Antixenotic and Antibiotic Resistance of Hybrid Poplars to the Poplar-and-Willow Borer, *Cryptorhynchus lapathi* (L.) (Coleoptera: Curculionidae)

by

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Thesis submitted in partial fulfillment of the requirements for the degree of

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In the Department of Biological Sciences

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ABSTRACT

Hybrid poplar clones show varying levels of attack by Cryptorhynchus lapathi (L.), a wood-boring weevil. I investigated olfaction, feeding, oviposition and larval survival as well as phenological and biochemical differences among four hybrid poplar clones in order to determine the underlying mechanisms of resistance. Weevils did not discriminate between resistant and susceptible clones based on olfaction in pitfall bioassays or antennal responses, but did discriminate against the most resistant hybrid, NM 6 (Populus nigra L. x Populus maximowiczii Henry), in choice and no-choice paired-twig feeding bioassays. In addition, the most susceptible hybrid, TN 302-9 (Populus trichocarpa Torrey and Gray x P. nigra), was preferred for feeding over Salix scouleriana Barratt ex Hooker, a preferred host in the wild. However, these feeding preferences were not consistently held, therefore resistance can only be partially based on antixenotic cues prior to oviposition. In choice and no-choice experiments involving potted or field-planted clones, oviposition occurred somewhat less frequently and abundantly on two clones with P. maximowiczii parentage. However, no larvae survived on field-planted NM 6 and only four emerged from TM 256-28 (P. trichocarpa x P. maximowiczii). In contrast, 50 and 140 adults emerged from the two susceptible clones, TD 52-226 (P. trichocarpa x Populus deltoides Bartram ex Marshall) and TN 302-9, respectively. Thus, resistance partially involves decreased levels of oviposition, but more significantly, antibiosis in resistant clones prevents the development of larvae, probably in early spring. The two resistant hybrids flushed earlier, but no differences were found with respect to sap flow or bark moisture content that could explain larval mortality. Similarly, constitutive levels of nutritive compounds (sitosterol, nitrogen, linoleic and
linolenic acids, and carbohydrates) were generally lower, or not significantly different, in
TN 302-9. Thus, all hybrids appear nutritionally sound. TN 302-9 contained high
constitutive levels of condensed tannins and catechin, but overall phenolic glycoside
levels (salicin and salicortin) were similar among all hybrids, with the exception of one
sampling date. Neither induced or constitutive polyphenol oxidase (PPO) activity nor
induced levels of secondary metabolites could explain resistance. Thus, the resistant
hybrids with *P. maximowiczii* parentage have a novel mechanism for resistance.
DEDICATION

There are two people to whom this thesis belongs.

First is John Borden
because without him,
this work would never have been completed.
(There are reasons no one has worked with this insect in
North America since the 1960’s!)

Second is Braeden,
for all the days I wish could have been different for you.
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The poplar-and-willow borer, *Cryptorhynchus lapathi* (L.) (Coleoptera: Curculionidae), primarily attacks hosts in the family Salicaceae, but it may also infest *Alnus* and *Betula* spp. (both Betulaceae) (Harris and Coppel 1967). In British Columbia, most adult weevils emerge in summer and oviposit throughout late summer and early fall; however, there is considerable longevity and asynchrony in the life cycle such that adults can be present year round (Smith and Stott 1964). Adults feed on bark tissues of host trees, but will feed on numerous fruits such as apple or plum as well (Harris and Coppel 1967). Eggs are deposited into niches created in the bark by females. Larvae mine in the bark while developing through approximately three instars, and then move into the xylem. Damage includes decreased wood quality, stem breakage and mortality (Broberg et al. 2001), and pathogen entry (Primm 1918, Abebe and Hart 1990). Weevil attack is devastating to native North American *Salix* spp. (Salicaceae) (Broberg et al. 2001), and plagues many *Populus* spp. and commercially-grown hybrids (Salicaceae) (Cadahia 1965, Dafauce 1976, Morris 1981, Moore et al. 1982, Abebe and Hart 1990, Johnson and Johnson 2003). This exotic weevil (Juelich 1887, Caesar 1916) is still expanding its range northward in North America (Broberg et al. 2002); it already covers the entire southern half of Canada and most of the continental United States, extending south into California (Furniss and Carolin 1977, Garbutt and Harris 1994).

There are records of unequal incidence of *C. lapathi* attack among hybrid poplar clones in North America (Morris 1981, Moore et al. 1982, Abebe and Hart 1990, Johnson and Johnson 2003); however, the mechanisms for these apparent resistance phenomena are unknown. To date, only Cadahia (1965) and Dafauce (1976) have tried to determine
the underlying mechanism(s) of resistance to *C. lapathi* in poplars. Both focused on pre-oviposition resistance to adult weevils by European-grown clones, and their experiments were not conclusive. Preference tests were variable with 6-16 choices for 9-20 days, only one no-choice assay involving only two clones was performed, $\alpha$ was frequently set at 0.10, no qualitative information about the clonal material was provided, and replication was often low. Oviposition was generally lower in clones derived from pure *P. alba* L.\footnote{Note, *P. alba* is an aspen belonging to the section *Populus* (formerly *Leuce*), unlike the other species which are either cottonwoods (*Aigeiros*) or balsam poplars (*Tacamahaca*). See Dickmann (2001) for more detail on sections within *Populus*.}, but also *P. simonii* Carrière, and *P. nigra* L. lineages compared to clones of parent lines involving *P. deltoides* Bartrani ex Marshall or *P. euramericana* Dode (Guinier) (*P. deltoides* x *P. nigra*). Dafauce (1976), unlike Cadahia (1965), found similar results among clones for feeding preferences. In North America, Johnson and Johnson (2003) performed a survey of 5-year-old poplar stands and found three clones with *P. nigra* parentage (two *P. nigra* x *P. nigra* and one *P. deltoides* x *P. nigra*) had less weevil attack than six different *P. trichocarpa* Torrey & Gray x *P. deltoides* hybrids. However, Abebe and Hart (1990) found *P. nigra* parentage did not protect two 7-year-old hybrids from *C. lapathi* attack nearly as well as a single hybrid with *P. maximowiczii* Henry parentage.

The different levels of attack observed among hybrid poplars (Morris 1981, Moore et al. 1982, Abebe and Hart 1990, Johnson and Johnson 2003) could be explained by antixenosis (non-preference) and antibiosis (Painter 1951, Kogan and Ortman 1978). Antixenosis would occur if hybrid poplars either lacked attractive stimuli or contained deterrent stimuli, and consequently were not preferred behaviorally by *C. lapathi*. For antibiosis, the host poplars would adversely affect herbivore fitness at a physiological
level, i.e., *C. lapathi* would suffer decreased fecundity, longevity, and/or developmental rate. Painter’s (1951) third method of resistance, tolerance, would not explain the absence of attack. The underlying mechanisms of antixenosis and antibiosis could be further categorized as physical or chemical, induced (present after a damage event) or constitutive (present all the time).

Because *C. lapathi* eggs and early instars are restricted to the bark tissue, mortality could be the result of physical hydraulic forces. For example, *Anoplophora glabripennis* (Motsch) (Coleoptera: Cerambycidae) larvae are drowned in sap of the golden rain tree, *Koelreuteria paniculata* Laxmann (Morewood et al. 2004), and Qin et al. (1996) reported that *Populus deltoides* cv. ‘Lux’ physically pitched out *A. glabripennis* eggs from oviposition punctures. High water content in bark has been implicated in conferring resistance to another wood boring cerambycid, *Phoracantha semipunctata* (F.) (Hanks et al. 1991).

Tree phenology may also affect the ability of hybrid poplars to mount physical (or chemical) defences against *C. lapathi*. For instance, Hulme (1995) found resistant Sitka spruce trees, *Picea sitchensis* Bong. Carr., to be susceptible to the white pine weevil, *Pissodes strobi* (Peck), if oviposition occurred before flushing, and susceptible trees became resistant if oviposition was delayed until after flushing. In all cases, linking resistance to phenology was high resin flow from wounds (Hulme 1995). Under natural conditions, resistant Sitka spruce trees tend to flush earlier than susceptible ones (Hulme 1995, Alfaro et al. 2000), i.e., before female *P. strobi* are gravid.

Chemical differences could involve nutrient levels and/or toxicity of secondary compounds. Insects require carbohydrate, protein and fat; essential are 10 amino acids,
sterols and linoleic acid (Chapman 1982). Berenbaum (1995) argues that low nutritive status of host plants would probably evolve as a defence mechanism against oligophagous\(^2\) herbivores with limited mobility or poor ability to compensate physiologically or behaviourally for low levels of nutrition. Because *C. lapathi* is oligophagous, and larvae are unable to disperse, low levels of nutrients could potentially explain the observed differences of *C. lapathi* attack and preferences among hybrid poplars (Morris 1981, Moore et al. 1982, Abebe and Hart 1990, Johnson and Johnson 2003). Furthermore, nutrition can be involved in mediating the effects of toxins. For example, high levels of protein in trembling aspen, *Populus tremuloides* Michaux, leaves ameliorated the adverse effects of phenolic glycosides to lepidopteran defoliators (Lindroth and Bloomer 1991, Osier et al. 2000a).


\(^2\) Oligophages typically feed on multiple species within a genus or family, or a few plants from different families (Bernays and Chapman 1994).
*Microtus pennsylvanicus* (Ord.) (Robison and Raffa 1998). Furthermore, one particular hybrid with *P. maximowiczii* parentage, NM 6, is also susceptible to the cottonwood leaf beetle, *Chrysomela scripta* F. (Coleoptera: Chrysomelidae) (Ramachandran 1993, Robison and Raffa 1998), and research suggests that phenolic glycosides which protect poplars from forest tent caterpillar and gypsy moth (Meyer and Montgomery 1987, Lindroth and Hemming 1990, Lindroth and Bloomer 1991, Hwang and Lindroth 1997, Osier et al. 2000a, Osier and Lindroth 2001), confer susceptibility to chrysomelid beetles which sequester glycosides for their own defence (Matsuda and Matsuo 1985, Smiley et al. 1985, Soetens et al. 1991, Bingaman and Hart 1993, Martinsen et al. 1998).

Condensed tannins are also present in poplar bark and are thought to bind and precipitate protein, restricting its availability to herbivores (Palo 1984). Direct toxic effects of condensed tannins have also been described (Blytt et al. 1988).

Poor nutrition or toxins in a host plant may also inhibit the growth of symbiotic fungi. Xylem-boring insects often have microorganisms associated with them that aid in the digestion of cellulose; the inner bark and cambium additionally contain starch and sugars (Haack and Slansky 1987). Microorganisms also produce vitamins. Although there are specific fungi associated with *C. lapathi* galleries (Kerrigan 2003), it is not known if they are necessary for *C. lapathi* development.

Phenolic levels are known to increase in bark (Fang et al. 2002) and leaves (Clausen et al. 1989b, Osier and Lindroth 2001, 2004, Arnold and Schultz 2002, Peters and Constabel 2002) after wounding and affect foliovore preference and/or performance (Clausen et al. 1989b, Robison and Raffa 1997, Havill and Raffa 1999, Peacock et al. 2001). Wounded poplars are also known to increase the levels of defensive proteins like

My ultimate goal was to determine the mechanism(s) of resistance and susceptibility among four hybrid poplar clones currently grown in North America. At the time this study commenced, there was no literature available to suggest which hybrid poplars currently cultivated in North America were resistant or susceptible to C. lapathi. Thus I selected four different hybrid poplars from different breeding backgrounds (Table 1.1). They are TN 302-9, TD 52-226, TM 256-28, and NM 6, and, for brevity, are herein referred to as TN, TD, TM and NM, respectively. My first step was to determine if resistance to C. lapathi attack occurred before or after oviposition. Specifically, I examined:

1. olfactory-based preferences among and between the hybrid poplar clones and two locally abundant salicaceous hosts (P. tremuloides and Salix scouleriana Barratt ex Hooker) in choice and no-choice situations;
2. feeding-based preferences among and between the hybrid poplar clones, P. tremuloides and S. scouleriana hosts in choice and no-choice situations;

---

3 The goal of my original thesis project was to conduct a comparative study of the chemical ecology of C. lapathi and Saperda calcarata Say (Coleoptera: Cerambycidae). Realization that this goal was unattainable (Appendix 1), as well as the results in Chapter 2, led me to shift my efforts toward the present goal.
Table 1.1  Parentage of the four hybrid poplar clones used in this investigation.

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>Maternal parent</th>
<th>Paternal parent</th>
<th>Breeding information</th>
</tr>
</thead>
<tbody>
<tr>
<td>TN 302-9</td>
<td><em>P. trichocarpa</em> 95-925 from Hoh Valley, Washington</td>
<td><em>P. nigra</em> L. 71.049/21 from V. Steenackers, Poplar Research Center, Geraardsbergen, Belgium</td>
<td>Bred by R.F. Stettler in 1988 at the University of Washington</td>
</tr>
<tr>
<td>TD 52-226</td>
<td><em>P. trichocarpa</em> 93-968 from Granite Falls, Washington</td>
<td><em>P. deltoides</em> 101 from Illinois</td>
<td>Bred by R.F. Stettler in 1981 at the University of Washington</td>
</tr>
<tr>
<td>TM 256-28</td>
<td><em>P. trichocarpa</em> 95-877 from Hoh Valley, Washington</td>
<td><em>P. maximowiczii</em> Henry 11863 from Japan</td>
<td>Bred by R.F. Stettler in 1987 at the University of Washington</td>
</tr>
<tr>
<td>NM 6</td>
<td><em>P. nigra</em></td>
<td><em>P. maximowiczii</em></td>
<td>Bred in Germany</td>
</tr>
</tbody>
</table>
3. oviposition levels among the four hybrid poplar clones in no-choice situations and preferences between two of these hybrids in choice situations; and

4. larval survival to adulthood in the four hybrid poplar clones.

Second, in an attempt to explain the biochemical and/or physical basis of antixenotic and antibiotic resistances, I quantified the following:

1. phenological differences of the four different hybrids with respect to time of bud burst, percent water in the bark, and sap flow during early spring;

2. constitutive levels of carbohydrate, total nitrogen (N) content, sterols, linoleic and linolenic acids, phenolic glycosides, condensed tannins and total phenolics among the four hybrid poplar clones at three different times corresponding to the periods of adult host selection, and before and after early instar larvae commence activity; and

3. constitutive and induced levels of phenolic glycosides, condensed tannins, total phenolics, and PPO activity among two hybrid poplar clones at two different times corresponding to the period of adult host selection, and first- or second-instar activity.
2 Olfactory and Feeding Preferences

2.1 Introduction

There are three phases to host selection (Schlyter and Birgersson 1999): location of host-containing habitat, location of suitable host species, and determination of host suitability or susceptibility. Most emergent *C. lapathi* adults would find themselves in a suitable habitat because their host trees support more than one generation and often have locally clumped distributions. Therefore, they would only need to find susceptible hosts. I tested the hypothesis that susceptible clones would be more attractive via olfaction and would sustain more feeding damage than resistant clones.

2.2 Materials and Methods

2.2.1 General strategy

Weevils were given a choice between two potential hosts, and were also exposed in no-choice experiments to a single host type in a series of laboratory olfactory and feeding bioassays. Because no quantitative information on resistance to *C. lapathi* was available for commercially planted clones when this study began in 2001, initial experiments compared responses by weevils to commercial clones vs. naturally-occurring ('susceptible') black cottonwood, *Populus trichocarpa* Torrey and Gray. To maximize the likelihood of encountering varying levels of resistance and susceptibility, the four clones were chosen from different breeding backgrounds (Table 1.1). Later experiments

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utilized information from field observations, and compared responses among native willows and two clones that showed apparent resistance and susceptibility, respectively.

2.2.2 Plant material

Clonal material for testing was pooled current year’s growth obtained from several vigorous coppiced ramets grown at Scott Paper Ltd.’s nursery in Harrison Mills, British Columbia (49° 15’ N, 121° 58’ W). The *P. trichocarpa* test material came from a single population in Langley, British Columbia (BC) (49° 5’ N, 122° 42’ W), and consisted of 1-year-old material on individuals coppiced two years previously. *Salix scouleriana* stems of assorted ages, growing from previously coppiced clumps, were collected from the Simon Fraser University campus, Burnaby, BC (49° 17’ N, 122° 56’ W). Leaves were from determinate branches (i.e., lacking active apical meristems).

2.2.3 Weevil colonies

To obtain adult *C. lapathi*, infested cut willow stems were collected in mid-July and kept in ventilated bins (30 x 35 x 50 cm$^3$) in an outdoor compound at Simon Fraser University. Voucher specimens have been submitted to the Pacific Forestry Centre (Victoria, BC). In 2001, material was collected from Coquitlam, BC (49° 20’ N, 122° 49’ W); in 2002, near Hope, BC (49° 33’ N, 121° 26’ W); and in 2003, from the Coquihalla summit area (49° 59’ N, 121° 00’ W). Emergent adults were manually removed from bins, sexed, and placed in ventilated containers (25 x 35 x 50 cm$^3$) with willow stems, water, and occasionally provided with sliced apple fruit. Weevils for bioassays were held at 4°C (where they fed less and were therefore easier to maintain).
2.2.4 Pitfall olfactometer bioassays

Pitfall olfactometers used to examine weevil responses to volatile stimuli were larger versions of those described by Prokopy et al. (1995). I used a 14 x 2.5 cm Petri dish fitted with two open 1.8 mL Eppendorf centrifuge tubes, spaced 8 cm apart. The bottoms of the Eppendorf tubes were opened with a 5 mm diameter drill, so they formed a tube through which weevils could pass into a pit below; from which escape was unlikely. Pits were glass jars 4.5 cm diameter and 6 cm high for stems alone or 4.3 cm diameter and 8.5 cm high when both stems and leaves were tested. Beetles were acclimated in the pitfall olfactometer for at least 10 min under the overturned bottom of a 3.5 x 1 cm Petri dish before the start of a bioassay. Bioassays ran overnight, ca. 18 h, in the dark with one female or male weevil per dish. Weevils were starved for 24 h prior to experiments in 2003, but in 2001 and 2002 small colony size and concern for increased mortality precluded using this procedure (weevils were not starved).

In choice bioassays, both pits contained stem sections cut to 3 cm in length. Diameters were matched between treatment and control pits and ranged from 7 to 20 mm. In no-choice bioassays, i.e., when only one pit contained plant material, both pits contained a 1 cm long section of water-saturated dental wick. In this manner, once beetles made a choice to enter a pit, there was stimulus for them to stay (Pierce et al. 1988). In experiments that included leaf material, a single leaf was placed in a small vial of water in addition to the stem material. I placed a vial of water in the control pit in the corresponding no-choice bioassays. Treatments were applied in a completely randomized design, and were repeated until sufficient response rates for statistical analysis were obtained.
I first tested responses to stem sections of each of the four clones paired with sections from *P. trichocarpa* or an empty (i.e., containing a wet dental wick) pit. After field-caging experiments (see Chapter 3) suggested that among the four clones, TN was highly susceptible and NM was most resistant, I repeated pitfall olfactometer bioassays using NM, TN, and *S. scouleriana*, a preferred host in the field.

2.2.5 GC-EAD analysis

Leafy shoots from the terminals of hybrid poplars were collected in late August 2001, returned to the laboratory, placed in water, and aerated (Rudinsky 1974). Volatiles were captured on 200 mg Porapak-Q (Byrne et al. 1975), eluted under pressure (N$_2$ gas) with 2 mL of distilled pentane. Both male and female adult *C. lapathi* were then subjected to gas chromatographic-electroantennographic detection (GC-EAD) analysis of the captured volatiles (Arn et al. 1975, Gries et al. 2002). Captured volatiles (1 µL) were injected splitless (Hewlett Packard 5890 gas chromatograph, injector port 250°C, detector port 260 °C, temperature program: held 1 min at 50°C, increased 10°C/min to 280°C; DB-5 GC column, 30 m x 0.32 mm ID, J & W Scientific, Folsom, CA 95630), and then passed over an electrophysiological antennal preparation (Arn et al. 1975, Gries et al. 2002) or a flame ionization detector. Antennally-active compounds were identified by GC-mass spectrometry (Varian Saturn 2000 Ion Trap, DB-5 column as above, electron impact mode) and confirmed by coelution and comparison of mass spectra with synthetic standards.
2.2.6 Paired-twig feeding bioassays

Paired-twig bioassays (Tomlin and Borden 1996) were performed to determine feeding preferences using 5 cm long cut stem sections suspended on a central (ca. 2 x 2 x 2 cm³) wax block. The exposed ends were sealed with paraffin wax, and each paired-twig assembly was placed in a 14 x 2.5 cm Petri dish. Dishes were arranged in a completely randomized design, and individual non-starved male or female weevils were allowed to feed for three days. Feeding was assessed by number of feeding punctures (determined under a dissecting microscope) and weight of frass (dried to constant mass) collected from under each of the twigs.

As in the pitfall bioassays, I first tested each of the four clones against *P. trichocarpa* ($n = 15$). No-choice experiments were also run on the four clones and *P. trichocarpa* ($n = 12$), in which the same material was placed on both sides of the paired-twig set up. Next, I used paired-twig bioassays to compare feeding responses among NM, TN and *S. scouleriana* in choice ($n = 15$) and no-choice ($n = 12$) situations.

