

Research Paper

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Author for correspondence:

P. C. Fern andez,
E-mail: pcfernan@agro.uba.ar

Previous herbivory alerts conspecific gravid
sawflies to avoid unsuitable host plants

G. A. Valladares^{1,2}, M. V. Coll-Ar oz³ , M. Alderete⁴, M. T. Vera^{1,2} 
and P. C. Fern andez^{2,5,6} 

¹Facultad de Agronom a y Zootecnia, Universidad Nacional de Tucum n, Florentino Ameghino S/N. B  Mercantil (4105), El Manantial, Tucum n, Argentina; ²Consejo Nacional de Investigaciones Cientificas y T cnicas (CONICET), CABA, Argentina; ³PROIMI-CONICET, Av. Manuel Belgrano 2960 (T4001MVB), S. M. de Tucum n, Tucum n, Argentina; ⁴Facultad de Ciencias Naturales e Instituto Miguel Lillo, Universidad Nacional de Tucum n, M. Lillo 205 (4000), S. M. de Tucum n, Tucum n, Argentina; ⁵INTA EEA Delta del Paran , Paran  de las Palmas y CI Comas S/N (2804), Campana, Buenos Aires, Argentina and ⁶C tedra de Qu mica de Biomol culas, Departamento de Qu mica Aplicada y Alimentos, Facultad de Agronom a, Universidad de Buenos Aires. Av. San Mart n 4453 (C1417DSE), Ciudad Aut noma de Buenos Aires, Argentina

Abstract

The willow sawfly, *Nematus oligospilus* (F rster), is a pest in *Salix* commercial forests and has been reported worldwide. Female adults must recognize a suitable host plant to oviposit, since her offspring lack the ability to move to another host. We evaluated the effect of conspecific herbivory on the oviposition choices of *N. oligospilus* females by providing damaged (DP) and undamaged (UP) plants of *Salix humboldtiana*, a native willow from South America, as oviposition substrates. Local and systemic effects were studied. For the local treatment, a twig from the DP with damaged leaves was contrasted to a twig from a UP in dual choice experiments. For systemic treatment, a twig from the DP with intact leaves was contrasted to a twig from a UP. We estimated the use of olfactory and contact cues by comparing volatile emission of DP and UP, and by analysing the behaviour of the females during host recognition after landing on the leaf surface. In the context of the preference–performance hypothesis (PPH), we also tested if oviposition site selection maximizes offspring fitness by evaluating neonate hatching, larval performance and survival of larvae that were born and bred on either DP or UP. Our results demonstrate that previous conspecific herbivory on *S. humboldtiana* has a dramatic impact on female oviposition choices and offspring performance of the sawfly *N. oligospilus*. Females showed a marked preference for laying eggs on UP of *S. humboldtiana*. This preference was found for both local and systemic treatments. Volatile emission was quantitatively changed after conspecific damage suggesting that it could be related to *N. oligospilus* avoidance. In the dual choice preference experiments, the analysis of the behaviour of the females once landing on the leaf surface suggested the use of contact cues triggering egg laying on leaves from UP and avoidance of leaves from DP. Furthermore, 48 h of previous conspecific feeding was sufficient to dramatically impair neonate hatching, as well as larval development and survival, suggesting a rapid and effective reaction of the induced resistance mechanisms of the tree. In agreement with the PPH, these results support the idea that decisions made by colonizing females may result in optimal outcomes for their offspring in a barely studied insect model, and also opens the opportunity for studying tree-induced defences in the unexplored South American willow *S. humboldtiana*.

Introduction

The preference–performance hypothesis (PPH) was first proposed by Jaenike (1990) and states that insect females maximize their own fitness by laying eggs on plants where their offspring perform best (reviewed in Gripenberg *et al.*, 2010). The theory, also known as ‘mother knows best’ (Valladares and Lawton, 1991) explains, from an evolutionary perspective, that natural selection should favour females with an ability to discriminate hosts of different suitability for larval development (Levins and MacArthur, 1969; Thompson, 1988; Gripenberg *et al.*, 2010). This female capacity is determinant for larvae that must start their development in the site selected by an adult female for oviposition. Evidence for this correlation ranges from excellent to poor (Jaenike, 1990; Forister *et al.*, 2009; Kim *et al.*, 2012) and has been explored among different hosts in polyphagous species (G mez Jim nez *et al.*, 2014), different varieties within a given host in monophagous species (Altesor and Gonz lez, 2018) and among different host qualities within a given plant species (Banchio *et al.*, 2007; McCormick *et al.*, 2016).

Choosing suitable oviposition sites involves processing different sensory cues from plants and other organisms associated with them (Schoonhoven *et al.*, 2005). Thus, gravid females should carefully assess cues that provide reliable information on the quality of the oviposition

site, the risk of natural enemies attack and the likelihood of competition. For many insects, the survival of the progeny is largely dependent on the capacity of the female to find a suitable host; especially in the case of low larval mobility and short adult lifespan (1–2 days) (Harris *et al.*, 1999).

In order to avoid herbivory, plants develop defences such as cuticles, trichomes, volatiles, toxins, repellent or anti-digestive compounds (Bennett and Wallsgrave, 1994; Kessler and Baldwin, 2002). Constitutive defences are always present in the plant, while induced defences are produced or mobilized to the site where a plant is injured (Walters, 2010). Induced defences have generally been regarded as mechanisms to reduce the cost of defence production (Steppuhn and Baldwin, 2008). However, in spite of the presence of defences, many of them very effective, insect females still lay eggs on these hosts. This mother's apparently 'bad choice' can have detrimental consequences, since its offspring may be negatively affected. The nutritional value of a leaf from a herbivore-damaged plant can be inferior compared to a leaf from an undamaged plant (Bergelson *et al.*, 1986; Fordyce, 2006). Therefore, larvae eat less and grow less, particularly at the beginning of development (Zalucki *et al.*, 2002) and older larvae might not be able to overcome the performance disadvantages suffered during early life. As a result, smaller larvae produce smaller adults of low reproductive potential (Awmack and Leather, 2002; Beyaert *et al.*, 2011).

