

# Host selection, oviposition behaviour and leaf traits in a specialist willow sawfly on species of *Salix* (Salicaceae)

CELINA L. BRACCINI,<sup>1</sup> ANDREA S. VEGA,<sup>2,3</sup> HUGO D. CHLUDIL,<sup>2</sup> SILVIA R. LEICACH<sup>2</sup> and PATRICIA C. FERNANDEZ<sup>2,3,4</sup> <sup>1</sup>INTA Instituto de Recursos Biológicos, Hurlingham, Argentina, <sup>2</sup>UBA, Facultad de Agronomía, Ciudad Autónoma de Buenos Aires, Argentina, <sup>3</sup>CONICET, Buenos Aires, Argentina and <sup>4</sup>INTA, EEA Delta del Paraná, Campana, Argentina

**Abstract.** 1. Plant genotype influences plant–herbivore interactions by affecting insect attraction, acceptance and development. Here we linked oviposition behaviour of the specialist willow sawfly *Nematus oligospilus* Förster (Hymenoptera: Tenthredinidae) with leaf traits on different *Salix* L. (Salicaceae) genotypes. This was done as a first step to find oviposition cues that guide females to their host plants.

2. By means of choice and no-choice bioassays we analysed host selection according to willow genotype and leaf surface. We also studied larval performance, adult fecundity and effect of experience on host selection. *Nematus oligospilus* prefers to oviposit on *S. nigra*. The least preferred genotype, *S. viminalis*, showed better larval performance and highest adult fecundity. Host preference was not modified by larval feeding experience.

3. By means of light and scanning electron microscopy we described ovipositor and leaf micromorphology. The egg is laid inside the epidermis or between the epidermis and adjacent chlorenchyma, showing a tight association with the leaf. Leaf toughness was lowest for *S. nigra* and higher for *S. viminalis* and *S. babylonica*. Total nitrogen and protein content were higher on *S. viminalis* and *S. babylonica*. Total phenolics and phenolic glycosides were the highest and more diverse on *S. nigra*. Salicin content levels correlate with oviposition preference, suggesting the role of salicylates as oviposition stimulants.

4. Results suggest that oviposition preference on *S. nigra* may be related to lower leaf toughness and ease of injection of female saw-like ovipositor, and motivated by the presence of phenolic glycosides. Nitrogen levels may explain better larval performance and adult fecundity in *S. viminalis*. Thus, a balance among the different leaf traits determines the outcomes observed in this study.

**Key words.** Genotype, micromorphology, *Nematus oligospilus*, oviposition preference, *Salix*, sawfly.

## Introduction

Most herbivorous insects start attacking a plant by laying eggs on it. Host plant selection can be crucial for the survival of the new insect generation, especially when larvae lack mobility to select their own feeding places (Hilker & Meiners, 2006). One of the attributes influencing plant–herbivore interactions that has received considerable attention is plant

genotype. Genotype variation in trees can lead to differences in host plant susceptibility, including factors that affect insect attraction, acceptance and development, and plant tolerance to damage. This can influence herbivore performance and assemblages, as well as community structure and diversity (Orians *et al.*, 1997; Hochwender & Fritz, 2004; Osier & Lindroth, 2004; Wimp *et al.*, 2005). Here, we studied the oviposition behaviour of the sawfly *Nematus oligospilus* Förster (Hymenoptera: Tenthredinidae), a serious pest of *Salix* spp., according to willow genotype. As different factors can affect host recognition and acceptance, we linked different leaf

Correspondence: Patricia Fernandez, INTA, EEA Delta del Paraná, Paraná de las Palmas y Cl Comas S/N, Campana 2804, Argentina. E-mail: pcfernan@agro.uba.ar

traits with egg laying to find cues that orientate females to oviposit. *Nematus oligospilus* is an external leaf feeder that feeds exclusively on species of *Salix* L. (Salicaceae) and has been reported worldwide (Dapoto & Giganti, 1994; Urban & Eardley, 1995; Charles & Allan, 2000; Smith, 2003). In Argentina, this sawfly is a pest of *Salix* spp. in the lower Delta of Paraná river. Nearly 80% of this area corresponds to wetlands with unique ecological conditions, due to its water regime and biodiversity. Here willow plants have a high potential for development; mainly in relation to paper pulp and wood-based panels, but also sawn wood, which makes this crop highly relevant for the economy of the area. *Nematus oligospilus* females lay single eggs under the leaf cuticle from October to March. Eggs hatch after 1 week and each larva establishes a feeding hole. They may complete development on the natal leaf, although they often move to a nearby leaf in later instars. Movement of larvae among plants is unfeasible for this small and sedentary insect (Carr *et al.*, 1998). This sawfly produces adults of both sexes in the northern hemisphere but is apparently thelytokous where it is found in the southern hemisphere (Urban & Eardley, 1995), including Argentina. Previous studies on this sawfly described differential damage according to willow genotype (Charles *et al.*, 1998) and host plant vigour (Carr *et al.*, 1998).

Once an insect alights on a plant, the outermost leaf surface constitutes the zone of initial contact. The structural properties and/or chemical composition of this surface layer may determine the insect acceptance of this plant, and within a given plant individual, a preference for specific localities such as the abaxial or adaxial leaf surfaces (Müller & Riederer, 2005; Reifenrath *et al.*, 2005). Adaxial and abaxial surfaces described in some *Salix* spp. can be considerably different. For example, by scanning electron microscopy Szafranek *et al.* (2008) showed the presence of epicuticular waxes in the form of conicoids only in abaxial surfaces of *S. alba* L. and *S. fragilis* L. These authors proposed similar wax structures are present on the abaxial surface in the majority of *Salix* spp. These marked structural differences can also reflect differences in the chemical composition of both leaf sides. A preference for egg deposition on the adaxial leaf surface by *N. oligospilus*, under both field and lab conditions, was previously reported on *S. babylonica* L. (Urban & Eardley, 1995) and other *Salix* spp. (Charles *et al.*, 1998). However, these reports were based only on observations performed on eggs from field-collected leaves or twigs collected from lab experiments. As these observations can be useful to determine oviposition cues used by the sawfly, we performed dual choice experiments exclusively designed to confirm them.