2.2.7 Statistical analyses

In all cases $\alpha = 0.05$. For pitfall olfactometer bioassays, responses were tested against the binomial distribution with $p = 0.5$ (Daniel 1995). Data from paired-twig feeding bioassays were tested with a series of paired t-tests (Daniel 1995). Data from no-choice feeding bioassays were analysed by ANOVA using PROC GLM, and when significant differences were found, the REGWQ test was used for means separation (SAS Institute 1990). If necessary, data were transformed ($x^{1/2}$) prior to analyses to correct for non-normality and heteroskedasticity.
2.3 Results and Discussion

2.3.1 Pitfall olfactometer bioassays

Weevils of both sexes responded strongly and consistently to pits containing poplar stems of any origin when the alternative was an empty pit (Table 2.1). These results suggest the presence of attractive volatiles in all five types of poplar, and absence of a repellent. In choice bioassays, male *C. lapathi* preferred cut stem sections of TN and TM, and females preferred TM to sections of ‘susceptible’ *P. trichocarpa* stem (Table 2.2). This discrimination was absent in late October (data not shown). There was also no evidence of preference among TN, NM and *S. scouleriana*, which included leaf material as well as stems (Table 2.1). Additional pitfall experiments including stem and leaf material during this same time period (Broberg et al. 2005b) showed that weevils preferred pits containing *S. scouleriana* over a series of field-collected alternate hosts and non-hosts: *P. trichocarpa*, *P. tremuloides*, *Alnus rubra* Bongard, *Acer macrophyllum* Pursh (Aceraceae), and *Picea abies* (L.) Karsten (Pinaceae). Clearly, olfactory discrimination does not explain resistance among hybrids. Dafauce (1976) also found no olfactory discrimination between ‘susceptible’ *P. deltoides* cv. ‘Missouriensis Zeeland’ and ‘resistant’ *P. alba* L. cv. ‘bolleana’ Lauche in the laboratory. It is probable that the volatile profiles of hybrid poplars are very similar and discrimination is not possible.

2.3.2 GC-EAD analysis

A lack of olfactory discrimination is supported by comparative GC-EAD analysis which showed very similar volatile profiles and antennal responses among clones (Figure 2.1). Male and female antennal responses were nearly identical; thus only males are
Table 2.1  Pitfall bioassay results of four hybrid poplar clones (see text for abbreviations), *Populus trichocarpa* (TT) and *Salix scouleriana* (SS).

Distributions that differed significantly from the binomial distribution (with \( p = 0.5 \)) indicated by * \( P < 0.05 \); ** \( P < 0.01 \); and *** \( P < 0.001 \).

<table>
<thead>
<tr>
<th>Dates performed</th>
<th>Plant material used</th>
<th>Weevil's choice (Side 1 vs. Side 2)</th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tails</td>
<td>N</td>
<td>Proportion entering side 1</td>
</tr>
<tr>
<td>20 Aug,</td>
<td>Stems alone</td>
<td>TN vs. TT</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>22 Sept,</td>
<td></td>
<td>TD vs. TT</td>
<td>2</td>
<td>19</td>
</tr>
<tr>
<td>12 Oct, 2001</td>
<td></td>
<td>TM vs. TT</td>
<td>2</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NM vs. TT</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>29 Aug, 7 Sept,</td>
<td>Stems alone</td>
<td>TT vs. Empty pit</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>2001, 3 Oct, 2002</td>
<td></td>
<td>TN vs. Empty pit</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TD vs. Empty pit</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TM vs. Empty pit</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NM vs. Empty pit</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>30 Aug, 25 Sept,</td>
<td>Stems and leaves</td>
<td>NM vs. TN</td>
<td>2</td>
<td>19</td>
</tr>
<tr>
<td>2003</td>
<td></td>
<td>NM vs. SS</td>
<td>2</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>TN vs. SS</td>
<td>2</td>
<td>20</td>
<td>0.35</td>
</tr>
</tbody>
</table>
Table 2.2  Results from paired-twig bioassays of four hybrid poplar clones (see text for abbreviations), and *Populus trichocarpa* (TT). Differences between side 1 and side 2 means were compared using a paired t-test.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Weevil's choice (Side 1 vs. Side 2)</th>
<th>Tails</th>
<th>Mean no. feeding punctures ± SE</th>
<th>Mean amount of frass produced ± SE (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Side 1</td>
<td>Side 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>t</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>TN vs. TT</td>
<td>2</td>
<td>20.0 ± 4.6</td>
<td>14.3 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>TD vs. TT</td>
<td>2</td>
<td>10.4 ± 1.7</td>
<td>26.6 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>TM vs. TT</td>
<td>2</td>
<td>20.1 ± 3.9</td>
<td>19.7 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>NM vs. TT</td>
<td>2</td>
<td>12.7 ± 1.8</td>
<td>22.9 ± 4.0</td>
</tr>
<tr>
<td>Male</td>
<td>TN vs. TT</td>
<td>2</td>
<td>15.6 ± 3.8</td>
<td>6.5 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>TD vs. TT</td>
<td>2</td>
<td>9.9 ± 3.6</td>
<td>13.0 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>TM vs. TT</td>
<td>2</td>
<td>21.1 ± 3.2</td>
<td>17.9 ± 6.4</td>
</tr>
<tr>
<td></td>
<td>NM vs. TT</td>
<td>2</td>
<td>13.2 ± 3.1</td>
<td>12.7 ± 3.3</td>
</tr>
</tbody>
</table>
Figure 2.1  Male Cryptorhynchus lapathi GC-EAD responses to five different Populus sources. Corresponding FID traces not shown. Antennal responses to: 1 = (2E)-hexenal; 2 = (3Z)-hexenol; 3 = (2E)-hexenol; 4 = hexanol; 5 = 1-octen-3-ol; 6 = benzyl alcohol; 7 = salicylaldehyde; 8 = conophthorin; 9 = mixture of (E) and (Z)-chalcogran and guaiacol; 10 = nonanal; 11 = unknown; 12 = nonanol; 13 = methyl salicylate.
shown. Antennal responses were primarily elicited by six-carbon alcohols, benzaldehyde, salicylaldehyde, conophthorin, nonanal, nonanol, methyl salicylate, and several unknown compounds. Low or high response to prominent volatiles were inconsistent with responses in the pitfall bioassays; e.g. response to salicylaldehyde was low in TM and high in TN, but both clones were preferred in pitfall experiments (Table 2.1). The strong antennal response to conophthorin by C. lapathi antennae despite its nearly undetectable level in the FID and MS supports its earlier discovery in P. trichocarpa (Huber et al. 1999), and reflects a new finding in willows (Broberg et al. 2005a). It further suggests that conophthorin may have behavioural activity for C. lapathi. In the closely related family Scolytidae, conophthorin acts as a repellent non-host kairomone (Huber et al. 1999, Zhang et al. 2001), a repellent or attractive pheromone (Kohnle et al. 1992, Dallara et al. 2000, Rappaport et al. 2000), and a synomone for competing congeners (Dallara et al. 2000). Other known bark beetle pheromones detected in the plant volatile profiles were 1-octen-3-ol (Pureswaran and Borden 2004), and chalcogran (Byers et al. 1989, 2000) which also occurs in Salix spp. (Broberg et al. 2005a).

2.3.3 Paired-twig feeding bioassays

Weevils fed on the inner bark at the cut ends of stem sections despite sealing with paraffin, making estimation of puncture numbers difficult. However, data on frass production and feeding punctures were generally consistent (Table 2.2, Figs. 2.2-2.4).

Female weevils alone fed less on TN or TD than on P. trichocarpa in choice situations (Table 2.2). In no-choice situations, both sexes generally fed the least on NM;
Figure 2.2  Results from feeding bioassay experiment in which no choice was given, comparing feeding on four hybrid poplar clones and wild *Populus trichocarpa*. ANOVA statistics as follows: A. $F = 4.61$, df = 4, 54, $P = 0.003$; B. $F = 3.92$, df = 4, 51, $P = 0.008$; C. $F = 1.12$, df = 4, 54, $P = 0.36$; and D. $F = 3.61$, df = 4, 52, $P = 0.01$. Within each sub-figure, bars with the same letter are not significantly different, REGWQ test, $P < 0.05$. 
Males Females

A

Females

B

Males

C

Mean Amount Frass Produced

Clone

TT TN TD TM NM

TT TN TD TM NM

Mean No. Feeding Punctures + SE

Clone

TT TN TD TM NM

TT TN TD TM NM

ab b ab a b

ab b ab a b

ab b ab a b

ab b ab a b
Figure 2.3  Number of punctures and amount of frass produced in the second round of feeding bioassays in which a choice was given between the pair wise combinations of NM, TN, and *Salix scouleriana* (SS). Results from paired t-tests are as given.
### FEEDING PUNCTURES

<table>
<thead>
<tr>
<th>Side 1</th>
<th>Side 2</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM</td>
<td>TN</td>
<td>-7.92</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>NM</td>
<td>SS</td>
<td>-2.00</td>
<td>0.03</td>
</tr>
<tr>
<td>TN</td>
<td>SS</td>
<td>8.63</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

### FRASS PRODUCTION

<table>
<thead>
<tr>
<th>Side 1</th>
<th>Side 2</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM</td>
<td>TN</td>
<td>-3.28</td>
<td>0.004</td>
</tr>
<tr>
<td>NM</td>
<td>SS</td>
<td>-1.90</td>
<td>0.04</td>
</tr>
<tr>
<td>TN</td>
<td>SS</td>
<td>7.16</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

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#### Mean Number of Punctures (+ SE)

- **Side 1 Females**: [Bar chart]
- **Side 1 Males**: [Bar chart]
- **Side 2 Females**: [Bar chart]
- **Side 2 Males**: [Bar chart]

#### Mean Amount of Frass Produced (mg + SE)

- **Side 1 Females**: [Bar chart]
- **Side 1 Males**: [Bar chart]
- **Side 2 Females**: [Bar chart]
- **Side 2 Males**: [Bar chart]
Figure 2.4  Results from feeding bioassay experiment in which no choice was given comparing feeding on wild *Salix scouleriana* and two hybrid poplar clones, one with and one without *Populus maximowiczii* parentage. ANOVA statistics as follows: \textbf{A}. $F = 7.43$, df = 2, 33, $P = 0.002$; \textbf{B}. $F = 12.74$, df = 2, 33, $P < 0.0001$; \textbf{C}. $F = 13.93$, df = 2, 32, $P < 0.0001$; \textbf{D}. $F = 7.69$, df = 2, 33, $P = 0.002$. Within each sub-figure, bars with the same letter are not significantly different, REGWQ test, $P < 0.05$. 
however, this feeding was not significantly different from that on TD, *P. trichocarpa*, or TN (Figure 2.2). When feeding preferences among NM, TN, and *S. scouleriana* were examined, responses became clear. When presented with a choice, weevils of both sexes avoided NM in favour of the more susceptible hosts TN or *S. scouleriana*. Furthermore, weevils selected TN over its preferred wild host, *S. scouleriana* (Figure 2.3). These results were reflected in the no-choice experiment, in which both feeding punctures and frass production on NM were significantly lower for both sexes than on TN or *S. scouleriana* (Figure 2.4). The preference for TN over *S. scouleriana* indicates that this hybrid could be very susceptible to attack in the field, especially when first planted. The lack of antixenosis towards TM, which is also highly resistant (see Chapter 3), and the lack of consistent preference for the susceptible TD and TN in choice and no-choice paired-twig feeding bioassays (Table 2.2, Figure 2.2) suggests that caution should be used in the use of feeding preference screens as indicators of resistance in the field.

Non-preference for *P. alba* cv. ‘bolleana’ remained constant over several choice feeding trials in Dafauce’s (1976) work, but not in that of Cadahia (1965). Also, in Dafauce’s (1976) only no-choice trial, there was equal feeding on the non-preferred *P. alba* cv. ‘bolleana’ and the [usually] highly preferred clone, *P. deltoides* Marsh cv. ‘Carolinensis’, on which adults also had the greatest longevity. Similarly, I observed that weevils make choices consistent with maximizing fitness by discriminating against NM and for TN, but often fail to do so (Table 2.2, Figs. 2.2-2.4), suggesting that either antixenotic signals or weevil preferences are unreliable. Accordingly, *C. lapathi* demonstrated only weak oviposition preference between TN and NM (see Chapter 3), yet the fitness costs of making a “wrong” decision were severe, as eggs did not complete
development in NM. All four hybrids in this study are from the two closely related *Populus* sections Aigeros (cottonwoods) and Tacamahaca (balsam poplars) (Eckenwalder 1996). Given the similarity in the volatile profiles of the different hybrids, and their evolutionary relatedness, it is possible that their bark chemistry is also quite similar, at least at the time of feeding and oviposition. Because I did not examine effects on weevil fitness directly, it is possible that reduced feeding on NM is not a result of antixenosis, but of enhanced nutritive value. This does not seem a likely alternate hypothesis as others have found NM to have antixenotic or antibiotic effects on a number of species including *C. lapathi* larvae (see Chapter 3), forest tent caterpillar larvae (Robison and Raffa 1994), gypsy moth larvae (Kruse and Raffa 1996), an aphid (Ramirez et al. 2004), and the meadow mouse (Robison and Raffa 1998). I conclude that *C. lapathi* is capable of discriminating against NM using gustatory cues (Table 2.2, Figures 2.2-2.4), but not olfactory cues (Table 2.1, Figure 2.1). Thus resistance is apparently based, at least in part, on antixenosis.
3 OVIPOSITION PREFERENCES AND LARVAL SUCCESS

3.1 Introduction

Earlier work with hybrid poplars suggested that oviposition preferences of C. lapathi for specific clones are generally stable, and were considered a correlate of resistance (Cadahia 1965, Dafauce 1976). I examined oviposition levels among TN, TD, TM and NM to determine if a similar trend existed among these hybrids, and hypothesized that clones expressing feeding deterrence would also sustain less oviposition. I also investigated larval survival to adulthood in the four clones (i.e. antibiosis), hypothesizing that clones expressing feeding and/or oviposition deterrence, would also produce fewer adult weevils.

3.2 Materials and Methods

3.2.1 Weevil colonies

To obtain adult C. lapathi, infested willows were collected in mid-July and kept in ventilated bins (30 x 35 x 50 cm³) in an outdoor compound at Simon Fraser University. Collections in 2001 were from Coquitlam, BC (49° 20’ N, 122° 49’ W); in 2002 and 2004 near Hope, BC (49° 33’ N, 121° 26’ W); and in 2003, from the Coquihalla summit area (49° 59’ N, 121° 00’ W). Emergent adults were manually removed, their sex

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determined, and held outdoors under cover in ventilated containers (25 x 35 x 50 cm³) with willow stems, water, and occasionally provided with slices of apple.

3.2.2 Choice and no-choice oviposition bioassays on potted poplars

Initial oviposition assays using cut stem sections in the laboratory were abandoned because response rates were very low. Subsequent oviposition experiments, two-choice and one no-choice, employed potted plants. In all potted plant experiments, the plants were irrigated with an automatic sprinkler, hand-weeded, treated with a single fertilizer treatment in mid-June following planting (ca. 13 g 14-29-5 + Minors: 5% S, 2.5 % Mg, 0.1% B, 0.1% Zn; Burgess Agri Supplies Ltd., Agassiz, BC), and pruned to one main stem in early July. Cages consisted of fibreglass screen cylinders sewn to fit around pots and were either 1.8 m high (2003) or 90 cm high (2004).

The first choice experiment involved caging a single pair of weevils for 3 wk, commencing 19 August 2003 in 10 assorted large containers, each containing one TN, NM, and Salix discolor Muhlenberg. This was repeated 3 and 6 weeks following the first caging; however, only 4 of these 20 replicates contained oviposition and are not included in analyses. The S. discolor was established in 2002 from ca. 30 cm long cuttings of unknown ages, planted to a depth of ca. 25 cm. For all potted plant experiments, clonal material was obtained from Scott Paper Ltd. (Harrison Mills, BC, 49° 15’ N, 121° 58’ W). Poplar clones were established in 2003 from 45 cm long 1-yr-old cuttings, planted to a depth of 25 cm. Oviposition was examined at the end of the 3-wk period by cutting each plant off at the root collar, and opening each puncture with a scalpel under a dissecting microscope.
The second choice oviposition experiment used 22 cm long 1-yr-old TN and NM cuttings planted to ca. 12 cm depth in 11 L pots in March 2004. A single pair of weevils was caged on each potted pair for up to 23 days, beginning 18 August 2004 (N = 40). Oviposition was assessed as above from 25 August to 10 September 2004.

The no-choice experiment (N = 60) was established in 2004 at the same times as the second choice experiment, except that TN and NM cuttings were planted separately into 3 L pots, and weevils were not removed from trees until oviposition was assessed on 43 replicates (20 December – 25 April 2005).

3.2.3 No choice oviposition and larval survival experiments in field plots

To examine oviposition and larval survival among clones, I established a field plot of TN, TD, TM, NM, and _P. trichocarpa_ cuttings (45 cm long 1-yr-old cuttings, planted to 30 cm depth) in April 2001, at a nursery run by Scott Paper Ltd. (Harrison Mills, BC). The design provided 60 replicates of each clone, and consisted of 12 consecutive 5 x 5 Latin squares. The field-collected _P. trichocarpa_ was improperly stored prior to planting and none survived, leaving a total population of 240 hybrid trees. Cuttings were 45 cm apart within rows; rows were 2 m apart. Every two Latin squares (i.e., 10 replicates) were completely bordered by another hybrid poplar, DTac7. Trees were pruned to one main stem in July, 2001, and secondary stems below a height of 1 m were periodically removed for the remainder of the experiment. Granular dichlobenil herbicide (Crompton Manufacturing Co., Middlebury, CT) was applied in May, 2001. Subsequent weeding was by hand within rows and by cultivator between rows. Irrigation followed Scott Paper's regular schedule. Approximately 37 g of 9-40-4 fertilizer
(Burgess Agri Supplies Ltd., Agassiz, BC) was buried at the base of each tree in June 2001.

A single pair of weevils was caged per tree on 15 August in both 2001 and 2002 (20 replicates per clone, for a total of 80 weevil pairs each year). The base of each cage consisted of a 15 cm long section of 20 cm diameter PVC pipe fitted around the root collar of each tree to a depth of ca. 8 cm. A screen sleeve was fitted around this collar, secured with a 90 cm cable tie, and soil was backfilled to the top of the collar. The top of the cage was closed around a pad of cotton batten about 1 m above ground level and tied tight to the stem with plastic flagging tape. (See Figure 3.1 for photograph). Four months after caging, 10 replicates were sacrificed in each year and all punctures were dissected as above to absolutely determine oviposition and life history stage encountered. Trees from the remaining 10 replicates per year were cut the following July, 11 months after the caging, kept individually in mesh bags outside, and monitored daily for adult emergence. Emergent weevils were separated by sex (Harris 1964), and measured under a dissecting microscope; head and elytra width were measured dorsally, and elytral length was measured laterally. In December 2001 and 2002, tree height was measured using a telescoping height pole, and diameter was taken at the root collar with a caliper.

3.2.4 Statistical analyses

In all cases $\alpha = 0.05$. Data from the first choice oviposition experiment and the Latin square field experiment (2001-2002 season) were compared by ANOVA using PROC GLM. When significant differences were found, LSMEANS with a Bonferroni correction was used for separation of means (SAS Institute Inc. 1990). For the first
Figure 3.1  Photograph of assembled field cage in August 2001 (A) and 2002 (B).
choice oviposition experiment, the model included host plant and replicate effects. The models for all data from field plots were based on that of a replicated Latin square design (Montgomery 2001), as long as assumptions of normality and homoskedasticity were upheld (oviposition and emergence data were transformed by $x^{1/2}$). Oviposition in two clones with $P. \text{maximowiczii}$ parentage was compared to that in two clones without this parentage in 2002 by orthogonal contrast (Sokal and Rolf 1981). Because of low oviposition and emergence in the 2002-2003 field season, data were analysed nonparametrically with a Kruskal-Wallis one-way analysis of variance by ranks (Daniel 1995) followed by the nonparametric equivalent of the Student-Newman-Keuls test (Zar 1984) for means comparison. Analogous to the orthogonal contrast in 2002, I used the Kruskal-Wallis test to compare oviposition in hybrids with and without $P. \text{maximowiczii}$ parentage. Data from the second choice oviposition experiment using potted plants with oviposition present were tested with a one-tailed paired t-test (Daniel 1995), and data from all 86 potted hybrids in the no-choice oviposition experiment were analysed by Kruskal-Wallis one-way analysis of variance by ranks (oviposition counts) or Chi-square test (oviposition frequency).

3.3 Results

3.3.1 Choice and no-choice oviposition bioassays on potted poplars

In the 2003 and 2004 potted plant experiments, there was no discrimination between hosts (2003 mean no. ovipositions for NM $7.3 \pm 2.4$, TN $6.6 \pm 2.0$, $S. \text{discolor}$ $2.9 \pm 1.0$, $F = 2.19$, df = 2, 18, $P = 0.14$; 2004 mean no. ovipositions for NM $1.5 \pm 0.6$, 2004 mean no. ovipositions for TN ...
TN 2.9 ± 1.0, N = 11, t = -1.23, P = 0.12). In 2004, oviposition was less frequent (11 of the 40 replicates had oviposition in 2004 vs. all 10 replicates in 2003) and the duration more protracted than in 2003, likely due to prolonged inclement weather. When analysis was restricted to the data from the ten pots from both years in which females oviposited in only one hybrid poplar clone (i.e. they made a choice), TN bore significantly more eggs than NM plants (2.8 ± 1.0, n = 7 vs. 0.3 ± 0.2, n = 3; t = -2.20; one-tail; P = 0.028).

When weevils were exposed to only a single potted hybrid, oviposition was clearly more abundant on TN than NM in terms of frequency (no. replicates with oviposition for TN = 16, NM = 4; χ² = 9.4; df = 1; P = 0.002), and mean no. ovipositions (TN 2.84 ± 0.85, NM 0.09 ± 0.04; H = 11.1; df = 1; P = 0.0008).

