Electrophysiological localization of plant factors affecting pea aphid (*Acyrthosiphon pisum*) compatibility to host and non-host plants

Dissertation

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by Dipl. Biol. Alexander Schwarzkopf

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in Düsseldorf

Reviewers:	Prof Dr Jonathan Gershenzon; Max-Planck-Institut für Chemische Ökologie; Jena, Germany
	Prof Dr Rolf Beutel; Friedrich-Schiller-Universität Jena; Institut für Spezielle Zoologie und Evolutionsbiologie mit Phyletischem Museum; Jena, Germany
	Prof Dr Jean-Christophe Simon; Institut National de la Recherche Agronomique (INRA); Institut de Génétique, Environnement et Protection des Plantes; Rennes, France
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Contents

1 General Introduction

One of the most fascinating questions in biology is which mechanisms drive speciation – the evolution of new species. The current and well recognized concept of ecological speciation describes the speciation process as emanating from the adaptation of populations of one species to different ecological conditions (Nosil, 2012). If this differential adaptation triggers genetic divergence between the subpopulations, this might lead to the reproductive isolation between the populations. This means the individuals of the populations of the original species are not able to mate successfully anymore and can be considered as new biological species.

But what initiates situations, in which populations of one species are forced to adapt to different ecological conditions? One possibility is a geographical separation of a species population resulting in subpopulations encountering different ecological conditions in spatially separated habitats. If this leads to genetic divergence and reproductive isolation between the subpopulations, the speciation mode is called allopatric speciation (Mayr, 1982). Another possibility is that populations of one species encounter different ecological conditions without any spatial separation, i.e. in one and the same habitat. If ecological conditions lead to genetic divergence and reproductive isolation without spatial separation, the speciation mode is called sympatric speciation (Berlocher & Feder, 2002). It is yet not well understood ecological factors influence speciation since individuals are in principal able to meet and mate in their habitat, which would lead to a continuing gene flow within the population. In plant-insect interactions, there is a mechanism which seems to be common to many examples of sympatric speciation: insect specialization on certain host plants, which may result in host shifts and possibly "host race" formation (Dres & Mallet, 2002). Such host races usually show differential plant preferences, differential host success (i.e. the ability to reproduce on a certain plant but not others), and other phenotypic differences. There are a couple of examples for plant-insect interactions in which the presence of host races and a sympatric mode of speciation has been documented (reviewed in Dres & Mallet, 2002) e.g. host races of the apple maggot fly *Rhagoletis pomonella* living on apple *(Malus pumila)* or hawthorn *(Crataegus mollis)*, host races of the willow leaf beetle *Lochmaea capreae* living on birch *(Betula pubescens)* or sallow *(Salix caprea),* and the host races of the pea aphid *Acyrthosiphon pisum* living on dozens of plant species belonging to the Fabaceae including *Medicago sativa*, *Pisum sativum*, *Trifolium pratense* an *Vicia faba* and many more (Ferrari *et al.*, 2006; Peccoud *et al.*, 2009a).

1.1 The pea aphid species complex

As all species belonging to the Aphididae, the pea aphid *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae) exhibits a piercing-sucking lifestyle. This means that aphids penetrate the plant tissue with their specialized mouthparts forming a straw-like structure in order to ingest their diet – the nutritious phloem sap. In contrast to other aphid species, the pea aphid is highly specialized and restricted to living and feeding on plants belonging to the Fabaceae (Peccoud *et al.*, 2009a). Ecological and modern molecular methods support the fact that the pea aphid represents not one species, but a species complex consisting of at least 15 genetically distinct host races, also called biotypes (Peccoud *et al.*, 2015). These biotypes show distinct plant preferences and distinct degrees of host fidelity on different legume species often co-occuring in one habitat. Hence, the pea aphid represents a good model organism for studying ecological speciation under sympatric conditions. This has been demonstrated during the last decades by an increasing number of studies (Via, 1999; Ferrari *et al.*, 2006; Peccoud *et al.*, 2009a; Peccoud *et al.*, 2009b; Peccoud & Simon, 2010). Moreover, three of the pea aphid biotypes characterized so far fulfill an important part of the biological species concept: hardly any hybrids could be detected amongst sympatric biotypes (Peccoud *et al.*, 2009a). The driving force for pea aphid speciation was most likely the adaptation of subpopulations to different legume plant species (Peccoud *et al.*, 2009b). A number of divergence events based on adaptive radiation could be documented using rapidly evolving sequences of the obligate pea aphid endosymbiont *Buchnera aphidicola* and dated to have happened about 9000 – 6000 years ago. The consequence of the repeated divergence events was a burst of diversification within the pea aphid species complex probably enhanced by anthropogenic range expansion of legume crops serving as pea aphid host plants in combination with global temperature rise (Peccoud *et al.*, 2009b). This scenario may have led to the variety of pea aphid biotypes one can observe in nature at present. Interestingly, there is one legume plant towards which all aphid biotypes characterized so far show similar preference as to their native host plant: *Vicia faba.* This species is suspected to play a special role for the evolution of the pea aphid species complex, as the universal preference of all biotypes for *V. faba* enables ongoing gene flow despite the specialization and restriction of the biotypes to other non-overlapping plants. *V. faba* serves as a universal host plant on which different pea aphid biotypes can survive and have the ability to meet and mate. A number of questions can be posed regarding the current situation, including which ecological factors related to the legume plants involved influence the speciation process. More precisely, which plant factors contribute to the maintenance of pea aphid host races despite the ongoing gene flow amongst pea aphid biotypes living in the same habitat?

1.2 Plant factors influence plant aphid interactions

The impact of plant factors on aphids can be characterized by measuring the performance (e.g. survival or growth) of aphids on various plant species or cultivars. If an aphid shows good performance on a certain species or cultivar, the plant is considered susceptible and the plant-aphid interaction can be called compatible. If the performance is instead poor, the plant is considered resistant and the plant-aphid interaction can be called incompatible. Knowledge about the degree of compatibility of aphid species or biotypes to a plant serves as a basis for elucidation of plant factors mediating such compatibility. Numerous plant factors influencing the compatibility of aphids to certain plant species were described previously (Goggin, 2007). The first contact between aphid and plant is with the aphid mouthparts (the "stylet bundle" consisting of the aphid's maxilla and mandible forming a straw-like structure and containing a food canal, a salivary canal and a neuronal canal (Myazaki, 1987; Tjallingii & Esch, 1993)) The second contact interface is the aphid gut, which is connected to the plant by the food canal in the stylet bundle. Hence, the aphid stylet bundle and the aphid gut contact the vast variety of plant factors which have the potential to influence the entire penetration process from the plant surface through the mesophyll to the sieve elements. When an aphid starts to insert its stylet bundle into the plant tissue, physical barriers might prevent the access to the plant tissue and thus to the aphid's diet, the phloem sap. Examples are the hairs of *Solanum* species (Alvarez *et al.*, 2006), or an impenetrable cell wall (Campbell *et al.*, 1986). Aphid nutrition can be impaired directly by poor nutrition, such as an unsuitable amino acid composition of the phloem sap (Sandstrom, 1994). Besides such physical or nutritional effects, chemical factors such as the presence of quinolizidine alkaloids in *Cytisus scoparius* (Wink et al., 1982) can lead to the incompatibility of an aphid with a plant. In turn, the recognition of chemical compounds specific to the aphid's host plant can positively influence the ability to establish feeding on the plant. Such chemical compounds important for aphid host choice have been found in plant epicuticular layers (Powell *et al.*, 1999). Other important factors influencing plant-aphid compatibility are associated with plant defence mechanisms and plant immunological factors. Prominent examples are proteins located in plant sieve elements called P-proteins which, if activated by aphid attack, block the sap flow in the sieve elements. In other words, P-proteins can mediate a shut-down of the aphid's diet flow (Will *et al.*, 2009). Moreover, plant R-gene products controlling downstream aphid resistance mechanisms (e.g. hypersensitive response and cell death) have been shown to have a high impact on aphid-plant compatibility (reviewed in Hogenhout & Bos, 2011).

To get information about the nature and mechanisms of plant factors influencing aphid plant compatibility in a certain plant-aphid interaction it is very useful to start by finding out where the putative plant factors are localized. Studies in this dissertation focus on the question of which tissues or cells of legume plants contain factors influencing feeding choice of pea aphid biotypes. Based on knowledge about the localization of these factors, it might be possible to infer the mechanism contributing to the continuity of pea aphid biotypes under sympatric conditions.

To localize the plant factors influencing pea aphid-plant compatibility, two basic questions should be asked: 1) in which tissue is the factor encountered, before the plant penetration process starts, in the epidermis, in the mesophyll, or in the sieve elements), and 2) is the factor encountered in the intracellular or extracellular space of the plant? An excellent method to answer these questions is the Electrical Penetration Graph (EPG) technique, which is an electrophysiological method allowing a localization of plant factors influencing the aphid's probing and feeding behavior with a very high temporal and spatial resolution (Tjallingii, 1988).

1.3 Electrophysiological plant factor localization using the Electrical Penetration Graph technique (EPG)

The ability of an individual aphid to reach the sieve elements and to start feeding is a crucial requirement for its ability to survive on the plant. To reach their diet – the sugar-rich phloem sap – and hence to survive on a plant, aphids insert their stylet bundle into the plant tissue and navigate towards the plant's vascular system (Hewer *et al.*, 2011). This process might take minutes or a couple of hours (Schwarzkopf *et al.*, 2013). During this penetration process, the stylet bundle is mainly moved through the plant apoplast, i.e. extracellularly (Tjallingii & Esch, 1993). However, nearly every cell along the stylet pathway through the tissue is punctured briefly, i.e. the stylet bundle tip is briefly moved into the cell lumen (Powell *et al.*, 1995). If the aphid reaches the vascular bundle and is able to insert its stylet bundle into a sieve element, it can start to ingest the phloem sap (Srivastava, 1987). The entire plant penetration process can be observed and recorded using the Electrical Penetration Graph (EPG) technique. This sophisticated technique was invented originally by McLean and Kinsey (1964) and remarkably elaborated by Dr. Freddy Tjallingii during the following decades (Tjallingii, 1978; Tjallingii, 1988; Tjallingii & Esch, 1993). The technique is based on an electrical circuit, in which an individual aphid as well as a plant are integrated (Chapter 2 Figure 1). The EPG device connected to a computer enables the real-time monitoring of aphid probing and feeding

behavior on the plant which manifest in voltage fluctuations measured over time (Tjallingii, 1985). As a result, in an EPG recording various waveforms can be observed which are known to correlate with specific behaviors occurring in specific plant tissue depending on where the stylet bundle tip is located (for an overview, please refer to Tjallingii, 1988). For an example, if an aphid does not insert its stylet bundle into the plant tissue, this results in a very long phase called non-probing (i.e. a flat-line in the EPG recording). This would point to plant factors located at the plant surface (e.g epicuticular waxes) which prevent the aphid from starting plant penetration. If the aphid is able to start penetrating the plant tissue, but withdraws its stylet bundle again and again after a couple of seconds, this might reflect encounters with plant factors located in the upper tissue layer (epidermis) that are repellent. Alternatively, plant factors which might be important for positively stimulating forward movement of the stylet bundle might be not present. An indication for factors located in the mesophyll that impair further penetration towards the sieve elements would be a very long duration of the aphid in the so called pathway phase, a phase reflecting aphid stylet movement in the mesophyll tissue. There are many more EPG waveforms that indicate specific behaviors such as salivation, feeding on the sieve elements, and whether the stylet bundle is intracellular or extracellular.