Experience of host plants can modify insect feeding (Karowe, 1989) and oviposition preferences (Prokopy *et al.*, 1986; Vet *et al.*, 1995; Bjorksten & Hoffmann, 1998; Zhang *et al.*, 2007). Many phytophagous and parasitic insects have a preference for the host species on which they had been laid and subsequently developed (Jaenike, 1983; Barron, 2001). This behaviour can lead to important ecological consequences such as the formation of new host races or sibling species (Barron, 2001). Here we evaluate the possibility of changes

in *N. oligospilus* host preferences depending on the willow species on which larvae developed.

The degree of tightness to which an egg is associated with the leaf varies greatly and may depend on the physicochemical characteristics of the leaf surface (Müller & Riederer, 2005). Plant anatomy can also influence oviposition behaviour (Lundgren *et al.*, 2008). The willow sawfly *N. oligospilus* damages the leaf at the site of oviposition. The ovipositor is distended to lift the leaf cuticle and to saw a flat pouch where the female lays a single egg (Dapoto & Giganti, 1994; Urban & Eardley, 1995). This saw-like ovipositor of sawflies is very delicate and can suffer from serious wear because it is inserted through plant tissue and flexed to allow accurate manoeuvring (Smith, 1972). Therefore, it was suggested that the toughness of the plant tissue being penetrated plays a critical role in a female sawfly's life (Ferrier & Price, 2004). We analysed ovipositor and leaf micromorphology, as well as leaf toughness in relation to oviposition preferences on the different experimental willow genotypes.

Host acceptability and suitability may depend on the presence of plant secondary compounds, as well as the distribution of nutrients, especially nitrogen. Plant nitrogen content is linked to the behaviour of herbivorous insects, as their performance and dynamics can be enhanced or impaired according to nitrogen levels in their host plants (Mattson, 1980; Cease *et al.*, 2012). Phenolic glycosides are the most abundant secondary metabolites known in Salicaceae plant tissues, and have been identified as important factors in many plant–herbivore studies. Phenolic glycosides normally function as feeding deterrents and reduce the fitness of generalist insects, but can stimulate feeding of specialists and benefit their performance (see Boeckler *et al.*, 2011 and references therein). In this study, we quantified total nitrogen and protein content, total phenolics, phenolic glycosides and salicin on the different experimental willow genotypes to correlate with oviposition preferences.

In summary, here we evaluate leaf traits affecting oviposition behaviour as a first step to find oviposition cues that can be later used in a control programme for this pest. To do this, we report host selection and larval performance. We describe egg deposition in relation to leaf micromorphology. We also quantify nutrient levels and secondary metabolites in different experimental willow genotypes. Finally, we discuss how leaf traits may affect oviposition behaviour and performance for this willow pest.

## Methods

### Insects

Adult *N. oligospilus* females from a laboratory population were used in all the bioassays. Every spring, the population was started from larvae and pupae collected in a field with a history of sawfly infestation in the lower Delta of Paraná river (34°10' 23.08"S, 58°45' 57.67"W). Larvae were reared until pupation on fresh cut twigs in transparent plastic boxes (46 × 30 × 32 cm) in a controlled environment chamber at 25 °C and LD 18:6 h photoperiod. Pupae were removed from the boxes and held in separate plastic jars until emergence

of adult females. During the season, the lab population was renewed several times with field-collected larvae to minimize selection of laboratory-adapted insects.

### Plants

Material from the National Program of Genetic Improvement for Salicaceae (willow genetic improvement area from Instituto Nacional de Tecnología Agropecuaria, INTA, Argentina) was used in this study. To perform the bioassays, six specific willow genotypes were chosen according to their economic or experimental relevance to the region. The origin of the genotypes corresponds to: *S. viminalis* ('Amarillo'), *S. babylonica* var. *Sacramenta* ('Soveny Americano'), *S. babylonica* × *S. alba* ('Ragonese 131–27 INTA'), *S. matsudana* × *S. alba* ('Barret 13–44 INTA') and *S. nigra* ('Alonzo nigra IV INTA'). In addition, an experimental genotype originated from [*S. babylonica* × *S. humboldtiana*] × *S. matsudana* (developed by T. Cerrillo, INTA) was included. An additional genotype from *S. alba* × *S. alba* (i.e. the breeding genotype) was employed for insect rearing. During late winter of 2009–2011, 10–15 cuttings (30 cm length) of each genotype were planted in consecutive rows in an experimental station in INTA Castellar (Buenos Aires, Argentina) to provide fresh twigs for bioassays.

### Oviposition preference

To investigate oviposition preferences, three different choice bioassays were carried out. (1) Multiple choice bioassays. The six willow genotypes previously mentioned were simultaneously offered to individual female sawflies. Bioassays were conducted inside transparent plastic boxes (33 × 23 × 14 cm) containing a fresh cut twig (10 cm length) of each genotype placed in small jars with tap water. The location of each genotype was randomized within the box. After this initial experiment, the rest of the bioassays and measurements were performed with three genotypes selected out of the six original ones: *S. viminalis*, *S. babylonica* and *S. nigra*. (2) Dual choice bioassays. To study leaf side preference, a two-leaf twig was offered to individual females. Both leaf surfaces were offered in an equivalent position (i.e. one of the leaves was offered upside down) to rule out position effects that may affect oviposition choice. (3) No-choice bioassays aimed to examine acceptability of each leaf side for each genotype. Two-leaf fresh cut twigs of each selected genotype with only one leaf side exposed (the other side was covered with paper tape) were offered to an individual female sawfly. In all three experimental designs, every freshly emerged or day-old female was released in the centre of the box and was allowed to oviposit until death (2–4 days). The number of eggs laid per female was recorded.