**3.3.2 No-Choice oviposition and larval survival experiments in field plots**

In the no-choice field caging experiments, both ANOVA (2001-2002) and the Kruskal-Wallis test (2002-2003) disclosed no significant differences in oviposition among clones (Figure 3.2). However, orthogonal contrast analysis in 2002 showed that the difference in oviposition frequency between two clones with *P. maximowiczii* parentage and the two without approached significance (F = 3.64; df = 1, 27; P = 0.07), and the nonparametric analysis in 2003 was significant (H = 7.10; df = 1; P = 0.008). Oviposition during the second year was lower and more variable than in the first year (Figure 3.2). The maximum oviposition by a single female in 2001 was 126 (mean = 30.1 ± 5.4), and in 2002, this dropped to 28 (mean = 1.9 ± 0.9).
Figure 3.2  Oviposition and emergence by *Cryptorrhynchus lapathi* from four hybrid poplar clones in two successive years. ANOVA and Kruskal-Wallis statistics as follows:  

**A.** $F = 1.5$, df = 3, 27, $P = 0.23$; **B.** $H = 7.3$, df = 3, $P = 0.06$; **C.** $F = 20.0$, df = 3, 26, $P < 0.0001$; **D.** $H = 15.0$, df = 3, $P = 0.002$.

Within a sub-figure, bars with the same letter are not significantly, $P < 0.05$, LSMEANS with Bonferroni correction (C), or nonparametric Student-Newman-Keuls analogue test (D).
In contrast to oviposition, there were highly significant differences in the number of emergent weevils among clones in both years (Figure 3.2). For both 2001 and 2002, approximately 63% of 1204 and 76 oviposition punctures, respectively, contained first instars (all head capsule widths less than 0.52 mm)\(^6\), and in December 2001, the proportion of hatched eggs (as determined by the presence of living larvae) was not significantly different among clones \((F = 1.86; \text{df} = 3, 27; n = 31; P = 0.16)\).

Among the three clones from which weevils emerged in 2002, over 40% of emergence from TN hosts had occurred by 15 August, while this level was not reached for TD until 1 September (Table 3.1). No emergence from TM had occurred by the latter date. The three measures of adult size were highly correlated and only head width is reported herein. The four weevils that did emerge from TM (total for both years) were not exceptionally smaller than those from TN or TD, and all were well within the range of head widths for the susceptible TN clones.

Feeding punctures (defined as a puncture not containing an egg or larva) were most abundant on the youngest available tissue (92% in 2001 and 91% in 2002). In contrast, oviposition was concentrated in the basal 2-yr-old bark in 2001 and the mid-bole 2-yr-old bark in 2002 (64% and 99%, respectively).

Phenology and size were different among clones. The two clones with a \(P.\) maximowiczii parent, NM and TM, flushed approximately three weeks earlier than TD

\(^6\) Note, for \(C.\) lapathi, the number of instars has been estimated from frequency distributions of head capsule widths. There is consensus only on the first three instars: \(L1 < 0.535\) mm wide, \(L2 < 0.708\) mm wide, \(L3 < 0.883\) mm wide (Harris 1959, Szalay-Marzsó 1962, Harris 1964).
Table 3.1  Emergence dynamics and size of emergent weevils from field-caged hybrid poplar clones. NM excluded because of lack of emergence.

<table>
<thead>
<tr>
<th>Year</th>
<th>Clone</th>
<th>No. trees producing adult weevils (N = 10)</th>
<th>Percent Emergence</th>
<th>Female head width (mm)*</th>
<th>Male head width (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15 August</td>
<td>1 September</td>
<td>n</td>
<td>Range</td>
</tr>
<tr>
<td>2002</td>
<td>TN</td>
<td>9†</td>
<td>42.0</td>
<td>89.7</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>TD</td>
<td>4</td>
<td>2.8</td>
<td>41.7</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>TM</td>
<td>2</td>
<td>0.0</td>
<td>0.0</td>
<td>1</td>
</tr>
<tr>
<td>2003</td>
<td>TN</td>
<td>7</td>
<td>63.6</td>
<td>87.9</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>TD</td>
<td>5</td>
<td>57.1</td>
<td>85.7</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>TM</td>
<td>1</td>
<td>100.0</td>
<td>100.0</td>
<td>1</td>
</tr>
</tbody>
</table>

* NA = not applicable
† One tree that might have produced weevils was attacked so severely that it died before weevils completed development. The main stems of a further four were broken off near the base due to larval feeding in the xylem, and had adventitious sprouts.
and TN. NM and TM were also taller than TD and TN, and both were of greater girth than TN (Table 3.2). The partially susceptible clone, TD, was similar in diameter to NM.

3.4 Discussion

My results partially support the hypothesis that hybrid poplars exerting pronounced feeding deterrence against *C. lapathi* (Figures 2.3, 2.4) would also show similar oviposition deterrence and post-oviposition antibiosis. While the association between feeding and oviposition deterrence was weak there was a pronounced correlation between feeding deterrence by clones with *P. maximowiczii* parentage and antibiosis expressed by the same clones against *C. lapathi* larvae.

The lack of equal feeding and oviposition deterrence is consistent with Cadahia (1965), but not with Dafauce (1976) who found that oviposition levels usually mirrored those of feeding preferences in detached stems. More importantly, both Dafauce (1976) and Cadahia (1965) described several of the same hybrids, and *C. lapathi* oviposition preferences were generally held across genotypes, unlike my results which suggest that oviposition preferences are not strongly maintained. Also in contrast to my results, Tomlin and Borden (1996) found that female white pine weevils, *Pissodes strobi* Peck, were consistently deterred from oviposition on several resistant clones of Sitka spruce, *Picea sitchensis* (Bongard) Carrière. However, in agreement with my results, resistant Sitka spruce clones were not consistently deterrent to these weevils with respect to feeding, and clones that expressed feeding deterrence did not uniformly deter oviposition (Tomlin and Borden 1996).
Table 3.2  Size measurements of four hybrid poplar clones over two growing seasons.

ANOVA statistics as follows: 2001 height $F = 102.6$, df $= 3, 216$, $P < 0.0001$; 2001 basal diameter $F_{3,178} = 79.9$, df $= 3, 178$, $P < 0.0001$; 2002 height $F = 140.1$, df $= 3, 139$, $P < 0.0001$; 2002 basal diameter $F = 38.1$, df $= 3, 27$, $P < 0.0001$. Means within a column and year followed by the same letter not significantly different, LSMEANS, $P < 0.05$.

<table>
<thead>
<tr>
<th>Date</th>
<th>Clone</th>
<th>Height (cm) ($\bar{x} \pm SE$)</th>
<th>Basal diameter (mm) ($\bar{x} \pm SE$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>December 2001</td>
<td>NM</td>
<td>$225 \pm 4$ b &amp; $23.4 \pm 0.4$ b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TM</td>
<td>$262 \pm 4$ a &amp; $26.7 \pm 0.6$ a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TD</td>
<td>$193 \pm 3$ c &amp; $23.6 \pm 0.5$ b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TN</td>
<td>$186 \pm 2$ c &amp; $17.8 \pm 0.3$ c</td>
<td></td>
</tr>
<tr>
<td>December 2002</td>
<td>NM</td>
<td>$439 \pm 8$ b &amp; $35.0 \pm 2.8$ b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TM</td>
<td>$527 \pm 6$ a &amp; $44.9 \pm 2.7$ a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TD</td>
<td>$382 \pm 5$ c &amp; $32.6 \pm 2.5$ b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TN</td>
<td>$357 \pm 6$ d &amp; $24.2 \pm 1.6$ c</td>
<td></td>
</tr>
</tbody>
</table>
The discrimination against NM for oviposition when weevils were offered only one potted host suggests that under constant exposure, NM may express induced antibiosis against adult females. One example of such antibiosis was observed by Sahota et al. (1998) who found that white pine weevils experienced ovarian regression when forced to feed on only resistant Sitka spruce. For *C. lapathi*, oviposition deterrence in resistant clones was most pronounced when oviposition levels in general were low, indicating that weevils were probably already experiencing suboptimal conditions. Because non-preference for oviposition on NM was not absolute, oviposition in the field would likely occur on both resistant and susceptible host types.

Oviposition was present in December 2001 in all four clones planted in the field and there was no difference among clones in the proportion of eggs that produced first instar larvae. Therefore, I conclude that clones with *P. maximowiczii* parentage that failed to produce large numbers of emergent adults in 2002 were resistant to larvae by some antibiotic mechanism. I observed no evidence of xylem feeding in NM clones indicating that larvae did not survive to the xylem penetration stage; thus the antibiosis must be expressed in early spring.

Resistance was not reflected in the size of emergent adults. In fact, competition among larvae in the small, heavily attacked TN plants could explain the small size of some emergent weevils, which contributed to the greatest range in head width (Table 3.1). Males were smaller than females, as previously reported (Harris 1964, Dafauce 1976).

Moore et al. (1982) found that attack moved up the stem, reaching mid-bole by the third year, and concluded that 1-yr-old bark is not generally acceptable for
oviposition. However, I observed that 36% of oviposition in 2001 occurred on 1-yr-old bark, and it was suitable for larval development.

The similar diameters of the highly resistant NM and the partially susceptible TD indicate that small size alone does not necessarily confer susceptibility. TN does not grow as fast as many other clones early in a rotation (G. Abebe, Scott Paper Ltd., Harrison Mills, B.C., pers. comm.), and its slower performance was probably exacerbated by removing its numerous sylleptic branches (Dickmann et al. 2001). Weevil abundance in natural settings and young plantations has been positively associated with large trees (Broberg et al. 2001, Safranyik 1963, Moore et al. 1982). These observations raise the possibility of non-preference by freely-attacking weevils for small TN, and strengthen the hypothesis that resistance in large TM and NM clones is physiological in nature. In older plantations, tree growth rate and attack by *C. lapathi* have not been related (Křítek 1989, Johnson and Johnson 2003). The large size of the young ramets of resistant NM and TM may be related to flushing three weeks earlier than the susceptible TD and TN clones, which would extend the growing season. Hulme (1995) found that phenology can be important in mediating Sitka spruce resistance to *P. strobi* (Hulme 1995) and may contribute to antibiosis simply by early activation of induced resistance mechanisms (Alfaro et al. 2000).

Morris (1981) states that hybrids with parents from the Aigeiros or Tacamahaca sections are susceptible and those of *Populus* (formerly Leuce) are predominantly resistant, which is consistent with the non-preference observed for *P. alba* clones (Cadahia 1965, Dafauce 1976). However, *C. lapathi* attack can be plentiful in *Populus tremuloides* Michx. (also *Populus*) (Thomas and Rose 1979, Vallée 1979). Johnson and
Johnson (2003) found five year old trees with *P. nigra* or *P. deltoides* x *P. nigra* parentage had less attack than those with a *P. trichocarpa* x *P. deltoides* (TD) background, and recommended *P. nigra* as a source of resistance to *C. lapathi*. I also found TD to be susceptible; however, TN was highly susceptible. The lack of agreement between my results and those of Johnson and Johnson (2003) may reside in differences in individual parent genotypes. Thus all clones need to be tested for resistance to *C. lapathi* individually, even if they share a common parentage.

Both clones with *P. maximowiczii* parentage in this study were highly resistant to *C. lapathi*. In agreement with my results Abebe and Hart (1990) found that after eight years, a third hybrid, *P. maximowiczii* x *P. x berolinensis* (NE 47), had only 21.8 % of its trees with *C. lapathi* attack, but two neighbouring clones, *P. nigra* var. *charkowiensis* x *P. nigra* Incrassata (NE 308) and *P. deltoides* x *P. nigra* Incrassata (NE 235), had 68.1% and 97.0% attack, respectively. Assuming that attack was random and independently distributed (Daniel 1995), I analysed the difference in proportional infestation between NE 47 and NE 308 (Abebe and Hart 1990) and found it to be highly significant (*z* = 13.23, *P* < 0.0001) and indicative of *P. maximowiczii* resistance. Two other clones with *P. maximowiczii* parentage, NM 6 and NC 1 1505 (*P. maximowiczii* x *P. trichocarpa*), are also resistant to the forest tent caterpillar, but susceptible to the cottonwood leaf beetle (Ramachandran 1993, Robison and Raffa 1994, 1998). NM 6 is also resistant to an aphid (Ramirez et al. 2004), the gypsy moth (Kruse and Raffa 1996), and the meadow mouse (Robison and Raffa 1998). Only Morris (1981) reported clones with *P. maximowiczii* parentage that had *C. lapathi* attack present.
In summary, I found varying levels of resistance among four hybrid poplar clones with differing genetic backgrounds: NM and TM are highly resistant, while TD and TN are susceptible. This resistance is largely driven by antibiotic effects directed towards early instar C. lapathi larvae. This is the first report that resistance to C. lapathi can occur after oviposition. For feeding, there is strong non-preference for NM and preference for TN (Figures 2.3, 2.4); however, these preferences are not absolute (Figure 2.2, Table 2.2), a finding consistent with that of Dafauce (1976) and Cadahia (1965). Oviposition deterrence on resistant clones containing P. maximowiczii parentage was weak.
4 PHENOLOGICAL DIFFERENCES

4.1 Introduction

Plant phenology and sap or resin flow is correlated with resistance for a number of wood boring insects (Hanks et al. 1991, Hulme 1995, Qin et al. 1996, Alfaro et al. 2000, Morewood et al. 2004). Since early instar *C. lapathi* feed in bark, close to the xylem, and larval mortality in NM and TM likely occurs in the spring, I hypothesized that resistant hybrids may have earlier or greater sap flow than the susceptible hybrids (TD and TN). Therefore, I documented the timing of bud burst, and monitored bark water content and sap production among the four hybrid poplar clones.

4.2 Materials and Methods

4.2.1 Timing of bud burst

To determine differences in timing of bud burst, I placed potted ramets of the four clones in a randomized block design (N = 10) outdoors in Burnaby, BC on 28 January 2005. Trees were established from 22 cm long 1-year old cuttings in March 2004 in 3 L pots (see Section 3.2.2). Bud burst was quantified by determining the proportion of buds that had green growth emerging through the bud scale at intervals throughout the spring of 2005. Growth was monitored by classifying the most mature bud(s) of individual trees on a scale of 0-6 referring respectively to: dormant, growth extending <5, <10, <20, or <30 mm past the bud, leaves beginning to separate, or leaves separate and expanding. The maximum length of new shoot growth was also measured on 21 March 2005.
4.2.2 Timing and quantity of sap flow and bark water content

To determine if poplars could drown weevils, I examined bark moisture and sap flow among the four hybrid poplar clones. On each of five sampling dates (6 and 25 February, 18 March, 9 April, 8 May, 2003), three ramets of each clone were randomly chosen from a pool of 60 saplings growing in monoclonal blocks (Scott Paper Limited’s nursery, Harrison Mills, BC, 49° 15’ N, 121° 58’ W). Blocks were established in spring 2002 from 45 cm long 1-year old cuttings, planted 30 cm deep. The selected ramets were drill-wounded on four sides (hole 1 mm diam. and 10 mm deep) near the root collar at locations preferred by weevils for oviposition. Drill wounds were more extreme than larval mining that would not normally penetrate the xylem during the sampling period. Three holes were located at the bases of different major branches growing from the planted cutting, and the fourth in the dominant stem at a bud within 10 cm of its base. A shortened 10 μL micropipette tip was inserted into the drill hole. The other end of the micropipette tip was fitted through the side of a closed 0.6 mL Eppendorf tube. The unit was secured to the tree with flagging tape. Sap was allowed to drain for 24 h into the Eppendorf tube, and the volume collected was calculated gravimetrically by water loss. After 24 h, eight bark samples were removed from each tree with a scalpel, four encompassed the previously drilled holes; the other four were from the opposite side of the tree. Bark samples were placed in Ziploc bags, returned to the laboratory on ice, weighed, dried to constant mass in a 50°C oven and reweighed. Water content determined as the amount of water lost per amount of dry weight remaining.
4.2.3 Statistical analyses

In all cases $\alpha = 0.05$ and residuals were checked for normality and homoskedasticity. Log transformations were performed if necessary.

The mean proportion of buds burst was analysed as a repeated measures ANOVA using PROC MIXED (Littell et al. 1996). The proportion of buds burst and shoot length data from 21 March 2005 were analysed by ANOVA using PROC GLM for a completely randomized block design (SAS Institute Inc. 1990). The mean percent wet weights for wounded and unwounded bark for each individual were analysed with a factorial model by ANOVA using PROC MIXED, and specifying the individual as random and nested within the clone x treatment interaction (Littell et al. 1996). The LSMEANS statement with a Bonferroni correction was used for multiple comparison analysis.

4.3 Results and Discussion

4.3.1 Timing of bud burst

By 10 February 2005, 68% and 7% of TM and NM buds, respectively, had burst, whereas bud burst for TN and TD occurred between 28 February and 3 March (Figure 4.1A). At the end of the sampling period, proportionately the most buds had burst on TM (Figs. 4.1A, 4.2A). The growth index tracked the proportion of buds burst before growth converged for all trees from all clones on 21 March (Figure 4.1B). At this time, the largest leafy shoots on TM were significantly longer than those of NM and TD, which were again longer than those of TN (Figure 4.2B). In terms of proportion of bud burst on 21 March, NM and TD were also not significantly different (Figure 4.2A), which
Figure 4.1  Phenology of four hybrid poplar clones during the spring of 2005 by cumulative proportion of buds burst (A), or by growth index (B).
A

Mean proportion of buds burst (± SE)

1-Jan 1-Feb 1-Mar 1-Apr 1-May

- NM
- TM
- TD
- TN

B

Mean growth index (± SE)

1-Jan 1-Feb 1-Mar 1-Apr 1-May

Sampling date
Figure 4.2 Maximum length of new shoots and proportion of buds burst on 21 March 2005 among four hybrid poplar clones. Bars within A or B with the same letter are not significantly different, LSMEANS with a Bonferroni correction, $P < 0.05$. 
Figure A: Mean proportion of buds burst + SE (mm)

Clone: NM, TM, TD, TN

Figure B: Mean shoot length + SE (mm)

Clone: NM, TM, TD, TN

Legend:
- b
- a
- ab
- c
indicates that although NM flushed considerably earlier than TD, TD clones were capable of sufficiently rapid growth to match NM.

If growth during the early spring alone explained resistance to *C. lapathi* larvae, NM should have developed earlier and more extensively than any other clone. Instead, TM developed the earliest, and displayed superior growth to NM and TD which had intermediate growth rates. TN was clearly the slowest growing of the clones (Figs. 4.1-4.2). The development rates displayed by these four clones in early spring are reflected in the height and diameter attained by these clones after their first and second growing season (Table 3.2).

4.3.2 Timing and quantity of sap flow and bark water content

Very little sap was collected from hybrid poplar clones during the spring of 2003, with two exceptions (Table 4.1), most notably 465.5 μL from a single TD ramet. More sap would have been collected had it not overflowed from the Eppendorf tube. Because this sapling had only two nodes, I had chosen a lenticel as an alternate third wound site, a location used secondarily for oviposition, in the 2-yr-old bark near the base of the tree. Sap began to flow freely from this internodal wound, and even slight movement of the sapling while attaching the apparatus caused sap to be forcibly ejected. I investigated sap flow from nodes and internodes further on a neighbouring stand of 2-yr-old trees. Significantly more sap visibly flowed from internodal wounds than nodal wounds 11 out of 12 times.

Restricted sap flow from nodal wounds is likely because vascular connections between main shoots and their branches develop after shoots emerge from their buds;
Table 4.1  Observations and collection of sap among four hybrid poplar clones during the spring of 2003 after drill-wounding.

<table>
<thead>
<tr>
<th>Date in 2003</th>
<th>Tree development</th>
<th>Sap Flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 February</td>
<td>Dormant</td>
<td>None observed.</td>
</tr>
<tr>
<td>25 February</td>
<td>Dormant</td>
<td>Stains present on bark of one TM, TD and NM, but no fluid present in the pipette tips or Eppendorf tubes.</td>
</tr>
<tr>
<td>18 March</td>
<td>Green growth 2-4 mm past bud on NM and TM plants. TD and TN still dormant.</td>
<td>One NM produced 198 μL of sap, another 1.7 μL. Heavy rains could have obscured traces of sap stain on bark.</td>
</tr>
<tr>
<td>9 April</td>
<td>Growth on NM and TM elongated (4.5-5 cm and 5-7 cm, respectively). On TD and TN, some buds had 1-2 mm of growth; others had none.</td>
<td>Collected 465.5 μL from one TD, another produced 0.6 μL of sap; two TN's produced 4.1 and 0.2 μL, respectively, and one NM produced 2.4 μL.</td>
</tr>
<tr>
<td>8 May</td>
<td>All buds burst, and leaves expanded.</td>
<td>None observed.</td>
</tr>
</tbody>
</table>
therefore connections are not direct (Dickmann et al. 2001). Thus, *C. lapathi* may oviposit at nodes to mitigate a potential defence mechanism.

Bark from the susceptible TD and TN clones typically contained more water than the resistant NM and TM (Figure 4.3). There were no differences between wounded and unwounded bark collected from wounded trees with an exception on 6 February (Table 4.2). On this date, unwounded bark contained 0.7-2.2% more moisture than wounded bark. Bark moisture tended to increase over the spring (Figure 4.3). In addition, the proportional wet weights of bark collected in July or August ranged from 61-74%, as opposed to 46-63% for bark collected in February-April (data not shown). The data showing that susceptible clones contained proportionally the most water, bark moisture increased during the time of rapid *C. lapathi* development in the spring, and that wounded bark was generally drier than unwounded bark, do not support the hypothesis that resistant clones could physically drown weevil larvae. Lastly, while performing dry weight measurements, I noticed that the bark from TN displayed less browning than the other three hybrids, indicative of lower levels of polyphenol oxidase (PPO) activity.