Volatile cues from plants are important during host selection as they carry information about the identity and quality of the host plant. Herbivore-induced plant volatiles (HIPVs) would thus represent an efficient indication of host quality (Arimura *et al.*, 2009; Dicke and Baldwin, 2010; Heil and Karban, 2010). In fact, some herbivores can sense HIPVs and prefer plants that do not emit these compounds (Kessler and Baldwin, 2001). Interestingly, insects are able to perceive these cues not only in the damaged leaves but also in the undamaged parts of the same plant and even in the neighbouring plants that had perceived the cue as well and had reacted in accordance (Holopainen *et al.*, 2013). Detection and avoidance of herbivore-induced plants have benefits for ovipositing females not only to avoid toxic plants, but also to avoid conspecific or heterospecific competitors for their offspring (Saad *et al.*, 2015). In addition, offspring growing on plants emitting HIPVs will likely suffer a higher level of parasitization and predation (Amo *et al.*, 2013; Lehrman *et al.*, 2013; Hatano *et al.*, 2015). Such factors exert a strong selection pressure on insects to distinguish between unsuitable and suitable host plants for feeding and reproduction (Agrawal and Fishbein, 2006; Hopkins *et al.*, 2009). Therefore, avoiding unsuitable plants, particularly those with strong defences, can be as important as finding suitable ones.

The willow sawfly, *Nematus oligospilus* Förster (Hymenoptera: Tenthredinidae) is an external leaf feeder that develops exclusively on the species of *Salix* (Salicaceae) (Urban and Earldley, 1995; Ede, 2009; Alderete *et al.*, 2010). It is native to the Northern Hemisphere but has invaded the Southern Hemisphere, where it causes serious defoliation (Urban and Earldley, 1995; Berry, 1997; Koch and Smith, 2000; Bruzese and McFadyen, 2006). Females of this species are parthenogenetic in the Southern Hemisphere and emerge sexually mature. Upon emergence, females forage alone and lay individual eggs under the cuticle or first layer of epidermal cells of willow leaves (Braccini *et al.*, 2013). Due to the low larval mobility, after hatching the larva starts its development on the leaf where its mother laid the egg. Thus, the nutritional value of the leaf from which the larvae

feed in its initial stage of development (i.e. 4–5 days) is crucial for its own fitness. Previous works have shown differential oviposition preferences in *N. oligospilus* on commercial willow genotypes, derived from exotic European and Asian *Salix* species. In several cases, that preference was associated both with volatiles and with contact cues (Braccini *et al.*, 2013). Previous observations have shown that *N. oligospilus* can also feed on a native species of the Salicaceae family present in the Southern Hemisphere: *Salix humboldtiana* Willd. (Gonzalez *et al.*, 1986; Hauenstein *et al.*, 2005; Dickmann and Kuzovkina, 2014). Its natural distribution range spans from Mexico to Argentina and Chile being one of the widest distribution ranges among South American native woody species (Tortorelli, 2009). *Salix humboldtiana* is a rustic species that presents an extraordinary adaptation to flooding and saline soils due to specific morphological and physiological traits, and whose resistance to insect damage is usually observed in the field (Leonardo Gallo, personal communication). *Salix humboldtiana* has not gone through domestication, thus its gene pool provides the potential to adjust to different environmental conditions. This makes *S. humboldtiana* a valuable species with potential for wood products and an important genetic resource in the context of global change (Cerrillo, 2009).

Here, we studied the effect of 48 h of previous conspecific herbivory on *S. humboldtiana* on oviposition choices by *N. oligospilus* females and its larval performance. In the first part of this work, we focused on host selection by gravid females. We determined if previous damage affects host finding and acceptance by studying females' preference for oviposition on previously damaged and undamaged plants. We also estimated the use of olfactory and contact cues by comparing headspace volatile emissions of damaged and undamaged plants, and by analysing female behaviour after landing on the leaf surface. In the second part, we tested the hypothesis that oviposition site selection increases offspring fitness. We thus, evaluated neonate hatching, larval performance and survival of larvae that were born and bred on either damaged or undamaged plants. The experimental approach followed is original and differs from all previous research conducted in this system, in which the oviposition substrate offered consisted of several host genotypes with different degrees of constitutive resistance against sawflies (Braccini *et al.*, 2013; Fernández *et al.*, 2019). Thus, we studied a novel aspect of the willow tree–sawfly interaction by including the role of the previous herbivory, with special focus on induced defences, on host selection. The nutritional quality or quantity of the host plant is not considered in this study and will be subject of a future research.

Methods

Plants

Salix humboldtiana Willd. saplings were derived from ten trees located along the streets of Yerba Buena (26°49'00"S 65°19'00"W), Tucumán, Argentina. Voucher specimens (LIL616910) were deposited in the herbarium of Instituto Miguel Lillo, San Miguel de Tucumán, Tucumán, Argentina. Stems 30 cm length were cut from the trees and transferred to a greenhouse. Stems from different plants were placed in containers with water until they were covered with roots. Once the roots reached 10 cm long, each stem was planted in a 5 l pot and kept under natural conditions. After 2 months growth, plants that had developed at least 3–4 twigs with approximately 12–16 leaves each were subjected to feeding damage treatment as described below.

