaf stages.

Many aphid species prefer immature sink leaves with high rates of nitrogen transport over mature leaves (Kennedy et al. 1950, Way and Cammell 1970, Dixon 1985, 1998). Willow aphids preferentially feed on growing leaves—with phloem sap high in amino acid variety and quantity—over mature leaves with lower phloem amino acid content (Kennedy and Booth 1951; Mittler 1953). Although aphids can compensate on low-quality leaves by feeding at a faster rate (Kennedy and Stroyan 1959, Prosser et al. 1992), their tolerance has limits. Indeed, high sucrose and low amino acid concentrations correlate with increased production of winged aphids and dispersal (White 1972). Dynamic tracking of leaf development by aphids has associated costs in terms of energy, risk of desiccation, dissociation from the plant, and lost feeding time (Klingauf 1987a, b, Risebrow and Dixon 1987, Montllor 1991). Despite costs, early-instar scale homopterans and birch aphids move from older to

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younger leaves or shoots to find suitable feeding sites (Wratten 1974, Luft and Paine 1997). Also, apterous *Myzus persicae* move from leaf to leaf as their host plants develop, apparently tracking sink leaves (Jepson 1983, Harrington and Taylor 1990). Hence, evidence from other systems suggests that aphids generally prefer and seek immature "sink leaves," perhaps because of higher free amino acid content in the phloem sap. Thus, if phloem amino acid content or concentration varies with *P. deltoids* leaf development, *C. populicola* may relocate feeding sites to obtain more amino acids.

Phenolic glycosides are the predominant secondary substances in Salicaceae, detrimental to generalist and attractive to specialist herbivores, and may be mobilized in phloem sap (Palo 1984, Rowell-Rahier and Pasteels 1989, Matsuki and MacLean 1994, Lindroth and Hwang 1996). The phenolic glycoside content of cottonwood leaves varies with developmental stage and is greatest in new leaves (Coleman 1986, Bingaman and Hart 1993,Wait et al. 1998). If phloem sap phenolic glycoside composition differs among leaf developmental stages, the specialist aphid *C. populicola* may be able to detect such differences to determine leaf age.

Although phytochemicals may be dominant mediators of herbivore behavioral response, the physical toughness of plant tissue also varies with development. Aphids penetrate plant tissues with the aid of digestive salivary components (Miles 1968, Pollard 1973, Montllor 1991). The distance aphid stylets must traverse, and the lignification of plant vascular bundles affect aphid access to and extraction of phloem; and both distance to and lignification of vascular bundles increase as leaves age (Risebrow and Dixon 1987, Elliot and Hodgson 1996, Loudon and McCulloh 1999). To determine *P. deltoides* leaf stage toughness for aphids, we measured surface to leaf vein distances and lignification.

Thus, the objectives of this study were to determine if *C. populicola* (1) can track *P. deltoides* leaf development; (2) selects leaf developmental stages with the greatest amino acid and phenolic glycoside content; and (3) avoids leaves with high lignin content and greater surface to vascular bundle distances.

Materials and Methods

Plants. Eastern cottonwood (*Populus deltoides*) ST109 clone cuttings (USDA, Stoneville, MS) were obtained from the Institute of Ecosystem Studies, Millbrook, NY. Cuttings were planted in 4-liter gravel-lined pots of Magic-Earth (Wal-Mart) potting mixture. They were watered two to four times weekly, as necessary. The trees in pots were at least 35 cm apart and were kept in a partially shaded area where they received ≈6–8 h of direct sunlight per day. One half of the trees had the aphid *C. populicola* introduced onto them, whereas the rest were kept aphid-free by daily manual inspection and removal. A pooled postexperimental soil sample, analyzed by Cornell Nutrient Analysis Laboratories, Ithaca, NY, revealed the following soil conditions: available (mg/kg) NO_3 0, P 179.4, K 384, Mg 1718.4, Ca 17780, Fe 0.2, Al 18.8, Mn 56.1, Zn 19.2, Cu 2.1, pH 7.8, 67% organic matter, and 7% moisture content.

Plant Growth and Leaf Developmental Stage. ST109 cottonwood heights, leaf initiation, and leaf lengths were measured weekly. Leaves were assigned to "age-stage" positions using a modified form of the plastochron index of Erickson and Michelini (1957), as applied to cottonwood saplings (Larson and Isebrands 1971, Jones et al. 1993). The youngest leaf that is completely uncurled and is 2 ± 0.2 cm long is designated leaf plastochron index 0 (LP0). The next leaf down is LP1, the following is LP2, and so on. Initial index leaves on each plant were marked with a small spot of paint. Growth rates of plant height and leaf length were calculated per Wait et al. (1998) using the following formula: $[\ln(h_t) - \ln(h_{t-1})]/[t - (t-1)]$. The average leaf initiation rate was 0.11 ± 0.01 leaves/d, with an initial average of 11.0 ± 0.2 leaves and height of 31.1 ± 1.2 cm and a 65-d final average of 18.2 ± 0.5 leaves and height of 42.7 ± 1.9 cm.

Leaves were assigned to one of four developmental stages based on their mean length expansion rate, location, and physical appearance. The new leaf category included LP0 and younger leaves that were not yet unfurled and had lengths <1.8 cm. Rapidly expanding leaves had length expansion rates >0.001 cm/d; these included LP1–LP3. The transition between rapidly expanding and mature leaves, at which point length growth rate dropped below 0.001 cm/d, occurred at LP3–LP4 for the cottonwoods under these growing conditions. Senescing leaves were turning yellow or brown and were typically the second to third oldest, nonabscised LPs at the bases of the plants.

Aphid Populations and Individual Aphid Tracking. Aphids were initially collected from cottonwood in San Antonio, TX. A board-certified entomologist (RVWE Consulting, Frenchtown, NJ) confirmed their identity as *C. populicola.* Mixed-age populations of roughly 20–25 *C. populicola* were introduced onto the ST109 cottonwoods using leaf cuttings from their original host. On cottonwoods with aphids, the number of aphids on each leaf, their developmental stage (first–third instar or adult), and their location on the leaf was recorded weekly. Individual aphids (24 total, 1–3 per plant, 6 separate sessions, 14 plants) were painted with small dots of different-colored toluene and formaldehyde-free nail polish (Bath and Body Works, New Albany, OH) on the dorsal thorax or abdomen. In a 3-d pilot study, these markings did not interfere with aphid feeding or movement. Marked aphids were observed every 6 h, through the production of a new leaf, to determine whether they tracked leaf development.