In summary, the most clear and unifying factor that the two resistant hybrids have in common is an earlier flushing date (Figure 4.1), but no physical mechanisms associated with flushing are correlated with resistance. This suggests that resistance in *P. maximowiczii* hybrids is by some other mechanism, possibly involving biochemical factors, or PPO.
Figure 4.3  Percent water content in bark sections from four different wounded hybrid poplar clones sampled during the spring of 2003.
Sampling date

Mean per cent wet weight (±SE)

- NM
- TM
- TD
- TN

Sampling date

3-Feb 17-Feb 3-Mar 17-Mar 31-Mar 14-Apr 28-Apr 12-May

44 46 48 50 52 54 56 58 60 62 64
Table 4.2  
Statistical summary of per cent wet weight in wounded and unwounded bark samples collected over the spring of 2003.  

$P < 0.05$ in bold.

<table>
<thead>
<tr>
<th>Model term</th>
<th>Source of variation</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clone</td>
<td>$F_{3,16}$</td>
<td>$P$</td>
<td>Clone</td>
<td>$F_{1,16}$</td>
<td>$P$</td>
</tr>
<tr>
<td>6 February</td>
<td>41.99$^c$</td>
<td></td>
<td>$&lt;0.0001$</td>
<td>5.43$^a$</td>
<td>0.03</td>
<td>0.41$^b$</td>
</tr>
<tr>
<td>26 February</td>
<td>63.56</td>
<td></td>
<td>$&lt;0.0001$</td>
<td>1.05</td>
<td>0.32</td>
<td>1.84</td>
</tr>
<tr>
<td>19 March</td>
<td>44.05</td>
<td></td>
<td>$&lt;0.0001$</td>
<td>3.88</td>
<td>0.07</td>
<td>0.55</td>
</tr>
<tr>
<td>12 April</td>
<td>4.45</td>
<td></td>
<td>0.02</td>
<td>0.18</td>
<td>0.67</td>
<td>0.07</td>
</tr>
<tr>
<td>8 May</td>
<td>26.19</td>
<td></td>
<td>$&lt;0.0001$</td>
<td>3.03</td>
<td>0.10</td>
<td>0.26</td>
</tr>
</tbody>
</table>

$^a$df = 3, 18  
$^b$df = 1, 18
5 DIFFERENCES IN CONSTITUTIVE CHEMISTRY

5.1 Introduction

There was no evidence of physical hydraulic mechanisms of resistance in TM and NM. Thus, antibiosis directed against larvae in the spring, as with antixenosis directed against adults in the summer, could be correlated with plant chemistry. Resistant clones could be nutritionally inadequate for weevils, or could contain toxins. To date, resistance in poplars has primarily been attributed to the presence of phenolic compounds, especially phenolic glycosides. Phenolic glycosides are known to protect poplars from forest tent caterpillars and gypsy moths (Meyer and Montgomery 1987, Lindroth and Hemming 1990, Lindroth and Bloomer 1991, Hwang and Lindroth 1997, Osier et al. 2000a, Osier and Lindroth 2001), and confer susceptibility to chrysomelid (leaf) beetles which sequester glycosides for their own defence (Matsuda and Matsuo 1985, Smiley et al. 1985, Soetens et al. 1991, Bingaman and Hart 1993, Martinsen et al. 1998).

Ramachandran (1993) has further shown that the youngest leaves of NM are high in total phenolics and most susceptible to the cottonwood leaf beetle. Because NM is resistant to the forest tent caterpillar, gypsy moth, and meadow mouse (Robison and Raffa 1994, 1998, Kruse and Raffa 1996), but susceptible to the cottonwood leaf beetle (Ramachandran 1993, Robison and Raffa 1998), phenolic glycosides may also play a role in resistance to C. lapathi. However, defence proteins could also be responsible for resistance, given that PPO activity may be suppressed in TN (Section 4.3.2). I tested the hypotheses that NM and TM are of inferior nutritional quality, and contain more secondary compounds than TD or TN, by quantifying levels of carbohydrate, total N,
sterols, linoleic and linolenic acids, phenolic glycosides, condensed tannins, total phenolics, and polyphenol oxidase (PPO) activity.

5.2 Materials and Methods

5.2.1 Clonal material for chemical analyses

Bark was obtained from the monoclonal blocks described in Section 4.2.2. Three or four randomly chosen ramets of each clone were destructively sampled on 6 February and 10 April 2003. Similar samples were collected on 30 July 2003 from additional monoclonal blocks planted in spring 2003. Trees sampled in February, when *C. lapathi* eggs and larvae would be overwintering in the bark, had closed buds. By April, buds on TN and TD had just flushed with approximately 1-2 mm of new growth, whereas new growth had already reached 5-7 cm in TM and 4.5-5 cm in NM. Larvae would be active by this time and possibly subjected to antibiosis expressed in the resistant clones (Figure 3.2). In July, all trees were actively growing and at this time early emergent females would be feeding on and possibly beginning to oviposit into hosts. Stems were cut, packed in dry ice, and transported to the laboratory. The bark was cut from the basal 20 cm above the root collar (including bark aged 1 and 2 years) over a bed of dry ice, ground under liquid N₂, and stored at -80°C until use. A portion of bark was freeze-dried (Labconco model no. 77540) and stored at -20°C.

I additionally examined constitutive levels of PPO activity (*N* = 5, 2005) and phenolic glycosides (*N* = 2, 2004) in bark obtained from trees potted (see Section 3.2.2 for planting methods) in 2004 and 2003, respectively, by removing plugs of 2-year old bark at the junction of a branch with the main stem.
5.2.2 Chemical Analyses

Percent dry weights were determined on ground bark samples as described above. Total nitrogen was determined by elemental analysis using a Carlo Erba model 1110 CHN analyzer (M. Yang, Department of Chemistry, Simon Fraser University, Burnaby, BC). Carbohydrate analyses were performed as described by Schoeneberger et al. (1991).

The method of Batta et al. (2002) was used to simultaneously extract, derivatize and quantify fatty acids, resin acids and sterols. Because these compounds are not usually found free in cells, classical methods involve acid treatments to free glycosides, then alkaline treatments to free esters, followed by clean up, which inevitably separates sterol and fatty acid fractions (e.g. Chen et al. 1994, Toivo et al. 2000). The Batta et al. (2002) method allowed rapid examination of lipid content in tree bark, despite the fact that abundant sugar fragments precluded concentrating compounds of interest further (Figure 5.1A). I added 200 μL of n-butanol spiked with 1 mg/mL each of palmitoleic acid and 5α-cholestan and 20 μL of concentrated HCl to 10-20 mg of freeze-dried bark (Batta et al. 2002). This was vortexed, capped and heated for 4 h at 60°C. Samples were then placed under vacuum for ca. 105 min, left in a dessicator overnight, then reacted with 300 μL of pyridine and 200 μL of N, O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA) with 10% trimethylchlorosilane (TMCS) (Pierce, Rockford, IL) for 30 min at 60°C, and finally diluted 20 μL into 1 mL pyridine for splitless GC injection. Levels of linoleic and linolenic acids were pooled and quantified based on standard curves with linoleic acid. Stigmasterol was used to generate standard curves for sterol quantification.
Figure 5.1 Representative GC-FID’s of silylated bark extract after acid-butanol treatment for quantification of linoleic and linolenic acids and sterols (A) or after methanoic extraction for quantification of phenolic glycosides (B). Carb. indicates peaks that coelute with carbohydrate fragments generated from acid-butanol treatment of synthetic sucrose. DB-5 column; temperature program 150°C for 1 min, increase to 240°C at 6°C/min (A), or 190°C with no hold, increase to 320°C at 10°C/min (B).
For both sterols and phenolic glycoside analysis, the GC was a HP 5890 Series II gas chromatograph with a DB5 GC column (30 m x 0.25 mm ID) attached to a FID. The injector port was held at 270°C and the detector at 320°C. For sterols, the temperature program was 150°C for 1 min, increased to 240°C at 6°C/min, then to 310°C at 10°C/min and held for 10 min. For glycosides, the temperature was 190°C with no hold, increased to 320°C at 10°C/min and held 25 min. Peaks were identified by cochromatography with authentic standards on GC-FID and some samples were secondarily confirmed by GC-MS analysis (Varian Saturn 2000 Ion Trap running in electron impact mode).

For phenolic glycosides, approximately 50 mg of freeze-dried bark was extracted three times into 1 mL 100% MeOH, spiked with 0.2 mg/mL of β-estradiol (internal standard), for 15 min intervals with sonication. Supernatants from pelleted bark (12,000 x g, 10 min, 4°C) were collected and pooled. One mL aliquots were removed, dried for 90 min on a SpeedVac (ThermoSavant DNA 120 SpeedVac®), and silylated overnight in 375 μL of pyridine and 125 μL of BSTFA 10% TMCS. The following day, samples were diluted 40 μL into 1 mL pyridine and immediately 1 μL was injected splitless into the GC. Salicin was used to generate the standard curve used to quantify glycosides and the unknowns; catechin was used to generate the curve to quantify catechin. To test for potential problems during sample handling and storage, I also analysed freshly collected bark from 4 NM and 4 TN 1-year old potted trees. Bark plugs were removed from potted trees, placed on ice, brought to the laboratory, ground under liquid N₂, and processed as above except they were dried in the Speedvac for a further 15-30 min to remove excess water. Because I detected salicortin and salicin, but no tremulacin or tremuloiden, in
bark samples (Figure 5.1B), I also analysed *P. tremuloides* leaves to confirm that the latter two glycosides could be detected using these methods.

Condensed tannins were extracted from dried bark (ca. 50 mg) into 70% acetone + 10 mM ascorbic acid (4 x 1 mL) with sonication over ice. Supernatants were collected following centrifugation. Tannins were then quantified using both an acid-butanol spectrophotometric assay (Porter et al. 1986) and an agarose disk assay (Hagerman 1987). Both assays gave similar results. Hydrolyzable tannins are not known from salicaceous species (Ayres et al. 1997). I used ytterbium-precipitated tannins (Giner-Chavez et al. 1997) from NM to create the standard curve in the spectrophotometric assay, and Sephadex-purified quebracho (Hagerman and Butler 1980) to generate curves for the agarose assay. The acetone extract was also used to determine total phenolics using catechin to generate a standard curve (Price and Butler 1977).

Total phenolics were determined on a 96 well plate using the Price and Butler (1977) method as described by Waterman and Mole (1994), using catechin to generate a standard curve. Samples were the 70% acetone extracts used in condensed tannin analyses.

For PPO assays, I followed the methods of Sherman et al. (1991), as modified by Constabel and Ryan (1998) and Constabel et al. (2000). I used supernatants of ca. 200 mg fresh bark extracted into 1 mL cold extraction buffer (100 mM NaPO₄ adjusted to pH 7 with 0.1% v/v Triton X-100 and 5% w/v polyvinylpyrrolidone), and kept on ice. The reaction buffer (ambient temperature) consisted of 100 mM NaPO₄ adjusted to pH 7, 5 mg/mL 3,4-dihydroxy-L-phenylalanine (DOPA), and 0.15 % sodium didodecyl sulfate (SDS). The buffer was saturated with O₂ by bubbling gas through it for 5 min and SDS
was added after bubbling. Catalase was 2800 units/mL in water. On a 96 well plate, I tested in duplicate 182 µL of reaction buffer, 18 µL of catalase, and 20 µL of sample into a spectrophotometer set at 490 nm and scanned every 10 sec for 5 min. The amount of protein in the extract was quantified using the Bradford (1976) method and bovine serum albumin (BSA) as an internal standard.

Solvents were of at least HPLC grade and water was nanopure. Reagents and standards were purchased from Sigma, except for salicortin, tremuloiden and tremulacin, which I purified from trembling aspen bark and/or leaf material (Clausen et al. 1989a) and confirmed by NMR (Lindroth et al. 1987a).

5.2.3 Statistical analyses

In all cases $\alpha = 0.05$ and residuals were checked for normality and homoskedasticity. Log transformations were performed if necessary.

Too few samples were quantified to analyze the biochemical data with a multivariate model. Because the biochemical survey was investigative, and the different time points are reflective of different phases of *C. lapathi* biology, I compared constitutive levels of biochemicals in clones to each other separately at the three time points by ANOVA using PROC GLM (SAS Institute Inc. 1990). When significant differences were found, the REGWQ test (Day and Quinn 1989) was used for means separation. Lastly, I subjected the biochemical dataset to principal components analysis using PROC PRINCOMP (SAS Institute Inc. 1990).
5.3 Results and Discussion

5.3.1 Lipids

No resin acids were found in my bark samples, consistent with reports for *P. tremuloides* bark and wood (Chen et al. 1994, Pakdel et al. 1994) and *P. deltoides* wood (Pakdel et al. 1994). The only sterol detected was sitosterol, a ubiquitous plant sterol, and there were no significant differences among clones at any time interval (Figure 5.2, Table 5.1). Thus neither sterols nor resin acids would limit *C. lapathi* development in resistant clones.

There were significant differences in the amount of linoleic and linolenic acids among clones in February and April (Figure 5.2, Table 5.1). In general, TN had the lowest levels of both essential fatty acids. In three instances, there was a decreasing trend that corresponded with increasing susceptibility of clones. This result does not support the hypothesis that low levels of essential linoleic and linolenic acids would confer resistance to *C. lapathi*. Rather, both acids are precursors of jasmonic acid and are therefore critical components of wound-induced defence responses (Porta and Rocha-Sosa 2002). Thus, low levels of linoleic and linolenic acids could reflect a reduced ability to mount a rapid and extensive defence response against larvae. Furthermore, linolenic acid induces wound responses in tomato (Narváez-Vásquez et al. 1994, Howe et al. 1996), and if production of linolenic acid is blocked, the wound response is inhibited (Martin et al. 1999).
Figure 5.2  Quantification of nutrients present constitutively in the bark of four hybrid poplar clones in decreasing order of resistance to Cryptorhynchus lapathi at three time periods. If ANOVA $P < 0.05$, bars within a subgraph with the same letter are not significantly different, REGWQ test, $P < 0.05$. fw = fresh weight, dw = dry weight.
Table 5.1 Summary of analysis of variances for constitutive levels of nutrients and secondary metabolites among four hybrid poplar clones varying in their susceptibility to *Cryptorhynchus lapathi*. Units for compounds as indicated on Figs. 5.2-5.3. *P* < 0.05 in bold.

<table>
<thead>
<tr>
<th>Compound</th>
<th>July</th>
<th></th>
<th></th>
<th>February</th>
<th></th>
<th></th>
<th>April</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>df</td>
<td><em>P</em></td>
<td>F</td>
<td>df</td>
<td><em>P</em></td>
<td>F</td>
<td>df</td>
</tr>
<tr>
<td>Sterols</td>
<td>1.75</td>
<td>3, 11</td>
<td>0.21</td>
<td>1.06</td>
<td>3, 9</td>
<td>0.41</td>
<td>2.35</td>
<td>3, 8</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>0.23</td>
<td>3, 11</td>
<td>0.87</td>
<td>10.97</td>
<td>3, 9</td>
<td><strong>0.002</strong></td>
<td>7.34</td>
<td>3, 8</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>4.58</td>
<td>3, 11</td>
<td><strong>0.03</strong></td>
<td>3.58</td>
<td>3, 9</td>
<td>0.06</td>
<td>4.69</td>
<td>3, 8</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>10.37</td>
<td>3, 11</td>
<td><strong>0.002</strong></td>
<td>28.87</td>
<td>3, 9</td>
<td>&lt;0.0001</td>
<td>2.05</td>
<td>3, 8</td>
</tr>
<tr>
<td>µg protein per mg fw</td>
<td>8.81</td>
<td>3, 11</td>
<td><strong>0.003</strong></td>
<td>5.45</td>
<td>3, 9</td>
<td><strong>0.02</strong></td>
<td>13.37</td>
<td>3, 8</td>
</tr>
<tr>
<td>Starch</td>
<td>3.31</td>
<td>3, 12</td>
<td><strong>0.06</strong></td>
<td>12.66</td>
<td>3, 9</td>
<td><strong>0.001</strong></td>
<td>3.50</td>
<td>3, 8</td>
</tr>
<tr>
<td>Hexoses</td>
<td>4.82</td>
<td>3, 12</td>
<td><strong>0.02</strong></td>
<td>4.07</td>
<td>3, 9</td>
<td><strong>0.04</strong></td>
<td>0.70</td>
<td>3, 7</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.85</td>
<td>3, 12</td>
<td>0.19</td>
<td>1.32</td>
<td>3, 9</td>
<td>0.33</td>
<td>1.25</td>
<td>3, 7</td>
</tr>
<tr>
<td>Sum carbohydrate</td>
<td>4.10</td>
<td>3, 12</td>
<td><strong>0.03</strong></td>
<td>3.31</td>
<td>3, 9</td>
<td>0.07</td>
<td>1.50</td>
<td>3, 8</td>
</tr>
<tr>
<td>CT, acid butanol</td>
<td>21.96</td>
<td>3, 12</td>
<td>&lt;0.0001</td>
<td>45.20</td>
<td>3, 9</td>
<td>&lt;0.0001</td>
<td>1.04</td>
<td>3, 8</td>
</tr>
<tr>
<td>CT, agarose disk</td>
<td>15.59</td>
<td>3, 12</td>
<td><strong>0.002</strong></td>
<td>12.95</td>
<td>3, 9</td>
<td><strong>0.001</strong></td>
<td>0.98</td>
<td>3, 8</td>
</tr>
<tr>
<td>Catechin</td>
<td>5.00</td>
<td>3, 11</td>
<td><strong>0.02</strong></td>
<td>9.30</td>
<td>3, 7</td>
<td><strong>0.008</strong></td>
<td>5.27</td>
<td>3, 8</td>
</tr>
<tr>
<td>CT binding: CT ionophores</td>
<td>1.36</td>
<td>3, 12</td>
<td>0.30</td>
<td>1.48</td>
<td>3, 9</td>
<td>0.29</td>
<td>6.41</td>
<td>3, 8</td>
</tr>
<tr>
<td>Total phenolics</td>
<td>1.82</td>
<td>3, 12</td>
<td>0.20</td>
<td>0.41</td>
<td>3, 9</td>
<td>0.75</td>
<td>0.96</td>
<td>3, 8</td>
</tr>
<tr>
<td>Salicin</td>
<td>7.60</td>
<td>3, 11</td>
<td><strong>0.005</strong></td>
<td>25.88</td>
<td>3, 7</td>
<td><strong>0.0004</strong></td>
<td>1.35</td>
<td>3, 8</td>
</tr>
<tr>
<td>Salicortin</td>
<td>2.86</td>
<td>3, 11</td>
<td><strong>0.09</strong></td>
<td>0.85</td>
<td>3, 7</td>
<td>0.51</td>
<td>0.83</td>
<td>3, 8</td>
</tr>
<tr>
<td>Salicin + Salicortin</td>
<td>2.81</td>
<td>3, 11</td>
<td>0.09</td>
<td>3.64</td>
<td>3, 7</td>
<td>0.07</td>
<td>0.92</td>
<td>3, 8</td>
</tr>
<tr>
<td>Unknown 1</td>
<td>14.50</td>
<td>3, 11</td>
<td><strong>0.004</strong></td>
<td>8.43</td>
<td>3, 7</td>
<td><strong>0.01</strong></td>
<td>15.13</td>
<td>3, 8</td>
</tr>
<tr>
<td>Unknown 2</td>
<td>7.13</td>
<td>3, 11</td>
<td><strong>0.006</strong></td>
<td>2.95</td>
<td>3, 7</td>
<td>0.11</td>
<td>1.91</td>
<td>3, 8</td>
</tr>
</tbody>
</table>


5.3.2 *N and protein*

In July, TD had more N than any other clone and, in February, both TM and TD had the highest levels (Figure 5.2). TN generally had low N levels. These values were somewhat similar to the amounts of protein present in buffered extractions; TN tended to have less and TD more protein than either NM or TM (Figure 5.2). Because not all plant N is in the form of protein, nor is extraction of protein into aqueous buffer 100% efficient, the differences found by the two techniques are not surprising.

I had predicted that clones high in N and protein would be more nutritious and therefore more susceptible than clones with low N and protein content. However, TN is clearly suitable for weevil development despite its low N and protein levels (Figure 3.2). It is possible that the high N and protein levels observed in TD may be partially responsible for susceptibility (Lindroth and Bloomer 1991). The smaller adults emerging from TN than from TD or TM (Table 3.1), may be linked to nutrient levels.

N and protein levels may correlate with vigor, as TN is the slowest growing of the four clones during early development (Table 3.2). If vigor is associated with resistance, TN’s susceptibility may be reversed over time. In a growth trial including 53 hybrids, TN’s rank moved from 20\textsuperscript{th} to 4\textsuperscript{th} over an 8 year period, surpassing TD, TM and NM (G. Abebe, Nursery Manager, Scott Paper Ltd., Harrison Mills, BC, pers. comm.). Change in susceptibility during development of this *P. trichocarpa x P. nigra* clone could explain the lack of consistency between my results and those of Dafauce (1976) and Johnson and Johnson (2003), who found that clones with *P. nigra* parentage often demonstrate some resistance to *C. lapathi*. 
5.3.3 Carbohydrates

There were few significant differences in the levels of carbohydrate between clones (Figure 5.2, Table 5.1). Sugars, not starch, were predominant in the bark. During the dormant period in February starch levels were especially low, consistent with the fact that sugars aid in cold hardiness (Gusta et al. 1996). Bark from April, when all clones were flushing, tended to have less overall carbohydrate (Figure 5.2). When females were selecting hosts in July, TD had the highest and TN the lowest level of total carbohydrate, reflecting the nonsignificant trend observed in February (Figure 5.2). Larvae of the western spruce budworm, Choristoneura occidentalis Freeman, develop best on artificial diets containing levels of sucrose reflecting the low end of the naturally occurring range in its host (Clancy 1992b). Furthermore, the ratio of specific nutrients underlies resistance and susceptibility to the western spruce budworm, as opposed to levels of individual compounds per se (Clancy 1992a, 1992b, 1993). Thus, although differences in the ratios of nutrients may occur between the two susceptible clones TD and TN, both ratios may promote susceptibility.