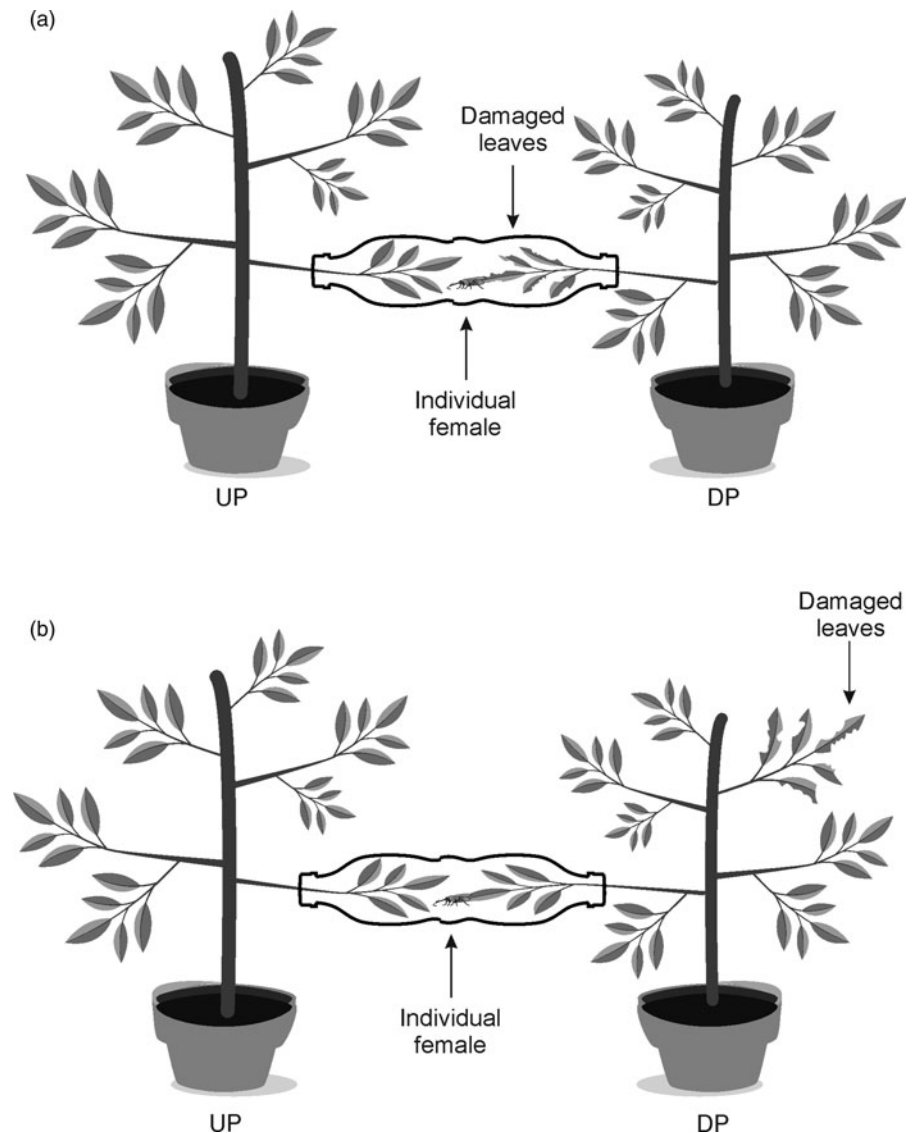


Figure 1. Scheme of the experimental design used for the *Nematus oligospilus* females oviposition preference experiment. Females were offered (a) intact leaves from an undamaged plant (UP) vs. damaged leaves with local damage (DP local) ($N=10$) or (b) intact leaves from UP vs. intact leaves from DP (systemic damage, DP systemic) ($N=10$).

Insects

Nematus oligospilus females derived from a laboratory established colony. The colony was established from larvae and pupae collected in Tafi del Valle (26°52'00"S 65°41'00"W), Tucumán, Argentina. Larvae were reared until pupation on fresh cut twigs of *S. humboldtiana* in transparent plastic boxes (23 × 30 × 18 cm) in an environment-controlled chamber at 25°C and 16:08 h L:D photoperiod. Fresh cut twigs were provided every 2 days. Pupae were removed from the boxes and held in separate glass tubes until adult emergence. See Alderete (2005) for more details in breeding methodology.

Plant treatments

In order to obtain damaged plants (DP), we placed four fourth-instar larvae on individual leaves from a selected twig containing 12–16 leaves. Larvae were allowed feeding for 48 h consuming approximately 50% of the individual leaf area. This resulted in a consumption of approximately 15% of the total leaf area of the twig. Larvae and faeces were removed from the twig and the plant was immediately used for the experiments. Two kinds of

twigs were used in experiments from DP: (1) twigs that had been in contact with larvae and had been subjected to herbivory themselves (i.e. DP local damage), and (2) twigs adjacent to the damaged ones that had not been subjected to herbivory themselves (DP systemic). This was accomplished by enclosing larvae in a PET tube (60 cm long, 15 cm diameter), and therefore restricting them to feed only in one part of the plant. Undamaged plants (UP) were not exposed to *N. oligospilus* larval feeding. Each plant was used only once.

Effect of previous conspecific damage on host finding by ovipositing females

Oviposition preference bioassay

Oviposition preference was evaluated in a dual-choice preference experiment in which female sawflies were simultaneously offered twigs from two different plants: UP and DP (either local or systemic). To avoid any influence on the choice behaviour of the female attributable to the amount of oviposition substrate offered, DP twigs had two leaves more than UP twigs to compensate for the loss of material produced by herbivory treatment.

Table 1. Behavioural units displayed by *Nematus oligospilus* females after landing on a willow leaf

Abbreviation	Behavioural units	Description
Q	Quiescent	Quiescent females
AW	Antennating while walking	Walking rapidly while tapping antennae on the leaf surface
A	Antennating	Touching the leaf surface with their antennae without walking
AR	Abdominal rubbing	The abdomen is bent into a comma shape, rubbing the leaf surface with it while slowly walking and simultaneously antennating
EG	Egg laying	Females remain still with their ovipositor inserted into the leaf while laying the egg, antennae are immobile facing up
G	Grooming	Grooming the antennae or legs with forelegs

The oviposition preference device consisted of a PET tube (60 × 15 cm) that enclosed the two twigs without detaching them from the plant (fig. 1). A newly emerged or a 1-day-old female was released in the centre of the device and was allowed to lay eggs until death (normally up to 3 days). The device was adjusted to the twigs of the plants by foams that surrounded the twigs and allowed the normal metabolism of the plant, but prevented the insect from escaping. The number of eggs laid on each plant was recorded. Each assay was conducted in a controlled environment at 22–26°C and 16:8 h L:D photoperiod. For each combination of plants, new PET tubes were used and the position of the DP and UP twigs was alternated to avoid side effects. A total of ten females were evaluated for each DP–UP combination (i.e. local and systemic damaged leaves).

Oviposition behaviour

In order to estimate the use of contact cues to discriminate between DP and UP, we recorded the oviposition behaviour sequence after landing on a leaf surface that ultimately led to egg laying or leaf rejection. Females were released in the same device used for the oviposition preference bioassays previously described. Two twigs were enclosed in the device: one belonged to a UP and the other to a DP with local damage. Once the female was released in the centre of the device, we observed its behaviour during 60 min and identified which of the six behavioural units described by Fernández *et al.* (2019) (table 1) was being performed on a given plant. We used a stopwatch to register the duration of each behaviour unit. The observer was able to move around the experimental arena, so the female was always visible. For each female, we quantified the time spent on the leaves of UP and DP, and the time spent performing each behavioural unit. Then, we estimated the relative frequency invested on a given behavioural unit as:

$$\frac{\text{Time spent performing a behavioural unit on a given plant}}{\text{Total time spent on leaves}} \times 100$$

Thus, the percentage was calculated based on the total time that females spent in contact with leaves. The time spent out of the leaves (i.e. on the observation device) was not taken into account. The experiment was performed once and a total of ten females were evaluated. To minimize any possible impact of positional effects, DP and UP occupied different positions in each observation.