For a detailed description of which EPG waveforms were used in plant factor localization in the framework of this dissertation, please refer to Figure 1 in the second chapter of this dissertation and to chapter 4 which especially deals with the disentanglement of the intra- and extracellular location of plant factors.

The great value of the EPG technique for localizing plant factors influencing aphid-plant compatibility was successfully demonstrated repeatedly by studies on various plant-aphid interactions (e.g. Klingler *et al.*, 1998; Kaloshian *et al.*, 2000; Alvarez *et al.*, 2006; Gao *et al.*, 2008; Pallipparambil *et al.*, 2010). These data show that the localization of factors influencing the plant compatibility of aphids seems not to be much conserved amongst the Aphididae, but to be localized to nearly all tissue levels which the aphid encounters from the beginning of the plant contact until the aphid reaches a sieve elements and starts feeding.

Studies on pea aphid biotypes that compared aphid behavior on host (in a broader sense susceptible or compatible) and non-host (in a broader sense resistant or incompatible) legume plants imply that plant factors influencing aphid behavior are located on the plant surface (Wilkinson & Douglas, 1998) or in the upper tissue levels (epidermis and mesophyll) (Caillaud & Via, 2000). This means that it is more likely that plant factors blocking the aphid's ability to reach the sieve elements and to establish feeding are responsible for the incompatibility of certain pea aphid biotypes on certain plants, than plant factors which might be toxic to the pea

aphid. However, both studies tested a restricted range of plant-aphid biotype combinations. Caillaud's investigation focused narrowly on the interaction of biotypes from *Trifolium pratense* or *Medicago sativa* on *T. pratense* and *M. sativa*. Wilkinson used a wider range of pea aphid biotypes (from *M. sativa, Pisum sativum* and *T. pratense*), but tested those only on *P. sativum* and *V. faba*. Differential plant preferences amongst sympatric pea aphid biotypes are accompanied by a continuum of genetic divergence(Peccoud *et al.* (2009a). Thus, it is important to conduct plant factor localization studies on a selection of legume plants to which the pea aphid biotypes show a differential compatibility. The classification of compatibility should range from plants on which the aphid biotypes show a high compatibility (called host plants), plants on which the aphid biotypes show an intermediate compatibility (called less suitable plants) and plants on which the compatibility is very low or not present (called non-host plants). Additionally, testing routinely all pea aphid biotypes on *V. faba* might give deeper insight into the putative role of *V. faba* as universal host plant.

There are several more open questions which were not well answered in previous pea aphid studies. Firstly, how does the plant species on which aphids were previously reared influence aphid probing and feeding behavior in the context of plant factor localization? It is known that aphid feeding experience is able to influence the subsequent plant preferences of other aphids and their probing and feeding behavior on different plant species (McCauley *et al.*, 1990; Liu *et al.*, 2008; van Emden *et al.*, 2009). Secondly, do intracellular aphid behavioral patterns contribute significantly to the aphid-plant compatibility? The intracellular behavioral patterns of aphids (salivation and ingestion of cell content) are difficult to study and, hence, not often investigated in the analysis of EPG experiments. However, there is indication that the salivation into the cell lumen and ingestion of cell content might enable delivery of aphid effectors to the plant (Hogenhout & Bos, 2011), modifying its physiology but providing important cues for plant recognition (Hewer *et al.*, 2011). As this might influence pea aphid-plant compatibility significantly, it appears to be essential to thoroughly analyze EPG parameters reflecting intracellular behavioral patterns.

1.4 Objectives of this study

This dissertation focuses on the electrophysiological localization of plant factors influencing the feeding choice of pea aphid biotypes using the Electrical Penetration Graph Technique (EPG).

In chapter 2 the degree of compatibility of a selection of pea aphid biotypes was determined in order to localize plant factors influencing probing and feeding behavior.

The work in chapter 3 investigated the role of plant experience during the aphid rearing period preceding the plant factor localization experiment to determine the experience influence on pea aphid probing and feeding behavior on plants different from the rearing plant.

Chapter 4 describes research to determine if plant factors influencing pea aphid-plant compatibility are localized extracellularly (i.e. in the plant apoplast) or intracellularly (i.e. in the plant cytoplasm or vacuole) and if intracellular behavioral patterns such as aphid salivation into the cell lumen or cell content ingestion contribute significantly to pea aphid biotype-plant compatibility.

The implications of the results for the maintenance of pea aphid host races occurring sympatrically are discussed in the last chapter (chapter 5) of this dissertation.

This thesis is based on the following manuscripts:

Chapter 2

To Feed or Not to Feed: Plant Factors Located in the Epidermis, Mesophyll, and Sieve Elements Influence Pea Aphid's Ability to Feed on Legume Species.

Alexander Schwarzkopf, Daniel Rosenberger, Martin Niebergall, Jonathan Gershenzon, Grit Kunert

Department of Biochemistry, Max Planck Institute for Chemical Ecology, Jena, Germany

Published in Plos One 8(9) 2013

Pea aphid biotypes show distinct plant preferences. In chapter 2, I first determined the performance of selected pea aphid biotypes on various legume species, and then employed the electrical penetration graph technique (EPG) to localize plant factors influencing probing and feeding behavior on these plants. The results show that plant factors influencing the ability of biotypes to feed on certain legumes are localized in epidermis, mesophyll and sieve elements. Potential mechanisms causing the altered behavior on certain plants are discussed.

Conceived by: Alexander Schwarzkopf and Grit Kunert

Designed the experiments: Alexander Schwarzkopf (80 %) and Grit Kunert

Performed the experiments: Alexander Schwarzkopf (90 %) and Daniel Rosenberger

Analyzed the data: Alexander Schwarzkopf (80 %) and Grit Kunert

Contributed analysis tools: Alexander Schwarzkopf (50 %) and Martin Niebergall

Wrote the paper: Alexander Schwarzkopf (70 %) Grit Kunert and Jonathan Gershenzon

Chapter 3

"You can't change the habits of a lifetime" or Feeding experience enhances host plant fidelity of pea aphid *(Acyrthosiphon pisum)* clones

Alexander Schwarzkopf, Daniel Rosenberger, Jonathan Gershenzon, Grit Kunert

Department of Biochemistry, Max Planck Institute for Chemical Ecology, Jena, Germany

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Usually, pea aphid biotypes are able to reach a sieve element and start feeding easily on their native and the universal host plant *Vicia faba*. It was observed however, that the Medicago biotype was not able to start feeding on its native host plant *Medicago sativa* after being reared on the native host *Vicia faba*. The work in chapter 3 investigated how feeding on various legumes during early development altered pea aphid probing and feeding behavior on different plant species at later stages.

Conceived by: Alexander Schwarzkopf and Grit Kunert

Designed the experiments: Alexander Schwarzkopf (80 %) and Grit Kunert

Performed the experiments: Alexander Schwarzkopf (90 %) and Daniel Rosenberger

Analyzed the data: Alexander Schwarzkopf (90 %) and Grit Kunert

Wrote the manuscript: Alexander Schwarzkopf (70 %) Grit Kunert and Jonathan Gershenzon

Chapter 4

Electrical Penetration Graph Technique (EPG) reveals a possible association of intracellular punctures and host-plant compatibility in the pea aphid (*Acyrthosiphon pisum*)

Alexander Schwarzkopf, Daniel Rosenberger, Jonathan Gershenzon, Grit Kunert

Department of Biochemistry, Max Planck Institute for Chemical Ecology, Jena, Germany

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The work in chapter 2 showed that plant factors are localized in multiple plant-tissue types. It is likely that plant factors influencing probing and feeding behavior of pea aphids are located inside the cells of the mentioned tissues. Chapter 4 describes whether aphid intracellular behavior, such as aphid salivation into the cell lumen or cell content ingestion, contributes significantly to feeding ability. The results did not show a significant connection between the duration of intracellular salivation or ingestion and feeding ability. However, there is evidence for the relevance of another yet unknown intracellular behavioral pattern for the feeding ability of biotypes on host plants.

Conceived by: Alexander Schwarzkopf and Grit Kunert

Designed the experiments: Alexander Schwarzkopf (80 %) and Grit Kunert

Performed the experiments: Alexander Schwarzkopf (90 %) and Daniel Rosenberger

Analyzed the data: Alexander Schwarzkopf (90 %) and Grit Kunert

Wrote the manuscript: Alexander Schwarzkopf (70 %) Grit Kunert and Jonathan Gershenzon

2 To Feed or Not to Feed: Plant Factors Located in the Epidermis, Mesophyll, and Sieve Elements Influence Pea Aphid's Ability to Feed on Legume Species

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To Feed or Not to Feed: Plant Factors Located in the Epidermis, Mesophyll, and Sieve Elements Influence Pea Aphid's Ability to Feed on Legume Species

Alexander Schwarzkopf, Daniel Rosenberger, Martin Niebergall, Jonathan Gershenzon, Grit Kunert*

Department of Biochemistry, Max Planck Institute for Chemical Ecology, Jena, Germany

Abstract

The pea aphid (*Acyrthosiphon pisum* Harris), a legume specialist, encompasses at least 11 genetically distinct sympatric host races. Each host race shows a preference for a certain legume species. Six pea aphid clones from three host races were used to localize plant factors influencing aphid probing and feeding behavior on four legume species. Aphid performance was tested by measuring survival and growth. The location of plant factors influencing aphid probing and feeding was determined using the electrical penetration graph (EPG) technique. Every aphid clone performed best on the plant species from which it was originally collected, as well as on *Vicia faba*. On other plant species, clones showed intermediate or poor performance. The most important plant factors influencing aphid probing and feeding behavior were localized in the epidermis and sieve elements. Repetitive puncturing of sieve elements might be relevant for establishing phloem feeding, since feeding periods appear nearly exclusively after these repetitive sieve element punctures. A combination of plant factors influences the behavior of pea aphid host races on different legume species and likely contributes to the maintenance of these races.