Because the susceptible TN tended toward low levels of many nutritive components, adequate nutrition for C. lapathi can likely be found in all Populus spp. as all poplars must store carbohydrate, lipids and proteins in twigs, bole and roots to support rapid spring growth of buds, vascular cambium and fine roots, respectively (Dickmann et al. 2001). Despite TD having high levels of N, protein, and carbohydrates at the time of adult feeding and oviposition (Figure 5.2), there is no evidence for concurrent feeding preferences for this clone (Figure 2.2, Table 2.2). Furthermore, the levels of these materials are often similar between TN and NM, despite the fact that weevils of both
sexes clearly prefer to feed on TN (Figures 2.3, 2.4). This suggests that secondary metabolites may play a role in antixenosis.

However, primary metabolites may still be important in antibiosis expressed towards larvae between February and April. At this time, TM and NM are most similar to each other and most different from TN in that they contain superior levels of fatty acids, N and protein (Figure 5.2). TD generally is intermediate between these two groups.

5.3.4 Condensed tannins

Condensed tannins negatively affect herbivore development in a highly specific, but generally weak, manner (Bryant et al. 1987, Ayres et al. 1997, Hwang et al. 1997). The highly susceptible TN had the highest levels of tannin and catechin, usually followed by NM, the most resistant clone (Figure 5.3). TM, of intermediate resistance, and the moderately susceptible TD typically had the lowest levels of condensed tannin and catechin (Figure 5.3). Thus, tannin and catechin levels do not appear to explain the observed antibiosis directed towards larvae by NM in particular (Figure 3.2). The correlation between tannin and catechin levels is likely because catechin units can comprise the tannin polymer (Hagerman 2002).

The antibiotic effects of condensed tannins are caused by variation in structure, number and configuration of subunits, factors which vary within the genera Salix and Populus (Clausen et al. 1990, Ayres et al. 1997). Thus tannins could differ quantitatively among the four clones, but still underlie resistance in two of them. However, standard curves generated with ytterbium-precipitated TN and NM tannins showed that the same
Figure 5.3 Quantification of secondary metabolites present constitutively in the bark of four hybrid poplar clones in decreasing order of resistance to *Cryptorrhynchus lupathi* at three time periods. Note differences in the scale on the y-axis for the two measures of condensed tannins, due to use of different CT's for standard curve generation. If ANOVA *P* < 0.05, bars within a subgraph with the same letter are not significantly different, REGWQ test, *P* < 0.05. CT = condensed tannins, fw = fresh weight, dw = dry weight.
weight of tannin from either source produced the same intensity of coloured ionophores (data not shown). Furthermore, there was high correlation between the ability of tannins to bind protein and form the coloured subunit ($r^2 = 0.79, P < 0.0001, n = 41$), and the ratio of binding ability to ionophore production was not generally different among clones (Figure 5.3). These findings suggest that the tannins are similar in nature (Clausen et al. 1989b), with the sole exception of the ratio comparison in April (Figure 5.3). In this instance, the most resistant clone has the least ability to bind protein, again not supporting any role of condensed tannins in resistance.

5.3.5 Phenolic glycosides

In general, GC-FID traces of methanol extracts were very similar among clones and time periods (Figure 5.1B). Tremuloiden and tremulacin were detected in aspen leaves, but not hybrid poplar bark. Two unknown compounds were detected, one of which was ubiquitous in February, but nearly absent at the other times (Figure 5.3).

Salicin levels varied significantly among clones in July and February, and were generally lowest in the susceptible TN (Figure 5.3); however levels in TN were never significantly different from those in NM. Furthermore, salicin is not highly toxic, even to nonadapted defoliators (Lindroth et al. 1988b, Lindroth and Peterson 1988), and is not a likely basis for resistance.

Although salicortin was the dominant glycoside in all methanol extracts, levels were never significantly different among field-planted clones. I examined the molar sum of salicin and salicortin to preclude any effect of salicortin degradation and again found no differences among clones at any time (Figure 5.3, Table 5.1). There is abundant
evidence linking high phenolic glycoside levels in salicaceous trees with resistance to most insect and mammalian herbivores and susceptibility to chrysomelids (Rowell-Rahier 1984, Tahvanainen et al. 1985, Matsuda and Matsuo 1985, Smiley et al. 1985, Bryant et al. 1987, Mattes et al. 1987, Meyer and Montgomery 1987, Lindroth et al. 1988a; Lindroth and Peterson, 1988; Lindroth and Hemming 1990, Reichardt et al. 1990, Lindroth and Bloomer 1991, Soetens et al. 1991, Bingaman and Hart 1993, Martinsen et al. 1998, Rank et al. 1998, Osier et al. 2000a, Pass and Foley 2000, Osier and Lindroth 2001). As indicated previously, generalist insect and mammalian herbivores perform poorly on NM 6, unlike the cottonwood leaf beetle (Robison and Raffa 1994, 1998, Kruse and Raffa 1996) suggesting that NM 6 too could be high in phenolic glycosides. Indeed, Ramachandran (1993) found higher constitutive levels of total phenolics in leaves of young NM 6 than in NC 5271 (an intraspecific *P. nigra* hybrid), and performances of *C. scripta* were superior on NM 6 and those of forest tent caterpillars were superior on NC 5271.

However, my results do not support the hypothesis that high phenolic glycoside levels in the bark of NM 6 would be responsible for antibiosis directed against *C. lapathi* larvae. Salicortin was an especially promising candidate because it was the more abundant glycoside (Figure 5.1B, 5.3) and, because it contains a cyclohexenone moiety, it is more deterrent and toxic to adapted herbivores like gypsy moth (Lindroth and Hemming 1990, Osier et al. 2000a, Osier and Lindroth 2001) and forest tent caterpillar (Lindroth and Bloomer 1991), as well as non-adapted herbivores like *Papilio glaucus* L. ssp. *glaucus* (Lindroth et al. 1988a) or the southern armyworm, *Spodoptera eridania* (Cramer) (Lindroth and Peterson 1988). The cyclohexenone moiety is apparently rapidly
converted to the toxic products 6-hydroxy-2-cyclohexenone and catechol (Mattes et al. 1987, Ruuhola et al. 2003) in the insect gut after ingestion (Lindroth et al. 1988a). Catechol is subsequently converted to o-quinone by PPO enzymes (Haruta et al. 2001).

There are possible explanations for the apparent NM 6 anomaly. First, *C. lapathi* attacks the bark and wood, not the foliage, and different defence strategies may exist in the different tissues. For instance, if *C. lapathi* uses microorganisms for nutrition, antimicrobial metabolites could kill larvae indirectly and phenolic glycoside levels need not be high. However, phenolics present in the bark have been implicated in resistance to mammalian herbivores (Tahvanainen et al. 1985, Reichardt et al. 1990, Pass and Foley 2000); thus observations of resistance against meadow mouse suggest that glycosides should be high in NM 6 bark (Robison and Raffa 1998). Alternately, it remains possible that induced rather than constitutive glycoside levels may impart resistance to *C. lapathi*. There is genotypic variation in the ability of individual *Populus* spp. or hybrids to produce glycosides following wounding (Clausen et al. 1989b, Robison and Raffa 1997, Havill and Raffa 1999, Osier and Lindroth 2001, 2004, Fang et al. 2002). Lastly, it is possible that resistance is best explained by the presence of defence proteins such as polyphenol oxidase (PPO) or proteinase inhibitors (PI’s), both of which are also known to be inducible in leaves after wounding in various poplars (Constabel et al. 2000, Haruta et al. 2001a, 2001b, Christopher et al. 2004, Wang and Constabel 2004). Resistance could also require an induced production of both substrate (phenolics) and enzyme (PPO) after wounding.
5.3.6  *PPO activity*

I assayed constitutive PPO activity of hybrids after noticing more rapid and intense browning (indicative of PPO activity) in NM, TM, and TD bark over that of TN (Section 4.3.2). Because I was concerned with bark quality after long-term storage, I assessed PPO activity the same day as bark was harvested in May 2005. In general, TN had lower levels of PPO activity than the other hybrid clones, although not always significantly different from the other three hybrids (Figure 5.4, Table 5.2), again not giving any clear indication of PPO activity is involved in resistance.

5.3.7  *Principal components analysis*

The temporal differences in plant chemistry were reflected in the principal components analysis (Figure 5.5). In July, the chemical profile of TN and TD, the two susceptible hybrids, are furthest from each other and the two resistant hybrids are intermediate. This could explain why adult weevils failed to feed and oviposit consistently among clones (Figures 2.2-2.4, 3.1, Tables 2.2, 3.2, Sections 3.3.1, 3.3.2). A similar trend is seen in April, when the chemical profile of TN most closely matches that of NM. Because by April buds have burst, and resistance is most likely being expressed against larvae, there is little support to a single hypothesis of constitutive chemical resistance, i.e., multiple mechanisms must exist, and their relationship to plant chemistry is not obvious. Only in February, when trees and larvae are still dormant, are clonal differences especially well resolved, and TN is nearly equidistant to both NM and TD. Because I have observed first instar larvae actively feeding in the bark of NM later in the
Figure 5.4  Quantification of constitutive levels of polyphenol oxidase (PPO) activity in the bark of four hybrid poplar clones in decreasing order of resistance to Cryptorhynchus lapathi at three time periods. If ANOVA $P < 0.05$, bars within a subgraph with the same letter are not significantly different, REGWQ test, $P < 0.05$. 
Table 5.2  Summary of analysis of variances for Figure 5.4 regarding the constitutive levels of polyphenol oxidase (PPO) activity among four hybrid poplar clones varying in their susceptibility to *Cryptorrhynchus lapathi*. $P < 0.05$ in bold.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Source of variation</th>
<th>July</th>
<th>February</th>
<th>April</th>
<th>May</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$F_{3,11}$ $P$</td>
<td>$F_{3,8}$ $P$</td>
<td>$F_{3,7}$ $P$</td>
<td>$F_{3,16} P$</td>
</tr>
<tr>
<td>PPO activity per µg protein</td>
<td>4.92 $0.02$</td>
<td>5.91 $0.02$</td>
<td>6.65 $0.02$</td>
<td>3.10 0.06</td>
<td></td>
</tr>
<tr>
<td>PPO activity per mg bark</td>
<td>12.10 $0.0008$</td>
<td>13.94 $0.002$</td>
<td>5.97 $0.02$</td>
<td>3.63 $0.04$</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.5  Principal components analysis of four hybrid poplar clones at three time periods according to constitutive levels of the following present in their bark: sitosterol, linoleic and linolenic acids, nitrogen, starch, hexose, sucrose, condensed tannins (from both acid butanol and agarose disk assays), catechin, total phenolics, salicin, salicortin, unknowns 1 and 2, PPO activity per μg protein, and percent wet weight.
spring, and equal levels of egg hatch among hybrids (Section 3.3.2), it is not logical that differences in plant chemistry in February could explain resistance.
6 DIFFERENCES IN INDUCED CHEMISTRY

6.1 Introduction

It is possible that resistance in NM and TM to early instar *C. lapathi* larvae is an induced phenomenon that is either absent or delayed in the later flushing TD and TN hybrids (Figure 4.1), similar to resistance in the white pine weevil (Hulme 1995, Alfaro 2000). Induced differences could also be responsible for feeding and oviposition deterrence. In addition, TN usually had lower levels of linoleic and linolenic acids which are required for induced wound responses (Figure 5.2, Narváez-Vásquez et al. 1994, Howe et al. 1996, Martín et al. 1999, Porta and Rocha-Sosa 2002). Although induced responses in salicaceous trees are genotype-specific (Clausen et al. 1989b, Robison and Raffa 1997, Havill and Raffa 1999, Osier and Lindroth 2001, 2004, Peacock et al. 2001, Fang et al. 2002, Peters and Constabel 2002), they can successfully inhibit the preferences and/or performances of glycoside-sensitive folivores (Robison and Raffa 1997, Havill and Raffa 1999, Peacock et al. 2001, Osier and Lindroth 2001, 2004). Induced defensive proteins like chitinases, Kunitz proteinase inhibitors (PI), and polyphenol oxidases (PPO) could also be responsible for resistance to herbivores; however, each genotype has its own suite of inducible products (Peters and Constabel 2002). Thus, I tested the hypothesis that resistance to *C. lapathi* by NM could be explained by higher induced levels of condensed tannins, catechin, total phenolics, phenolic glycosides, and PPO activity than TN.
6.2 Materials and Methods

6.2.1 Clonal material for chemical analyses

I examined bark from two series of potted TN and NM plants. First, 1-year-old TN and NM 45 cm long cuttings were planted 25 cm deep in 3 L pots fitted with milk cartons [for added depth] in spring 2003 as described in Section 3.2.2. These were grown until 24 July when they were removed to a greenhouse facility at the University of British Columbia, Vancouver, BC, where they were maintained with daily flood fertirrigation. On 26 August 2003, 10 weevil pairs, previously starved for 48 h, were caged onto the basal 1 m of the current year's growth (previously clipped to have only one main stem). Empty mesh cages were used on controls (N = 4). After 48 h, all bark from the caged portion of the stem was removed and immediately frozen in liquid N₂.

The second source of trees examined for induced responses comprised 22 cm long 1-year old cuttings potted 12 cm deep in 3 L pots trees in the spring of 2004, and maintained as described (Section 3.2.2). The wounding treatment consisted of caging a single pair of weevils onto the basal 60 cm of the trees throughout the fall of 2004. Control trees were bagged without weevils (N = 5). Oviposition was infrequent during this year, so treated trees were additionally mechanically wounded by puncturing the bark 100 times on each of 3 and 4 March 2005 using an 18 gauge needle (200 punctures per tree total). At this time, new growth had emerged only 1-2 mm past the bud from TN clones, but was ca. 2 cm long on NM clones. Bark was harvested 48 h after the second piercing, separated into 2- and 1-year old tissue, and immediately flash frozen in liquid N₂. All bark samples were stored at −80°C until analysis in 2005.
6.2.2 Chemical analyses

Percent dry weights, total phenolics, condensed tannins, phenolic glycosides, and PPO activity were quantified as described in Section 5.2.2 using both constitutive and locally wounded bark samples.

6.2.3 Statistical analyses

In all cases $\alpha = 0.05$ and residuals were checked for normality and homoskedasticity. Log transformations were performed if necessary. Too few samples were quantified to analyze the data with a multivariate model. I used a $2 \times 2$ factorial model to test for the main effects of clone, wounding, and their potential interaction using PROC GLM (SAS Institute Inc. 1990). To remain consistent and comparable with the earlier constitutive analyses, and because weevils would never be exposed to both genotypes or treatments at any one time, I again used REGWQ for multiple comparisons and did not pool data even if the factorial analysis indicated that it would be appropriate. This data set was subjected to principal components analysis using PROC PRINCOMP (SAS Institute Inc. 1990).

6.3 Results and Discussion

6.3.1 Condensed tannins

The levels of condensed tannins and catechin were typically higher in potted TN than NM (Figure 6.1), similar to earlier results on field-planted individuals (Figure 5.3); however, the magnitude of difference was less profound. In two instances, minor increases in tannin or catechin levels were detected in response to wounding (Figure 6.1, Table 6.1). Bark from
Figure 6.1  Locally induced and constitutive levels of secondary metabolites and PPO activity in two hybrid poplar clones that are resistant (NM) and susceptible (TN) to *Cryptorhynchus lapathi*. Effects from factorial model indicated as clone, treatment (Trt), and clone x trt if significant, NS if non-significant. If $P < 0.05$ for any effect, bars within a subgraph with the same letter are not significantly different, REGWQ test, $P < 0.05$. 
Table 6.1 Summary of analysis of variances for secondary metabolites and defense protein levels in control and wounded NM and TN, two hybrid poplar clones varying in their susceptibility to Cryptorhynchus lapathi. Units for compounds as indicated on Figure 6.1. \( P < 0.05 \) in bold.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Compound</th>
<th>Source of variation</th>
<th>Clone</th>
<th>Treatment</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>( F^a )</td>
<td>( P )</td>
<td>( F^a )</td>
</tr>
<tr>
<td>August 2003</td>
<td>Total phenolics (mg/g dw)</td>
<td>Clone</td>
<td>2.37</td>
<td>0.15</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>Salicin (mg/g dw)</td>
<td>Treatment</td>
<td>2.14</td>
<td>0.17</td>
<td>8.75</td>
</tr>
<tr>
<td></td>
<td>Salicortin (mg/g dw)</td>
<td>Treatment</td>
<td>0.39</td>
<td>0.54</td>
<td>2.47</td>
</tr>
<tr>
<td></td>
<td>Salicin and salicortin (mmol/g dw)</td>
<td>Treatment</td>
<td>0.68</td>
<td>0.43</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>Unknown 1 (mg/g dw)</td>
<td>Treatment</td>
<td>19.17</td>
<td>0.0009</td>
<td>4.21</td>
</tr>
<tr>
<td></td>
<td>Condensed tannins (% dw)</td>
<td>Treatment</td>
<td>4.14</td>
<td>0.06</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>Catechin (mg/g dw)</td>
<td>Treatment</td>
<td>0.04</td>
<td>0.84</td>
<td>0.00</td>
</tr>
<tr>
<td>March 2005, lower bark</td>
<td>Total phenolics</td>
<td>Clone</td>
<td>3.38</td>
<td>0.08</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>Salicin</td>
<td>Treatment</td>
<td>51.67</td>
<td>&lt;0.0001</td>
<td>19.84</td>
</tr>
<tr>
<td></td>
<td>Salicortin</td>
<td>Treatment</td>
<td>20.83</td>
<td>0.003</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>Salicin and salicortin</td>
<td>Treatment</td>
<td>39.49</td>
<td>&lt;0.0001</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>Unknown 1</td>
<td>Treatment</td>
<td>119.04</td>
<td>&lt;0.0001</td>
<td>2.02</td>
</tr>
<tr>
<td></td>
<td>Condensed tannins</td>
<td>Treatment</td>
<td>7.41</td>
<td>0.02</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>Catechin</td>
<td>Treatment</td>
<td>6.53</td>
<td>0.02</td>
<td>4.91</td>
</tr>
<tr>
<td></td>
<td>PPO activity per µg protein</td>
<td>Treatment</td>
<td>15.80&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.001</td>
<td>0.00&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>PPO activity per mg bark</td>
<td>Treatment</td>
<td>21.59&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0003</td>
<td>0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>March 2005, upper bark</td>
<td>Total phenolics</td>
<td>Salicin</td>
<td>Salicortin</td>
<td>Salicin and salicortin</td>
<td>Unknown 1</td>
</tr>
<tr>
<td>-----------------------</td>
<td>----------------</td>
<td>---------</td>
<td>------------</td>
<td>------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td></td>
<td>4.89</td>
<td>0.04</td>
<td>0.24</td>
<td>0.63</td>
<td>0.00</td>
</tr>
<tr>
<td>Salicin</td>
<td>3.55</td>
<td>0.08</td>
<td>0.09</td>
<td>0.77</td>
<td>0.04</td>
</tr>
<tr>
<td>Salicortin</td>
<td>72.43</td>
<td>&lt;0.0001</td>
<td>5.30</td>
<td>0.04</td>
<td>0.03</td>
</tr>
<tr>
<td>Salicin and salicortin</td>
<td>74.81</td>
<td>&lt;0.0001</td>
<td>2.64</td>
<td>0.12</td>
<td>0.08</td>
</tr>
<tr>
<td>Unknown 1</td>
<td>75.90</td>
<td>&lt;0.0001</td>
<td>0.07</td>
<td>0.79</td>
<td>0.41</td>
</tr>
<tr>
<td>Condensed tannins</td>
<td>16.57</td>
<td>0.0009</td>
<td>8.93</td>
<td>0.009</td>
<td>1.78</td>
</tr>
<tr>
<td>Catechin</td>
<td>5.20</td>
<td>0.04</td>
<td>1.84</td>
<td>0.19</td>
<td>1.38</td>
</tr>
<tr>
<td>PPO activity per µg protein</td>
<td>1.80</td>
<td>0.20</td>
<td>0.07</td>
<td>0.80</td>
<td>3.63</td>
</tr>
<tr>
<td>PPO activity per mg bark</td>
<td>6.16</td>
<td>0.02</td>
<td>0.13</td>
<td>0.72</td>
<td>5.38</td>
</tr>
</tbody>
</table>

Superscript a: df for August 2003 are 1, 12 and for March 2005 are 1,16

Superscript b: because of depleted sample, df for this analysis are 1, 15
fertirrigated trees (i.e., collected in August) generally appeared to contain relatively lower tannin and catechin levels. These findings are consistent with previous work which has documented that tannin levels differ between genotypes, and increase in response to wounding and low-nutrient environments (Osier and Lindroth 2001).

### 6.3.2 Phenolic glycosides

Constitutive levels of salicortin, salicin, and the molar sum of salicin and salicortin were higher in NM than TN in bark collected from potted trees in March (Figure 6.1), in contrast to the other four collection periods (Figure 5.3). There were three differences between bark collected in March and the other four periods: growing conditions, length of time bark was stored, and age. Bark collected in March was from potted trees growing outside under ambient conditions, bark from August was from potted trees growing in a greenhouse with daily fertigation, and bark from July, February and April was from planted stock at Scott Paper Nursery. Bark from March was analysed within a month of collection, whereas other samples were stored for at least 10 months. Because phenolic glycoside levels within trembling aspen are under strong genotypic control, and do not change much in response to fertilizer treatments (Osier and Lindroth 2001, 2004), I would have expected glycoside levels to be consistently different between NM and TN at all five collection periods. Degradation during storage at −80°C also does not explain higher glycoside levels in NM than TN. First, decomposition rates should be consistent within samples collected on a given date. Second, the molar sum of salicortin and salicin and total phenolic levels were not significantly different between clones. With respect to age, phenolic glycoside levels are known to change over time in trembling aspen leaves in a genotype-specific manner (Lindroth et al. 1987b, Osier et al. 2000b); thus it is possible that certain hybrid clones could have higher levels at
some times of the year and not at others. However, restricting significantly higher glycoside levels in NM over TN to March, but not February or April seems highly suspect. In sum, the presence of high glycosides in NM in March does not seem a good explanation for resistance.