Volatile collection

In order to identify possible chemical cues emitted by DP subjected to conspecific larval herbivory, we collected volatiles from 2-month-old plants of *S. humboldtiana*. To assure we collected the sufficient amount of volatiles required for the chemical characterization and quantification (Braccini *et al.*, 2015), we used plants three times bigger (150–200 leaves) than those used in the previous experiments. Thus, we adjusted the number of feeding larvae to keep the same proportion of larvae per leaf as in the behavioural assays, where plants were smaller. UP had intact leaves, while DP had been previously exposed to 10–12 larvae for a period of 24 h before volatile collection. After this time, larvae were removed. To provide adequate lighting conditions for volatile collection, pots were placed outdoors at 25–30°C, 70% RH and 25,000–35,000 Lux. Headspace samples were taken by enclosing the aerial part of the whole plant into a polyethylene (PET) bag. Air was pushed into the PET bag with an aquarium air pump and then pulled by a suction pump at a constant rate of 0.5 l per min. Air leaving the bag through an outlet passed through a volatile collection trap with 30 mg HayeSep Q (Grace, Deerfield, IL, USA) where volatiles were retained. After a sampling period of 6 h (between 10:00 and 16:00), the volatile collection traps were wrapped in Teflon tape and stored in the freezer until elution. Empty PET bags were used as blanks. Volatile compounds were eluted from the filters with 150 µl of dichloromethane containing 5 ng µl⁻¹ of dodecane as an internal standard. Samples were analysed by gas chromatography (Agilent 7890 A) coupled to mass spectrometry (Agilent 5977). A DB5 ms capillary column was used (0.25 mm i.d., film thickness 0.25 µm). Samples (1 µl) were injected at 240°C in a splitless mode. Helium was used as carrier gas at 0.75 ml per min (inlet pressure: 20.48 kPa). The column temperature was held at 35°C for 1 min, then increased at a constant rate of 5°C per min until it reached 100°C, then 12°C per min until 230°C. Finally, the temperature was held at 230°C for 10 min. In order to confirm compound identities, further injections on a DB5 column were performed with the following conditions: injector temperature 280°C and the oven programmed from 60 to 300°C at a constant rate of 3 then held at 300°C for 8 min. Compounds were identified by computer matching with commercial mass spectra libraries (NBS75K, NIST 98, WILEY275) and published data (Adams, 2007); comparison of their Kovats retention index (RI) on a DB5ms column and by comparison of retention times with authentic standards: trans-β-Caryophyllene and the alkane series C8 to C40 (Sigma Aldrich, EEUU). Data were collected with ChemStation software (Hewlett-Packard) and the detected volatiles

were quantified on the basis of their peak area in comparison with the area of the internal standard. We collected volatiles from $N = 19$ UP and $N = 11$ DP. The number of UP was higher than DP because of the reduced availability of larvae for the feeding damage treatment at that time of the year.

Effect of previous conspecific damage on larval performance and female fecundity

Larval performance

The effect of starting development on plants that had previously been subjected to herbivory on the subsequent larval performance was examined on different variables along the course of larval development. To do so, one female was enclosed in a cage containing one potted plant either with a UP or DP. Each female was allowed to lay eggs for approximately 3 h. The eggs were counted and the plants were kept in a controlled environment at 22–26°C and 16:8 h L:D photoperiod. Plants were inspected every day to register the emergence of neonates. Larvae were allowed to feed for 5 days, and after this time, the twigs containing the 5-day-old larvae were cut from the plant and transferred to small jars with tap water. Rearing took place in transparent plastic boxes (33 × 23 × 14 cm). Once larvae consumed the leaves on which they had hatched (6–8 days approximately after hatching), larvae were transferred to new twigs from UP until they completed development. Therefore, larvae did not suffer food shortage and there was no food source competition effect. Fresh twigs were offered every other day. The number of eggs, larvae and pupae on each twig were recorded daily and five variables were calculated: percentage of hatching, 2-day-old larval size, larval survival, larval developmental time and pupal weight. Percentage of hatching was estimated as: Number of neonates/number of eggs × 100. To measure larval size, 2-day-old larvae were photographed with a digital camera adjusted to a magnifying glass. In the visual field, we placed a ruler to scale the measure. Larval area was quantified by the image processing software, ImageJ version 1.45 s (National Institute of Health, Bethesda, Maryland, USA). We chose this kind of indirect size measurement because 2-day-old larvae are too small and delicate and their manipulation for weighting could cause their death. A total of 26 and 24 larvae were measured for UP and DP, respectively. Larval developmental time was estimated as the number of days that larvae from UP and DP took to pupate. Pre-pupal weight was recorded 4 days after larvae set up their cocoons by removing the prepupae from the cocoon. Larval survival was estimated as the number of hatching larvae/number of cocoons.

Female fecundity

The aerial part of a potted UP was enclosed on a PET tube of 40 × 15 × 15 containing approximately 30 leaves and offered to individual females derived either from UP or DP. One of the ends of the tube was closed around the trunk of the plant and the other end was enclosed with a voile fabric to allow air entrance into the PET tube. Twelve and nine sawflies derived from UP and DP, respectively, were evaluated. The total number of eggs laid per female was counted after female's death (i.e. 2 days).

Statistical analyses

For all cases, if parametric assumptions were not met, we run the corresponding non-parametric test.

In the oviposition preference bioassay, differences between the number of eggs laid in UP and DP were analysed with a paired *t*-test. The *t*-value was estimated using the Satterthwaite method for unequal variances. The same statistical method was used to analyse the oviposition behaviour observations (i.e. the percentage of time spent on each plant and the time performing each specific behaviour). The frequencies of the different possible behavioural transitions (i.e. from one behaviour to another) between UP and DP treatments on those transitions involving abdomen rubbing, were compared by means of a χ^2 homogeneity test.

In the larval performance experiment, we used the following statistical tests: for larval hatching, larval survival percentages and pre-pupal weight, we used a Mann–Whitney–Wilcoxon test. For larval size and developmental time, we used a *t*-test for independent samples. In all cases, the type of plant in which the eggs were laid was the main factor. We also applied the non-parametric Kaplan–Meier procedure to obtain the estimates of survival functions and survival curves of *N. oligospilus* larvae that initiated development on UP or DP (Caesar, 2003). This analysis compares the time required to achieve a specific outcome (often death) for individuals exposed to an explanatory factor (i.e. dietary regime in our case) (Caesar, 2003). Individuals that moulted from larvae to pupae were considered as censored data. Results plotted from this univariate survival analysis procedure were used to determine the time in which 50% of the larvae survived the treatments (the median lethal time, LT50). For female fecundity, we used a Mann–Whitney–Wilcoxon test.

The volatile collection data were subjected to the Mann–Whitney–Wilcoxon test to determine differences between individual compounds and total volatiles emitted by UP and DP.

All analyses were made using Infostat (Di Rienzo *et al.*, 2018).