### Larval performance, adult fecundity, and *F*<sub>1</sub> oviposition preference

The relationship between host plant quality and plant genotype was examined in larvae by monitoring survival

to pupation, time from larval hatch to pupation, time from larval hatch to adult eclosion, pupal weight and fecundity on different genotypes. Performance of larvae on different willow genotypes was investigated by rearing groups of 83 larvae on *S. viminalis*, 64 on *S. babylonica*, and 80 on *S. nigra* twigs. Larvae were transferred 2–3 days after hatching as neonates have a very low survival rate after manipulation. Only successfully established larvae were used for bioassays. Larvae were assigned randomly to the different experimental willow genotypes. Rearing took place in transparent plastic boxes (33 × 23 × 14 cm) where twigs were kept in small jars with tap water. The twigs were replaced by fresh ones every other day. The survival of larvae and pupae and adult appearance were registered daily. Immediately after pupation, individuals were assigned to one of three different treatments: (1) pupal weight: individual pupae were taken out of their cocoon and weighed; (2) adult fecundity: freshly emerged individual females were exposed to the breeding genotype fresh cut twigs for oviposition until death (the total number of eggs laid per female was counted); (3) *F*<sub>1</sub> oviposition preference: dual choice bioassays were performed on individual female sawflies. Females reared on *S. viminalis* or *S. nigra* were given the choice to oviposit on *S. viminalis* or *S. nigra*, while females reared on *S. babylonica* were given the choice to oviposit on *S. babylonica* or *S. nigra*. The procedure was identical to the one described in the previous section (point 2).

### Egg deposition and leaf traits

**Scanning electron microscopy studies.** The ovipositor of newly emerged females was dissected under a stereoscopic microscope. The material was air-dried, mounted and coated with a gold-palladium (40–60%) alloy by a Thermo VG Scientific (West Sussex, England) and then observed using a Phillips XL 30 scanning electron microscope (Phillips, Eindhoven, The Netherlands) at the Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia', Argentina.

**Leaf anatomy.** Segments of the middle portion of the penultimate leaf blade of an innovation were used in anatomical studies. Materials were embedded in paraffin and cut with a rotary microtome, dehydrated in an ethanol series and double stained with Safranin-Fast Green (D'Ambrogio de Argüeso, 1986). Transverse sections were studied with a Light Microscope (Zeiss Axioplan, Zeiss, Oberkochen, Germany) and photographed with a photographic camera 35 mm Mot.

**Leaf toughness.** The index most widely used as an indicator of leaf toughness is leaf specific mass (LSM), defined as the ratio of leaf dry mass to area (Witkowski & Lamont, 1991; Groom & Lamont, 1999). LSM ( $\mu\text{g mm}^{-2}$ ) was calculated from 17 fully expanded and non-damaged leaves of each genotype (*S. viminalis*, *S. babylonica* and *S. nigra*). Leaf area was measured by using a scanner and image processing software, *ImageJ* version 1.45 s (National Institutes of Health, Bethesda, Maryland) (Abramoff *et al.*, 2004). After that, leaves were oven-dried at 70 °C for 6 days and weighed.

**Leaf chemistry.** Fully expanded and non-damaged leaves from 6-month-old plants were sampled from the mid-section of each genotype. Total nitrogen and protein content were measured by AOAC International Official Method and data were expressed as percentage dry weight (AOAC International, 1995). Phenolics were extracted by combining 0.1 g fresh leaves with 1 ml of methanol in 2 ml vials for 1 h in an ice bath before been spun down in a centrifuge for 5 min at 3000 g. Total phenolic content was determined according to Swain and Hillis (1959) modified method. Twenty microlitres of each extract were mixed with 50  $\mu$ l 2 N Folin-Ciocalteu reagent and 500  $\mu$ l 1 N sodium carbonate solution, and then brought to 5.0 ml with distilled water. Each solution was allowed to stand at room temperature for 1 h and its absorbance was measured at 725 nm using a spectrophotometer (HP/Agilent 8453 UV-Visible, Agilent Technologies, Inc., Waldbronn, Germany). Phenolic content data were expressed as chlorogenic acid mg equivalents  $g^{-1}$  FW, by extrapolation from the correlation curve. To estimate total phenolic glycosides, methanolic leaf extracts were analysed with a high performance liquid chromatography (Serie 1200, Agilent) equipped with a diode array detector (G1365D, Agilent) and a reverse-phase column, Zorbax Eclipse XDB C18 (Agilent Technologies, Inc.) (4.6  $\times$  150 mm, 5  $\mu$ m) used for the separation of individual compounds. Detection was performed at 270–320 nm. Salicin and total phenolic glycosides were expressed as salicin  $\mu$ g equivalents  $g^{-1}$  FW, by extrapolation from the correlation curve using authentic samples (Sigma-Aldrich).