To determine the amount of time associated with aphid relocation, 17 leaves containing 1–20 aphids were cut and attached onto new, rapidly expanding, mature or senescing leaves of new, aphid-free, ST109 cottonwood host plants. Aphids were observed hourly until they produced honeydew, a sign of phloem ingestion (Miles 1989, Prado and Tjallingii 1997). Once

the aphids were observed producing honeydew, their feeding site selection was recorded.

Phloem Sap Collection. Phloem sap from new, rapidly expanding, mature and senescent leaves (one each) from three cottonwoods with aphids and three aphid-free plants was collected at the same time each week, yielding 24 sap samples per week. Plants were never reused for phloem sap samples in subsequent weeks. We examined exudates from plants with and without aphids, because large aphid populations may bring about early senescence and increase mobile nitrogen content in their hosts (Miles 1989, Dorschner 1990, Sandstrom et al. 2000). Samples were collected between 18 May and 30 June 1997. Leaf petioles were cut, and EDTA (Sigma, St. Louis, MO) was used as a chelating agent to prevent callose formation on phloem sieve plates, allowing the phloem sap to exude from the cut petiole into water (King and Zeevaart 1974). Leaves were cut from the plant near the petiole attachment point to the stem in a randomized leaf-stage order, once weekly, always beginning at 2100 hours, to avoid any confounding effect of diurnal fluctuations in phloem components that might otherwise occur. Petioles were recut under water and placed in a 4-ml solution of 20 mM EDTA, pH 7.4, in the dark for 2 h (2200–2400 hours). They were then transferred to 4-ml vials of deionized water for 12 h of sap collection in the dark. The phloem sap samples exuded in diH_2O for 12 h were used for chemical analysis. Each sample was filtered (0.22 or 0.45 μM polyvinylidenefluoride syringe filter) into a microcentrifuge tube and stored at −20°C until assays were performed.

Dry Leaf Weight. After phloem sap exudate collection, leaves were pressed at room temperature for 72 h and dried in a 65°C oven for 2 h. Dried leaves were weighed to the nearest 0.01 mg.

Sucrose Assays. Sucrose content of the exudates was used as relative indicator of the rate of phloem sap exudation from cut leaf petioles. Sucrose, the predominant translocatable photoassymilate, is used as a standard indicator of phloem sap flow rate in translocation studies (Martin and Edgington 1981, Ziegler 1986, Hopkins 1995). If exudation rates were similar among leaves, exudate sucrose content will be similar. If exudate sucrose concentration differed significantly among leaves, a proportion based on sucrose content would be used to adjust concentrations of other constituents, as in Lichtner (1984).

Phloem exudates were analyzed for sucrose content by enzymatic assay as per Jones et al. (1977) with minor modifications. A sucrose assay kit (SCA-20; Sigma) was used, and the enzyme P-glucoisomerase (converts fructose to glucose) was not included (Sigma 1996). Absorption of samples at 340 nM (UV lamp) was measured on a Beckman DU 640 spectrophotometer in 1.5 ml methacrylate microcuvettes (PGC Scientifics, Frederick, MD). Standard curves were generated for each assay, and absorption was converted to milligram sucrose.

High-Performance Liquid Chromatography for Salicin. Phloem exudates were analyzed for the phenolic glycoside salicin by high-performance liquid chromatography (HPLC). Salicin standard was obtained from Sigma. We used an isocratic HPLC system following the procedure of Robison (1993) that was modified from Lindroth and Pajutee (1987). Phloem sap samples were run through a reverse phase ODS-Hypersil CPG column (250 mm long by 4.6 mm i.d.; 5-μm particles; Keystone Scientific, Bellefonte, PA), equipped with a javelin filter and a 10 by 4-mm ODS Hypersil precolumn. A Waters (Milford, MA) WISP 701 B auto-injector loaded 100 μl of sample every 15 min. The flow rate of the Waters 510 pump was 1.8 ml/min (3 psi), and the solvent system used was 1:3 MEOH: H_2O. The UV absorption detector was a LDC/Milton Roy UV Spectromonitor 3000 set for 254 nm. Detector output went to a PC for analysis with Millipore Dynamic Solutions MAXIMA 820 software through a Waters System Interface Module. Salicin retention times ranged from 2.72 to 3.25 min. Salicin was quantified by comparison of sample peak heights against a standard calibration curve produced by linear regression of standard peak heights versus concentrations forced through the origin. The detection limit was 0.2 nmol.

Amino Acid Analysis. Phloem exudates were analyzed for free amino acid content by HPLC. Phloem samples were dried in 1.5-ml microcentrifuge tubes from an initial volume of 500 μl in a Savant (Holbrook, NY) SVC 100H speed-vac and stored until use at −20°C. Standard solutions were made from Beckman system 7300/6300 amino acid standard (Fullerton, CA) containing 2.5 mM of the L-amino acids: alanine, methionine, phenylalanine, proline, leucine, lysine, arginine, aspartic acid, cystine (cysteine diamer, 1.25 mM), serine, threonine, tyrosine, valine, glutamic acid, glycine, histidine, and isoleucine. These were supplemented with additional standards for asparagine, tryptophan, γ-amino-n-butyric acid (GABA), ornithine, and glutamine (Sigma). The standard solution mixture was diluted to 0.01 nmol/μl and aliquotted at 2, 1, 0.5, and 0.25 nmol/tube. Standard solutions were dried in a speed-vac and stored at −20°C until use.

Phloem sap samples and standards were derivatized with the fluorescent reagent 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate using the Waters AccQ-Tag chemistry package (AccQ-Fluor reagent kit; Waters, Milford, MA). Samples and standards were reconstituted in 5 μl of 100 μM norleucine (internal standard) in 20 mM HCl plus 35 μl AccQ-Fluor borate buffer, and 10 μl of AccQ-Fluor reagent. Each tube was vortexed, heated in a 55°C heating block for 15 min, and stored at room temperature for 16–20 h.

Samples and standards were loaded into a 48-vial carousel of a Waters 712 Millipore WISP. The injection volume was 10 μl. A mobile phase gradient of degassed A (140 mM sodium acetate, 1.7 mM triethylamine, pH to 5.05 with phosphoric acid), and B (60% acetonitrile MeCN in deionized water) buffers was achieved using two Waters 510 pumps over 40 min (100% A to 100% B), with 25 min of column equilibration between runs. The system ran at 1.0 ml/min on a 3.1 by 150-cm Waters AQC C18 column, heated to 37°C. Detection of the AQC amino acids in the phloem

sap samples and standards was by UV absorption with a Waters 484 detector at 254 nm. A Waters System Interface Module transferred signals from the detector to a 386/16 personal computer. Amino acid peaks were quantified by linear regression of heights versus standard curves using Millipore Maxima 820 Software. Peaks were identified by the Maxima 820 program and verified manually. The detection limit was 5 pmol.