Results

Effect of previous conspecific damage on host finding and acceptance by ovipositing females

Oviposition preference

Sawfly females preferred laying eggs on twigs from UP instead of twigs from plants that were locally or systemically damaged (DP) (fig. 2, *t*-test for paired samples, $T = -4.97$, $P = 0.001$ and $T = -2.28$, $P = 0.049$ for local and systemic treatments, respectively, $N = 10$).

Oviposition behaviour

Females spent a total of 34.73 ± 6.71 min in contact with a leaf and 25.26 ± 6.71 min (mean ± standard error) without contacting a leaf. Once on a leaf, females spent significantly more time on leaves from UP and also more time laying eggs (EG) on those leaves, than on DP (paired *t*-test, Q, $T = 1.90$, $P = 0.094$; AW, $T = 1.87$, $P = 0.085$; A, $T = 1.91$, $P = 0.093$; AR, $T = 1.89$, $P = 0.096$; EG, $T = 2.46$, $P = 0.040$; G, $T = 1.85$, $P = 0.102$ and total time on leaf, $T = 3.29$, $P = 0.011$, table 2). For the remaining types of behaviours, there were no significant differences between treatments (table 2). Since it was suggested that contact chemosensilla from female cercus may play a key role in triggering EG (Fernández *et al.*, 2019), the frequency of the different types of behaviours performed before and after abdomen rubbing (AR) were analysed. Before AR, females performed the same types of behaviours at the same frequency in UP and DP ($\chi^2 = 2.39$, $P = 0.496$; fig. 3 upper panel). However, after AR, the frequency

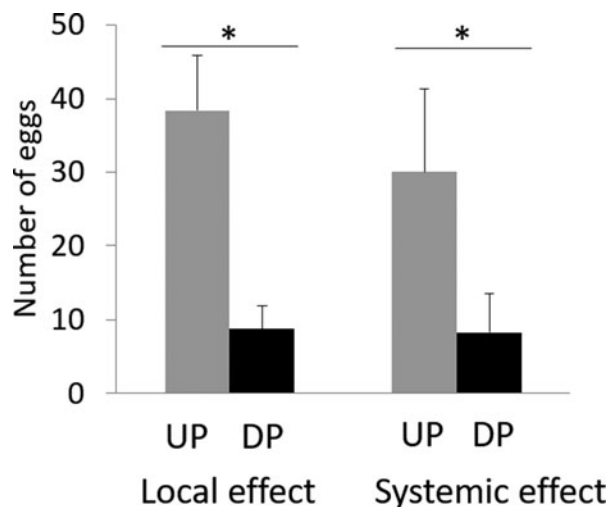


Figure 2. Number of eggs laid by *Nematus oligospilus* females on leaves from an *S. humboldtiana* undamaged (UP, grey bars) or a damaged plant (DP, black bars) either locally or systemically. ($N=10$). *Significant differences at $P < 0.05$.

Table 2. Relative frequency of behavioural units displayed by *Nematus oligospilus* females on *Salix humboldtiana* UP (undamaged) and DP (feeding damaged) plants

Behavioural units	Percentage of time invested		P
	UP	DP	
Q	22.28 ± 10.4	1.53 ± 1.4	0.094
AW	26.37 ± 7.2	6.16 ± 4.7	0.085
A	1.98 ± 1.0	0	0.093
AR	18.42 ± 5.2	4.6 ± 3.6	0.096
EG	10.43 ± 3.5	1.33 ± 1.0	0.040*
G	6.54 ± 3.2	0.4 ± 0.4	0.102
TOTAL	85.97	14.03	0.011*

t -Test for paired samples.*Significance with $P < 0.05$, $N = 10$.

of behaviours significantly changed between UP and DP ($\chi^2 = 8.45$, $P = 0.038$, fig. 3 lower panel). More specifically, on UP females spent more time EG while on DP they spent more time sensing with their antenna while walking (AW).

Volatile collection

A total of eight compounds were identified from volatiles collected from UP and DP of *S. humboldtiana* (table 3). The volatile profile included aromatic hydrocarbons, alkanes, mono- and sesquiterpenes. The DP emitted significantly higher amounts of Germacrene-D compared with control plants (Wilcoxon test, $N = 19$ for UP and $N = 11$ for DP, $W = 126$, $P = 0.012$). For the rest of the compounds, we found no statistically significant differences between UP and DP nor for the total of volatiles collected [Mann-Whitney-Wilcoxon test, (-) α -Pinene, $W = 66$, $P = 0.867$; β -Ocimene, $W = 68$, $P = 0.598$; Decane, $W = 73$, $P = 0.732$; Tetradecane, $W = 67$, $P = 0.693$; β -Caryophyllene, $W = 93$, $P = 0.069$; Non-identified Sesquiterpene, $W = 58$, $P = 0.367$; Valerianol,

$W = 58$, $P = 0.653$, total volatiles, $W = 75$, $P = 0.818$ (table 3)]. The compounds α -Pinene, β -Ocimene and β -Caryophyllene were reported for willow and poplar species in Füssel *et al.*, 2007 and Clavijo *et al.*, 2014.

Effect of previous conspecific damage on larval performance and female fecundity

The development of *N. oligospilus* larvae was dramatically affected by the plant treatment in which larvae developed from egg to 6–8 days old. The percentage of hatching neonates was higher (Mann-Whitney-Wilcoxon test, $W = 39$, $P = 0.03$), 2-day-old larval size was bigger (t -test for independent samples, $T = -3.29$, $P = 0.002$), percentage of larval survival to pupation was higher (Mann-Whitney-Wilcoxon test, $W = 51$, $P = 0.01$) and larval rate of development was faster (t -test for independent samples, $T = 2.02$, $P = 0.047$) when eggs were laid on UP compared to those laid on DP (fig. 4a, b, c, d). On the contrary, pre-pupal weight (Mann-Whitney-Wilcoxon test, $W = 85$, $P = 0.701$) and the number of eggs laid by the progeny (as an indicator of female fecundity, Mann-Whitney-Wilcoxon test, $W = 110$, $P = 0.433$) were not affected by the plant in which eggs were laid (fig. 4e, f).

Larval survival analysis revealed significant differences between the two treatments. Larvae from eggs laid on DP reached their LT50 on day 4, while larvae from UP did on the eighth day of development (Kaplan-Meier analysis, $\chi^2 = 28.81$, $P \leq 0.0001$, fig. 5).

Discussion

Here, we demonstrated that previous conspecific herbivory on *S. humboldtiana* has a dramatic impact on the willow sawfly *N. oligospilus* female oviposition choices and its offspring performance. There was a marked preference of ovipositing females for laying eggs on undamaged plants of *S. humboldtiana* and avoiding damaged plants in both local and systemic conditions.