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* E-mail: gkunert@ice.mpg.de

Introduction

The pea aphid (*Acyrthosiphon pisum* Harris) is confined to plants of the family Fabaceae. Within the last 6500–9500 years, this aphid underwent a rapid genetic diversification involving host plant shifts [1], probably influenced by global warming and anthropogenic range expansion of potential hosts. As a consequence, pea aphid populations now occur sympatrically on legume crop plants [2] as well as on legume species in natural habitats [3]. Pea aphid populations are often very specialized, performing best on the particular legume species on which they are found (called native host plant), but showing significantly reduced performance, or not surviving at all on other legumes [4,5]. However, all pea aphid populations tested so far perform as well on *Vicia faba* as on their native host plant. Thus, *V. faba* can be considered as a "universal host plant" for the genetically diverse populations of this species [4,5]. By investigating more than 1000 wingless pea aphids from 19 legume species in western Europe, Peccoud et al. [3] identified 11 genetically distinct and sympatrically occurring pea aphid races associated with different legume host plants. Analysis of migration and hybridization among these races led to delineation of three possible species and eight host races.

The distinct plant preferences of pea aphid host races lead to assortative mating which reduces gene-flow [2,6]. On the other hand the presence of *V. faba* as a universal host allows the different races to meet and mate [5]. The presence of ongoing gene flow amongst host races raises the question of how the host races are maintained. Plant factors are very likely to be involved as aphid feeding behavior involves an intimate relationship with its host. Numerous studies on different plant-aphid systems have shown that a range of plant factors influence the plant-aphid interaction (reviewed in [7–9]). Relevant plant factors differ among various plant-aphid combinations and can function at different stages of host selection as aphids land on the plant, penetrate tissues with their stylets and establish feeding sites in phloem. For example, plant factors that influence aphid host selection can be located at the plant surface in the form of attractive [10] or repellent volatiles [11], deterrent epicuticular lipids [12] or glandular trichomes [13]. Such factors can also be located elsewhere in the plant including deterrent gustatory cues in the epidermis [14], or compounds inhibiting stylet penetration in the mesophyll [13]. In sieve elements (SEs), phloem sap may have low nutritional value for the aphid [15], and barriers that prevent the aphid from starting to feed [13,16–19]. In addition to attractive or deterrent plant factors, different nutrient levels or the presence or absence of certain compounds may also influence aphid host selection [20]. The variety of such factors and their distinct mode of action in different plant species may have been critical in driving aphid speciation. Thus to understand the diversification among pea aphid lineages, the nature of factors affecting host selection among closely related aphid taxa must be better investigated. The results should be applicable to other polymorphic aphid species or species complexes that feed on an assortment of different plant species.

An excellent method to investigate plant-aphid interactions and to localize plant factors that influence these interactions is the electrical penetration graph (EPG) technique [13,14,16 19,21,22] (Figure 1) which monitors aphid probing and feeding behavior in detail. By comparing numerous parameters of the probing and feeding behavior of aphid individuals on resistant and susceptible plant species it is possible to detect and locate plant factors influencing plant resistance or susceptibility. This approach has also been used to detect and localize plant factors influencing the host range of pea aphids on different legume species [22 24]. Whilst Wilkinson and Douglas [23] investigated mainly interclonal differences in the probing and feeding behavior of pea aphid clones from the Medicago, Pisum and Trifolium races on *Pisum sativum* and the universal host plant *V. faba*, other authors (Caillaud [24]; Caillaud and Via [22]) focused on susceptible and resistant plants by using clones from the Medicago and Trifolium races on *Medicago sativa* and *Trifolium pratense*. However, to more fully account for plant specific factors a broader overview of different host plants in combination with different aphid clones from multiple host races is needed.

A previous study on two pea aphid races revealed that individuals are able to recognize the suitability of a potential host plant by briefly puncturing epidermal or subepidermal plant cells [22]. Later it was shown by Del Campo et al. that this behavior depends on recognition of chemical substances from the native host plant [25]. Pea aphid clones with either *M. sativa* or *T. pratense* as native hosts were able to recognize stimulants in the extract from their respective native host. Although both studies [22,25] made important advances in understanding pea aphid host plant use, many questions remain unsolved, such as where the recognized chemical substances of Del Campo et al. [25] are located. Additionally, beyond simple host vs. non-host recognition [22], pea aphid races can accept plants other than their native or universal host plant and show an intermediate performance [3]. Feeding of aphids on such intermediate hosts might facilitate hybridization among host races and act against speciation.

Plant factors influencing aphid performance after initial host/non-host choices have been made are more likely to be in deeper plant layers including the phloem. Previous studies demonstrated that features of the sieve elements (SEs) themselves were responsible for reduced feeding in various plant-aphid associations:

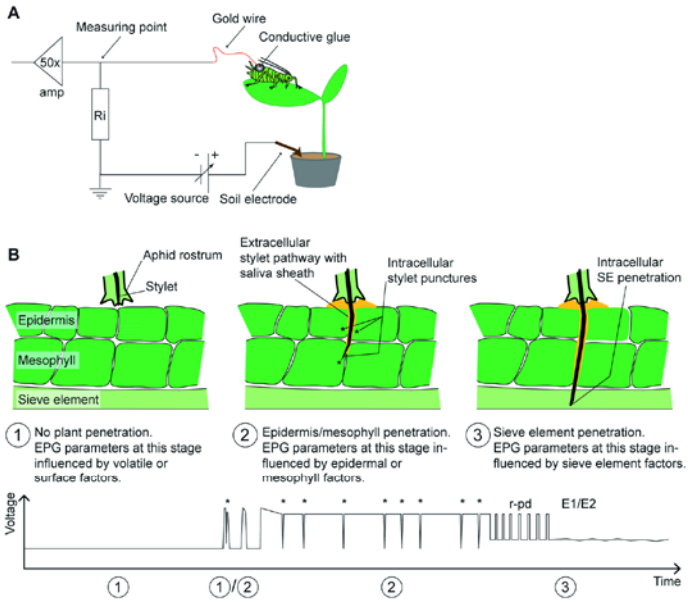

Figure 1. Principle of EPG technique and plant factor localization modified after Tjallingii [53]. A) Principle of EPG technique: the aphid is connected to the EPG device using conductive glue and a thin gold wire. The plant is connected to the EPG device by inserting an electrode into the soil next to the plant. As soon as the aphid starts plant penetration the electrical circuit is closed and EPG waveforms can be observed and recorded. Amp = amplifier, Ri = input resistor. B) Simplified illustration of various stages in the penetration of plant tissue by the aphid stylets correlated with a schematic representation of EPG recordings below. The parameters derived from the EPG recordings (listed in Table S1) indicate the tissue location of plant factors influencing stylet penetration and feeding behavior. (1) As long as the aphid does not penetrate the plant a flat line, called non-probing waveform (np) is visible. EPG parameters from this stage are influenced by volatile or surface plant factors influencing aphid feeding behavior (Table S1, parameters #1–#4). (1)/(2) When the aphid starts penetration, short probes can often be observed, some with cell punctures (potential drops (pd) marked by asterisks) and separated by non-probing periods. The number and duration of short probes are influenced by factors in the epidermis (parameter #5) or the mesophyll (#8). (2) During the pathway phase (#15) the aphid navigates its stylet bundle through the plant apoplast towards the sieve elements (SEs). Almost every single cell along the stylet pathway is punctured (pd, marked by asterisks) by the aphid's stylet (#16). Aphid activities during epidermis and mesophyll penetration are reflected in parameters #5–#24. (3) Parameters #25–#51 reflect SE factors, important as the SEs are the aphid's ultimate feeding target. Before SE salivation (E1) and ingestion (E2), *A. pisum* often carries out extended and repetitive cell punctures (r-pd).
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in the pea aphid and in *Acyrthosiphon kondoi* on *Medicago truncatula* [19,26], in *Macrosiphum euphorbiae* on *Solanum lycopersicum* [17,27], and in *Aphis gossypii* on *Cucumis melo* [28]. In these cases, plant resistance genes (*R*-genes) were found to be involved in SE-specific aphid resistance. *R*-genes mediate the recognition of effector proteins delivered by the aphid saliva to the plant (reviewed in [29]). On the other hand, aphid saliva effector molecules suppressing plant defense responses that are specific to aphid and host plant species were also characterized [30–33]. However, it remains unclear if plant *R*-genes and aphid salivary effectors play a role in determining the acceptance of host plants to the various pea aphid host races.

Our study focuses on the detection and localization of plant factors influencing probing and feeding behavior of various pea aphid host races on different legume species. These plant factors may contribute to the maintenance of host races in the pea aphid species complex. As it is crucial to know the performance of each clone on each plant species, we firstly characterized the performance (survival, growth) of six pea aphid clones belonging to three races on four legume species, including the native host plant, the universal host-plant *V. faba* and non-host plants. Secondly, we performed EPG recordings for each aphid clone-plant combination to localize putative plant factors responsible for differential performance.

Materials and Methods

Plants

Four different legume species were used in in this study: *Medicago sativa* cv. "Giulia" (Appels Wilde Samen GmbH, Darmstadt, Germany), *Pisum sativum* cv. "Baccara" (S.A.S. Florimond Desprez, Cappelle-en-Pévèle, France), *Trifolium pratense* cv. "Dajana" (Appels Wilde Samen GmbH, Darmstadt, Germany), and *Vicia faba* cv. "The Sutton" (Nickerson-Zwaan, Made, The Netherlands). All plants were reared in 10 cm diameter pots on plant substrate "Klasmann Tonsubstrat" (Klasmann-Deilmann GmbH, Geeste, Germany) in a climate chamber under the following conditions: 20°C, 70% relative humidity, 16 hours light per day.