Leaf Toughness Assessment. High fiber content in leaves hinders chewing insects and can be measured by penetrometer (Sands and Brancatini 1991, Casher 1996), but such methods are not appropriate for determining leaf toughness for sap-feeding aphids (Hysell et al. 1996). Thus, we took a different approach to assessing leaf toughness for aphids. Petioles from four leaves, one each of senescing, mature, rapidly expanding, and new, were cut from four different ST109 cottonwoods, providing a sample size of four for each leaf age class. Single petiole segments of 0.5 cm in length were excised, starting 1 cm from the base of the leaf blade, where the vascular bundles are most discrete (Isebrands et al. 1976). Petiole segments were fixed in 2.5% gluteraldehyde in 0.05 M sodium phosphate buffer, pH 6.8, per Stein et al. (1992) and Hayat (1972). Petioles in fixative were shipped to the Histology Laboratory, Veterinary Pathobiology Department, Texas A&M University, College Station, TX, to be embedded in JB-4 plastic (a glycol methacrylate) and sliced into 2-μm-thick cross-sections. Sections were stained in 0.05% toludine blue for 10 s at 50°C to differentiate lignified petiolar areas. With toludine blue, lignified areas stained bright blue, whereas other plant structures appeared violet (Stockert et al. 1984).

Digital images (TIFF) of stained petiole sections were captured on a Nikon microphot-SA microscope at a total magnification of ×12.5, along with a 1-mm micrometer standard with 10-μm divisions. Digital image analysis was performed using Macintosh IIci and NIH Image shareware (available at http://rsb.info.nih.gov/nih-image/, version Scion Image 1.60c). Anatomy of petiolar structures was derived from Isebrands and Larson (1973, 1980) and Isebrands et al. (1976). Two sets of measurements were taken from each petiole section: one each from dorsal and ventral petiolar bundles. The shortest distance from the petiole surface to each vascular bundle was measured. Grayscale measurements for lignin density were taken of bundle sheath (0.0016 mm^2), and background grayscale value was subtracted from each measurement.

Statistical Analyses. Data were analyzed using the multivariate analysis of variance (MANVA), nonparametric and discriminant function analysis units of Statistica for Windows (StatSoft 1997). Aphid population leaf stage selection pattern was determined from three consecutive observations per plant, staggered across nine weekly observation periods for 18 plants, and analyzed by repeated-measures analysis of variance (ANOVA). Also, the number and developmental stage of aphids on every leaf of the 18 plants was tallied weekly (18 May to 30 June 1997) before cutting petioles for phloem exudation. These data were transformed into the mean percentage of the total aphid population in each instar per leaf developmental stage and analyzed by nonparametric Mann–Whitney *U* tests. Distributions of marked individual aphids before and after new leaf initiation were compared by χ^2 tests. Finally, time for aphids to reinitiate phloem ingestion was analyzed by nonparametric ANOVA because of its non-normal distribution. Plant phloem sap content and leaf toughness data were analyzed using MANOVA or ANOVA, and discriminant function analysis. Outliers with values >3 SD above the mean were dropped. Because the four leaf age classes always came from the same plants, they were not truly independent.

Forward stepwise discriminant function analysis was performed to determine which of the 20 amino acids measured in phloem sap were the most valuable for discriminating between leaf developmental stages. This stepwise analysis works by adding amino acids to the discriminant funtions one by one until adding further amino acids does not provide significantly better discrimination among leaf stages, such that amino acids excluded from the model made little contribution. The amino acids included in the model are therefore most indicative of a leaf stage grouping. The initial model included nine amino acids to separate the four leaf stages; it used three cannonical roots, with rapidly expanding and new leaves not being significantly different from one another (Mahalanobis $F = 0.58$; df = 9,116; $P = 0.8$). The model had a cumulative 43% correct, and root 2 and root 3 made no significant contribution toward separating leaf stage groups (root 2 removed: $\chi^2 = 20$; df = 16; $P = 0.2$; root 3 removed: $\chi^2 = 3.3$; df = 7; $P = 0.85$). To develop a simpler model with greater explanatory power, new and rapidly expanding leaves were pooled into one group, and the procedure was repeated.

Results

Aphid Selection Patterns For Leaf Developmental Stages. Aphid populations observed weekly for 3 wk on the ST109 cottonwoods displayed a distinct preference for rapidly expanding leaves over mature leaves. Aphids were significantly ($F = 4.5$; df = 3,108; $P < 0.005$; Scheffé $P < 0.01$) more numerous on rapidly expanding leaves than on mature leaves (Fig. 1). By the third observation, there were significantly more aphids on all leaf stages than at the first ($F = 7.1$; df = 2,216; $P < 0.001$; Scheffé $P < 0.001$). There was no significant interaction between leaf developmental stage and observation ($F = 0.1$; df = 6,216; $P < 0.99$).

The average aphid age distribution differed somewhat among leaf developmental stages (Fig. 2). First-instar nymphs were always significantly ($Z > 3.2$; Mann-Whitney $U < 34$; $P < 0.001$) more numerous than other stages of aphid development on all leaf developmental stages, with the sole exception of second-instar nymphs on mature leaves ($Z = 1.6$; Mann-Whitney $U = 28$; $P < 0.09$). Proportions of adults and third instars were similar across leaf developmental stages. There was a significantly ($Z > 1.9$; Mann-Whitney $U < 46$; $P < 0.05$) higher percentage of

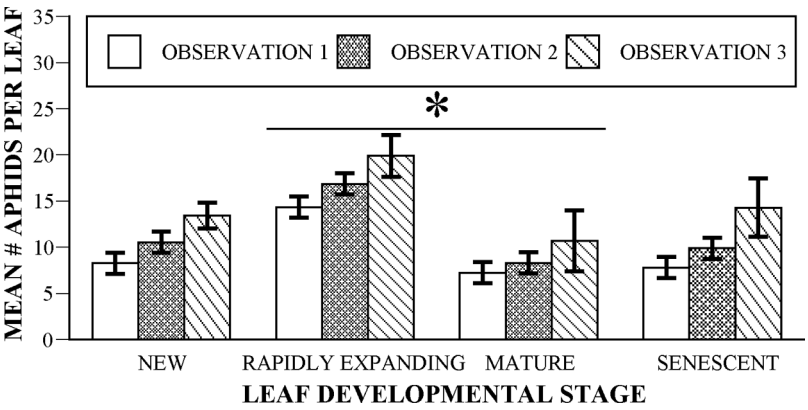

Fig. 1. Aphid population distribution among Eastern cottonwood leaf developmental stages. Data are from three consecutive observations of 18 *C. populicola*–infested plants occurring on or between 11 May and 17 July 1997. Bars represent mean ± SE. *Significantly fewer aphids were present on mature leaves than on rapidly expanding leaves (Scheffé, $P < 0.01$).

first-instar aphids found on new leaves than on either mature or senescing leaves.