A similar conclusion can be drawn when examining actual salicortin levels. In March, salicortin levels were ca. 41 mg/g dw in NM and ca. 26 mg/g dw in TN (or 2.0 and 1.3% fw, respectively), and the mean range observed for all clones sampled in February and April was 16-32 mg/g dw (or 0.7-1.4% fw). These values are similar to the naturally occurring range of trembling aspen leaf salicortin levels (4 – 40 mg/g dw, Lindroth and Hwang 1996); however, clonal resistance among aspen to herbivores is correlated to the combined levels of tremulacin and salicortin (Hwang et al. 1997, Osier et al. 2000a), making a comparison of salicortin levels and resistance to _C. lapathi_ difficult. Tremulacin is at least as toxic as salicortin because its structure is the same with an additional benzoyl moiety (Lindroth et al. 1988a, Lindroth and Peterson 1988, Lindroth and Hemming 1990).

Salicortin levels as low as 1.5% fw have deleterious affects on non-adapted herbivores like _Papilio glaucus_ L. _glaucus_ (Lindroth et al. 1988a) and _Spodoptera eridania_ (Cramer) (Lindroth and Peterson 1988), but levels of 3.5% fw (and in low protein environments) are required for even minor deleterious response in forest tent caterpillars (Lindroth and Hemming 1991). Because the highest salicortin level observed was 2% fw, and _C. lapathi_ is well adapted to salicaceous plants (Harris et al. 1967), it seems unlikely that salicortin levels are responsible for lack of emergence from NM (Figure 3.2). This conclusion is in contrast to Ramachandran’s (1993) observation of high total phenolic levels in NM leaves and susceptibility to _C. scripta_, suggesting NM 6 is indeed high in phenolic glycosides, but is consistent with Robison and Raffa (1994) who describe retarded developmental rates and
constant, moderate mortality rates among forest tent caterpillars when reared on NM 6 leaves. This latter pattern suggests that foliage is only mildly toxic or deterrent, or of low digestibility or nutritive value to larvae. Given my biochemical and behavioural findings (Figures 2.2-2.4, 3.1, 5.2-5.5, Tables 2.2, 3.1, Section 3.3.1), and Robison and Raffa’s (1994) assessment of N levels and insect feeding behaviour, the former hypothesis of toxicity or deterrenency in NM is most likely.

In general, I observed few locally induced increases in phenolic glycosides (Figure 6.1, Table 6.1). Specifically, wounding did not lead to increased salicortin levels, similar to some reports (Osier and Lindroth 2001, 2004), but not others (Clausen et al. 1989b, Robison and Raffa 1997, Havill et al. 1999, Fang et al. 2002). However, in two instances there were detectable increases in salicin (Figure 6.1), possibly due to local salicortin decomposition in wounded (disrupted) tissues.

6.3.3 PPO activity

NM and especially TN demonstrated induced PPO activity due to local weevil feeding in August (Figure 6.1). However, mechanical wounding in March did not lead to increased PPO activity. In fact, PPO activity levels dropped near to zero during this time period. Because bark from both experiments was assayed for PPO activity simultaneously and previous results from bark collected in February and April had obvious PPO activity, the lack of activity in March is even more unusual.

6.3.4 Principal components analysis

The chemical profiles of control bark of NM and TN harvested in August are different from each other (Figure 6.2); however, bark collected from TN individuals
Figure 6.2  Principal components analysis of locally induced and constitutive levels of secondary metabolites and PPO activity in two hybrid poplar clones that are resistant (NM) and susceptible (TN) to Cryptorhynchus lapathi. Bark collected in August was wounded by adult weevils, and bark collected in March was mechanically wounded. Condensed tannin, catechin, PPO activity per μg protein, percent wet weight, total phenolics, salicin, salicortin, and unknown I were included in the analysis.
previously exposed to weevil feeding tends to cluster more closely with both induced and control bark from NM individuals. This supports the earlier observation that TN shows more of an induced response than NM. In March, the chemical profiles from the lower bark of each hybrid are highly similar to each other, and wounding did not dramatically alter the overall chemical profile. Since most oviposition occurs in this part of the tree (Section 3.3.2), it is unlikely resistance is induced. Wounding in the upper bark did elicit a change in the chemical profile of NM; however, the profile in induced bark became more similar to TN which is not in the direction one would expect if NM were mounting an effective induced defence.

In sum, under the wounding conditions tested in NM and TN, especially considering lack of a strong response to adult feeding in July, there is no evidence to suggest that resistance in NM is an induced phenomenon. However, given that bark collected in March was from trees which essentially only had been mechanically wounded, one cannot exclude the possibility that larval damage could elicit an effective resistance response. It remains possible that other defence proteins are induced, or that other secondary compounds are present in NM and TM which would be responsible for resistance.
The following pertinent conclusions can been drawn from my research.

1. *Cryptorhynchus lapathi* does not appear to rely on olfactory cues to discriminate among hybrid poplar clones of varying resistance. This is possibly because hosts tend to be locally abundant in distribution, and can sustain multiple generations of weevils, making long-distance dispersal and long-range orientation toward new susceptible hosts unnecessary.

2. Weevils can discriminate between hybrid poplar clones and other salicaceous species based on feeding preferences, but preferences are not consistently held, and therefore are not reliable for resistance screening programs.

3. The relationship between hybrid susceptibility and oviposition preference was weak, and likely expressed when females are experiencing sub-optimal conditions.

4. Larval survival to adulthood was low in two hybrids with *P. maximowiczii* parentage, and high in *P. trichocarpa* x *P. nigra* and *P. trichocarpa* x *P. deltoides* hybrids. These results indicate that hybrid poplar breeding programs need to screen trees for susceptibility at all stages of plant colonization, and not just feeding or oviposition preferences. Breeders may also choose to utilize *P. maximowiczii* as a source of resistance.

5. NM and TM were highly resistant and TD and TN were susceptible to *C. lapathi* attack. As long as site conditions are considered, both clones with *P.*
maximowiczii parentage would be suitable for planting where C. lapathi is prevalent. The other two would not be suitable.

6. Larval mortality was the strongest and most consistent factor explaining resistance. It apparently occurs during early spring (between February and April) as there were equal levels of egg hatch in the winter following oviposition, and there was no evidence of larvae reaching the xylem-feeding stage in NM.

7. The two resistant hybrids, NM and TM, flushed significantly earlier than the susceptible TD and TN. While early flushing is thus correlated with resistance, no specific resistance mechanism could be found that was associated with early flushing.

8. Bark moisture content was 7-10% higher in susceptible clones. There was no evidence of mortality caused by excessive or early sap flow in resistant hybrids. Thus, physical hydraulic mechanisms cannot explain resistance. Rather, it is likely that C. lapathi females prefer to oviposit at nodes rather than internodes to avoid high levels of sap flow.

9. Nutritive compounds (sitosterol, linoleic and linolenic acids, N, and carbohydrates) tended to be lowest in the most susceptible hybrid; therefore all hybrids appear nutritionally sound for C. lapathi development.

10. Of the secondary metabolites, condensed tannins and catechin were highest in TN, the most susceptible clone. They were slightly inducible after feeding by
adults or mechanical wounding. Resistance is not correlated with tannin levels.

11. Salicortin, the most abundant and toxic phenolic glycoside found in the bark of these four hybrids, was significantly higher in NM over TN in March, 2005. However, the difference was not likely great enough to explain resistance, and there were no significant differences at four other time periods: February, April, July and August, 2003. There was also no evidence that salicortin levels increased after wounding. Therefore, phenolic glycosides do not appear to be responsible for resistance.

12. Polyphenol oxidase (PPO) activity was also not clearly correlated with resistance. PPO activity tended to be low in TN, but sometimes also NM, and was frequently high in TD hybrids. Also, PPO activity was induced to a greater degree in response to adult feeding in TN than NM.

13. In principal components analyses of plant biochemistry, TN’s nearest neighbour was often NM, indicating that multiple, independent mechanisms must be responsible for resistance and susceptibility.

14. With regard to future research, the mechanism of resistance shared by *P. maximowiczii* hybrids could involve compounds present in non-polar or low or high molecular weight polar bark fractions, bark toughness, or defence proteins other than PPO. Further information will probably be learned from an ongoing investigation into changes in mRNA expression at the University of British Columbia using microarray analysis. It is also not known at what stage of attack other hybrid poplars demonstrate resistance to *C. lapathi*. If
the resistance that I have shown to be imparted by *P. maximowiczii* parentage could be integrated with resistance mechanisms imparted by other species, the development of hybrid genotypes highly resistant to *C. lapathi* and other herbivores could be facilitated.
Abebe, G., and J.H. Hart. 1990. The relationship of site factors to the incidence of
_Cytospora_ and _Septoria_ cankers and poplar and willow borer in hybrid poplar

2000. Budburst phenology of Sitka spruce and its relationship to white pine weevil

selective and sensitive tool in the gas chromatographic analysis of insect pheromones.

Arnold, T.M., and J.C. Schultz. 2002. Induced sink strength as a prerequisite for induced
tannin biosynthesis in developing leaves of _Populus_. Oecologia 130:585-593.

Diversity of structure and antiherbivore activity in condensed tannins. Ecology
78:1696-1712.

Simultaneous quantitation of fatty acids, sterols and bile acids in human stool by

Berenbaum, M.R. 1995. Turnabout is fair play: secondary roles for primary compounds. J.


(Eds.), Nutritional ecology of insects, mites, spiders, and related invertebrates. Wiley, Toronto.


Hulme, M. A. 1995. Resistance by translocated Sitka spruce to damage by Pissodes strobi (Coleoptera: Curculionidae) related to tree phenology. J. Econ. Entomol. 88:1525-1530.


Morris, R. C. 1981. The poplar-and-willow borer of hybrid poplars in Ontario


Morris, R. C. 1982. Preliminary tests for control of the poplar and willow borer,

*Cryptorrhynchus lapathi* L. in Ontario (Curculionidae: Coleoptera), pp. 63-68. In J. Zavitkovski and E.A. Hansen (Eds.), Proc. North American Poplar Coun. Meeting, Kansas State University, Manhattan, KS.


APPENDIX 1

The original goal of my Ph.D. research was to conduct a comparative study of the chemical ecology of *C. lapathi* and *S. calcarata*. In this study, I first worked with Ms. Regine Gries to pursue GC-EAD analysis of host and beetle-produced volatiles for both species. Numerous compounds were found to be antennally-active (Table A-1). However, behavioural responses were obtained only for *C. lapathi* in a laboratory olfactometer to a 19:1 ratio of conophthorin:frontalin (males only), and to a 1:1:1:1:2:6 ratio of hexanal:nonanal:benzaldehyde:(Z)-hex-3-en-1-ol:conophthorin:hexanol (females only), but it was impossible to obtain similar results consistently over time. In three years of intensive field trapping experiments for *C. lupathi* and two for *S. calcarata*, no beetles were captured to any beetle-produced or host compounds, or combinations thereof. Therefore, this thesis project was abandoned.

Despite the overall lack of success, some components of the original study led to publishable results or to projects still underway, as outlined below.

1. **Broberg, C.L., J.H. Borden, R. Gries. 2005.** Antennae of *Cryptorhynchus lapathi* (L.) (Coleoptera: Curculionidae) detect two pheromone components of coniferophagous bark beetles in the stems of *Salix sitchensis* Sanson ex Bong. and *Salix scouleriana* Barratt ex Hook. The Canadian Entomologist (in press).

As part of the more comprehensive GC-EAD study, the first discovery of the scolytid pheromones conophthorin and chalcogran in willows led to publication of this note (See Appendix 1A).
2. Comparative study of the occurrence of pheromone components of coniferophagous bark beetles in the bark of angiosperm trees.

In a study stimulated by No. 1 above, captured bark volatiles from the bark of 42 species in 24 families at the VanDusen Botanical Garden, Vancouver, BC, are being analyzed by GC-MS. A publication is planned for 2006 in the journal Chemoecology.


This research documents the olfactory and gustatory preferences of *C. lapathi* among willows, black cottonwood, trembling aspen, red alder, and big leaf maple, and oviposition and emergence among these species (see Appendix 1B).


This investigation led to the conclusion that *S. calcarata* preference for trembling aspen as a host over black cottonwood or Scouler’s willow is based primarily on gustatory cues rather than olfactory attraction or oviposition (see Appendix 1C).
Table A 1. Summary of antennally-active compounds found by GC-EAD analysis for *C. lapathi* and *S. calcarata*. Compounds identified by GC-MS, and confirmed by coelution of authentic synthetic standards. Chirality reported where known. Antennae from male and female weevils responded similarly, unless otherwise noted.

<table>
<thead>
<tr>
<th>Species</th>
<th>Source of volatiles</th>
<th>Antennally-active compounds</th>
<th>Qualitative estimate of response strength of male and female antennae</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. lapathi</em></td>
<td><em>Salix</em> spp. stems, plus female C. <em>lapathi</em></td>
<td>(Z)-hex-3-en-1-ol, hexanol, benzaldehyde, β-pinene, benzyl alcohol, salicylaldehyde, (E)-conophthorin, (E)-chalcogran, (Z)-chalcogran, nonanal</td>
<td>++, ++, +, +, ++, +, ++</td>
</tr>
<tr>
<td></td>
<td><em>Salix</em> spp. stems, plus male C. <em>lapathi</em></td>
<td>(Z)-hex-3-en-1-ol, hexanol, benzaldehyde, β-pinene, benzyl alcohol, salicylaldehyde, (E)-conophthorin, (E)-chalcogran, (Z)-chalcogran, nonanal</td>
<td>++, ++, +, +, ++, +, ++</td>
</tr>
<tr>
<td><em>Salix</em> spp. stems, plus female and male <em>C. lapathi</em></td>
<td>(E)-hex-2-en-1-al, (Z)-hex-3-en-1-ol, hexanol, benzaldehyde, β-pinene, benzyl alcohol, salicylaldehyde, (E)-conophthorin, (E)-chalcogran, (Z)-chalcogran, nonanal</td>
<td>++, ++, +, +, ++, ++, ++, ++, ++</td>
<td></td>
</tr>
</tbody>
</table>
oats, plus female, male, or both sexes of C. lapathi

Salix spp. stems

P. tremuloides bark

virgin female C. lapathi on oats
<table>
<thead>
<tr>
<th>Compound</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>hexanal</td>
<td>♂</td>
<td>♀</td>
</tr>
<tr>
<td>(E)-hex-2-en-1-al</td>
<td>+</td>
<td>♀ ++</td>
</tr>
<tr>
<td>(Z)-hex-3-en-1-ol</td>
<td>♂ ++++</td>
<td>♀ ++</td>
</tr>
<tr>
<td>hexanol</td>
<td>♂ ++</td>
<td>♀</td>
</tr>
<tr>
<td>(17:1+)-α-pinene</td>
<td>♂ +++</td>
<td>♀ ++</td>
</tr>
<tr>
<td>frontalin</td>
<td>♂ +</td>
<td>♀ +++</td>
</tr>
<tr>
<td>benzaldehyde</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>(−)-β-pinene</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>2-hydroxycyclohexenone</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>benzyl alcohol</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>salicylaldehyde</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td>conophthorin</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>octanol</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>guaiacol</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>nonanal</td>
<td>♀ +</td>
<td>♂ ++</td>
</tr>
<tr>
<td>(−)-trans-verbenol</td>
<td>++</td>
<td></td>
</tr>
</tbody>
</table>

*a* absent in aerations on oats containing males alone, mixed males and females, or no weevils

*b* absent in aerations on oats containing males alone, or no weevils, and trace amount detected in an aeration containing mixed males and females
APPENDIX 1A. ANTENNAE OF *CRYPTORHYNCHUS LAPATHI* (L.) (COLEOPTERA: CURCULIONIDAE) DETECT TWO PHEROMONES COMPONENTS OF CONIFEROPHAGOUS BARK BEETLES IN THE STEMS OF *SALIX SITCHENSIS* SANSON EX BONG. AND *SALIX SCOULERIANA* BARRATT EX HOOK.

Several scolytid beetles (Coleoptera: Scolytidae) produce conophthorin, (E)-7-methyl-1,6-dioxaspiro[4.5]decane, and use it as an aggregation or antiaggregation pheromone or competition-mediating synomone (Francke et al. 1979; Kohnle et al. 1992; Birgersson et al. 1995; Pierce et al. 1995; de Groot et al. 1998; Dallara et al. 2000; Rappaport et al. 2000). Predators/associates of these beetles may use conophthorin as a host/habitat-finding kairomone (Kohnle et al. 1992). Other conifer-infesting scolytid and predator species use chalcogran, 2-ethyl-1,6-dioxaspiro[4.4]nonane, as a semiochemical with similar functions as conophthorin (Francke et al. 1977; Heuer & Vité 1984; Baader 1989; Byers 1993; Byers et al. 1989, 2000).

Recently, conophthorin has been found in several north-temperate angiosperm trees in the genera *Betula*, *Populus*, *Acer*, and *Quercus* (Byers et al. 1998; Huber et al. 1999; Francke et al. 1995). Several coniferophagous bark beetles and two woodborers, *Monochamus* spp. (Coleoptera: Cerambycidae), have been shown to be repelled by conophthorin (Huber et al. 1999; Morewood et al. 2003; Zhang et al. 2001; Zhang & Schlyter 2003), presumably as an avoidance response to non-hosts. Because its presence in angiosperm trees presumably predates its occurrence in coniferophagous scolytids, its role as a synomone in bark beetles has been postulated to be a form of chemical Batesian mimicry (Huber et al. 1999).

Chalcogran has not been identified in angiosperm trees to date, but has been found in five
species of tropical orchid, once in conjunction with \((E)\)- and \((Z)\)-conophthorin (Kaiser 1991, 1993).

We report the discovery of both of these compounds in the stem volatiles of \textit{Salix} spp. as detected by antennae of the poplar and willow borer, \textit{Cryptorhynchus lapathi} (L.) (Coleoptera: Curculionidae).

\textit{Salix} spp. stems ca. 3 cm in diameter were collected in mid-November, 1999, and cut into ca. 25 cm lengths. They were aerated (Rudinsky 1974) for 6 days in a plastic chamber fitted with a water aspirator. Volatiles were captured on Porapak-Q (Byrne \textit{et al.} 1975), eluted with 4 mL distilled pentane, and subjected to gas chromatographic-electroantennographic detection (GC-EAD) analysis (Arn \textit{et al.} 1975) using both male and female \textit{C. lapathi} antennae. Captured volatiles (1\,\mu L) were injected splitless into a Hewlett Packard 5890 gas chromatograph (injector port 250°C, detector port 260 °C, temperature program: held 1 min. at 50°C, increased 10°C/min to 280°C) fitted with a DB-5 fused silica column (30 m x 0.32 mm ID, J \& W Scientific, Folsom, CA 95630), then passed over an antenna or a flame ionization detector. Antennally active compounds were identified by GC-mass spectrometry (Varian Saturn 2000 Ion Trap, DB-5 column as above, electron impact mode) and confirmed by coelution and mass spectra with synthetic standards.

EAD traces disclosed a strong antennal response to \((E)\)-conophthorin and a weaker response to \((E)\)- and \((Z)\)-chalcogran (Fig. 1). The responses occurred repeatedly in different antennae. Conophthorin comprised 0.05% of the volatiles; and the two isomers of chalcogran \((E)-\) and \((Z)-\), 0.07% and 0.09%. Too little material was present to determine the enantiomeric composition of these compounds.
Because the original willow stems collected in November had no leaves and could not be identified to species, we repeated aerations in November 2004 using verified Salix sitchensis Sanson ex Bong. and Salix scouleriana Barratt ex Hook. Based on GC-MS comparisons with authentic standards, (E)-conophthorin and (E)- and (Z)-chalcogran were present in both species, but not in a solvent control.

These findings extend the presence of conophthorin into a new angiosperm genus, and document for the first time the occurrence of chalcogran in a dicotyledonous species. The consistent strong responses by antennae of both sexes to conophthorin, despite its being a minor volatile component suggests that it may be behaviorally important in C. lapathi as it is for numerous bark beetles.

References


Morewood WD, Simmonds KE, Gries R, Allison JD, Borden JH. 2003. Disruption by


Figure 1. Representative FID (upper) and GC-EAD (lower) traces for *Salix* sp. stem volatiles against female and male *C. lapathi* antennae. Identified peaks are (1) \((E)\)-conophthorin; and (2) \((E)\)- and \((Z)\)-chalcogran, respectively.
APPENDIX 1B. HOST SELECTION BY *CRYPTORHYNCHUS Lapathi* (L.)
(COLEOPTERA: CURCULIONIDAE) AMONG HOSTS AND NONHOSTS

Abstract

In British Columbia, native willows, *Salix* spp., and to a lesser extent black cottonwood, *P. trichocarpa* Torrey & Grey, are frequently attacked by the poplar and willow borer, *Cryptorhynchus lapathi* (L.) (Coleoptera: Curculionidae). Red alder, *Alnus rubra* Bongard, trembling aspen, *P. tremuloides* Michx., and big leaf maple, *Acer macrophyllum* Pursh, are not attacked. We studied olfaction and feeding preferences in the laboratory, and feeding, oviposition, and emergence in the field. Female *C. lapathi* preferred Scouler’s willow, *Salix scouleriana* Barret ex Hooker, over all other species by olfaction; males did not discriminate between Scouler’s willow and black cottonwood or trembling aspen. All species elicited at least some attraction in no-choice situations. Willow was generally preferred for feeding, but black cottonwood and red alder were also acceptable, unlike trembling aspen or big leaf maple. In field caging experiments, adult weevils emerged from willow, cottonwood, and red alder. We conclude that olfaction and feeding preferences are sufficiently powerful to mediate the frequent attack observed on native willows, intermediate levels on cottonwood, and absence on red alder. Successful development on red alder suggests that *C. lapathi* could expand its host range to include this species.