Aphids

Six different *Acyrthosiphon pisum* HARRIS clones occurring sympatrically in Western Europe were used. The aphid clones were collected in the field from three legume species: clones "L1_22" and "L84" (called M1 and M2) from *M. sativa*; clones "P136" and "Colmar" (P1 and P2) from *P. sativum*; clones "YR2" and "T3_8V1" (T1 and T2) from *T. pratense* (for detailed clone information see Table S1 in [1]). All aphid clones were maintained on *V. faba* cv. "The Sutton" covered with air-permeable cellophane bags (Armin Zeller, Nachf. Schütz & Co, Langenthal, Switzerland) to prevent aphid cross-contamination. Conditions for all aphid rearing in this study were: 20°C, 70% relative humidity, 16 hours light per day.

For each experiment aphid clones were reared on *V. faba* starting from one apterous adult aphid which was placed on a *V. faba* plant and allowed to reproduce for two days. After two days, the adult aphid was removed and larvae kept on the plant until adulthood. These adult aphids were transferred to new *V. faba* plants (one aphid per plant). This rearing process was repeated several times until a sufficient number of aphids for the experiments were obtained.

Aphid Performance

Each aphid clone listed in the previous section was tested on the four legume species: *M. sativa*, *P. sativum*, *T. pratense*, and *V. faba*. At the beginning of the experiment ten first-instar larvae from each clone were placed on the soil close to the base of a 27 day-old plant. Each aphid clone-plant combination was replicated five times and set up in a spatially randomized pattern in a climate chamber. After nine days, all surviving individuals per plant were counted and weighed, and the average weight per surviving individual was calculated. Statistical analysis was performed by using R version 2.12.2 [34]. The effect of plant species, aphid clone and plant species-aphid clone interaction on the number of surviving aphids was tested using generalized linear models with a poisson/quasipoisson error family. The effect of plant species, aphid clone and plant species-aphid clone interaction on aphid survivor weight was tested with a two-factorial ANOVA.

Monitoring Aphid Probing and Feeding Behavior by the EPG Technique

Each aphid clone was tested on the four legume species: *M. sativa*, *P. sativum*, *T. pratense*, and *V. faba*. For each EPG recording, a 9–11 day old adult aphid was immobilized on a disposable pipette tip connected to a vacuum pump. A small droplet of conductive silver-glue (EPG Systems, Wageningen, The Netherlands) was applied to the aphid's dorsum. The tip of a 2 cm long gold-wire (diameter 20 µm) connected to an insect electrode (prepared from a 1.5 cm long copper pin) was inserted into the glue droplet. The wired aphid was placed on a 27–32 day-old experimental plant at the edge of the adaxial side of the uppermost fully developed leaf, which was fixed by a hair-clip. The soil electrode was inserted into the soil. This procedure was repeated eight times to equip each of the eight EPG probes of the direct current-EPG device ("GIGA-8", EPG Systems, Wageningen, The Netherlands). The experimental plants and the EPG device equipped with aphids were then placed in a Faraday cage. The EPG device was connected via an USB analog-digital converter device ("DI 710", DATAQ Instruments, Akron OH, USA) to a computer. As the aphid starts penetrating the plant by inserting its stylet bundle into the plant tissue, the electrical circuit is closed and EPG waveforms (i.e. voltage changes over time) can be recorded (Figure 1). EPG recordings were conducted for 4 hours using the software "Probe 3.5" (EPG Systems, Wageningen, The Netherlands). For each aphid clone-plant combination, 17–24 4 h EPG recordings were conducted in which aphids were successful in initiating probing during the recording time. In preliminary experiments, a time of 4 h was found to be sufficient for nearly all of our experimental aphid clones to reach a sustained feeding phase on their native host plants. The exceptions, clones of the Medicago host race, are described in the discussion section.

The beginning and the end of each EPG waveform (Table S1, "EPG waveforms"; Figure 1) in all EPG recordings were marked manually using "Stylet a+" software (version v01.00 26.08.2010, EPG Systems, Wageningen, The Netherlands). Subsequently, 54 EPG parameters representing aphid probing and feeding behaviors were calculated (Table S1, "EPG parameter") by using a Microsoft Office Excel Macro designed for our purposes. It calculates a wide range of standard EPG parameters like other Macros available for EPG data processing (e.g. [35]), but also calculates the number and total duration of repetitive SE puncture periods (r-pd) and their association with SE salivation (E1), SE feeding (E2) and sustained (longer than 10 min) SE feeding (Table S1, #41–#51). Statistical analysis was performed by using R version 2.12.2 [34]. The effect of each plant species on each of the 54 EPG parameters was tested for every clone separately. If an EPG parameter was observed in less than five replicates in an aphid clone-plant combination, the respective combination was excluded from the analysis. The effect of plant species on the

proportion of individuals showing a certain EPG parameter was tested by using the test for equality of proportions. Plant effects on the total time an aphid clone spent in a certain waveform during 4 h recording time were tested using one-factorial ANOVA (after appropriate data transformation, if necessary). In case of non-normality of the errors or inequality of variances, the non-parametric Kruskal-Wallis-Test was applied. Plant effects on the value of EPG parameters for each aphid clone during the 4 h recording time were tested by using generalized linear models with a poisson/quasipoisson error family. For full information about test statistics for each parameter and applied transformations please refer to Table S3. Plant effects on the average number of repetitive SE puncture periods with and without subsequent SE feeding phases and on the average number of SE feeding phases with and without preceding repetitive SE puncture periods were tested using generalized linear models with a binomial/quasibinomial error family (Table 3, Table S2).

Results

Aphid Performance

Survival. Aphid survival for each clone was assessed on *M. sativa*, *P. sativum*, *T. pratense*, and *V. faba*. When the performance of the various aphid clones was compared on the four legume species, nearly all of the aphids survived on their native host plants. This was also true for aphids on the universal host plant *V. faba* regardless of host race or clone (Figure 2 A, Table 1). On other plant species normally not used as hosts, aphid survivor numbers were significantly lower than on the native or universal host plant, as for the Pisum and Trifolium race clones on *M. sativa* plants. These plant species can be considered non-hosts for the respective aphid clones. Besides native and universal host plants on the one hand and non-host plants on the other, there is a third group of plant species on which aphid survival is essentially as good as on host plants (like clone P2 on *T. pratense*) or in between the survival on host and non-hosts (like clone M2 on *P. sativum* and *T. pratense* or clone P1 on *T. pratense*). These plant species are designated as less suitable or intermediate hosts. Within an aphid host race, the number of survivors of each clone was similar on the universal and the native host plants, but on less suitable or non-host plants the number of survivors sometimes differed. For example within the Medicago race, clone M1 showed a significantly lower survivor number on *T. pratense* compared to clone M2. Within the Pisum race, survival of clone P1 on *T. pratense* was significantly lower than survival of clone P2, which survived on *T. pratense* as well as on the native and universal host plants.

Survivor weight. In general, the weight of aphid survivors was at least twice as high on the native and universal host plants compared to aphid weight on less suitable or non-host plants (Figure 2 B, Table 1). However, the two clones of each host race did not always respond in the same way. Within the Pisum race, clone P2 showed nearly the same weight on *T. pratense* as on the native and universal host plants whereas clone P1 showed a significantly lower weight on *T. pratense*. Within the Trifolium race, clone T2 showed a significantly lower weight than clone T1 on the native and the universal host plant.

Aphid Probing and Feeding Behavior

EPG recordings were conducted for each aphid clone on each of the four plant species to localize the factors important for aphid feeding (Figure 1 B). Parameters derived from analysis of EPG waveforms were used to assess aphid behavior in specific plant tissues (Table S1).

General parameters reflecting multiple tissue levels. During the 4 h recording time, 70–95% of aphid individuals from all clone-plant combinations started to penetrate the plant with their stylet (Figure 3). The identity of the plant species did not have any influence on the proportion of individuals starting stylet penetration (Table 2, Parameter #1). The total duration of stylet penetration of the aphid clones ranged between ~2000 and ~12000 s during the 4 h (= 14400 s) EPG recording. But for clones P1, P2 and T1, the total stylet penetration times on native and universal host plants were significantly (two to four times) longer than on less-suitable or non-host plants (Table 2, #2).

Volatile and plant surface-related parameters. For most aphid clones the plant species did not influence the time from the start of experiment until first plant penetration (Table 2, #3), which ranged between 1000 and 6000 s. However, clones M2 and P1 took about 500–1000 s from the start of the experiment until first penetration on the universal host plant. This was significantly shorter (two to four times) than on the less suitable plant *T. pratense* (M2), and on all other plants (P1) (Table 2, #3).

Epidermis and mesophyll-related parameters. Aphids sometimes penetrate the plant tissue only briefly with very short probes of <30 sec. During this behavior only epidermal cells are likely to be punctured. Trifolium clones made significantly more (two-fold) very short probes on less-suitable and non-host plants than on native and universal host plants (Figure 4 A; Table 2, #5). The same effect was also observed in Medicago clone M2 (Table 2, #5).

For the Trifolium clones, we tested whether the very short probes on different plant species involve intracellular punctures. When both Trifolium clones fed upon *M. sativa*, *T. pratense* and *V. faba* most very short probes indeed contained intracellular punctures, whereas on *P. sativum* the proportion of very short probes without an intracellular puncture was significantly higher (Figure 4 B; Table 2, #6).

During probes longer than 30 s but shorter than 3 min, aphids very likely penetrate not only epidermal, but also upper mesophyll cells [21,36]. In this parameter we could not detect significant differences among any aphid clone-plant combinations (Table 2, #8).

The pathway phase (Figure 1 B) is characterized by sheath salivation, cell puncturing and stylet bundle movement towards the sieve elements (SEs). It excludes xylem phase, penetration difficulty periods, and all SE-related phases. Aphids on their native and universal host plants spent two times longer in the pathway phase than they did on less suitable and non-host plants (Figure 5). This effect was significant for all clones except for P2 (Table 2, #15).