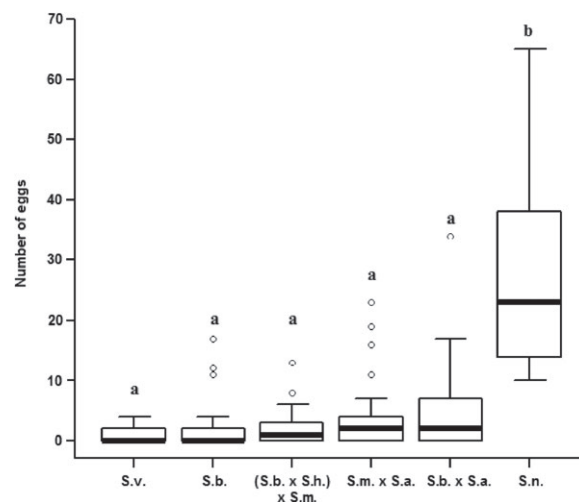
#### Statistical analysis

When possible after testing the assumptions, data were compared by ANOVA. Otherwise, non-parametric statistical tests were performed. Homogeneity of variance was checked using the Levene test and normality using the Shapiro–Wilk test. The number of eggs laid per genotype in multiple-choice bioassays was compared using the Friedman test (Conover, 1999), followed by multiple comparisons tests. The number of eggs laid per genotype in dual choice bioassays was analysed by Wilcoxon matched-pairs test, while for no-choice bioassays data were analysed by Mann–Whitney *U*-tests. Pupal weight and adult fecundity were analysed with ANOVA followed by multiple comparisons using the Tukey adjustment. *F*<sub>1</sub> oviposition preference was examined using the Wilcoxon matched-pairs test. LSM of three genotypes was compared using a Kruskal–Wallis test, followed by multiple comparisons tests. All statistical procedures were conducted with the software package R 2.15.1 for Windows (R Core Team, 2012).

## Results and discussion

### Oviposition preference

There is a clear effect of willow genotype on the number of eggs laid by a female in a multiple-choice array (Friedman  $\chi^2 = 47.8997$ , d.f. = 5,  $P < 0.001$ , followed by multiple comparisons test  $P < 0.05$ ; Fig. 1). These results support previous

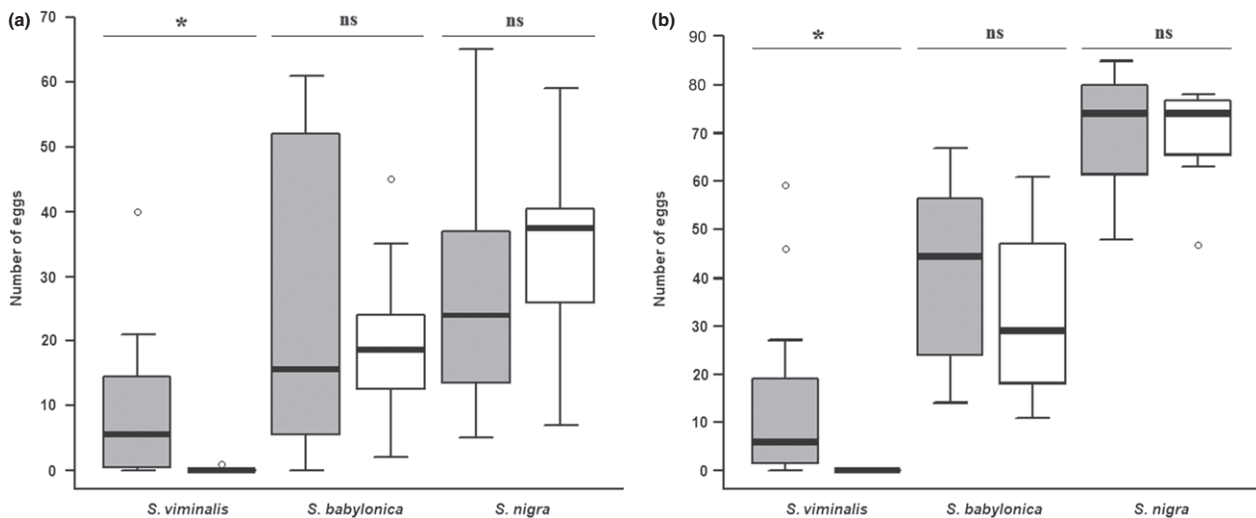


**Fig. 1.** Oviposition preference of *Nematus oligospilus* on six different willow genotypes in a multiple-choice bioassay. The boxes represent the first to third quartile range with the median indicated by a line across the box. The whiskers represent the range. The circles denote outliers. Friedman test,  $\chi^2 = 47.8997$ , d.f. = 5,  $n = 21$ ,  $P < 0.001$  followed by multiple comparisons tests. Different letters indicate significant differences at  $P < 0.05$ . S.v. = *Salix viminalis*, S.b. = *S. babylonica*, (S.b.  $\times$  S.h.)  $\times$  S.m. = (*S. babylonica*  $\times$  *S. humboldtiana*)  $\times$  *S. matsudana*, S.m.  $\times$  S.a. = *S. matsudana*  $\times$  *S. alba*, S.b.  $\times$  S.a. = *S. babylonica*  $\times$  *S. alba*, S.n. = *S. nigra*.

findings under field conditions showing a high level of feeding damage in *S. nigra*, very low feeding damage in *S. viminalis* and intermediate levels of damage for the rest of the genotypes used in the study (Cerrillo *et al.*, 2011). These data also suggest that the pattern of attack observed in the field results from oviposition choices of individual females, as was previously suggested by Carr *et al.* (1998) for this insect. Further analysis was performed by using three genotypes only: *S. nigra* (the preferred genotype), *S. viminalis* (the non-preferred one) and *S. babylonica* (intermediate preference and high commercial value). Egg deposition did not differ among leaf sides in *S. nigra* and *S. babylonica* (Wilcoxon matched-pairs test, NS; Fig. 2a). However, it was significantly lower in the abaxial leaf side of *S. viminalis* (Wilcoxon matched-pairs test,  $P < 0.05$ ; Fig. 2a). The same results were found in no-choice bioassays (Mann–Whitney *U*-test for *S. viminalis*,  $P < 0.05$ , *S. babylonica* and *S. nigra*, NS; Fig. 2b). Contrary to our results, Urban and Eardley (1995) found 90% of the eggs on the adaxial surface of *S. babylonica*. These differences could be simply due to leaf position. *Nematus oligospilus* normally walks or flies up to the light. Inside lab boxes, while not on leaves, they spend most of the time in the upper side of the box, upside down. This behaviour makes adaxial surfaces more accessible both under field and lab conditions.