The volatile profile of both kinds of plants showed quantitative differences that could be related to *N. oligospilus* orientation. Additionally, the behaviour of the female once landing on the leaf surface suggests the use of contact cues for triggering egg laying on leaves from undamaged plants or avoidance of leaves from damaged ones. Furthermore, 48 h of herbivory was sufficient to induce changes in the plant that dramatically impaired neonate hatching, as well as larval development and survival, suggesting a rapid and effective reaction of induced resistance mechanisms of the tree. This positive correlation between female oviposition preference and offspring performance is in agreement with the PPH.

Different chemical cues may be acting in a damaged plant in order to avoid the settling of a new generation of herbivores, by the induction of plant volatiles (Dicke and van Loon, 2000; Hilker and Meiners, 2006; Allman *et al.*, 2013) or by changing the chemical composition of the leaf surface (Blenn *et al.*, 2012). While volatiles may act as a long distance orientation cue, contact cues from the leaf surface can be the determining factor for female final oviposition decisions (Schoonhoven *et al.*, 2005). In our study, a higher concentration of Germacrene-D was emitted by damaged plants as reported in previous studies in poplar trees after gypsy moth (*Lymantria dispar* L.) (Frost *et al.*, 2007) and forest tent caterpillars (*Malacosoma disstria*) (Arimura *et al.*, 2004) larval damage. The fact that volatiles emitted by poplar trees, including Germacrene-D, function as

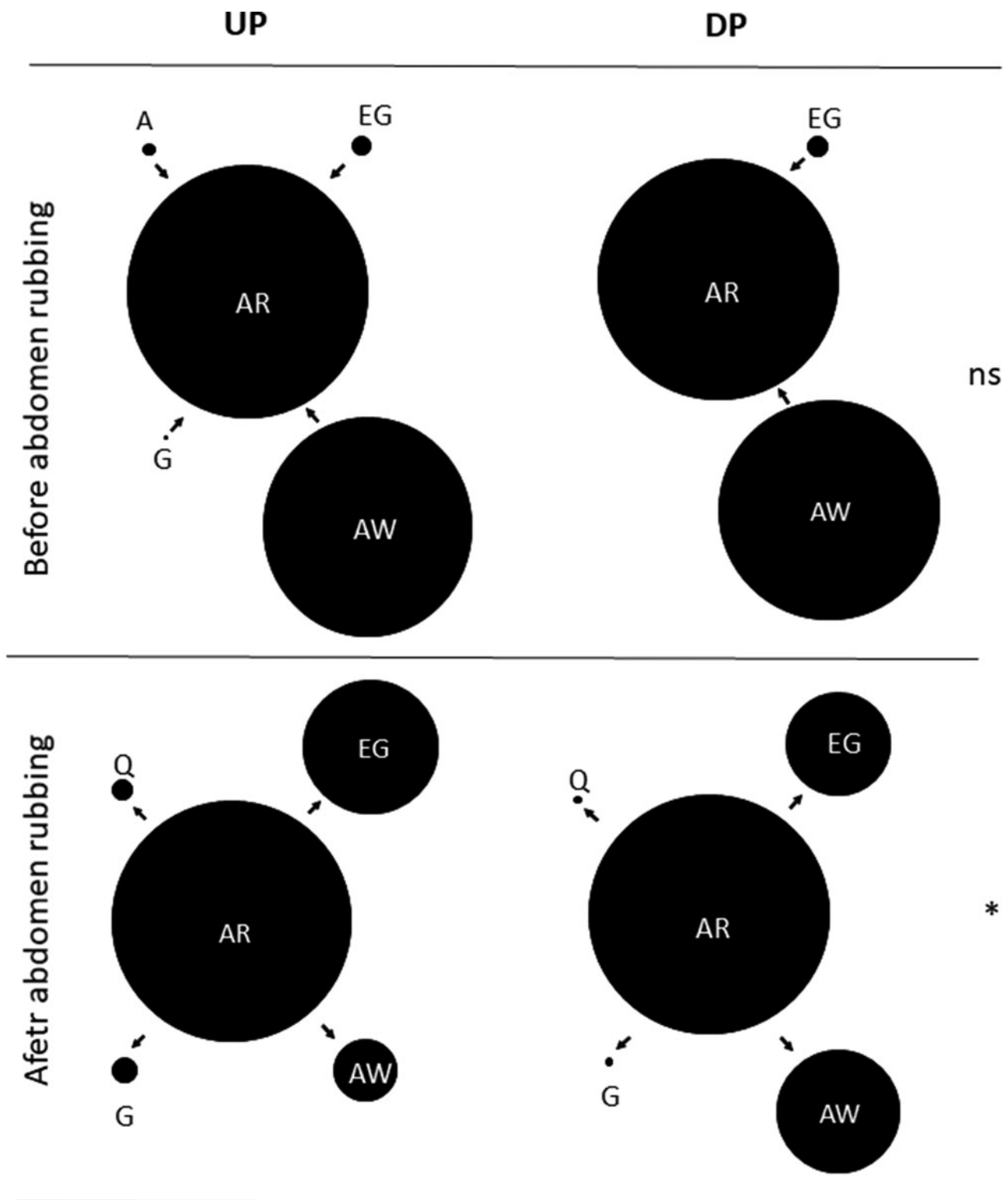


Figure 3. *Nematus oligospilus* females behavioural units displayed before and after abdomen rubbing on leaves from *Salix humboldtiana* undamaged (UP) and damaged plants (DP) ($N = 10$). *Significant difference at $P < 0.05$; ns, non-significant differences. AR (abdomen rubbing), Q (quiescent), EG (egg laying), G (grooming), AW (antennal walking). The size of the circles is proportional to the time spent on each type of behaviour.

chemical cues to prime plant defence (Frost *et al.*, 2007) encourages us to suggest that this compound could be also acting as a possible volatile honest signal for *N. oligospilus* females. Yet, the repellent effect of this specific compound should be tested in

order to confirm this hypothesis. Moreover, the significant change in the behaviour of the female after contacting the leaf surface strongly suggests the use of contact cues as the last step of decision making during the oviposition process as proposed by Fernández

Table 3. Volatile compounds emitted by *Salix humboldtiana* undamaged (UP) and damaged (DP) plants for 6 h (mean \pm SE expressed as ng μl^{-1}) ($N = 19$ and 11 for UP and DP, respectively)