Key Words

*Cryptorhynchus lapathi*, host selection
Introduction

The poplar and willow borer, *Cryptorhynchus lapathi* (L.) (Coleoptera: Curculionidae), is a serious pest of willows and poplars (Harris 1964; Smith & Stott 1964; Cadahia 1965; Harris & Coppel 1967; Dafauce 1976; Broberg et al. 2001, 2005; Johnson & Johnson 2003; Broberg & Borden 2005), and since 1963 has more than doubled its range in British Columbia (BC) (Broberg et al. 2002). Adult *C. lapathi* are very long-lived, and development can take two years within a stem (Smith & Stott 1964). Throughout southern BC, most eggs are laid in August and September. Adults emerge the following August, and oviposit into niches chewed in the bark; eggs and first instars are the predominant overwintering stages (Harris & Coppel 1967). Larvae mine in the bark before moving into the xylem to complete development and pupate.

*Cryptorhynchus lapathi* has numerous hosts, primarily within the Salicaceae (*Salix* and *Populus* spp.), but some Betulaceae are also attacked. *Salix* spp. native to BC are all highly susceptible (Broberg et al. 2001); golden willow, *Salix alba* var. *vitellina* (L.) Stokes, and black cottonwood, *P. trichocarpa* Torrey & Grey, are also attacked. Attack occurs only infrequently on trembling aspen, *Populus tremuloides* Michx. (Thomas & Rose 1979; Vallée 1979) and has not been reported on red alder, *Alnus rubra* Bongard. Within the generally susceptible sections of Aigeiros and Tacamahaca in the genus *Populus*, antixenotic resistance in hybrids with *P. maximowiczii* Henry parentage leads to decreased levels of feeding and oviposition (Cadahia 1965; Dafauce 1976; Broberg et al. 2005). More importantly, antibiosis is directly expressed against early instar larvae which are unable to complete development (Broberg & Borden 2005). We report the results of investigations concerning the roles of
olfaction and feeding on host selection by *C. lapathi* among potential host genera *Salix*, *Populus*, *Alnus* and the nonhost genus *Acer* (Aceraceae).

**Materials and Methods**

**Weevil colonies.** Willow stems infested with *C. lapathi* were harvested in mid-July and kept outside in shaded, ventilated 30 x 35 x 50 cm³ bins at Simon Fraser University (49° 17' N, 122° 56’ W). In 2001, material was collected from Coquitlam, BC (49° 20’ N, 122° 49’ W); in 2002 and 2004, near Hope, BC (49° 33’ N, 121° 26’ W); and in 2003, from the Coquihalla summit area (49° 59’ N, 121° 00’ W). Emergent adults were collected weekly and sexed, placed in 25 x 35 x 50 cm³ ventilated containers, and allowed access to willow stems, water, and occasionally sliced apple fruit. Weevils for laboratory bioassays were held at 4°C; those for oviposition trials were maintained outside. Fresh food was supplied monthly for refrigerated weevils and weekly for weevils kept outside. At least one week between experiments was given to weevils used repeatedly in laboratory bioassays.

**Plant material.** Plant material (branches) was collected locally, except for trembling aspen branches which were collected from various interior BC locations, stored in water at 4°C, and used within one week of collection. For all other species, leafy material was either used within 24 h of collection, or stored immediately at 4°C in water. Stems of trees bioassayed in the winter of 2002 were kept outside and generally used within one week of collection. Willows collected in winter lacked leaves making accurate identification impossible, but were probably Sitka willow, *Salix sitchensis* Sanson ex Bongard. Golden willow, *Scouler’s willow, Salix scouleriana* Barratt ex Hooker, and big leaf maple, *Acer macrophyllum* Pursh (Aceraceae), were of assorted ages, growing from coppiced clumps. Red alder stems and leaves were from young, wild-seeded trees. Trembling aspen, black
cottonwood, and Norway spruce, *Picea abies* (L.) Karsten (Pinaceae), material consisted of branches growing from large trees; in the case of aspen, small suckers were also used.

**Pitfall olfactometer bioassays.** Pitfall olfactometers (Broberg et al. 2005) consisted of a Petri dish arena with two holes in the floor, which weevils could enter but not leave. One hole led into a glass “pit” containing a treatment stimulus; the other contained a control stimulus. Beetles were acclimated in the olfactometer for ca. 10 min before a bioassay. Each bioassay ran ca. 18 h overnight in the dark with one weevil per dish. Weevils were starved for 24 h prior to experiments in 2003 and 2004, but not in 2001 and 2002 because of small colony size and concern for increased mortality.

We first tested the hypothesis that species in the host genera *Salix* and *Populus* contain attractive stimuli, which other deciduous or coniferous non-hosts lack. Treatment pits were provided with a 3 cm section of stem plus a 1 cm piece of water-saturated dental cotton wick. Control pits contained the wick alone. In later bioassays, a single leaf was placed into the treatment pit in a small vial of water along with the stem section, and the control pit contained a small vial of water with no leaf or stem material. We then tested the hypothesis that *C. lapathi* could discriminate preferentially for willow over other potential hosts or non-hosts. In this case, the control pit contained a 3 cm section of willow stem, of equal diameter to that of another species in the treatment pit. Again, later bioassays involved the addition of a single leaf to the stem material. Treatments were applied to each olfactometer in a completely randomized design, and bioassays were repeated until sufficient responses for statistical analysis were obtained.

**Paired-twig feeding bioassays.** In January and February 2002, paired-twig bioassays (Tomlin & Borden 1996; Broberg et al. 2005) were performed to determine feeding
preferences among hosts and non-hosts. We suspended two 5 cm long cut stem sections, diam. 8-13 mm, on opposite sides of a 2 x 2 x 2 cm³ paraffin wax block, placed each assembly in a Petri dish, and allowed a single non-starved male or female weevil (N = 20) to feed for three days. Feeding was assessed by counting the number of feeding punctures under a dissecting microscope and drying frass collected from below each twig to constant mass, and weighing it. As in the pitfall bioassays, we tested whether C. lapathi would feed more on willows than other hosts or non-hosts in no-choice (two stems of the same species) and choice (one stem of each of two species) situations.

**Field caging experiments.** In 2002 and 2003, a single pair of weevils was caged onto willow (Sitka in 2002 and Scouler's in 2003), black cottonwood, red alder, and big leaf maple trees. Cages were 1.3 m long fibreglass screen cylinders (internal length ca. 1 m) with a zippered opening, cinched tight at the top and bottom around the boles of small trees starting at 5 to 50 cm above the ground. Secondary branches were removed prior to caging. Trees were 2 - 10 cm basal diam., with each species growing naturally at a minimum of two locations between Harrison Bay and 70 Mile House, BC. For trembling aspen, each replicate was a different clone. In 2002, 20 replicates were caged on 24-26 August and half sacrificed the following winter for oviposition counts. The other half were sacrificed the following July to determine emergence. In 2003, a further 15 replicates of each species were caged on 9-10 September and again assessed the following July for emergence.

**Statistical analyses.** In all cases α = 0.05. Within a bioassay period, the proportions of male and female weevils that entered a pit in pitfall olfactometers were tested for differences by Chi-square, and compared when appropriate with the Student-Newman-Keuls multiple comparison procedure for proportions (Zar 1984). The actual responses were tested against
the binomial distribution with $p = 0.5$ (Daniel 1995) (one-tailed for single host and two-tailed for two host bioassays). Data from paired-twig choice feeding bioassays were similarly tested with a series of two-tailed paired t-tests, transforming the data ($x^{1/2}$) if necessary to meet assumptions of normality (Daniel 1995). Data from no-choice feeding bioassays were also transformed by $x^{1/2}$, and the number of feeding punctures created in field cages were transformed by $\log(x+1)$, then both were analyzed by ANOVA using PROC GLM. When significant differences were found, the REGWQ test was used for means separation (SAS Institute 1990).

**Results and Discussion**

**Pitfall olfactometer bioassays.** Response rates were generally best in 2003 and 2004 when weevils were starved before bioassays; however, all bioassays performed during these years always contained the highly preferred host, Scouler’s willow.

We are cautious with how we interpret our data. In the pitfall olfactometer bioassays, 1,338 pits contained willow. Thus, for practical reasons, several cut stem sections from the same plant were used in bioassays, thus limiting the number of genotypes represented.

*Cryptorrhynchus lapathi* adults of both sexes were attracted to volatiles present in deciduous hosts and non-hosts over an empty pit (Table 1). Big leaf maple was more attractive than an empty pit in only two of four instances, so apparently contains some attractive volatiles. There was also a trend for high overall response rates when attractive stimuli were present. In contrast, Norway spruce was not attractive and even repellent in one instance and had the lowest proportional response among the test population. Thus it lacks attractive and/or contains repellent volatiles. When choices were offered in pitfall
olfactometers, response rates remained generally consistent (Table 2), likely because a suitable host was always present.

The possibility that *C. lapathi* used vision to locate pits with cut stem sections is unlikely because the bioassays were done in the dark. Other evidence in support of attraction to both host and non-host volatiles includes positive responses to pits with a rolled paper containing a stem section vs. an empty roll, and to trapped willow volatiles vs. solvent controls (*C. Broberg*, unpubl. data), as well as coupled gas chromatographic-electroantennographic detection analysis that showed similar antennally-active compounds in both host and non-host angiosperm trees (*C. Broberg* and *R. Gries*, unpubl. data). Based on this latter finding, we developed a synthetic blend in pentane containing the six most common antennally-active volatiles: hexanal, nonanal, hexanol, (Z)-3-hexenol, benzaldehyde, and conophthorin. When mixed in the approximate ratio found in willow and tested in pitfall olfactometers against a solvent control across a seven-fold concentration series, there was no evidence that *C. lapathi* adults were attracted to these volatiles (*C. Broberg*, unpub. data).

In host choice bioassays, *C. lapathi* consistently discriminated against the coniferophagous non-host, Norway spruce (Table 2), further supporting the observed lack of attraction (Table 1). Females chose black cottonwood and red alder over winter-collected willow of undetermined species, and both sexes selected trembling aspen over golden willow. The only species *C. lapathi* preferred less than the strongly-scented golden willow was Norway spruce; therefore, we conclude that this willow is not highly attractive. In contrast, Scouler’s willow was preferred by females over all other species, and by males over red alder, big leaf maple and Norway spruce (Table 2). The fact that females found black cottonwood and red alder stems more attractive than willow in the winter of 2002 (Table 2)
indicate that both leaves and the phenological state of the plant may be important mediators of attraction

In the field, attack is frequent on Scouler's willow (Broberg et al. 2001). We have observed attack on golden willow, but there are no records of attack on trembling aspen in BC. The strong attraction to trembling aspen in pitfall bioassays suggests that volatiles from leafy trembling aspen are more similar to those of Scouler’s willow than golden willow, and further suggests that rejection of trembling aspen as a host occurs because of perception of non-volatile chemical stimuli.

**Paired-twig feeding bioassays.** When females were given a choice in paired-twig bioassays, they clearly preferred willow over all other deciduous trees, with the exception of black cottonwood on which they produced less frass, but not significantly fewer feeding punctures (Fig. 1A, B). However, this was not consistent with feeding responses in no-choice assays in which red alder, not black cottonwood, was as acceptable as willow (Fig. 2A, B). Males were not as discriminatory as females in feeding choice, producing more feeding punctures on willow than on trembling aspen, but consistently and strongly discriminating against big leaf maple in favour of willow (Fig. 1C, D). These results are consistent with those of no-choice assays in which males fed little on big leaf maple compared to the other species (Fig. 2C, D).

These results clearly show that big leaf maple is not an acceptable host for *C. lapathi*, and that willow is highly preferred for feeding. In contrast, trembling aspen, black cottonwood and red alder were variable. Cadahia (1965), Dafauce (1976), and Broberg et al. (2005) also found that *C. lapathi* does not consistently maintain feeding preferences, even within a single genotype. This suggests that among potential hosts, discrimination by *C. lapathi* is
unreliable, or that small differences in tree quality can affect palatability. Harris (1964) also reports that red alder and trembling aspen were not fed upon by caged weevils when alternate hosts were present, but would feed on them when no other species was available.

**Field Caging Experiments.** No weevils emerged from trees on which adults were caged in 2003. In 2002, successful emergence was found from Sitka willow (4 of 9 replicates produced 11 weevils), black cottonwood (2 of 8 replicates produced 8 weevils), and red alder (1 of 7 replicates produced 2 weevils). This is the first record of *C. lapathi* developing successfully on red alder. Oviposition was observed in one of seven remaining black cottonwood replicates and in two of 11 Sitka willow replicates. The low success in this experiment may have been due to high adult mortality. Adults confined to a cage above ground would have been unable to seek refuge during hot days and would have been at risk of desiccation. Under natural conditions, weevils oviposit at the base of trees (Broberg et al. 2001), but tend to feed at the terminals of succulent shoots (Smith & Stott 1964; C. Broberg, unpub. obs.) to which the cages denied them access.

While determining oviposition, we also counted feeding punctures (Table 3). These results show significantly higher feeding levels on black cottonwood, Sitka willow, and red alder, and almost no feeding on trembling aspen and big leaf maple. These results are consistent with observations from paired-twig bioassays in which trembling aspen and big leaf maple were typically fed upon less in choice (Fig. 1) and no-choice (Fig. 2) situations.

**Conclusion**

Our results demonstrate that *C. lapathi* can discriminate by olfaction between highly preferred hosts like Scouler’s willow and other potential hosts and nonhosts. There is evidence that most angiosperm trees contain some attractive components that are lacking in
coniferous non-hosts like Norway spruce. Feeding preference bioassays and field observations on feeding and development showed clear and consistent discrimination against big leaf maple, partial discrimination against trembling aspen, and a general preference for willow, but inconsistent acceptance and rejection of black cottonwood and red alder. Thus, *C. lapathi* could discriminate among highly preferred hosts and non-preferred non-hosts using olfaction alone, but in other cases may need to combine olfactory information with feeding cues. Black cottonwood is a common host in BC, although not to the extent of native willows (Harris & Coppel 1967). Trembling aspen is generally considered a non-host, was highly attractive in some pitfall bioassays, but not fed upon readily. This suggests that the general trend of resistance in the Leuce section of *Populus* may be dominated by antixenosis that results in decreased feeding and probably oviposition, unlike the combined antixenotic and antibiotic resistance observed in hybrid poplars containing *P. maximowiczii* parentage (Broberg & Borden 2005, Broberg et al. 2005). The successful emergence of two adult progeny from adults caged on red alder suggests that *C. lapathi* may have the potential to expand its host range to include this species.
Acknowledgements

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References


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Table 1. Results from pitfall bioassays in which *C. lapathi* adults were tested for response to pits containing host or non-host cut stem sections or an empty, water-containing control pit. Percent responders followed by the same letter (within each of the three time periods for each sex) are not significantly different, Student-Newmann-Keuls multiple comparison procedure. Distributions of responders entering Side 1 that differed from the one-tailed binomial distribution (with \( p = 0.5 \)) indicated by * \( P < 0.05 \); ** \( P < 0.01 \); *** \( P < 0.001 \). Abbreviations as follows: Wil = *Salix* spp., CW = black cottonwood, TA = trembling aspen, RA = red alder, BLM = big leaf maple, NSpr = Norway spruce, ScWil = Scouler’s willow, and GWil = golden willow.

<table>
<thead>
<tr>
<th>Plant material</th>
<th>Dates performed</th>
<th>Experiment (Side 1 vs. Side 2)</th>
<th>No. test weevils</th>
<th>Percent response</th>
<th>Proportion of responders entering Side 1</th>
<th>No. test weevils</th>
<th>Percent response</th>
<th>Proportion of responders entering Side 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>stems only</td>
<td>January, March &amp; October, 2002</td>
<td>Wil vs. empty</td>
<td>33</td>
<td>57.6 a</td>
<td>0.789**</td>
<td>43</td>
<td>76.7 a</td>
<td>0.576</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CW vs. empty</td>
<td>39</td>
<td>53.8 a</td>
<td>0.905***</td>
<td>39</td>
<td>51.3 bc</td>
<td>0.850***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TA vs. empty</td>
<td>29</td>
<td>75.9 a</td>
<td>0.773**</td>
<td>28</td>
<td>57.1 abc</td>
<td>0.875***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RA vs. empty</td>
<td>33</td>
<td>60.6 a</td>
<td>0.700</td>
<td>30</td>
<td>66.7 ab</td>
<td>0.900***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BLM vs. empty</td>
<td>34</td>
<td>61.8 a</td>
<td>0.762*</td>
<td>29</td>
<td>58.6 abc</td>
<td>0.647</td>
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<tr>
<td></td>
<td></td>
<td>NSpr vs. empty</td>
<td>68</td>
<td>47.1 a</td>
<td>0.469</td>
<td>70</td>
<td>41.4 c</td>
<td>0.483</td>
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<tr>
<td>stem and leaf material</td>
<td>August 2004</td>
<td>SeWil vs. empty</td>
<td>30</td>
<td>93.3</td>
<td>0.964***</td>
<td>30</td>
<td>80.0</td>
<td>0.917***</td>
</tr>
<tr>
<td>August to October, 2002</td>
<td>GWil vs. empty</td>
<td>62</td>
<td>19.4 c</td>
<td>0.833*</td>
<td>41</td>
<td>39.0 bc</td>
<td>0.813*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CW vs. empty</td>
<td>60</td>
<td>26.7 bc</td>
<td>0.875**</td>
<td>54</td>
<td>35.2 cd</td>
<td>0.737*</td>
</tr>
<tr>
<td></td>
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<td>TA vs. empty</td>
<td>29</td>
<td>55.2 a</td>
<td>0.938***</td>
<td>44</td>
<td>59.1 a</td>
<td>0.923***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RA vs. empty</td>
<td>49</td>
<td>32.7 b</td>
<td>0.813*</td>
<td>36</td>
<td>52.8 ab</td>
<td>0.789**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BLM vs. empty</td>
<td>89</td>
<td>20.2 c</td>
<td>0.611</td>
<td>50</td>
<td>38.0 bc</td>
<td>0.789*</td>
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<tr>
<td></td>
<td></td>
<td>NSpr vs. empty</td>
<td>90</td>
<td>17.8 c</td>
<td>0.313</td>
<td>60</td>
<td>25.0 d</td>
<td>0.133**</td>
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</table>
Table 2. Results from pitfall bioassays in which *C. lapathi* were tested for response to pits containing host or non-host cut stem sections over a pit with a cut willow stem section. Percent responders followed by the same letter (within each of the three time periods for each sex) are not significantly different, Student-Newmann-Keuls multiple comparison procedure. Distributions of responders entering Side 1 that differed from the two-tailed binomial distribution (with $p = 0.5$) indicated by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Abbreviations as follows: Wil = *Salix* spp., CW = black cottonwood, TA = trembling aspen, RA = red alder, BLM = big leaf maple, NSpr = Norway spruce, ScWil = Scouler’s willow, and GWil = golden willow.

<table>
<thead>
<tr>
<th>Plant material</th>
<th>Dates performed</th>
<th>Experiment (Side 1 vs. Side 2)</th>
<th>Proportion of responders entering Side 1</th>
<th>Proportion of responders entering Side 1</th>
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<tr>
<td></td>
<td></td>
<td>Females</td>
<td>Males</td>
<td></td>
</tr>
<tr>
<td>stems only</td>
<td>January and</td>
<td>CW vs. Wil</td>
<td>33</td>
<td>0.636***</td>
</tr>
<tr>
<td></td>
<td>October, 2002</td>
<td>TA vs. Wil</td>
<td>34</td>
<td>0.636*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RA vs. Wil</td>
<td>44</td>
<td>0.750**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BLM vs. Wil</td>
<td>32</td>
<td>0.500</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NSpr vs. Wil</td>
<td>39</td>
<td>0.190**</td>
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<tr>
<td>stems and</td>
<td>August and</td>
<td>CW vs. GWil</td>
<td>42</td>
<td>0.400</td>
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<td>leaves</td>
<td>September, 2002</td>
<td>TA vs. GWil</td>
<td>30</td>
<td>0.467</td>
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<tr>
<td></td>
<td></td>
<td>RA vs. GWil</td>
<td>29</td>
<td>0.467</td>
</tr>
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<td></td>
<td>September,</td>
<td>BLM vs. GWil</td>
<td>59</td>
<td>0.579</td>
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<td></td>
<td>2003</td>
<td>NSpr vs. GWil</td>
<td>40</td>
<td>0.118**</td>
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<td></td>
<td>CW vs. ScWil</td>
<td>30</td>
<td>0.200**</td>
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<tr>
<td></td>
<td></td>
<td>TA vs. ScWil</td>
<td>30</td>
<td>0.211*</td>
</tr>
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<td></td>
<td></td>
<td>RA vs. ScWil</td>
<td>30</td>
<td>0.207**</td>
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<td>BLM vs. ScWil</td>
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<td></td>
<td></td>
<td>NSpr vs. ScWil</td>
<td>30</td>
<td>0.000***</td>
</tr>
</tbody>
</table>
Table 3. Comparison of number of feeding punctures produced by *C. lapathi* adult pairs caged on host and non-host deciduous tree species in the field. Means followed by the same letter are not significantly different, REGWQ test.