Tracking and Moving Time Costs for Individual Aphids on ST109 Saplings. Before new leaf initiation, the percentage of marked aphids migrating up to younger leaves, down to older leaves, or staying on original leaves was 9, 9, and 82%, respectively, on new and rapidly expanding leaves; 57, 14, and 29% on mature; or 50, 0, and 50% on senescent leaves. Hours after new leaf initiation, the percentage of marked aphids migrating up to younger leaves, down to older leaves, or staying on the same leaves was 60, 6, and 33%, respectively, on new and rapidly expanding leaves. Aphids on both mature and senescent leaves migrated up to younger leaf positions within hours of leaf initiation 100% of the time. The locations of the marked *C. populicola* individuals on ST109 cottonwoods differed significantly between pre- and postleaf initiation on new/rapidly expanding, mature, and senescing leaves ($\chi^2 = 314$, 75, and 100; df = 2; $P < 0.05$, respectively), indicating aphid migration, primarily to younger leaves. Aphids on new and rapidly expanding leaves were more likely to move up to younger leaves after the initiation of a new leaf, yet this tendency was less pronounced than in aphids on mature and senescent leaves. Hence, most *C. populicola* individuals ob-

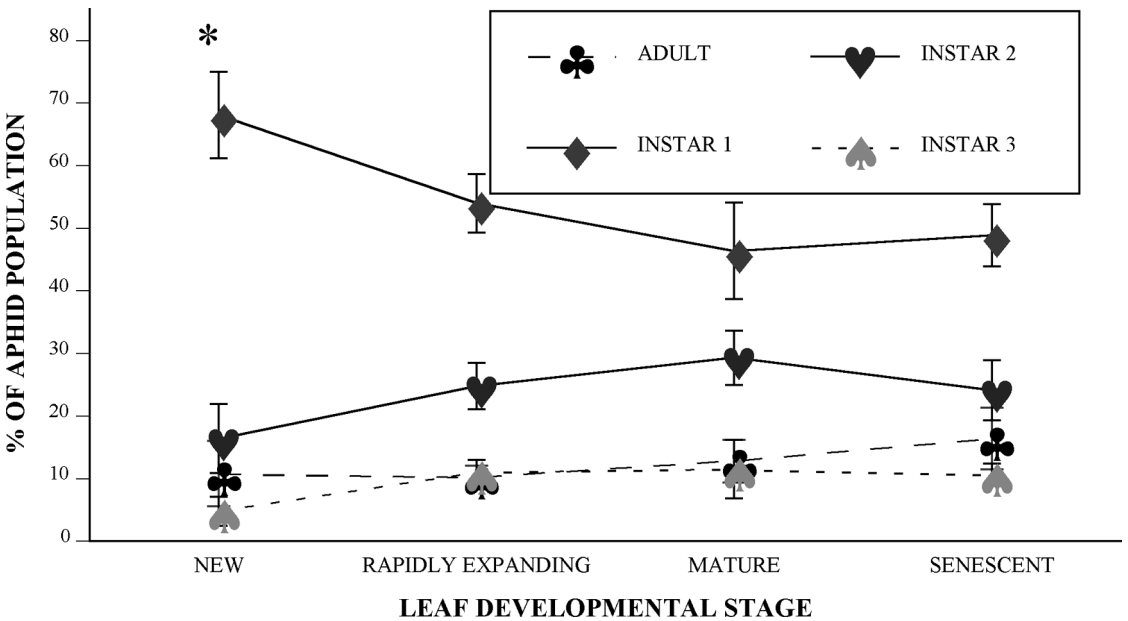

Fig. 2. Average proportion of the *C. populicola* population, by instar per leaf, for different leaf developmental stages of Eastern cottonwood. Mean percent ± SE of per leaf aphid age distribution is shown for $N = 18$ plants. First-instar nymphs were always more numerous than other stages of aphid development, and there was a significantly (Mann-Whitney U, $*P < 0.05$) higher percentage of first-instar aphids found on new leaves than on either mature or senescing leaves.

Table 1. Amino acid analysis of phloem sap exudates

Amino acid	Cottonwood leaf developmental stage			
	New	Rapidly expanding	Mature	Senescent
Aspartic acid	317 ± 42[a]	260 ± 30[ab]	196 ± 24[b]	225 ± 35[ab]
Asparagine/serine	607 ± 50	546 ± 60	460 ± 56	489 ± 63
Glutamic acid	95 ± 23	68 ± 16	61 ± 15	125 ± 38
Glycine	652 ± 90	592 ± 97	550 ± 97	543 ± 102
Histidine/glutamine	89 ± 23	68 ± 14	50 ± 12	113 ± 41
Arginine	34 ± 12	34 ± 13	41 ± 13	39 ± 9
Threonine	238 ± 30	219 ± 31	181 ± 28	199 ± 31
Alanine	462 ± 51	396 ± 49	301 ± 32	342 ± 48
Proline	176 ± 29	109 ± 15	116 ± 20	139 ± 40
GABA	232 ± 48[a]	252 ± 54[a]	529 ± 94[b]	338 ± 69[ab]
Tyrosine	102 ± 19	73 ± 11	59 ± 10	64 ± 10
Valine	183 ± 17	158 ± 16	131 ± 15	159 ± 25
Methionine	49 ± 7	48 ± 7	56 ± 14	76 ± 23
Ornithine	269 ± 23	273 ± 34	234 ± 24	229 ± 28
Lysine	89 ± 18	69 ± 9	53 ± 6	55 ± 9
Isoleucine	105 ± 18	79 ± 9	61 ± 9	85 ± 16
Leucine	128 ± 20	96 ± 13	80 ± 12	99 ± 16
Phenylalanine	64 ± 12	47 ± 9	30 ± 7	71 ± 30
Tryptophan	15 ± 4	22 ± 7	12 ± 3	37 ± 20
Total	3,955 ± 372	3,476 ± 397	3,258 ± 351	3,500 ± 465

Mean ± SEM amino acid concentration (nM) in exudate. Amino acid concentrations from aphid-infested and aphid-free plants are pooled in the leaf stage averages for all amino acids measured, except for cystine, which differed significantly between these two groups. $N = 31–32$ cottonwoods. Aspartic acid was significantly higher in new leaves than in mature leaves, and GABA was significantly higher in mature leaves than in new and rapidly expanding ones (Tukey HSD, $P < 0.05$). Superscript a or b indicates significant differences between means, whereas ab indicates no significant mean separation.

served tracked leaf development on *P. deltoides* saplings through a period of host plant new leaf initiation.