Sieve element-related parameters: *repetitive SE punctures*. During repetitive SE punctures the aphid inserts its stylet repeatedly into the intracellular lumen of the SE. Approximately 40–100% of aphid individuals on the native and universal host plants showed repetitive SE puncture periods during the experiment. On less suitable and non-host plants, these proportions were significantly lower (10–30%; Figure 6 A, Table 2, #41). Successful SE feeding periods often co-occur with repetitive SE puncture periods. On native and universal host plants, most or sometimes all (clone P1 on the universal host and clone P2 on the native host plant) feeding periods followed a period of repetitive SE punctures (Figure 6 C; Table 3). However, the clones from the Medicago race which fed exclusively on the universal host but not on their native host plant, repetitively punctured SEs without subsequent feeding (Figure 6 B; Table 3). For all other clones, repetitive SE puncture periods without subsequent feeding were

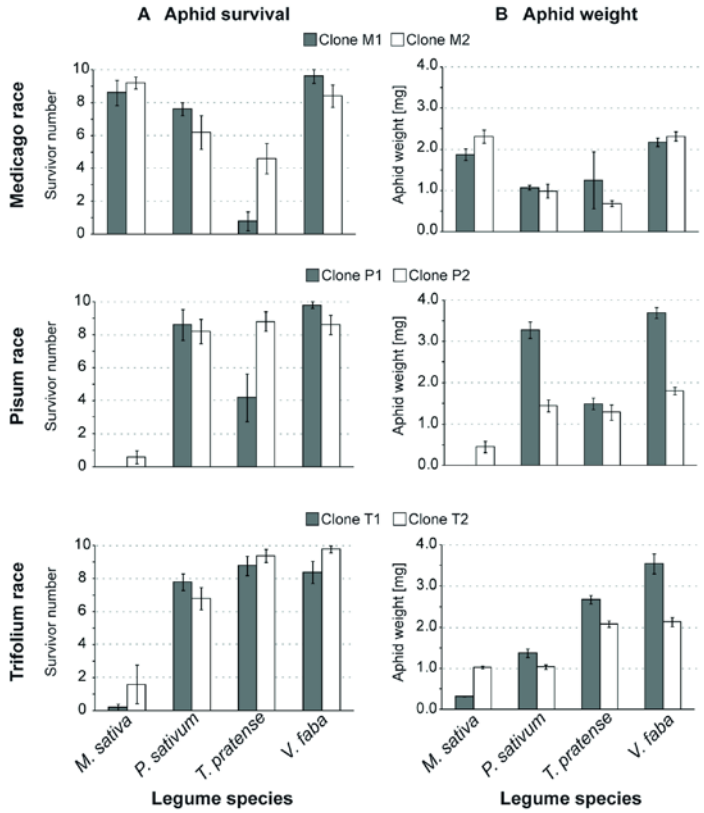

Figure 2. Performance of each clone of the pea aphid host races on the four legume species. Performance was best on the plant the aphid clones were originally collected from (those plants called native host plants). On *V. faba* all aphid clones were able to perform well (universal host plant). On other plants, aphid clones were able to perform intermediately (less-suitable plants) or were not able to survive at all (non-host plants). Bars represent mean +/− Std. Error. Number of aphid nymphs at start of experiment was 10. Each treatment was replicated five times. A) Survivor number after nine days. B) Aphid weight [mg] after nine days. For test statistics, see Table 1.
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also observed (Figure 6 B; Table 3). The number of these events was higher compared to the number of repetitive SE puncturing periods with subsequent feeding especially on non-host plants.

Sieve element-related parameters: *SE salivation and SE feeding*. The proportion of individuals that salivated into and/or fed on SEs during the experiment followed a uniform pattern throughout most aphid clone-plant combinations: on native and universal host plants 40 80% of individuals salivated into and/or fed on the SEs (Figure 7; Table 2, #25, 36, 39). On less suitable or non-host plants, a significantly lower proportion of individuals (0 10%) showed this behavior. Interestingly, aphid clones belonging to the Medicago race showed a pattern different from clones belonging to the other two races. On their native host plant *M. sativa*, only 0 10% of individuals of clones M1 and M2 salivated into and/or fed on SEs during the experiment, which is more similar to the behavior of other host races on the less suitable or non-host plants. On the universal host plant *V. faba*, 40 60% of the Medicago clone individuals showed SE salivation and SE feeding.

Discussion

Performance Differences

The clones of the three pea aphid host races tested (the Medicago, Pisum and Trifolium races) had similar patterns of performance with higher survival and greater weight on their native and the universal host *V. faba* than on non-hosts (Figure 2). Certain clones showed an intermediate performance on some legume species (Figure 2), indicating that these were less-suitable than the native and universal hosts. Performance was well-correlated with a clone's ability to establish feeding on the sieve elements (SEs) of each plant (Figure 7). On non-host plants, few individuals (0 10%) of each clone were able to establish sustained SE feeding during the 4 hour EPG recording times, whilst on native and universal host plants many individuals (40 80%) established feeding. These results are consistent with the findings of Caillaud and Via [22] who found that Medicago and Trifolium clones established feeding on their native host, but not on a non-host plant. In our experiments, we observed a remarkable phenomenon for the Medicago clones. Both performed well on

Table 1. Test statistics on performance data of clones of aphid host races feeding on the various legume species.

	Medicago race		Pisum race		Trifolium race	
Survivor number	*F*	*P*	*F*	*P*	*F*	*P*
Plant	19.466	**<0.001**	30.268	**<0.001**	36.054	**<0.001**
Clone	0.805	0.376	2.690	0.110	1.638	0.209
Plant:Clone	5.244	**0.005**	4.883	**0.007**	2.478	0.079
Average weight						
Plant	50.690	**<0.001**	45.127	**<0.001**	70.568	**<0.001**
Clone	0.239	0.629	119.567	**<0.001**	58.771	**<0.001**
Plant:Clone	3.696	**0.023**	19.273	**<0.001**	10.342	**<0.001**

The influence of plant species and aphid clone identity on the survivor number was analyzed separately for the three aphid races using generalized linear models with a quasipoisson error structure. The influence of plant species and clone identity on the average aphid weight was analyzed separately for the three aphid races using two-factorial ANOVA. *P*-values below significance level ($P<0.05$) are printed in bold letters. Mean values and standard errors shown in Figure 2.

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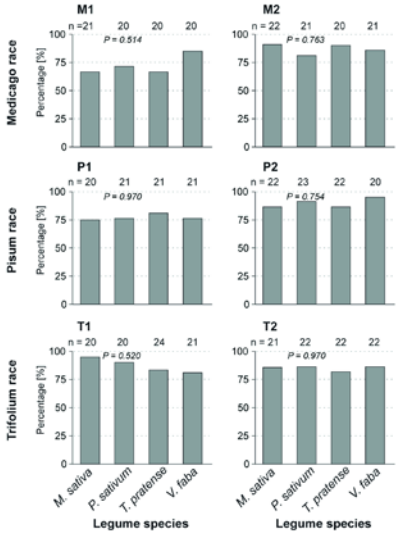

Figure 3. Percentage of aphid individuals initiating plant penetration. The graph shows the percentage of individuals for clones (M1, M2, P1, P2, T1 and T2) of each pea aphid host race initiating plant penetration (Table 2, parameter #1) on all four legume species throughout the entire EPG recording period. For details about test statistics, see Table S3.

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their native host plant *M. sativa* but were not able to establish SE feeding during the 4 h experiment. A similar observation was made for other clones on less suitable plants, such as P2 on *T. pratense*, which were rarely able to establish SE feeding. These inconsistencies between feeding and EPG performance might not been seen in longer EPG recordings if some clones need more time to repress plant defense responses and subsequently establish SE feeding. Another explanation might be the different age of the aphids used in the two experiments. Aphids at the start of the performance experiment were recently born, while those used in the EPG experiment were young adults (age~10 days). First instar larvae might have a greater ability to adapt to less suitable plants, whereas young adults might have lost this ability, as shown for lepidopteran larvae [37]. In general, an intermediate number of individuals were able to establish feeding on less suitable plants (clone M2 on *T. pratense* about 12.5%, clone T1 on *P. sativum* about 25%) (Figure 7). Most pea aphid clones did not feed on non-host plants during the EPG experiment, and their low survival on these plants might be a consequence of starvation. However, the Trifolium clones on the less suitable plant *P. sativum* survived well but showed a reduced weight (Figure 2) which might be explained by various plant factors like feeding deterrents [38–41], low nutritional quality of the phloem sap [4,15], or SE-located plant factors (e.g. [18,19,27]).

Plant Factors Influencing Aphid Feeding

Volatile and surface factors. Plant volatiles or surface factors did not play an important role in host race choice on these legumes since most aphids started probing during the EPG recording regardless of clone or plant (Table 2, Parameter #1). This result confirms the findings of Caillaud [24] that there is a necessity for the pea aphid to taste the plant to discriminate among potential hosts. However, plant volatiles or surface factors could influence the time an aphid takes from being placed on the plant until first probe. In two clones, this parameter (Table 2, #3) differed significantly depending on the experimental plant. Clones M2 and P1 needed more time until beginning probing on less suitable plants compared to the native and universal hosts. Attractive substances may shorten the time to first probe on native and universal host plants, as reported for *V. faba* volatiles that had an attractive effect on *Aphis fabae* [10]. On the other hand, the lack of attractive stimuli or the presence of repellent stimuli may delay probing by *Aphis fabae* reacting to epicuticular lipids of the non-host plant *Avena sativa* [12]. The effect of volatiles or surface factors might be more widespread than can be seen in the EPG experiment since attaching wires to aphids leads to a decrease of the behavioral differences related to stylet penetration and feeding on host and non-host plants especially in the first 30 min of the aphid-plant interaction [24,42].

Epidermal factors. Stylet penetration speed has been assumed to occur at a rate of approximately 0.5 cell layers per minute through the plant tissue [21,36]. Thus, probes shorter than 30 sec should reflect factors in the epidermis. Both Trifolium clones showed a significantly higher number of these short probes on less suitable and non-host plants compared to the number on the native and universal host plant (Figure 4 A). Notably, most of the very short probes involved an intracellular puncture (Figure 4 B). Hence, intracellular epidermal factors can be assumed to be important for plant recognition and discrimination in the Trifolium clones. This result is supported by previous studies implying that factors located in peripheral plant tissue stimulate further stylet penetration of pea aphids on their native host plants [22]. Early plant recognition with subsequent rejection of less suitable or non-host plants might be one reason for the low survival or weight of the Trifolium race on these legume species during the performance experiment. In nature, early plant recognition might be advantageous since aphids with this ability might not spend as long on unsuitable plants. However, there were no hints for such behavior in the other aphid clones.