Leaf side preference in *S. nigra* and *S. babylonica* suggest similar oviposition cues within species but different oviposition cues between them, as *S. babylonica* received fewer eggs independently of the experimental array. In the case of *S. viminalis*, results suggest a strong repellence, as was previously reported for this sawfly (Charles *et al.*, 1998;





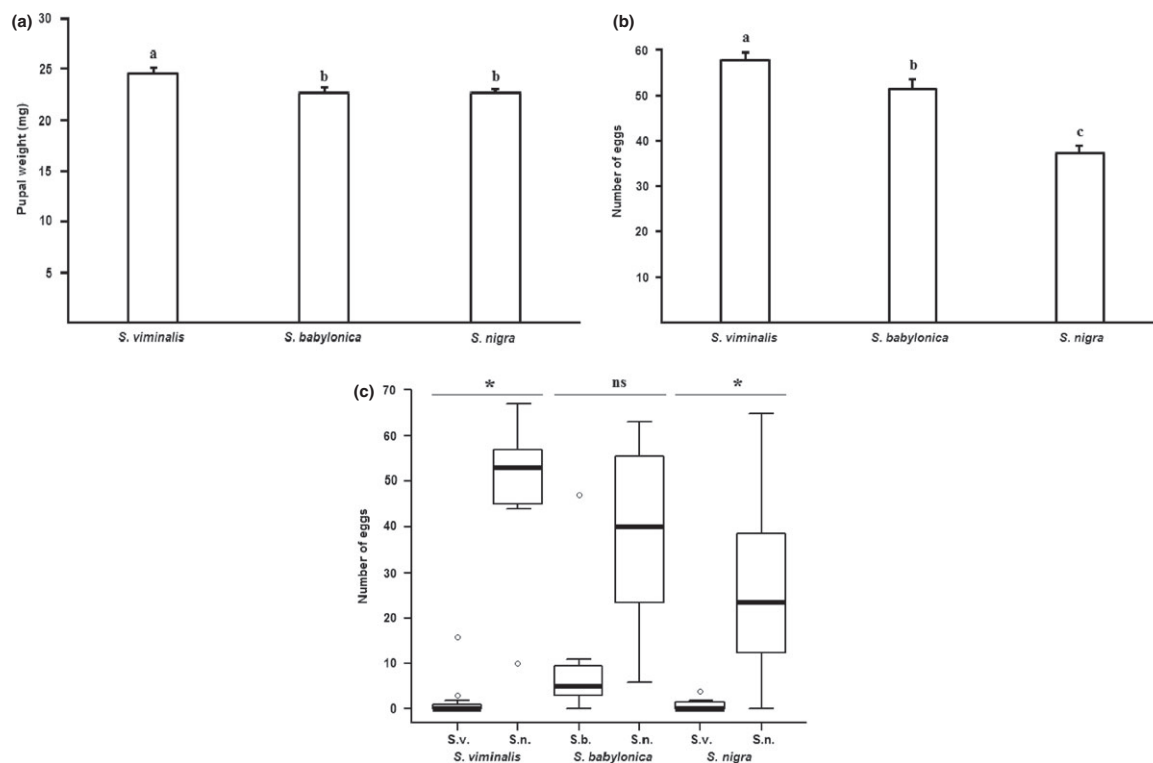
**Fig. 2.** Leaf side oviposition preference of *Nematus oligospilus* in (a) Dual choice bioassays (Wilcoxon matched-pairs test,  $n = 12$  for each bioassay). (b) No-choice bioassays (Mann–Whitney  $U$ -test,  $n = 12$  for each bioassay). Comparisons are made between adaxial and abaxial leaf sides (grey boxes and white boxes respectively) within each *Salix* genotype. The boxes represent the first to third quartile range with the median indicated by a line across the box. The whiskers represent the range. The circles denote outliers. \*Significant difference at  $P < 0.05$ ; ns, no significant difference.

Cerrillo *et al.*, 2011). When a specialist insect evaluates an oviposition site, it relies on plant-associated stimuli indicating host presence. This decision might be based on the presence/absence of a particular chemical profile or even a single chemical that is diagnostic of its particular host plant (Bernays & Chapman, 1994). Our experiments with *S. viminalis* reflect the high selectivity of this sawfly towards critical stimuli and its sensitivity to inhibitory effects, as was previously reported in *N. salicis* L., another oligophagous sawfly (Roininen & Tahvanainen, 1989). Different compounds, including cuticular waxes and phenolic glycosides have been reported as oviposition stimulants or deterrents of willow sawflies (Craig *et al.*, 1988; Roininen *et al.*, 1999). Rejection to oviposition can be related to the presence of one or a few of these compounds. Considering the overall number of eggs laid in dual choice and no-choice bioassays, the percentage declined from about 50 to 60% on *S. nigra*, to 30–40% on *S. babylonica*, and 6–8% on *S. viminalis*. All of the sawflies laid eggs on *S. nigra* and *S. babylonica*; whereas 21% died refusing to lay any eggs on the adaxial leaf side of *S. viminalis* and only one egg was found on the abaxial leaf side of *S. viminalis* throughout the whole study. Moreover, on dissecting *N. oligospilus* soon after their emergence, we found an average of 60 mature eggs in females hatched from larvae fed on genotypes of *S. alba* origin (data not shown). In dual choice and no-choice bioassays, females deposited fewer than 60 eggs on leaves from *S. viminalis* and *S. babylonica* (an average of  $7.97 \pm 2.37$  and  $39.86 \pm 3.54$ , respectively), refusing to lay the rest of their eggs even when they were mature. On the other hand they deposited an average of  $66.94 \pm 2.34$  eggs on *S. nigra*. These results suggest that plant genotype determines not only the probability of egg deposition, but also the number of eggs laid by individual females.