Aphids forced to relocate on leaves of different developmental stages on new ST109 cottonwood hosts began ingesting phloem sap and producing honeydew between 6 and 22 h on average after relocation, depending on the developmental stage of their replacement leaf. Times to initiate phloem ingestion were all significantly different among leaf developmental stages (Kruskal-Wallis, H = 61, df = 3; $P < 0.05$; Mann-Whitney *U*, $P < 0.05$). Aphids on new leaves took the longest to initiate phloem ingestion after relocation, with an average of 22 ± 5 h ($N = 55$), followed by those on rapidly expanding leaves with 12 ± 5 h ($N = 13$) and mature leaves at 9 ± 4 h ($N = 15$). Aphids on senescent leaves reinitiated feeding more rapidly than any of the other groups, averaging 6 ± 2 h ($N = 9$).

Phloem Sap Exudation Rates. Sucrose content was used as an indicator of phloem sap flow rate (Martin and Edgington 1981). There were no significant differences in sucrose content among ST109 phloem sap exudates collected from leaves of different developmental stages, nor between plants with or without aphids (two-way ANOVA, $F = 0.016$; df = 1,120; $P = 0.90$ and $F = 0.19$; df = 3,120; $P = 0.90$, respectively), indicating a constant exudation rate. Furthermore, no significant interaction occurred ($F = 0.14$; df = 3,120; $P = 0.93$). The mean ± SEM sucrose content of 12-h exudate from new leaves without aphids was 1.22 ± 0.38 mg/ml. Sucrose content of phloem sap exudates was not correlated with leaf dry weight for ST109 cottonwoods ($R^2 = 0.01$), suggesting that exudation rate is independent of leaf size. Thus, sucrose content of phloem sap exudates did not differ significantly among leaves of different developmental stages, indicating a relatively constant phloem sap flow rate among leaves of all developmental stages. Based on these results, no adjustments were made to the data.

Salicin Content. ST109 cottonwood phloem sap exudates were analyzed by HPLC for the phenolic glycoside salicin. There was significantly more salicin (two-way ANOVA, $F = 3.7$; df = 3,120; $P < 0.02$; Tukey honest significant difference [HSD] $P < 0.05$) present in new leaves than in any other leaf developmental stage. Mean ± SEM salicin content of exudates for plants without aphids was 30 ± 13 μM from new, 8 ± 2 μM from rapidly expanding, 8 ± 4 μM from mature, and 5 ± 2 μM from senescent leaves ($N = 14$). There was no significant difference ($F = 0.86$; df = 1,120; $P = 0.36$) between plants with and without aphids and no significant interaction ($F = 1.3$; df = 3,120; $P = 0.27$) between leaf stage and aphid presence. Cottonwoods with aphids ($N = 18$) had lower salicin concentrations in their phloem sap exudates on new leaves (14 ± 6 versus 30 ± 13 μM), but this trend was not significant.

Amino Acid Content. We analyzed phloem sap exudates from ST109 leaves of different developmental stages in the presence or absence of aphids by HPLC for the concentrations of 20 amino acids. Four peaks were unresolved, so asparagine/serine and histidine/glutamine peaks were combined. Mean amino acid content of phloem sap for leaf stages, pooled among plants with and without aphids, is shown in Table 1. There were significant differences in the concentration of two amino acids, aspartic acid (two-way MANOVA, Wilks' λ = 0.75; df = 20,101; $P = 0.05$; $F = 2.6$; df = 3,120; $P < 0.05$) and GABA ($F = 4.1$; df = 3,120; $P < 0.01$) among leaf stages. Aspartic acid content was significantly higher in new versus mature

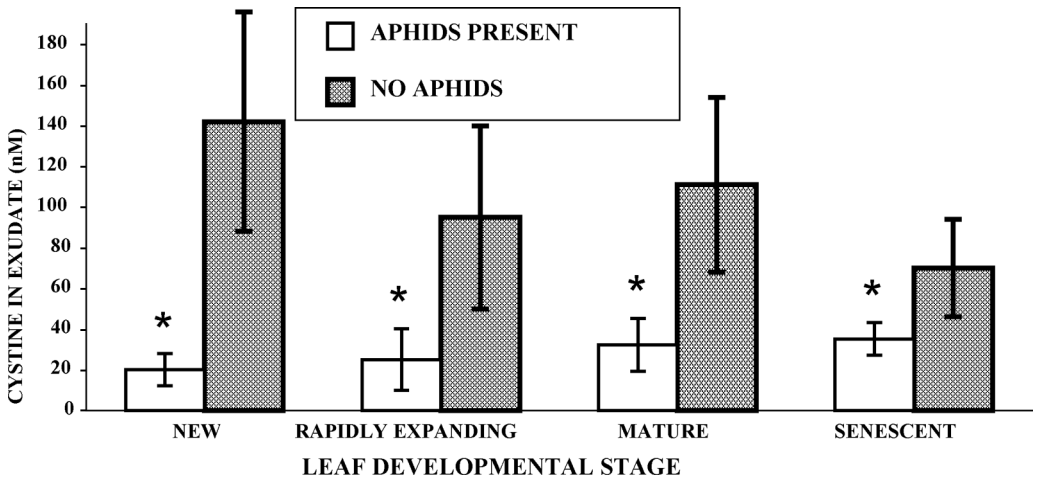

Fig. 3. Amino acid content of phloem sap exudate reveals cystine concentrations are significantly lower in ST109 cottonwoods with populations of the aphid *C. populicola* present. Mean ± SEM. *Tukey HSD, $P < 0.001$.

leaves, and GABA was significantly higher in mature than in new or rapidly expanding leaves (Tukey HSD, $P < 0.05$). Alanine and tyrosine content tended to be highest in new leaves ($F = 2.5$ and 2.2, df = 3,120; $P = 0.07$ and $P = 0.09$, respectively), but this trend was not significant. All other amino acid concentrations did not differ significantly among leaf developmental stages. No significant interactions occurred between leaf stage and aphid presence (Wilks' $\lambda = 0.54$; df = 60,302; $P = 0.2$). However, phloem sap exudate cystine content was significantly higher (Wilks' $\lambda = 0.44$; df = 60,302; $P < 0.01$; $F = 11.8$; df = 1,120; $P < 0.005$) in plants without aphids (104 ± 48 nM) than in plants with aphid infestations (28 ± 15 nM), as shown in Fig. 3.