Mesophyll factors. After the epidermis, the next plant tissue contacted by the aphid stylets is the upper mesophyll which is

Table 2. *P*-values of EPG parameters discussed in text.

			Medicago race		Pisum race		Trifolium race	
Tissue	**#**	**EPG Parameter**	**M1**	**M2**	**P1**	**P2**	**T1**	**T2**
Multiple	1	Proportion of individuals starting penetration	0.514	0.763	0.970	0.754	0.520	0.970
	2	Total penetration time	0.074	0.110	**<0.001**	**<0.001**	**<0.001**	0.071
Volatile/Surface	3	Time from start of experiment to first probe	0.710	**0.025**	**0.031**	0.167	0.145	0.501
Epidermis	5	Number of probes shorter than 30 s	0.659	**0.021**	0.651	0.278	**0.017**	**0.005**
	6	Number of probes shorter than 30 s without/with cell puncture	–	–	–	–	**0.008**	**<0.001**
Epidermis/Mesophyll	8	Number of probes shorter than 3 min	0.389	0.171	0.162	0.218	0.563	0.283
Mesophyll	15	Total duration of pathway phase	**0.001**	**<0.001**	**0.009**	0.138	**0.042**	**0.004**
Sieve elements	25	Proportion of individuals showing SE salivation	**<0.001**	**0.014**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	36	Proportion of individuals showing SE feeding	**<0.001**	**0.009**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	39	Proportion of individuals showing sustained SE feeding	**<0.001**	**0.003**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	41	Proportion of individuals showing repetitive SE punctures	**0.003**	**<0.001**	**0.002**	**0.003**	**<0.001**	**<0.001**

The influence of the legume species on the selected parameters was analyzed separately for each of the six aphid clones using appropriate statistical tests, i.e. parameters #1, 25, 36, 39, 41: test for equality of proportions; parameters #1, 2, 15: ANOVA; parameters #5, 8: generalized linear models with poisson/quasipoisson error structure; parameter #6: generalized linear models with quasibinomial error structure. *P*-values below significance level (<0.05) are printed in bold letters. Mean values and standard errors (or proportion data expressed as percentages, respectively) are shown in Figures 3–5, Figure 7. For details about test statistics and corresponding values, see Table S3.

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probably reached between 30 sec and 3 min after plant probing starts. However, since there were no differences in the number of probes longer than 30 sec, but shorter than 3 min in any aphid clone-plant combination, factors responsible for host choice are likely not associated with the upper mesophyll tissue. The stimulating plant factors located in peripheral plant tissue proposed by Caillaud and Via [22] and Del Campo et al. [25], are likely to be located in the epidermal tissue, since the authors did not distinguish between epidermal and mesophyll located factors.

From the upper mesophyll, the aphid navigates its stylets through the plant apoplast towards the SEs. This phase of stylet penetration, known as the pathway phase, was significantly longer for most clones on their native and the universal host plant than on other plants, possibly due to plant factors stimulating further probing in native and universal host plants. These factors may be located intracellularly since during the pathway phase the aphid punctures nearly every cell it contacts [43]. Whilst puncturing cells, aphids ingest cell content and inject watery saliva [44]. Puncturing cells might serve as orientation towards the SEs [20]. Alternatively, injection of watery saliva into the cell lumen upon

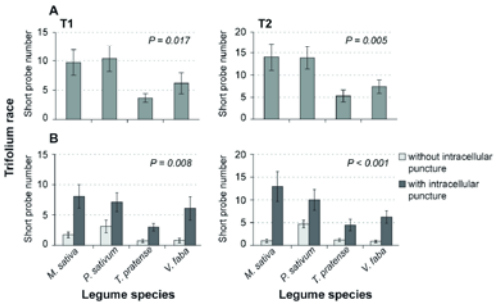

Figure 4. Number of very short probes (<30 s) during 4 h EPG recording. All bars represent mean numbers +/− standard errors. $n = 14–21$. A) The graph shows the numbers of very short probes (Table 2, parameter #5) made by two clones (T1, T2) of the Trifolium host race on the four legume species. B) The graph shows the proportion of very short probes without (light bars) and with (dark bars) intracellular punctures. For details about test statistics, see Table S3.

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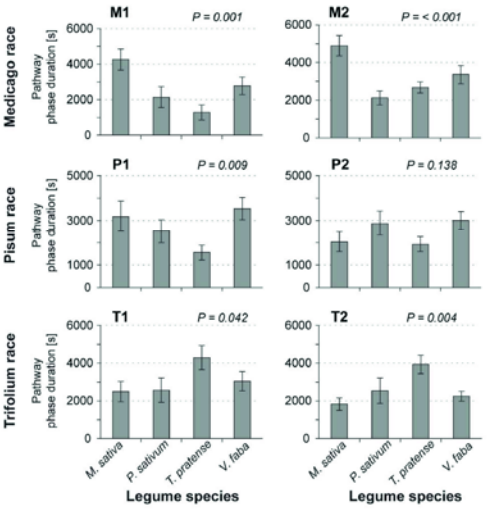

Figure 5. Mean duration of pathway phase during 4 h EPG recording. The graph shows the mean duration of pathway phase (Table 2, parameter #15) for clones (M1, M2, P1, P2, T1 and T2) of each pea aphid host race on the four legume species. Bars represent mean duration +/− Std. Error. $n = 14–21$. For details about test statistics, see Table S3.

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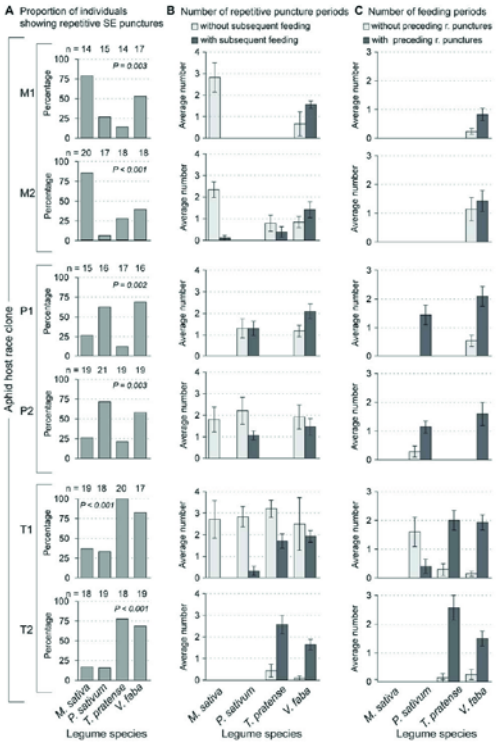

Figure 6. Co-occurrence of repetitive SE punctures and SE feeding. The graph shows the occurrence of repetitive SE punctures and SE feeding for clones (M1, M2, P1, P2, T1 and T2) of each pea aphid host race on the four legume species. A) Percentage of individuals showing repetitive SE punctures throughout the entire experiment (Table 2, parameter #41). B) Mean number +/− std. error of repetitive SE puncture periods per aphid with and without subsequent feeding periods (Table 3). C) Mean number +/− std. error of feeding periods per aphid with and without preceding repetitive SE puncture periods (Table 3). Missing bars in (B) and (C) are due to low replicate number caused by very low observation frequency (< five individuals showing repetitive SE punctures or SE feeding).
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puncturing might condition a plant for feeding since aphid saliva is known to harbor numerous effector molecules [45]. These effector molecules interact with plant *R*-gene products or other compounds of the plant defense system [29].

Sieve element factors. When pea aphids reach the SEs, they often repetitively puncture these cells [46,47]. Just as during intracellular punctures of mesophyll cells, aphids salivate into SEs and ingest at least some cell content, most likely to identify it as nutrition source [48,49]. Individuals of every pea aphid clone reached the SEs and carried out this behavior on every species of legume tested (Figure 6A). Thus repetitive SE punctures seem to be a conserved behavior that all pea aphid clones share [19,22]. However, the percentage of individuals reaching this step on less suitable or non-host plants was significantly lower (10–25%) than on native and universal hosts (65–100%). Thus, there must be a factor earlier in the penetration process, e.g. the lack of a stimulant factor in less suitable or non-host plants, which diminishes further probing towards the SEs. The high percentage of aphids that repetitively punctured SEs on their native or universal host plant indicates that this behavior is linked to the pea aphid's ability to feed on a plant as proposed previously [19,22]. In both of these previous studies, the total duration of repetitive SE puncture periods was considered rather than the number of aphids showing repetitive SE puncture periods. This total duration was shorter for aphids on non-hosts. In the present study, the analysis of repetitive SE puncture periods showed the same trend. However, sometimes it was not possible to compare the durations of repetitive SE puncture periods on host and non-host plants due to the low replicate number (<five) of repetitive SE puncture periods on non-host plants.

To find out whether repetitive SE punctures are linked to feeding on the various legume species, the number of repetitive SE puncture periods that ended up with feeding was compared to the number that ended without feeding (Figure 6 B). On less suitable or non-host plants, repetitive SE puncture periods often ended without subsequent feeding (clones P2 and T1 on less suitable plants or the non-host *M. sativa*). This behavior might reflect sampling of SE elements that were subsequently rejected for a variety of reasons, due to imbalanced amino acid composition [4], or the presence of active defense mechanisms that shut down the flow of phloem sap [50]. One mechanism to shut down phloem flow in legumes is the activation of proteins called forisomes which can block sieve plates [18,51]. However, on native and universal host plants not every repetitive SE puncture period ended with feeding. This implies that not every SE is suitable for feeding, and multiple SEs are sampled before feeding is established. The Medicago race seems to be an extreme example of this. For both clones studied, all repetitive SE puncture periods on their native host plant ended without feeding. The fact that Medicago clones are not able to establish SE feeding on their native host plant while being able to reach and repetitively puncture SEs, points to a factor located in the SE that prevents feeding. This non-compatibility could be due to a lack of sufficient time to overcome plant defense during the 4 h EPG experiment. Alternatively, non-compatibility of the Medicago race on its native host plant might

Table 3. Test statistics for comparing the proportions of repetitive SE puncturing periods without and with subsequent feeding period and the number of feeding periods without and with preceding repetitive SE puncture periods.