Retention Time	RI	Compound	UP	DP	<i>P</i>
7.9	913	α -Pinene	0.17 \pm 0.05	0.12 \pm 0.05	0.867
10.2		Decane	0.05 \pm 0.03	0.02 \pm 0.02	0.732
11.3	1049	β -Ocimene	0.11 \pm 0.01	0.35 \pm 0.28	0.598
18.8		Tetradecane	0.28 \pm 0.08	0.22 \pm 0.13	0.693
19.1	1425	β -Caryophyllene	0.06 \pm 0.04	0.10 \pm 0.04	0.069
19.86	1488	Germacrene-D	0.12 \pm 0.04	0.35 \pm 0.07	0.012 *
20.14	1520	Non-identified ST	0.19 \pm 0.06	0.45 \pm 0.20	0.367
21.7	1668	Valerianol	0.09 \pm 0.03	0.06 \pm 0.03	0.653
		Total volatiles	1.08 \pm 0.39	1.42 \pm 0.61	0.818

Wilcoxon–Mann–Whitney *U* test.*Significant difference at $P < 0.05$.

et al. (2019). These results are also in agreement with our previous studies (Braccini *et al.*, 2013, 2015) reinforcing the idea that orientation of *N. oligospilus* females on *Salix* spp. is driven by volatile cues and final decision for egg laying is determined by a balance among leaf structure, plant nutrients and secondary metabolites. Additionally our results contributed to increase our understanding of the factors that modulate oviposition behaviour in the poorly studied South American native willow *S. humboldtiana*, in this case, by revealing an important role of conspecific feeding in modulating female oviposition choices.

Our study also showed that neonate hatching and larval performance was severely impaired on previously damaged plants. Herbivorous insects laying their eggs on plant tissue usually face the risk of aggressive plant responses that are detrimental to the eggs and/or larvae. Egg-induced plant defence strategies directly targeting the eggs may include plant-mediated desiccation of eggs, egg dropping, egg crushing and egg killing (Hilker and Fatouros, 2015). After hatching, larval performance and its rate of development can be affected by the quality of the food they received at very early stages. In Lepidoptera, for example, it was demonstrated that young larvae are especially sensitive to low dietary N or the presence of secondary metabolites (Zalucki *et al.*, 2002). A variety of secondary metabolites commonly found in *Salix* spp. like phenolics, tremulacin, protein inhibitors and tannins affect the performance of neonates from different species (Zalucki *et al.*, 2002 and references there in). These changes could be mediated by phytohormones (like jasmonic or salicylic acid). Actually, jasmonic acid, not salicylic, was induced in poplar trees after feeding of leaf-chewing herbivores in damaged and adjacent undamaged leaves (Fabisch *et al.*, 2019). Moreover, defoliation by sawflies is known to modify the N content of the foliage and consequently its nutritive value [i.e. *Pinus resinosa* (Reich *et al.*, 1990) and *Picea glauca* (Cook *et al.*, 1978)]. Thus, it appears that primary compounds, such as N, play important roles in sawflies development. Likewise, the growth of certain species of sawflies larvae is reduced when they feed on plants that were previously defoliated [i.e. *Oporinia autumnata*-*Betula pubescens*; *Lymantria dispar*-*Quercus robur* (Wagner and Raffa, 1993)]. Niemela *et al.* (1984) studied the short-term effects of defoliation on *Neodiprion sertifer*, *Gilpinia virens* Klug, *Microdiprion pallipes* Fall and *Diprion pini*, as well as the long-term effects on *N. sertifer*. They observed an increase in the mortality of *M. pallipes* and an increase in the rate of development of

N. sertifer and *G. virens*. Here, we show that *N. oligospilus* responds in the same way as other sawflies and both egg and larval stages, at least during the first days of development, are affected by previous herbivory.

It is usually assumed that plant domestication for agricultural usage has strongly affected plant–insect interactions (Chaudhary, 2013; Chen *et al.*, 2015). Plant chemical defences are generally expected to be weakened as a result of selective breeding because the defences may be costly to produce (Herms and Mattson, 1992) and also divert resources away from plant growth and yield (Rosenthal and Dirzo, 1997). As it was mentioned before, *S. humboldtiana* is a rustic species that has not gone through domestication. Our results showed that only a 15% defoliation by larval feeding initiated 48 h before the experiments elicited a significant female rejection (even on leaves adjacent to the site of damage) and a dramatic drop in performance and survival of the progeny. This observation agrees with the hypothesis that monophagous insects are more affected by food quality than polyphagous ones (Wallner, 1987).

The relationship between female choice for oviposition and the resulting offspring survival and development has often been considered in the context of the PPH. Here, *N. oligospilus* females avoided damaged plants as oviposition substrates suggesting that they were able to differentiate damaged from undamaged plants. Moreover, a strong positive correlation between female oviposition preference and offspring performance was found. Four out of six parameters of performance evaluated in this study were affected by the substrate where sawflies developed as embryos and during the first days of larval development. The affected parameters included critical steps of insect development, such as hatching and larval growth and survival. This good correlation between female's selection and offspring performance was recently confirmed for another specialist phytophagous sawfly, *Tequus schrottkyi* (Konow) (Altesor and Gonzalez, 2018). On the contrary, in a previous study, we found that *N. oligospilus* female's selection for oviposition between different *Salix* commercial varieties clearly did not maximize larval and adult performance (Braccini *et al.*, 2013). Thus, at least within the *Salix* genus, the preference–performance theory seems to match in the context of plants with herbivory-induced defences.

Our study suggests both a quick and intense reaction of the plant to insect damage and a high sensitivity of the insect to the up-regulated defences. Plant defence priming is a