<table>
<thead>
<tr>
<th>Tree species</th>
<th>No. replicates</th>
<th>Mean no. feeding punctures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sitka willow</td>
<td>11</td>
<td>50.9 ± 14.0 a</td>
</tr>
<tr>
<td>Black cottonwood</td>
<td>7</td>
<td>76.6 ± 16.1 a</td>
</tr>
<tr>
<td>Red alder</td>
<td>8</td>
<td>30.9 ± 7.5 a</td>
</tr>
<tr>
<td>Trembling aspen</td>
<td>9</td>
<td>1.1 ± 0.8 b</td>
</tr>
<tr>
<td>Big leaf maple</td>
<td>9</td>
<td>5.8 ± 2.2 b</td>
</tr>
</tbody>
</table>
Figure 1. Comparison of frass production and number of feeding punctures among different species of deciduous hosts and non-hosts when *C. lapathi* was given a choice between willow (Wil) and black cottonwood (CW), trembling aspen (TA), red alder (RA) or big leaf maple (BLM). *P*-values for paired t-tests appear above paired bars.
Weevil's Choice (Side 1 vs. Side 2)
Figure 2. Ranked amounts of frass produced and numbers of feeding punctures on
different deciduous hosts and non-hosts when *C. lapathi* was exposed in a paired-twig
bioassay to only one species: willow (Wil), black cottonwood (CW), trembling aspen
(TA), red alder (RA) or big leaf maple (BLM). Bars within a subgraph with the same
letter are not significantly different, REGWQ test, *P* < 0.05. ANOVA statistics as
follows: A) $F_{4/81} = 12.83, P < 0.0001$; B) $F_{4/86} = 15.79, P < 0.0001$; C) $F_{4/84} = 3.38, P =
0.01$; D) $F_{4/84} = 8.05, P < 0.0001$. 
Species presented for feeding

Females

Males

Mean amount frass produced (mg) + SE

Mean no. feeding punctures + SE

Legend:
- 'a': Group A
- 'b': Group B
- 'c': Group C
- 'd': Group D
APPENDIX 1C. INVESTIGATION OF HOST PREFERENCE BY SAPERDA CALCARATA SAY (COLEOPTERA: CERAMBYCIDAE)

Abstract

We conducted five laboratory and one field experiment to examine potential host selection mechanisms of *Saperda calcarata* Say in British Columbia. Olfactory bioassays indicated that female (and possibly male) beetles were attracted to volatiles from leafy twigs of trembling aspen, *Populus tremuloides* Michx. However, wounding of the bole, ethanol baiting, or both, did not result in significant orientation toward or attack of trembling aspens in the field. Feeding preferences for trembling aspen were strong for both sexes in choice bioassays, but in no-choice bioassays, females did not discriminate between trembling aspen and black cottonwood, *P. trichocarpa* Torr. & Gray. Scouler’s willow, *Salix scouleriana* Barrat in Hooker, was fed upon the least by both sexes. When diameter of bolts offered as oviposition hosts was equalized, frequency of oviposition was similar among the three hosts. Our data suggest that feeding preference is the predominant mechanism of host selection by *S. calcarata*.

Introduction

and willows (Baker 1972). *Populus* spp. are susceptible from approximately 3 years of age (Abrahamson & Newsome 1972), or 4-5 cm diameter at breast height (dbh = 1.3 m) (Drouin & Wong 1975; Hofer 1920; Nebeker et al. 1985). *Saperda calcarata* adults reportedly discriminate among poplar hybrids for feeding (Garland & Worden 1969) and there are differences in attack rates among *P. deltoides* clones (Nebeker et al. 1985).

In British Columbia (BC), *S. calcarata* adults emerge in late June - July, undergo a short period of maturation feeding (Linsley 1959) and mate. Females oviposit into oblong niches chewed in the bark of host trees. Young larvae mine in the inner bark and sapwood, then move deeper creating large, irregular galleries throughout the sapwood and heartwood (Hofer 1920). Frequently a single tree is repeatedly attacked forming a ‘brood’ tree. Attacked trees are identified by their deformed bole, oviposition scars, sap stains spreading down the bark, and frass piles at their base. The life cycle takes 3 - 4 years in Canada, but is probably shorter in the south (Hofer 1920; Peterson 1947; Baker 1972).

*Saperda calcarata* is considered a major pest of poplars (Solomon 1987) and frequently becomes prevalent within stands (Bird 1930; Nebeker et al. 1985). Physical damage to the boles from larval galleries makes trees susceptible to breakage. Openings from oviposition niches and woodpeckers lead to increased incidence of pathogens like *Hypoxylon mammatum* (Wahlenb.) P. Karst. (Graham & Harrison 1954) or *Phellinus tremulae* (Boandartsev) Bondartsev & Borisov (Hofer 1920) which girdle the bark or stain and rot the wood, and attacked trees may be further damaged by other insects, e.g., *Agrilus anxius* Gory or *Poecilonota cyanipes* (Say) (Hofer 1920).
Because attack may be prevalent in poor sites, e.g., dry slopes (Hofer 1920; Bird 1930; Morris 1963), or in decadent hosts (Graham & Harrison 1954), *S. calcarata* is assumed to prefer weakened hosts that remain alive during attack (Hanks 1999). In agreement with this hypothesis, less attack was observed on *P. deltoides* clones from southern provenances which grew most vigorously (Nebeker et al. 1985). In contrast, Baker (1972) noted that brood trees were larger and faster growing than neighbouring trees, and Abrahamson & Newsome (1972) concluded there was no difference in attack level on different quality sites. Olfaction is generally believed to play a large role in cerambycid host location (Linsley 1961; Hanks 1999; Allison et al. 2004).

We commonly observe *S. calcarata* attack on trembling aspen, *P. tremuloides*, in BC, but not in black cottonwood, *P. trichocarpa*, or willow, *Salix* spp. Our objectives were to determine: if olfactory attraction occurs to trembling aspen, the apparent preferred host, and if there were different levels of feeding or oviposition among these three hosts.

**Materials and Methods**

*Saperda Colonies*. We collected ca. 2.5 m³ *S. calcarata*-attacked trembling aspen bolts from trees felled near 70 Mile House, BC in April or May of 2002-2004. Adults emerged from the caged bolts during June and July for two successive years. A total of 76 and 101, 46 and 30, and 11 beetles emerged each year from bolts harvested in 2002, 2003 and 2004, respectively. Timing of emergence was in agreement with Garland & Worden (1969). Adults were kept on aspen branches in water in 1.2 x 1.8 x 0.6 m outdoor enclosures until used in bioassays. Beetles were used once in any one type of
bioassay, except for feeding bioassays in 2002, when tested beetles were returned to the holding cage from which test subjects were removed.

**Plant material.** Leafy aspen branches were collected periodically, mostly from various interior BC locations, but also from Burnaby and Maple Ridge on the coast. Branches were kept with the cut ends in water at $4^\circ\text{C}$, and used in bioassays within one week. Both Scouler’s willow, *Salix scouleriana* Barrat in Hooker, and black cottonwood branches were collected in Burnaby, BC the same day bioassays were performed. In total, four to five genotypes of each species were tested.

**Olfaction experiments.** Responses to volatiles were investigated in the laboratory using a still air olfactometer. A circular arena was enclosed by a black paper cylinder 60 cm high. The 60 cm diam. arena floor was a white coroplast (GE Polymershapes, Coquitlam, BC) platform with two holes, 5.5 cm in diam. with centres 28 cm apart. This platform was covered with white paper perforated 41 times with a pin in a uniform, radial pattern above the openings. Three concentric rings 5.5, 12.5, and 19.5 cm diam. were drawn on the paper above each hole. The platform rested on glass cookie jars (aperture 11 cm diam.), centred below each hole. Randomly assigned treatment and control jars contained either a small jar of water with a small, leafy branch of aspen, or just water, respectively. The arena ceiling was a sheet of clear Lexan (GE Polymershapes, Coquitlam, BC). A video camera was positioned above the arena. To induce the photopositive beetles to approach the stimuli, a fluorescent light was placed under the platform between the two jars. The entire apparatus was covered with a black cloth. Three to five adults of a single sex taken directly from the holding cage were placed into each arena. Presence of beetles in the concentric rings above the treatment or
control jars were determined at 30 sec intervals for 2 h. Some trials were not video recorded. Feeding damage to the perforated centres above control and treatment stimuli was assessed for both recorded and unrecorded trials. Jars were washed and the paper covering replaced before each assay. Assays commenced any time between 0800 and 2400 h.

A field experiment was set up in a trembling aspen grove near Sabiston Lake, northeast of Savona, BC, on 28 June, 2002. Apparently healthy trees, spaced approximately 20 m apart, received one of four treatments in a randomized block design (n = 14): 4 axe cuts on opposite sides of the tree at ca. 1.5 m; ethanol bait stapled at ca. 2m; 4 axe cuts plus ethanol bait; or no treatment. Ethanol is a ubiquitous kairomonal indicator of stressed trees (Kelsey & Joseph 1998 and references therein). The basal 3 m of the trees were examined for oviposition in 2003 and 2004.

**Feeding bioassays.** Choice and no-choice feeding bioassay experiments were performed to investigate feeding preferences of *S. calcarata* among trembling aspen, black cottonwood and Scouler’s willow. Three small branches, each with three to five leaves, were placed in water-filled vials inside 17 x 16 x 12 cm plexiglass boxes. A single *S. calcarata* adult was allowed to freely feed on plant material overnight. No-choice bioassays contained one of the three potential hosts, and choice bioassays contained one branch of each. Before an assay, the leaves were traced onto paper. After the bioassay ended, leaves were attached to their traced counterparts, scanned, and the leaf area consumed was quantified using Scion Image software (Scion Corporation, Frederick, Maryland). A total of 17 choice and 14 no-choice bioassays (replicates) were performed during July of 2002 and 2003.
**Oviposition bioassays.** In 2002 we tested oviposition by *S. calcarata* in holding cages on eight freshly cut bolts of varying diameter of each of the above three species. In 2003, two apparently healthy trembling aspen, black cottonwood, and Scouler’s willow trees of similar dbh were felled on 1 and 18 July 2003, bucked and transported to SFU where they were kept refrigerated until needed. Six to eight beetle pairs were placed in 13 outdoor cages, 90 x 90 x 90 cm, with one randomly positioned bolt of each species and a central water jar containing leafy trembling aspen branches for 7 days. Bolts were ca. 50 cm in length, and diameters taken from their midpoint. Oviposition was determined in both experiments by opening all niches cut in the bark.

**Statistical analyses.** In all cases \( \alpha = 0.05 \). For olfaction bioassays, one-tailed paired t-tests were used to determine if the frequency of observations in each of the concentric rings was greater over treatment than control jars. Chi-square tests were performed for each sex to compare the frequency of feeding damage above treatment vs. control stimuli against the null hypothesis of no discrimination between stimuli. Data from feeding and oviposition bioassays were transformed by \( x^{\frac{1}{2}} \) and \( \log(x+1) \), respectively, to correct for non-normality and heteroskedasticity, then analyzed as randomized complete blocks by ANOVA with PROC GLM (SAS Institute 1990). Because it was not possible to perform all no-choice feeding assays at once, the analysis included trial date and host species effects. Since no interaction was found (females, \( F_{12,22} = 0.90, P = 0.56 \); males, \( F_{12,21} = 0.82, P = 0.63 \)), interactions were not included in the final model. Multiple comparisons were performed with REGWQ (SAS Institute 1990).
Results

**Olfaction experiments.** There were no differences in the occurrence of beetles in the middle and outside rings bordering the perforated area above treatment and control stimuli for both sexes (data not shown). Females (but not males) were present more frequently above the treatment than the control stimulus (Table 1). Often, females and males fed on the perforated paper directly above the treatment stimulus, but in one instance females fed above the control as well (Table 1). Females also chewed curvilinear patterns in the paper covering the arena floor that were reminiscent of oviposition niches, but no eggs were found.

There were too few attacks on trembling aspen treated to release host volatiles (axe cuts) or baited with ethanol to draw any definitive conclusion. A total of 8 oviposition chambers in two replicates were found: 1 niche on an ethanol-treated tree; 1 and 3 niches on two axe-cut trees; 2 and 1 niches on two trees with both treatments and none on control trees. None of these niches developed into successful larval galleries.

**Feeding bioassays.** We observed feeding on both the petioles and leaves as did Garland & Worden (1969), but only quantified the more abundant foliar damage.

When given a choice (Fig. 1), both sexes clearly preferred trembling aspen over both black cottonwood and Scouler’s willow (females $F_{2,32} = 41.15, P < 0.0001$; males $F_{2,32} = 42.68, P < 0.0001$). There were also significant differences in feeding in the no-choice experiment (females $F_{2,34} = 20.53, P < 0.0001$; males $F_{2,33} = 26.96, P < 0.0001$). However, females accepted trembling aspen and black cottonwood equally, and males fed on black cottonwood more vigorously than on Scouler’s willow (Fig. 1).
Oviposition bioassays. When bolt diameter was not controlled in 2002, *S. calcarata* females oviposited preferentially in trembling aspen and black cottonwood, the species with the largest diameter bolts (Table 2). When bolt diameter was equalized in 2003, there was no preference in oviposition among the three host species (Table 2).

We observed some larvae feeding in the bark of all three species, but they did not survive long as bark quality deteriorated rapidly because of infection by *Cytospera chrysosperma* (*Pers.:Fr.*) Fr., distinguished by characteristic orange conidial tendrils (Callan 1998).

Discussion

Our results indicate that *S. calcarata* can find potential hosts by olfaction, can discriminate among tree species through gustatory cues, and may reject trees for oviposition if their diameter is too small.

Females were attracted to the volatiles from leave-bearing twigs of trembling aspen in an arena olfactometer (Table 1). The fact that males chewed the paper above treatment but not control stimuli suggests they too are attracted to host volatiles. In the field experiment, we had hypothesized that *S. calcarata* brood trees would produce ethanol, and possibly other metabolites caused by wounding, and that the combination would be attractive. The positive response to leafy twigs, and the failure to induce significant attack on trees that were wounded, ethanol-baited, or both, suggests that initial
orientation is to volatiles from leaves on which adults feed. Oviposition tends to be located in the upper parts of the bole beneath the canopy (Peterson 1947), requiring little movement by feeding beetles.

Lack of strong olfactory attraction is not surprising. As a specialist on weakened hosts that can support multiple generations on the same tree (Hanks 1999), *S. calcarata* is not subject to strong selection pressure to adapt to finding new hosts. Furthermore, *Populus* spp. are pioneer species and often occur in locally abundant populations. Thus emergent *S. calcarata* may not need to disperse long distances to find a suitable host. In contrast, stressed hosts which are moribund and can only support one generation of beetle (Hanks 1999) are often rare and/or patchy in distribution. Cerambycid specialists on these hosts have evolved strong long-distance response mechanisms that often involve orientation to host volatiles from recently downed or injured trees, smoke volatiles from burned trees, or pheromones produced by secondary bark beetles pheromones (Allison et al. 2004). Although these cerambycids mate and oviposit on these newly found hosts, they engage in maturation feeding on healthy trees (Hanks 1999). Weakened host specialists like *S. calcarata* can use a single individual for all functions; thus even the crippled emergent adults observed by us and others (Peterson 1947; Drouin & Wong 1975), can experience reproductive success without long range dispersal and olfactory orientation to suitable hosts.

7 In an unpublished study conducted by C.L. Broberg and R. Gries (SFU), 15 antennally-active volatiles from the bark of trembling aspen were identified by coupled gas chromatographic electro-antennographic detection analysis. However, in six field-trapping experiments, no *S. calcarata* were captured to various partial or complete blends of these compounds. These results may indicate that *S. calcarata* used bole volatiles to reinforce gustatory stimuli during feeding, but that leaf volatiles are used in orientation toward suitable hosts by flying beetles.
Both sexes clearly preferred trembling aspen in choice feeding bioassays and rejected other species, but in the no-choice bioassays, females did not discriminate between trembling aspen and black cottonwood, and males accepted black cottonwood more than Scouler’s willow (Figure 1). Females may have higher nutritive requirements than males and therefore cannot afford to be as selective. Preference for trembling aspen could be a result of local adaptation to a species that comprises 4.6 times more wood volume in BC than black cottonwood (BC Ministry of Forests 1998). Because *S. calcarata* is a strong flyer, it is mobile enough to sample numerous trees before finding one that is suitable for feeding.

There are few records in BC of *S. calcarata* attack on *Salix* spp. In Saskatchewan, however, both *Salix* and *Populus* spp. were reported to be “readily eaten” by adults (Peterson 1947). Thus, there could be host-related ecotypes in different regions of North America.

The lack of discrimination between hosts for oviposition when trembling aspen leaves were available for maturation feeding, and diameters of bolts from the three species were equalized, indicates that host volume is more important than species for larval feeding and development (Table 2). Oviposition in large-diameter hosts would be adaptive in ensuring that most hosts did not suffer breakage during the 3-4 years required for larval development.

In conclusion, lack of evidence for long-range olfactory orientation to new hosts, correlation between *S. calcarata* incidence in BC and gustatory preferences, high mobility, and lack of discrimination between hosts for oviposition, suggest that feeding
preference constitutes the predominant mechanism of host selection by *S. calcarata* in BC.

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**References Cited**


Natural Resources Canada, Canadian Forest Service, Canada.

Drouin, J.A. and H.R. Wong. 1975. Biology, damage and chemical control of the poplar 
borer (Saperda calcarata) in the junction of the root and stem of balsam poplar in 

Garland, J.A. and H.A. Worden. 1969. Feeding and mating of the longhorn beetle, 
Saperda calcarata Say. (Coleoptera: Cerambycidae). The Manitoba Entomologist 3:  
81-84.


Hanks, L.M. 1999. Influence of the larval host plant on reproductive strategies of 

Hofer, G. 1920. The aspen borer and how to control it. USDA Farmer's Bulletin 1154.


and incidence of the poplar borer in southern cottonwood plantations. pp. 247-251 in 
Proceedings of the Third Biennial Southern Silvicultural Research Conference 


Table 1. Comparison of behavioural activity by male and female *S. calcarata* within perforated centres of arena floor above treatment and control jars in the still-air olfactometer.

<table>
<thead>
<tr>
<th>Observations</th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Observations of beetles within arena circle circumscribing perforated area above treatment or control stimulus, 30 sec intervals for 2 h.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no. replicates</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>mean no. observations (± SE)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>treatment stimulus</td>
<td>55.4 ± 18.5</td>
<td>41.9 ± 8.8</td>
</tr>
<tr>
<td>control stimulus</td>
<td>26.3 ± 10.8</td>
<td>41.9 ± 18.5</td>
</tr>
<tr>
<td>t-value</td>
<td>1.99</td>
<td>0.00</td>
</tr>
<tr>
<td>probability</td>
<td>0.047</td>
<td>0.50</td>
</tr>
</tbody>
</table>

| Observations of feeding on perforated area of arena floor above treatment or control stimulus | | |
| no. replicates | 8 | 11 |
| no. times most feeding above treatment stimulus | 7<sup>a</sup> | 6 |
| no. times most feeding above control stimulus | 0 | 0 |
| no. times no feeding damage observed | 1 | 5 |
| Chi-square value | 7.00 | 6.00 |
| probability | 0.008 | 0.01 |

<sup>a</sup> In one trial feeding damage was observed above both stimuli, but damage was much greater over the treatment stimulus.
Table 2. Comparison of oviposition by *S. calcarata* on bolts from three different hosts when bolt diameters were unequal or similar.

<table>
<thead>
<tr>
<th>Experimental description</th>
<th>Species</th>
<th>Bolt diameter</th>
<th>Mean ± SE&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean no. ovipositions (± SE)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bolt diameters unequal (2002)</td>
<td>Trembling aspen</td>
<td>6.3 - 13.2</td>
<td>9.5 ± 0.8 a</td>
<td>21.0 ± 9.6 b</td>
</tr>
<tr>
<td></td>
<td>Black</td>
<td>8.9 - 16.7</td>
<td>12.2 ± 0.9 a</td>
<td>27.5 ± 9.4 a</td>
</tr>
<tr>
<td></td>
<td>cottonwood</td>
<td>3.2 - 5.5</td>
<td>4.6 ± 0.3 b</td>
<td>0.4 ± 0.2 c</td>
</tr>
<tr>
<td></td>
<td>Scouler’s willow</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bolt diameters similar (2003)</td>
<td>Trembling aspen</td>
<td>7.8 - 17.1</td>
<td>13.5 ± 0.6 a</td>
<td>4.4 ± 2.1 a</td>
</tr>
<tr>
<td></td>
<td>Black</td>
<td>8.1 - 17.3</td>
<td>13.1 ± 0.6 a</td>
<td>8.0 ± 3.2 a</td>
</tr>
<tr>
<td></td>
<td>cottonwood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Scouler’s willow</td>
<td>9.2 - 17.0</td>
<td>12.6 ± 0.6 a</td>
<td>8.3 ± 2.8 a</td>
</tr>
</tbody>
</table>

<sup>a</sup> Means within an experiment and column followed by the same letter are not significantly different, REGWQ test, *P* < 0.05. ANOVA statistics as follows: 2002 bolt diameter *F*<sub>2,18</sub> = 33.83, *P* < 0.0001; oviposition *F*<sub>2,18</sub> = 21.17, *P* < 0.0001; 2003 bolt diameter *F*<sub>2,24</sub> = 2.57, *P* = 0.10; oviposition *F*<sub>2,24</sub> = 1.17, *P* = 0.33.
Figure 1. Leaf area consumed by female and male *S. calcarata* when presented with the three potential hosts simultaneously (choice experiment) or separately (no-choice experiment). Bars within an experiment and sex with the same letter are not significantly different, REGWQ test, $P < 0.05$. TA = trembling aspen, CW = black cottonwood, Wil = Scouler’s willow.
CHOICE EXPERIMENT

Females

Males

NO-CHOICE EXPERIMENT

Host species

Mean leaf area consumed (cm²) + SE

TA CW Wil

TA CW Wil

TA CW Wil