Forward, stepwise discriminant function analysis of amino acid concentrations revealed that seven amino acids, GABA, aspartic acid, alanine, glutamic acid, histidine/glutamine, glycine, and methionine, can be used to separate three leaf stage groups, new/rapidly expanding, mature, and senescent, on two roots (axes). Separation along canonical root 1 accounted for 75% of total variance, whereas root 2 accounted for the remaining 25%. Both roots contribute significantly toward separating leaf stage groups (root 1 removed: $\chi^2 = 46$; df = 14; $P < 0.0001$; root 2 removed: $\chi^2 = 12$; df = 6; $P = 0.05$). A cumulative 63% of leaf stage groups were correctly classified with this model. New/rapidly expanding leaves are distinct from mature or senescent ones (Mahalanobis $F = 4.2$; df = 7,119; $P < 0.001$), but mature and senescent leaves did not significantly differ (Mahalanobis $F = 1.8$; df = 7,119; $P < 0.09$).

Standardized coefficients and the factor structure (loadings) matrix are shown in Table 2. Based on the standardized coefficients, for which the larger the absolute magnitude, the greater the importance of an amino acid to the model, alanine (+), glutamic acid (−), and GABA (−) had the greatest polarizing importance for root 1, whereas glycine (+), GABA (+), and glutamic acid (−) had the greatest importance for root 2. The loadings matrix provides a meaning or label for the discriminant functions because the variables with the highest coefficients shape the functions the most. Based on the loadings matrix, GABA (−) versus alanine and aspartic acid (+) contribute the most to separating leaf stages along root 1, whereas GABA (+) versus glutamic acid and His/Gln (−) separate the most along root 2. Because of root 1 having the most explanatory power, phloem content of GABA, aspartic acid, glutamic acid, and alanine are the most useful to distinguish leaf stages because of their magnitudes in root 1 standardized coefficients or loadings matrix. High concentrations of aspartic acid and alanine (+) are characteristic of new or rapidly expanding leaves, whereas GABA and glutamic acid (−) are characteristic of mature or senescent leaves.

Table 2. Coefficients of the discriminant functions based on forward stepwise analysis of phloem sap exudate amino acid concentrations for selected amino acids

Select amino acids	Standardized coefficients		Loadings: factor structure matrix	
	Root 1	Root 2	Root 1	Root 2
GABA	**−0.693**	**0.523**	**−0.443**	**0.540**
Alanine	**1.153**	0.066	**0.370**	−0.172
Glutamic acid	**−0.703**	**−0.825**	−0.070	**−0.491**
Methionine	−0.490	−0.361	−0.192	−0.260
Histidine/glutamine	−0.398	−0.467	−0.018	**−0.480**
Glycine	−0.528	**0.537**	0.123	0.015
Aspartic acid	0.497	0.015	**0.363**	−0.167
Eigenvalues	0.322	0.107	—	—
Percent total variance	75.1	24.9	—	—

Discriminant function analysis was performed to determine how well amino acid concentration differences could be used to classify leaves into one of three developmental stages (new + rapidly expanding, mature, or senescent). The roots on the left are standardized coefficients (weightings) for cannonical variables, and on the right are the loadings for the correlations variables (cannonical roots) resulting from pooled within-groups correlations.

The largest, most influential coefficients (in absolute value) in each function are in bold.

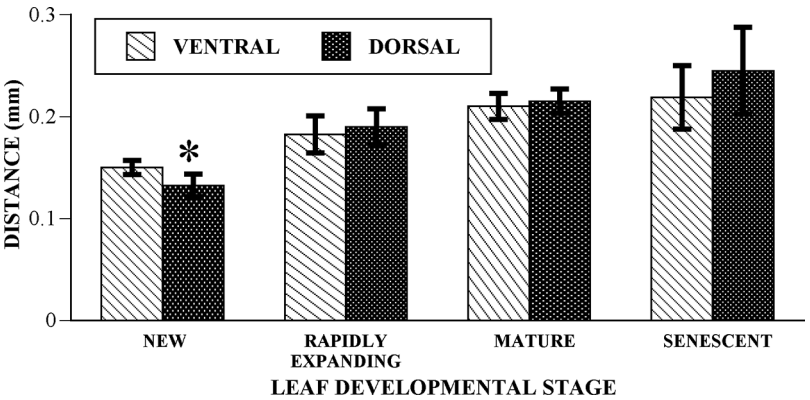

Fig. 4. Distance from epidermis to vascular bundle in ST109 cottonwood leaf petioles of different developmental stages. Distance from the epidermis to the vascular bundle was significantly (Tukey HSD, $P < 0.05$) shorter to dorsal leaf veins in new petioles than in mature or senescent petioles. There were no significant differences among leaf developmental stages in epidermis to ventral petiolar bundle distances.

Leaf Toughness Assessment Distance and Density Measurements. In dorsal petiolar bundles, epidermal surface to vascular bundle distances are significantly shorter (ANOVA, $F = 3.8$; $P < 0.05$; Tukey HSD, $P < 0.05$) in new petioles than in mature or senescing petioles (Fig. 4). In ventral petiolar bundles, surface to vascular bundle distances did not significantly differ ($F = 2.5$; df = 3,12; $P = 0.1$) between leaf developmental stages. Ventral petiolar bundle sheath grayscale values (optical density) were significantly higher in senescent leaves (ANOVA, $F = 6.9$; df = 3,12; $P < 0.01$; Tukey HSD, $P < 0.05$), indicating a greater amount of lignification (Fig. 5). In dorsal petiolar bundle sheaths, the grayscale values of rapidly expanding leaf petioles are significantly lower ($F = 5.4$; df = 3,12; $P < 0.02$; Tukey HSD, $P < 0.01$) than in other stages, except for new ones. Thus, as leaves mature, both distance from surface to vascular bundles and the extent of lignification increase.