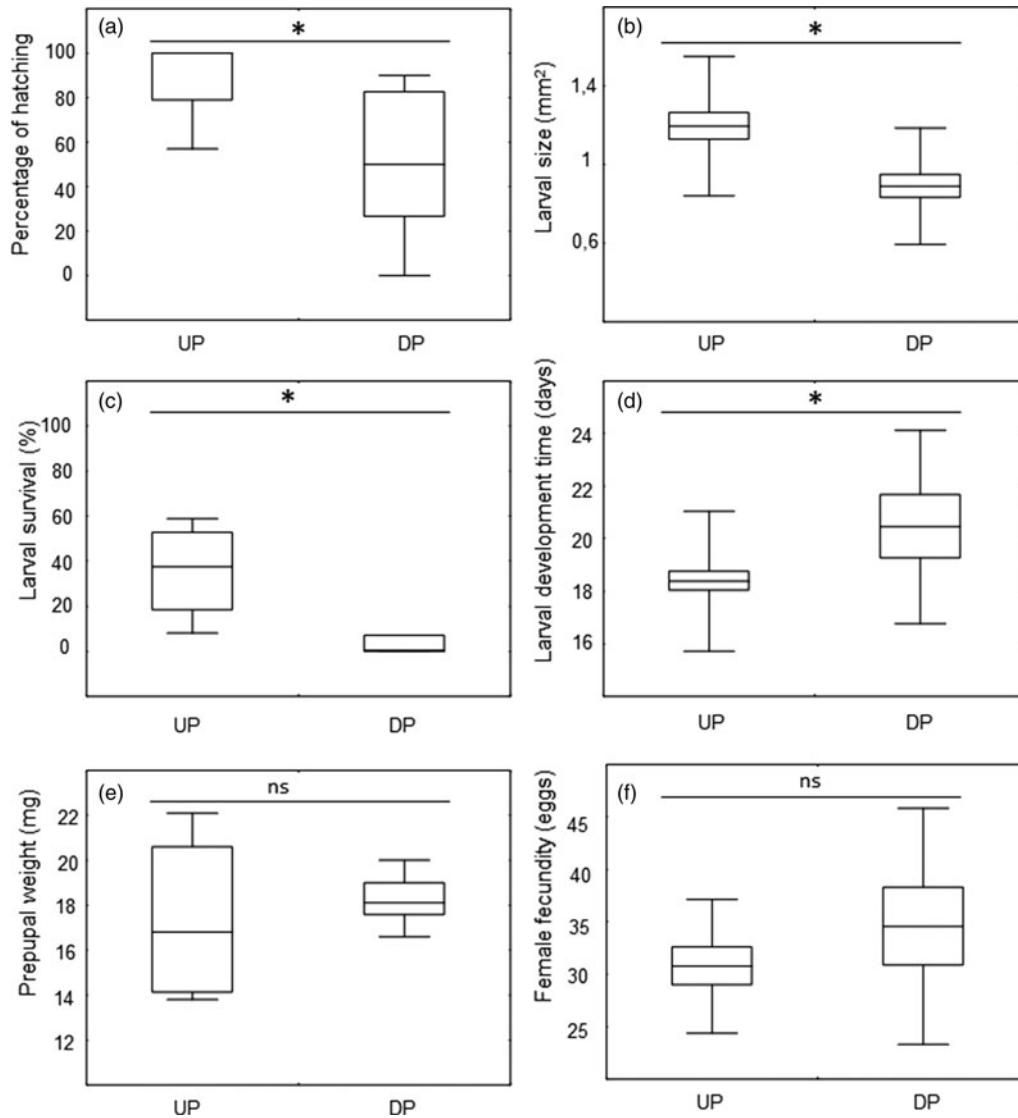


Figure 4. Larval performance and female fecundity of *Nematus oligospilus* insects developed on UP (undamaged) and DP (feeding damage) from *Salix humboldtiana*. (a) Percentage of hatching, $N = 4$ plants, $n = 173$ eggs and $N = 8$ plants, $n = 294$ eggs respectively for UP and DP. (b) Larval size (mm^2), $N = 24$ and $N = 26$, respectively, for UP and DP. (c) Larval survival (%), $N = 4$ and $N = 11$, respectively, for UP and DP. (d) Larval development time (days), $N = 52$ and $N = 9$, respectively, for UP and DP. (e) Pre-pupal weight (mg; $N = 11$ and $N = 8$ for UP and DP. (f) Female fecundity (number of eggs), $N = 12$ and $N = 9$, respectively, for UP and DP. For percentage of hatching, larval survival, pre-pupal weight and female fecundity, boxes represent the first to third quartiles range with the median indicated by a line across the box. For larval size and larval development time boxes represent the standard error and the mean is indicated by a line across the box. *Significant difference at $P < 0.05$.

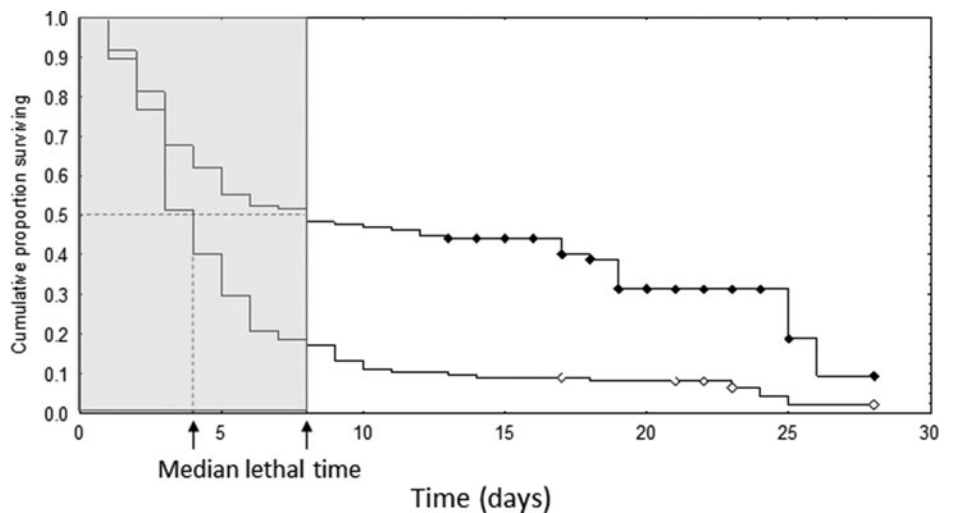


Figure 5. Cumulative proportion of surviving *Nematus oligospilus* larvae that initiated development on undamaged (UP, black dots) or damaged (DP, white dots) *Salix humboldtiana* plants. For larvae that started development in DP, the grey zone indicates the time spent feeding on DP, while the white zone indicates the time spent feeding on UP. Dots indicate the transition from larva to pupa of a given individual. Decrease in survival from day 1 to 28 is significantly different between treatments (Kaplan–Meier survival analysis, initial $N = 52$ for UP and $N = 9$ for DP, $P \leq 0.0001$).

physiological process by which a plant prepares itself for a quicker or more aggressive response to future biotic or abiotic stress (Frost *et al.*, 2008a). Our results suggest that the plant was possibly primed by the previous larval feeding that led to the high reduction in egg and larval survival we observed. This priming response has already been described for other species of the Salicaceae family (Frost *et al.*, 2008b).

To conclude, this work provided new insights into the role of previous herbivory on female behaviour in a poorly explored *Salix*-sawfly system. Further work should concentrate on the analysis of the effects of secondary metabolites produced by the tree after herbivore damage on the sawfly, which opens the possibility to contribute to pest management and to the implementation of willow genetic improvement programmes. Particularly, Germacrene-D emerges as a candidate compound to be evaluated as an allomone.

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