#### Larval performance, adult fecundity and $F_1$ oviposition preference

A total of 85.5%, 87.5% and 87.5% larvae survived to pupation when reared on *S. viminalis*, *S. babylonica* and *S. nigra*, respectively. These results indicate ability to breed on any of the experimental genotypes presumably because all of them provide sufficient nutrients for larvae to survive (Price *et al.*, 1999). Time from larval hatch to pupation and time from larval hatch to adult eclosion were slightly faster for *S. viminalis*, the non-preferred genotype (time to pupation for 50% of larvae was 12, 14 and 15 days; time to eclosion of 50% of adults was 19, 21 and 21 days; respectively for *S. viminalis*, *S. babylonica* and *S. nigra*). Pupal weight was significantly higher for insects reared on *S. viminalis* (ANOVA followed by Tukey contrasts, d.f. = 2,  $P < 0.05$ ; Fig. 3a). Fecundity varied accordingly: females laid more eggs on leaves of a different genotype (i.e. *S. alba*  $\times$  *S. alba*) when larvae were fed on *S. viminalis*, intermediate when fed on *S. babylonica* and lower when fed on *S. nigra* (ANOVA followed by Tukey contrasts, d.f. = 2,  $P < 0.05$ ; Fig. 3b). This suggests that the food plant spectra of *Nematus* larvae are wider than those of ovipositing females, as pointed out by Roininen and Tahvanainen (1989). Interestingly, our results also suggest better performance for larvae that have been reared on the least preferred genotype (see below).

As host preferences can often be modified by experience and conditioning (Prokopy *et al.*, 1982, 1986; Boller *et al.*, 1998), we studied oviposition preference in females hatched from larvae reared on each of the three experimental genotypes. Results show that females laid more eggs on *S. nigra* genotypes regardless of the diet upon which larvae fed (Wilcoxon matched-pairs test,  $P < 0.01$  for *S. viminalis* and *S. nigra*,  $P = 0.079$  for *S. babylonica*; Fig. 3c). This shows clearly that



**Fig. 3.** (a) Mean ( $\pm$ SE) pupal weight of females reared on *Salix viminialis* ( $n = 12$ ), *S. babylonica* ( $n = 14$ ) and *S. nigra* ( $n = 15$ ). Bars with different letters indicate significant differences (ANOVA:  $d.f. = 2$ ,  $F = 4.2686$ ,  $P < 0.05$  followed by Tukey contrasts:  $P < 0.05$ ). (b) Fecundity of females reared on *S. viminialis* ( $n = 36$ ), *S. babylonica* ( $n = 31$ ), and *S. nigra* ( $n = 33$ ) measured as mean number of eggs ( $\pm$ SE) laid per female on *S. alba* × *S. alba*. Bars with different letters indicate significant differences (ANOVA:  $d.f. = 2$ ,  $F = 35.375$ ,  $P < 0.001$  followed by Tukey contrasts:  $P < 0.05$ ). (c) Dual choice bioassays performed on females reared on different *Salix* genotypes (rearing genotype in bold letters). Wilcoxon matched-pairs test,  $n = 11$ , 8 and 12 females reared on *S. viminialis*, *S. babylonica* and *S. nigra*, respectively. The boxes represent the first to third quartile range with the median indicated by a line across the box. The whiskers represent the range. The circles denote outliers. \*Significant difference at  $P < 0.05$ ; ns, non-significant differences.

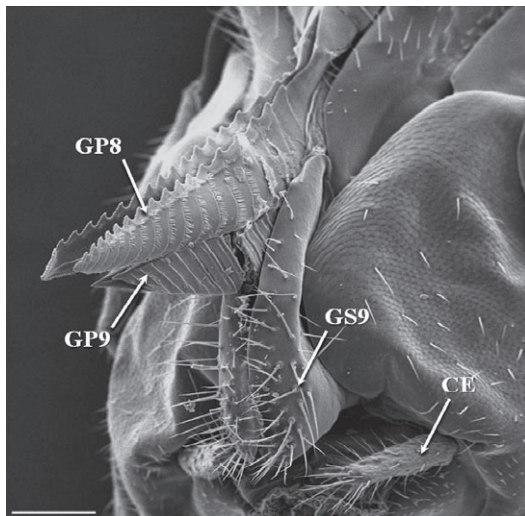
host preference was not modified by larval feeding experience and strengthens the hypothesis that it would be related to the presence of critical stimulating/repellent plant stimuli and/or a physical barrier (i.e. leaf toughness).

In insect–plant interactions, the relationship between maternal choice of host plant for offspring and the resulting survival and development of progeny has often been considered in the context of the preference–performance hypothesis, first proposed by Jaenike (1978). It states that female insects will preferentially oviposit on plants that maximise the survival and performance of their larvae. This is particularly relevant to insects whose larvae possess little or no ability to relocate (Clark *et al.*, 2011). Evidence for a preference–performance linkage has been found in many sawflies (Price, 2003; but see Ferrier & Price, 2004). More specifically, a strong relationship between oviposition preference and larval performance was reported in *N. oligospilus* that was mediated by the bottom-up effects of host plant vigour on larval survival and development time (Carr *et al.*, 1998). Our results showed that the diet breadth of the larvae is wider than the range of plants that adult females accept for oviposition, as was already reported in sawflies (Roininen & Tahvanainen, 1989) and many other

families (Schoonhoven *et al.*, 2005). Moreover, even when it was not the aim of this work, our results suggest a negative association between oviposition choices and larval performance. However, different facts make this study incomplete to discuss the preference–performance linkage. First, we did not evaluate mortality before establishment as we transferred larvae after 2–3 days of hatching. According to Carr *et al.* (1998), larval mortality was higher in neonates during the establishment phase on *S. lasiolepis* Benth., associated with inability to pierce leaf tissue or abnormal feeding behaviour. Second, we did not estimate female cost for oviposition. Besides, females may oviposit on ‘poor-quality’ host plants because their offspring obtain protection from natural enemies, as was already shown for the sawfly *Neodiprion sertifer* (Bjorkman *et al.*, 1997). Alternatively, females may fail largely to oviposit on *S. viminialis* simply because they fail to recognize it in the absence of appropriate oviposition cues (Price *et al.*, 2011).