Discussion

Aphid Selection Patterns Among Leaf Developmental Stages. Populations of the aphid *C. populicola* primarily fed on new, rapidly expanding, and senescent over mature leaves on ST109 *P. deltoides* clones. This pattern in *C. populicola* populations was initially observed by Coleman and Jones (1988) and Wait et al. (1998) for beetles *Chrysomela scripta* and *Plagiodera versicolora* on ST109 cottonwood clones. The difference between these results and previous studies is that the cottonwoods were not given fertilizer, so they exhibited slower growth. In comparison to Coleman and Jones (1988) and Wait et al. (1998), in which ST109s had 20 leaves and a transition from rapidly expanding to the mature leaf stage at LP5, the ST109s in this study had ≈15 leaves and a transition from rapidly expanding to mature at LP3. Accordingly, LP3, closest to the transition point from sink to source, was most preferred by

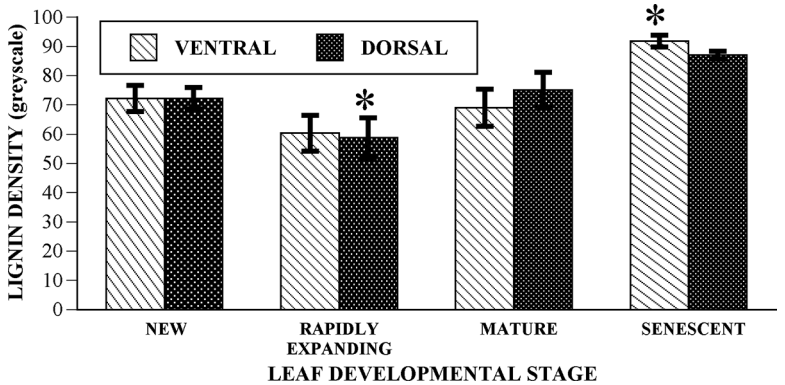

Fig. 5. Lignin density of ST109 cottonwood petiolar leaf veins. Ventral bundle sheaths in senescent petioles had significantly (Tukey HSD, $P < 0.05$) higher grayscale values, indicating more lignin, than bundle sheaths in other petioles. Dorsal bundle sheaths in rapidly expanding petioles had significantly (Tukey HSD, $P < 0.05$) lower grayscale values (less lignification) than all other petioles, except new ones.

C. populicola instead of LP5, as predicted by Coleman (1986).

Furthermore, *C. populicola* populations tracked leaf development over time to maintain feeding sites at preferred leaf stages. Apterous aphid migration is associated with host plant maturation and is not always caused by conspecific overcrowding (Hodgson 1991). Jepson (1983) and Harrington and Taylor (1990) observed the aphid *M. persicae* tracking leaf development in sugar beet and cabbage. Both predicted that aphid settling and movement relates to changes in the content of phloem sap during leaf development, so that as a leaf matures, it becomes less favorable, and aphids move to a younger feeding site. Given that the leaf stage selection pattern of *C. populicola* for feeding sites is reasonably consistent under different plant growth conditions and over time, we sought to understand what phloem-borne factors associated with leaf developmental stages could account for this pattern of behavior.

Phloem Sap Analysis. Quality and quantity of phloem sap constituents are probably the main factors associated with aphids either continuing to occupy a feeding site or abandoning it for a new one. Free amino acids in phloem (Atkins et al. 1983, Chino et al. 1987, Douglas 1993) and plant secondary chemistry (Niemeyer 1990, Pickett et al. 1992, Merritt 1996) can vary with leaf developmental stage. Preingestion behavior and putative gustatory chemoreceptors suggest that aphids have some capacity to "taste" phloem sap and other plant fluids (Miles 1958, Wensler and Filshie 1969, Anderson and Bromley 1987, Pickett et al. 1992, Mullin et al. 1994). Aphids probe potential feeding sites extensively and frequently move before committing to phloem ingestion (Pollard 1973, Montllor 1991, Girma et al. 1994). On some resistant hosts, aphids penetrate sieve elements as usual, but terminate phloem ingestion early, indicating resistance factors can occur in phloem sap (Sauge et al. 1998, Lei et al. 1999, Kaloshian et al. 2000).

EDTA-Enhanced Phloem Exudation. EDTA chelation represents a convenient time and cost-effective phloem collection method compared with the severed aphid stylet technique of Kennedy and Mittler (1953). Studies of the two techniques indicate reasonably high correlations for phloem amino acids, such that EDTA-enhanced phloem exudation is adequate for relative comparisons (Urquhart and Joy 1981, Weibull et al. 1990, Douglas 1993, Telang et al. 1999, Sandstrom et al. 2000). Furthermore, phloem sampling by severed aphid stylets introduces the bias that only sieve elements accepted by aphids can be sampled (Ponder et al. 2000, Wilkinson and Douglas 2003), and it is not clear that such samples are any more representative of a host plant or a particular leaf developmental stage. Because this study involved relative comparisons of phloem sap composition between leaves of different developmental stages and required sufficient sample volumes, we used the EDTA exudation method.

***Chaitophorous populicola* Feeding Does Not Substantially Alter Phloem Quality.** We compared phloem sap chemistry in plants with and without aphids. Feeding aphids can alter host plant quality and performance while showing few external signs of damage (Maschinski and Whitham 1989, Miles 1989, Dixon 1985). Aphid aggregations of sufficient size can redirect photosynthate toward themselves, acting as a resource "sink," much like a developing shoot (Way and Cammell 1970, Bianchi et al. 1989, Larson and Whitham 1997). Whether this is caused by increased phloem transport or a change in phloem sap quality in the host plant, or both remains unclear. Locally or systemically, high-density aphid populations bring about early senescence and increase mobile nitrogen, thereby improving host plant nutritional quality (Kennedy 1951, Miles 1989, Dorschner 1990, Larson and Bjorkman 1993, Sandstrom et al. 2000).

In the case of *C. populicola* feeding on ST109 cottonwood saplings, there was only a significant overall reduction in phloem cystine concentration from plants sustaining aphid populations compared with exudates from aphid-free plants. There was a nonsignificant trend toward lower salicin concentrations from new leaves with aphids present. However, the mean difference was a 50% reduction that, while not statistically relevant (because of a large SEM), may have had some biological meaning. Thus, *C. populicola* depleted cystine and reduced new leaf salicin content in phloem exudates but otherwise had little influence on *P. deltoides* phloem quality with respect to the other amino acids measured. Our EDTA-enhanced collection of phloem sap exudates would have been capable of detecting systemic but not localized effects. However, in wheat, feeding damage by *Diuraphis noxia* resulted in higher concentrations of essential amino acids that were detected in both the severed aphid stylets and from phloem sap exudates (Telang et al. 1999). It is also possible that our *C. populicola* population density, averaging >20 aphids per leaf, was insufficient to produce greater changes in phloem sap.

Leaf Developmental Stage and Amino Acids. The majority of amino acids measured in ST109 cottonwood phloem exudates from leaves of various developmental stages did not differ significantly in concentration. As melon and mustard plants develop and age, there are two peak levels in total amino acid content in phloem sap: a large peak in young plants or leaves and a second smaller peak in senescent plants or leaves (Merritt 1996, Blackmer and Byrne 1999). Similarly, we expected to find that total amino acid content—or at least essential amino acid content—in cottonwood phloem sap would be greatest in new and senescent leaves and lowest in mature leaves. However, we did not observe this pattern among leaf developmental stages.