	Medicago race		Pisum race		Trifolium race	
	M1	M2	P1	P2	T1	T2
Repetitive SE punctures without vs.repetitive SE punctures with subsequent feeding	**<0.001**	**<0.001**	0.273	**0.007**	**<0.001**	0.331
Feeding periods without vs. feeding periods with preceding repetitive SE punctures	–	–	**0.027**	**0.024**	**<0.001**	0.256

For details about test statistics and corresponding values, see Table S2.
doi:10.1371/journal.pone.0075298.t003

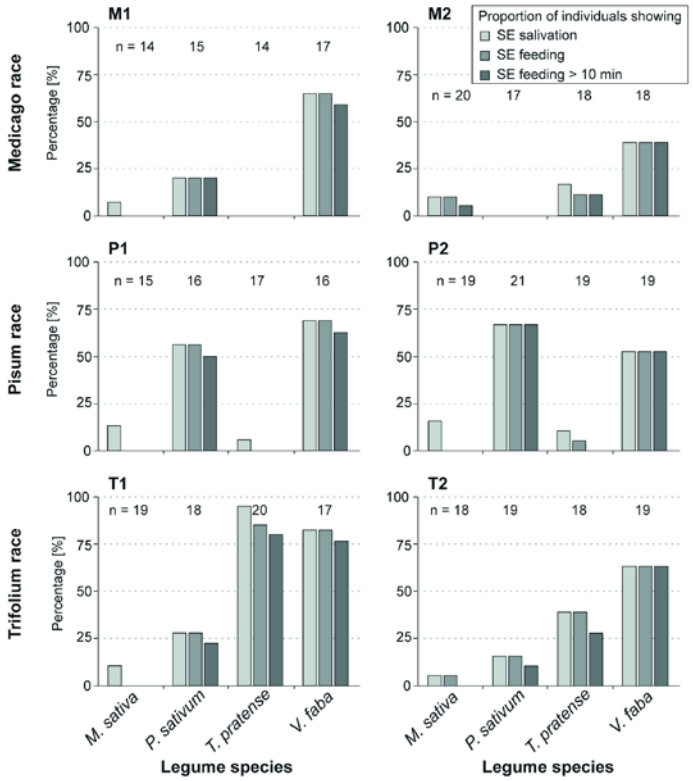

Figure 7. Percentage of aphid individuals showing SE related waveforms. The graph shows the percentage of individuals for clones (M1, M2, P1, P2, T1 and T2) of each pea aphid host race showing SE related waveforms on the four legume species. The influence of the plant species on proportion of individuals showing SE salivation, SE feeding and SE feeding >10 min was analyzed separately for each parameter. *P*-values all <0.001 except clone M2 ($P<0.02$) For details about test statistics, see Table 2 and Table S3.
doi:10.1371/journal.pone.0075298.g007

result from the lack of experience with this species as all clones had been reared on the universal host *V. faba* rather than their native host plants. However, the other host races were able to overcome lack of experience on their native hosts and establish feeding.

The occurrence of SE feeding mainly on native and universal host plants raises the question of whether repetitive puncturing of SE is a prerequisite for feeding. On universal and native hosts, SE feeding preceded by repetitive SE puncturing occurred significantly more frequently than feeding without puncturing preceding it (Figure 6 C). In some cases there was no single feeding event without repetitive SE punctures (P1 on native host plant, P2 on universal host plant). The same pattern was found for *Brevicoryne brassicae* on its host plant *Sinapis alba* where most feeding periods were preceded by repetitive SE puncture periods [47]. This pattern implies that repetitive SE punctures might fulfill a role in conditioning SEs for subsequent feeding. Salivation into SEs might also play an important role in overcoming the defense mechanisms of plants during repetitive SE puncturing. Salivary compounds can for instance suppress calcium influx into the SE right before and during SE feeding and therefore suppress SE occlusion [18]. In addition, when a salivary protein important for pea aphid survival on *V. faba* was knocked down by RNAi, ingestion from SEs was limited [30]. Notably, there is evidence from a recent study that aphid salivary effectors influence aphid performance in a plant-specific way [52]. However, so far it is not possible to answer the intriguing question of the function of repetitive SE punctures in pea aphid-legume interactions.

In general we can conclude that SE-based factors are critical in whether or not pea aphids can establish SE feeding. Their role as an ultimate barrier to feeding for certain aphid clone-legume species combinations may have driven selection for aphids to discriminate soon after initiation of penetration, which has resulted in the recognition of factors in the epidermis and the mesophyll that stimulate or deter continuation of probing towards the SEs. The observed continuum of pea aphid race ability to establish feeding on different legume species, which ranged from good to intermediate to poor, was mirrored in the performance of the different races on these same plants. This connection between host selection behavior and physiology provides a strong basis for pea aphid speciation.

Supporting Information

Table S1 Parameters derived from EPG recordings used to indicate the location of plant factors affecting pea aphid penetration and feeding. For detailed information about EPG waveform standard terms and corresponding aphid behavioral correlates, please refer to Tjallingii and Esch [43], Tjallingii and Gabrys [47], and Tjallingii [53]. Additional abbreviations in column "EPG waveform": r-pdsg = single repetitive potential drop period, E1sg = single sieve element salivation period, E1fr = fraction SE salivation period (SE salivation associated with SE feeding period).
(PDF)

Table S2 Test statistics for comparing the proportions of repetitive SE puncture periods without and with subsequent feeding period and the proportion of feeding periods without and with preceding repetitive SE puncture periods. KW = Kruskal-Wallis test; GLM B = Generalized linear model with binomial error structure (P-values calculated by $\chi 2$ -test, deviance values printed in regular letters); GLM Q = Generalized linear model with quasibinomial error structure (P-values calculated by F-test, F-values printed in italic letters).
(PDF)

Table S3 Test statistics of all EPG parameters. Statistical tests: ANOVA = Analysis of variance; KW = Kruskal-Wallis test; GLM QB = generalized linear model with quasibinomial error structure; GLM QP = generalized linear model with quasipoisson error structure; GLM P = generalized linear model with poisson error structure; EQP = test for equality of proportions. Transformations: (−) no transformation; log = logarithmic; sqrt = square root; 1/y = reciprocal transformation; asinsqrt = arcsine square root transformation. $\chi 2$ and F-values: $\chi 2$ printed in regular letters, F-values printed in italic letters. P-values: P-values <0.05 are printed in bold letters. Further signs/abbreviations: (−) = not analyzed as replicate number<five or no contrasts available. no = parameter not observed. na = parameter not analyzed.
(PDF)

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Author Contributions

Conceived and designed the experiments: AS GK. Performed the experiments: AS DR. Analyzed the data: AS GK. Contributed reagents/materials/analysis tools: AS MN. Wrote the paper: AS GK JG.

References

1. Peccoud J, Simon JC, McLaughlin HJ, Moran NA (2009) Post-Pleistocene radiation of the pea aphid complex revealed by rapidly evolving endosymbionts. Proceedings of the National Academy of Sciences of the United States of America 106: 16315 16320.
2. Via S (1999) Reproductive isolation between sympatric races of pea aphids. I. Gene flow restriction and habitat choice. Evolution 53: 1446 1457.
3. Peccoud J, Ollivier A, Plantegenest M, Simon JC (2009) A continuum of genetic divergence from sympatric host races to species in the pea aphid complex. Proceedings of the National Academy of Sciences of the United States of America 106: 7495 7500.
4. Sandstrom J, Pettersson J (1994) Amino acid composition of phloem sap and the relation to intraspecific variation in pea aphid (*Acyrthosiphon pisum*) performance. Journal of Insect Physiology 40: 947 955.
5. Ferrari J, Via S, Godfray HCJ (2008) Population differentiation and genetic variation in performance on eight hosts in the pea aphid complex. Evolution 62: 2508 2524.
6. Peccoud J, Simon JC (2010) The pea aphid complex as a model of ecological speciation. Ecological Entomology 35: 119 130.
7. Powell G, Tosh CR, Hardie J (2006) Host plant selection by aphids: Behavioral, evolutionary, and applied perspectives. Annual Review of Entomology 51: 309 330.
8. Goggin FL (2007) Plant-aphid interactions: molecular and ecological perspectives. Current Opinion in Plant Biology 10: 399 408.
9. Walling LL (2008) Avoiding effective defenses: Strategies employed by phloem-feeding insects. Plant Physiology 146: 859 866.
10. Webster B, Bruce T, Dufour S, Birkemeyer C, Birkett M, et al. (2008) Identification of volatile compounds used in host location by the black bean aphid, Aphis fabae. Journal of Chemical Ecology 34: 1153 1161.
11. Gibson RW, Pickett JA (1983) Wild potato repels aphids by release of aphid alarm pheromone. Nature 302: 608 609.
12. Powell G, Maniar SP, Pickett JA, Hardie J (1999) Aphid responses to non-host epicuticular lipids. Entomologia Experimentalis Et Applicata 91: 115 123.
13. Alvarez AE, Tjallingii WF, Garzo E, Vleeshouwers V, Dicke M, et al. (2006) Location of resistance factors in the leaves of potato and wild tuber-bearing *Solanum* species to the aphid *Myzus persicae*. Entomologia Experimentalis Et Applicata 121: 145 157.
14. Powell G, Hardie J (2000) Host-selection behaviour by genetically identical aphids with different plant preferences. Physiological Entomology 25: 54 62.
15. Karley AJ, Douglas AE, Parker WE (2002) Amino acid composition and nutritional quality of potato leaf phloem sap for aphids. Journal of Experimental Biology 205: 3009 3018.
16. Vanhelden M, Tjallingii WF (1993) Tissue localization of lettuce resistance to the aphid *Nasonovia ribisnigri* using Electrical penetration graphs. Entomologia Experimentalis Et Applicata 68: 269 278.
17. Kaloshian I, Kinsey MG, Williamson VM, Ullman DE (2000) *Mi*-mediated resistance against the potato aphid *Macrosiphum euphorbiae* (Hemiptera : Aphididae) limits sieve element ingestion. Environmental Entomology 29: 690 695.
18. Will T, Tjallingii WF, Thonnessen A, van Bel AJE (2007) Molecular sabotage of plant defense by aphid saliva. PNAS 104: 10536 10541.
19. Gao LL, Klingler JP, Anderson JP, Edwards OR, Singh KB (2008) Characterization of pea aphid resistance in *Medicago truncatula*. Plant Physiology 146: 996 1009.
20. Hewer A, Becker A, van Bel AJE (2011) An aphid's Odyssey - the cortical quest for the vascular bundle. Journal of Experimental Biology 214: 3868 3879.
21. Gabrys B, Tjallingii WF, vanBeek TA (1997) Analysis of EPG recorded probing by cabbage aphid on host plant parts with different glucosinolate contents. Journal of Chemical Ecology 23: 1661 1673.
22. Caillaud MC, Via S (2000) Specialized feeding behavior influences both ecological specialization and assortative mating in sympatric host races of pea aphids. American Naturalist 156: 606 621.
23. Wilkinson TL, Douglas AE (1998) Plant penetration by pea aphids (*Acyrthosiphon pisum*) of different plant range. Entomologia Experimentalis Et Applicata 87: 43 50.
24. Caillaud MC (1999) Behavioural correlates of genetic divergence due to host specialization in the pea aphid, *Acyrthosiphon pisum*. Entomologia Experimentalis Et Applicata 91: 227 232.
25. Del Campo ML, Via S, Caillaud MC (2003) Recognition of host-specific chemical stimulants in two sympatric host races of the pea aphid *Acyrthosiphon pisum*. Ecological Entomology 28: 405 412.
26. Klingler J, Creasy R, Gao LL, Nair RM, Calix AS, et al. (2005) Aphid resistance in *Medicago truncatula* involves antixenosis and phloem-specific, inducible antibiosis, and maps to a single locus flanked by NBS-LRR resistance gene analogs. Plant Physiology 137: 1445 1455.
27. Pallipparambil GR, Reese JC, Avila CA, Louis JM, Goggin FL (2010) *Mi*-mediated aphid resistance in tomato: tissue localization and impact on the feeding behavior of two potato aphid clones with differing levels of virulence. Entomologia Experimentalis Et Applicata 135: 295 307.
28. Klingler J, Powell G, Thompson GA, Isaacs R (1998) Phloem specific aphid resistance in *Cucumis melo* line AR 5: effects on feeding behaviour and performance of *Aphis gossypii*. Entomologia Experimentalis Et Applicata 86: 79 88.
29. Hogenhout SA, Bos JIB (2011) Effector proteins that modulate plant-insect interactions. Current Opinion in Plant Biology 14: 422 428.
30. Mutti NS, Park Y, Reese JC, Reeck GR (2006) RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, *Acyrthosiphon pisum*. Journal of Insect Science 6.