#### Egg deposition and leaf traits

Before egg deposition, a *N. oligospilus* female walks along the leaf tapping several times over its surface with antennae.



**Fig. 4.** Scanning electron micrograph of the ovipositor of *Nematus oligospilus* in dorsolateral view. CE, cercus; GP8, gonapophysis VIII (saw); GP9, gonapophysis IX; GS9, gonostylus (sheath). Scale bar: 200  $\mu$ m.

Occasionally it stops, bends its abdomen into a comma shape, touches the leaf surface and waddles over the leaf. When she finds a suitable location, she faces down and lowers the ovipositor to lay the egg. An intimate physical contact between the different parts of the ovipositor was described for galling sawflies from the genus *Euura* (Smith, 1972). A scanning electron microscopy photograph shows the morphology of the serrated ovipositor used by the female to cut into plant tissue (nomenclature according to Smith, 1972, Fig. 4). Females probe with the gonapophyses before oviposition. This structure is highly sensitive to dilute chemicals placed on the ovipositor blades suggesting the presence of chemoreceptors (Dethier, 1947 in Smith, 1972). The sheaths of the gonostyli are rubbed over the plant surface before and during oviposition. Gonostyli and cerci possess sensilia that are directly related to host plant discrimination (Smith, 1972). Micromorphological studies investigated how tightly an egg is associated with the leaf on the different experimental genotypes. The egg is laid inside the epidermis (Fig. 5a) or between the epidermis and the adjacent chlorenchyma showing a tight association with the leaf. The same oviposition pattern was observed in the six different experimental willow genotypes, suggesting the same oviposition procedure. Chlorenchyma cells were observed always intact. This suggests a similar oviposition mechanism as described for *Euura*, in which after the ovipositor has penetrated about one-third of its length, the abdomen is straightened out parallel to the plant surface from its deflexed position (Smith, 1972). The incision opening is covered presumably by a colleterial secretion from female accessory glands (Smith, 1972). To investigate leaf toughness or a possible mechanical trait that can impede or prevent oviposition, both leaf micromorphology as well as LSM were studied.

The leaf blade anatomical structure differs among the studied genotypes. *Salix babylonica* as well as *S. nigra* showed an

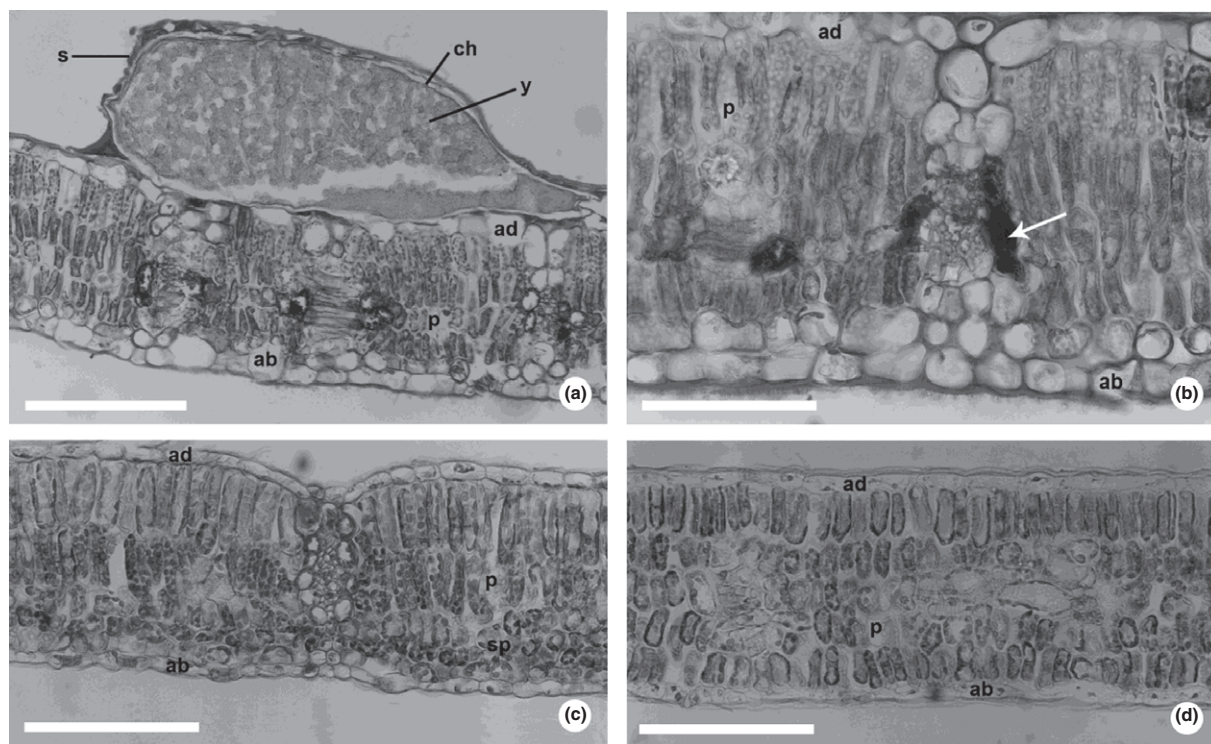
isolateral anatomical structure of leaf blades (i.e. palisade chlorenchyma beneath both adaxial and abaxial surfaces; Fig. 5b,d) and *S. viminalis* exhibit a dorsiventral anatomical structure of leaf blade (i.e. palisade and spongy chlorenchyma beneath adaxial and abaxial epidermis, respectively; Fig. 5c). Leaf blade cross-sections of the three experimental genotypes showed thicker leaves for *S. babylonica*. *Salix viminalis* and *S. nigra* possess leaf blades approximately half of that observed in *S. babylonica* (Fig. 5b–d). None of the genotypes shows typical sclerophyllous leaf traits, like a great proportion of mechanical tissue, thick or rigid living cell walls, thick cuticle or multilayer leaf tissue. LSM values were similar in *S. viminalis* and *S. babylonica*, and significantly lower in *S. nigra* (Kruskal–Wallis  $\chi^2 = 30.4142$ , d.f. = 2,  $P < 0.05$  followed by a multiple comparisons test, Table 1). This result indicates the lowest leaf toughness for *S. nigra*, suggesting that female oviposition preference may be based, in part, on ease of injection of her saw-like ovipositor, as was previously suggested by Ferrier and Price (2004) for a galling sawfly.