However, even phloem sap variability of single amino acids can influence aphid feeding behavior. In barley and oat, for example, although no relationship existed between total free amino acid content and aphid resistance, glutamic acid content was higher in resistant cultivars (Weibull 1988). The only amino acids with significant concentration differences between leaf stages were aspartic acid and GABA in our ST109 cottonwood exudates. Concentrations of aspar-

tic acid were highest in new leaves, and GABA concentrations were highest in mature leaves. Although aspartic acid is not an essential amino acid for aphids (Ponder et al. 2000, Wilkinson and Douglas 2003), it could indicate to *C. populicola* feeding on *P. deltoides* the developmental stage or nutritional quality of a particular leaf.

In higher plants, GABA is produced in response to temperature change, darkness, and mechanical stimulation and may function in defense against insect herbivores (Wallace et al. 1984, Ramputh and Bown 1996, Bown and Shelp 1997). It has been suggested that GABA may be an artifact of the EDTA phloem exudation technique (Girousse et al. 1991). However, in a comparative study on lettuce, phloem samples collected by aphid stylectomy contained roughly 10-fold more GABA than samples from EDTA exudation (van Helden et al., 1994). In whole leaf analyses, GABA was negatively correlated with population survival and growth of the aphid *M. persicae* (van Emden 1973), although more recent studies indicate that L-DOPA (L-3,4-dihydroxyphenylalanine) and ornithine have a stronger negative correlation with aphid performance than GABA (Ciepiela and Sempruch 1999). GABA may be deterring *C. populicola* from feeding on mature leaves, but we could not directly test this hypothesis because we could not induce *C. populicola* to feed on artificial diets in parafilm sachets.

Aphid dietary requirements vary because of interspecific differences or differing symbiotic nutrient-producing bacteria (Houk 1987). Generally, phloem sap composed of 2–4% free amino acids containing the essentials (threonine, histidine, isoleucine, leucine, lysine, methionine, valine, tryptophan, and phenylalanine) is optimal for aphid growth (Dadd and Mittler 1965, 1966, Dadd and Krieger 1968, Adams and Emden 1972, Srivastava and Auclair 1974, Turner 1977, Sandstrom and Pettersson 1994, Sandstrom and Moran 1999). Given the importance of amino acids in the diet of aphids, it is reasonable to hypothesize that changes in amino acid composition or concentration might prompt aphids to relocate. Based on the results of this study, the only amino acids in *P. deltoides* phloem with the potential to cue aphids to relocate are GABA and aspartic acid, because they differed significantly between leaves of different developmental stages. The discriminant function analysis model was consistent with the ANOVA results, and it also indicated that five other amino acids (alanine, glutamic acid, histidine/glutamine, glycine, and methionine) could be potential indicators of leaf stage when examined in combination with GABA and aspartic acid. However, although these amino acid concentration differences are present in *P. deltoides* phloem sap, it remains unclear if they are important factors for the aphid *C. populicola.*

Leaf Developmental Stage and Phenolic Glycosides. The phenolic glycoside salicin was found in significantly higher concentrations in new leaves of ST109 cottonwoods. Based on whole leaf extract analysis, the highest concentrations of phenolic glycosides occur in immature, actively growing, and expanding leaves, and specialist herbivores tend to prefer them (Rowell-Rahier 1984, Coleman 1986, Rowell-Rahier et al. 1987, Soetens et al. 1991, Bingaman and Hart 1992, 1993). However, the galling aphid *Pemphigus betae,* which is a *Populus angustifolia* specialist, is more likely to induce galls on new leaves and shoots that contain the lowest levels of phenolic glycosides (Zucker 1982). Phenolic glycoside concentrations wane as a leaf ages (Coleman 1986, Bingaman and Hart 1993), and they are involved in poplar short-term–induced response to leaf damage (Clausen et al. 1989). Aside from being present in the leaf material, based on our results, they are also transported in the phloem sap of ST109 cottonwoods, with highest concentrations in new leaves. *M. persicae,* a generalist aphid, was less likely to feed on artificial diets containing salicin (Schoonhoven and Derksen-Koppers 1976). We were unable to directly examine *C. populicola*'s response to salicin, because we were unable to induce the aphid to feed on artificial diets in parafilm sachets. However, the cottonwood specialist aphid *C. populicola* did not seem to be deterred by the higher presence of phenolic glycosides in new leaves. However, it did take *C. populicola* a significantly longer time to begin producing honeydew when introduced onto new leaves as opposed to other leaf developmental stages. Perhaps the delay in initiation of feeding and honeydew production occurred because *C. populicola* on new leaves must suppress or otherwise contend with high salicin or phenolic glycoside levels in general before successfully feeding.

Leaf Developmental Stage and Toughness. In the ST109 cottonwoods, the amount of lignification of the vascular bundles increased with age, as did the distance that must be traversed to reach the vascular bundles. Thus, lignification and distance to vascular bundles together should reflect the toughness of a leaf to an aphid and hinder access to phloem sap. Leaf toughness has been shown to discourage feeding on otherwise palatable hosts (Raupp 1985, Berdegue and Trumble 1996). Leaf toughness for phloem sap feeding aphids is somewhat different; the time it takes to tap the phloem sap becomes longer if thick cuticle and lignified vascular bundles are encountered (Risebrow and Dixon 1987). The distance that the stylets must traverse can also lengthen the time to reach the phloem (Pollard 1973, Loudon and McCulloh 1999) or may determine whether it is reached at all if phloem depth exceeds stylet length (Elliot and Hodgson 1996). However, other factors may influence leaf toughness for aphids. For example, some species of aphids take less time to reach phloem on plants in locations previously infested by aphids, probably because of physiological changes induced by saliva and stylet tracks from the previous colony (Prado and Tjallingii 1997). This might also explain why *C. populicola* colonizing new leaves (untouched by other aphids) took longer to begin producing honeydew. However, based on distance and lignin density measures alone, the "toughness" associated with cottonwood leaf petioles that might delay aphids reaching

phloem seem less formidable in new and/or rapidly expanding leaves than in mature and senescent leaves.

In conclusion, for *C. populicola* feeding on cottonwood, phytochemical and physiological differences associated with leaf developmental stage may result in differential suitability of feeding sites within the host plant. The aphid seems to track leaf development to avoid mature leaves and to preferably feed on rapidly expanding leaves. Concentrations of the amino acids GABA and aspartic acid, as well as the phenolic glycoside salicin, differ in leaves of different developmental stages and may be used by *C. populicola* to determine leaf age. Of the other amino acids measured, most concentrations did not vary significantly with leaf developmental stage. However, discriminant function analysis revealed some patterns of variability in seven amino acids among the leaf developmental stages. The distance to vascular bundles and lignification of vascular bundles varied significantly with leaf developmental stage and might also influence *C. populicola* leaf selection patterns.

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