*Salix viminalis* showed a high content of total nitrogen as well as total protein (Table 1) indicating a high nutritional value for this genotype. Total phenolics and phenolic glycosides were low in *S. viminalis* and *S. babylonica*. *Salix nigra* showed the lowest nitrogen and protein content and the highest content of total phenolics and phenolic glycosides (Table 1). Salicin levels were not detectable in *S. viminalis*, intermediate in *S. babylonica* and the highest in *S. nigra* (Table 1), showing a close correlation with the oviposition preference. The fact that salicin is the breakdown product of higher molecular weight salicylates (Ruuhola *et al.*, 2003; Lehrman *et al.*, 2012) suggests an important role of salicylates in host selection for egg deposition. The different traits measured in this study may not solely explain the differences in oviposition observed, so further studies should look into leaf surface morphology and epicuticular wax chemistry that can also offer additional stimuli for host acceptance and recognition (Müller & Riederer, 2005). Chemical identity of phenolic glycosides combined with bioassays can help to find an oviposition stimulant present in *S. nigra* or a deterrent present in *S. viminalis*. The relative importance of nutrient levels and secondary metabolites should also be evaluated in future experiments.

## Conclusion

A ‘balance model’ is a concept that has been introduced to understand selection behaviour. It states that the stimulatory and inhibitory effects that plant chemicals exert on host seeking behaviour of herbivores counteract one another, and their balance determines the outcome of the decision-making process (Schoonhoven *et al.*, 2005). We propose that the oviposition preference of *N. oligospilus* females is determined by a balance among leaf structure, nutrients and secondary metabolites. On the one hand, the higher nitrogen content of *S. viminalis* leads to the best larval performance and the highest adult fecundity. However, due to its leaf toughness and probable lack of stimulating signals, it is unsuitable for egg deposition. The typically low phenolic glycoside content





**Fig. 5.** Leaf blades of *Salix* in transverse section, light microscopy photographs. (a) Leaf blade of *Salix babylonica* bearing an egg deposition of *Nematus oligospilus*, (b) *S. babylonica*, (c) *S. viminalis*, (d) *S. nigra*. ab, abaxial epidermis; ad, adaxial epidermis; ch, egg chorion; p, palisade chlorenchyma; s, female secretion; sp, spongy chlorenchyma; y, egg yolk. Arrow, tannic compounds in bundle sheath. Scale bars: a–d, 100  $\mu\text{m}$ .

**Table 1.** Leaf-specific mass (mean  $\pm$  SE) and total content of nitrogen, proteins, phenolics, phenolic glycosides and salicin in leaves of three *Salix* genotypes.

	<i>S. viminalis</i>	<i>S. babylonica</i>	<i>S. nigra</i>
Leaf-specific mass ( $\mu\text{g mm}^{-2}$ )	80.55 $\pm$ 0.48	79.79 $\pm$ 1.83	62.55 $\pm$ 0.75
Total nitrogen (% DW)	2.40	2.36	1.43
Protein (% DW)	15.03	14.72	8.92
Total phenolic content* ( $\text{mg g}^{-1}$ FW)	6.78	6.10	19.13
Total phenolic glycosides† ( $\mu\text{g g}^{-1}$ FW)	686	788	4175
Salicin ( $\mu\text{g g}^{-1}$ FW)	ND	27.7	44.8

\*Chlorogenic acid mg equivalent  $\text{g}^{-1}$  FW.

†Salicin  $\mu\text{g}$  equivalent  $\text{g}^{-1}$  FW.

ND, not detected.

of *S. viminalis* (Julkunen-Tiitto, 1986) may explain (at least in part) the lack of a stimulating oviposition signal. The high concentrations of condensed tannins reported for this species (Julkunen-Tiitto, 1986; Glynn *et al.*, 2004) does not seem negatively to affect larval growth. The strong repellence observed in the abaxial surface of *S. viminalis* leaves indicates a strong oviposition inhibitory trait that might be useful in a control programme for this pest. *Salix babylonica* showed similar nutrient and total phenolic content to *S. viminalis*.

However, its salicin levels might provide the cue that leads to oviposition in this genotype. On the other hand, *S. nigra* was highly preferred for egg deposition probably due to its low leaf toughness and the presence and abundance of phenolic glycosides and salicin (salicylates) content that may act as oviposition stimulants. Its low nitrogen content may explain why it is less suitable for larval growth and adult fecundity. Overall, while egg deposition in the sawfly *N. oligospilus* seems to be driven by a balance among different sensory cues, offspring performance seems to be related directly to the nutrients available for development.

## Acknowledgements

We thank Ing Teresa Cerrillo for provision of *Salix* clones and Dr Eduardo Botto for lending the chamber for insects breeding. We appreciate the valuable technical help of Ing Lucas Landi along the whole study. Appreciation is also expressed to Dr Micaela Buteler for her valuable comments in an earlier version of the manuscript. This work was funded by means of Prestamo BID–PICT 247 ANPCyT, PNFOR 2212 042121 INTA and UBACyT 20020100100229 and 20020110200266.

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Accepted 26 June 2013

First published online 24 September 2013