31. Mutti NS, Louis J, Pappan LK, Pappan K, Begum K, et al. (2008) A protein from the salivary glands of the pea aphid, *Acyrthosiphon pisum*, is essential in feeding on a host plant. Proceedings of the National Academy of Sciences of the United States of America 105: 9965 9969.
32. Bos JIB, Prince D, Pitino M, Maffei ME, Win J, et al. (2010) A functional genomics approach identifies candidate effectors from the aphid species *Myzus persicae* (Green Peach Aphid). Plos Genetics 6.
33. Carolan JC, Caragea D, Reardon KT, Mutti NS, Dittmer N, et al. (2011) Predicted effector molecules in the salivary secretome of the pea aphid (*Acyrthosiphon pisum*): A dual transcriptomic/proteomic approach. Journal of Proteome Research 10: 1505 1518.
34. R Core Team (2011) R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.
35. Sarria E, Cid M, Garzo E, Fereres A (2009) Excel Workbook for automatic parameter calculation of EPG data. Computers and Electronics in Agriculture 67: 35 42.
36. Van Hoof HA (1958) An investigation of the biological transmission of a nonpersistent virus [PhD]. WageningenThe Netherlands: The Agricultural University. 81 112 p.
37. Yamamoto RT (1974) Induction of host-plant specificity in tobacco hornworm, Manduca sexta. Journal of Insect Physiology 20: 641 650.
38. Dreyer DL, Jones KC, Jurd L, Campbell BC (1987) Feeding deterrency of some 4-hydroxycoumarins and related compounds relationship to host-plant resistance of alfalfa towards pea aphid *Acyrthosiphon pisum*. Journal of Chemical Ecology 13: 925 930.
39. Gururani MA, Venkatesh J, Upadhyaya CP, Nookaraju A, Pandey SK, et al. (2012) Plant disease resistance genes: Current status and future directions. Physiological and Molecular Plant Pathology 78: 51 65.
40. Golawska S, Leszczynski B, Oleszek W (2006) Effect of low and high-saponin lines of alfalfa on pea aphid. Journal of Insect Physiology 52: 737 743.
41. Golawska S, Lukasik I (2009) Acceptance of low-saponin lines of alfalfa with varied phenolic concentrations by pea aphid (Homoptera: Aphididae). Biologia 64: 377 382.
42. Tjallingii WF (1986) Wire Effects on Aphids During Electrical Recording of Stylet Penetration. Entomologia Experimentalis Et Applicata 40: 89 98.
43. Tjallingii WF, Esch TH (1993) Fine-structure of aphid stylet routes in plant tissues in correlation with EPG signals. Physiological Entomology 18: 317 328.
44. Martin B, Collar JL, Tjallingii WF, Fereres A (1997) Intracellular ingestion and salivation by aphids may cause the acquisition and inoculation of non-persistently transmitted plant viruses. Journal of General Virology 78: 2701 2705.
45. Carolan JC, Fitzroy CIJ, Ashton PD, Douglas AE, Wilkinson TL (2009) The secreted salivary proteome of the pea aphid *Acyrthosiphon pisum* characterised by mass spectrometry. Proteomics 9: 2457 2467.
46. McLean DL, Kinsey MG (1968) Probing behavior of pea aphid *Acyrthosiphon pisum*. 3. Effect of temperature differences on certain probing activities. Annals of the Entomological Society of America 61: 927-&.
47. Tjallingii WF, Gabrys B (1999) Anomalous stylet punctures of phloem sieve elements by aphids. Entomologia Experimentalis Et Applicata 91: 97 103.
48. Wensler RJ, Filshie BK (1969) Gustatory sense organs in food canal of aphids. Journal of Morphology 129: 473-&.
49. Backus EA, McLean DL (1985) Behavioral evidence that the precibarial sensilla of leafhoppers are chemosensory and function in host discrimination. Entomologia Experimentalis Et Applicata 37: 219 228.
50. Will T, van Bel AJE (2006) Physical and chemical interactions between aphids and plants. Journal of Experimental Botany 57: 729 737.
51. Will T, Kornemann SR, Furch ACU, Tjallingii WF, van Bel AJE (2009) Aphid watery saliva counteracts sieve-tube occlusion: a universal phenomenon? Journal of Experimental Biology 212: 3305 3312.
52. Pitino M, Hogenhout SA (2013) Aphid Protein Effectors Promote Aphid Colonization in a Plant Species-Specific Manner. Molecular Plant-Microbe Interactions 26: 130 139.
53. Tjallingii WF (2006) Salivary secretions by aphids interacting with proteins of phloem wound responses. Journal of Experimental Botany 57: 739 745.

3 "You can't change the habits of a lifetime" *or* Feeding experience enhances host plant fidelity of pea aphid (*Acyrthosiphon pisum*) clones

3.1 Abstract

The pea aphid (*Acyrthosiphon pisum* Harris) consists of over ten genetically distinct host races, each native to a single species of the Leguminosae. Yet all thrive on the universal host broad bean (*Vicia faba*). It is widely reported that prior experience on a host plant influences herbivore success on subsequent hosts. This study investigates whether prior feeding experience of two pea aphid clones of the Medicago race affects their ability to use their native and universal host plants.

Both aphid clones were reared on either *Medicago sativa* or *V. faba* and tested on either *M. sativa* or *V. faba* while penetration and feeding behavior were recorded by the Electrical Penetration Graph (EPG) technique. Feeding experience on the rearing plant significantly affected the overall ability to feed on the test plant, but not the time required to establish feeding. The proportion of individuals able to establish feeding on a host was much higher for aphids that had already had experience on the same host. Prior experience facilitated penetration of the epidermis and mesophyll, and feeding on the sieve elements (SEs). Experience effects manifested in the SEs were most crucial for aphids trying to feed on *M. sativa*. Both experienced and inexperienced individuals carried out cycles of repeated sieve element punctures, but these were followed by feeding most often in experienced individuals.

Previous feeding experience could have acted by influencing the chemoreceptive abilities of aphids or the composition of salivary effector proteins. By conditioning future acceptance of the same host, feeding experience will reinforce separation of the different pea aphid host races on various legumes and thus maintain their differences

3.2 Introduction

The pea aphid *Acyrthosiphon pisum* Harris is considered to be a species complex consisting of at least 11 host races, each native to a certain species of legume (Ferrari *et al.*, 2008; Peccoud *et*

al., 2009a). Yet all pea aphid host races are able to feed on the universal host plant *Vicia faba* (Sandstrom & Pettersson, 1994; Ferrari *et al.*, 2008; Schwarzkopf *et al.*, 2013). Since all the potential host plants can be found in the same geographic region, the pea aphid is an excellent example for sympatric speciation. One aim in studying sympatric speciation is to identify factors which are responsible for reproductive isolation among races. Such factors can act either from top-down, like natural enemies which might create an enemy free space only on the native host plant (Balog & Schmitz, 2013), or from bottom-up via the plant species. The latter is known to be the most important driver for host race separation (Peccoud *et al.*, 2010). But the occurrence of a universal host plant, such as *V. faba*, suitable for feeding by all pea aphid host races, will act against host race separation (Ferrari *et al.*, 2008).

The success of an herbivorous insect on a certain host plant may be influenced by its previous feeding experience which can alter host choice or host plant suitability. Such alterations may be caused by habituation to deterrents, increased ability to process xenobiotics, acquisition of specific positive responses, or associative learning (reviewed in Bernays & Weiss, 1996). Evidence for the effect of previous feeding comes from studies on plants and phytophagous insects from the Coleoptera, Lepidoptera, Heteroptera, Homoptera and Phasmatodea, where feeding experience affected the performance on a subsequent plant, or induced a preference for a certain plant (Bernays & Chapman, 1994). This phenomenon has been shown in a few aphid-plant interactions: *Schizaphis graminum* feeding on resistant and susceptible cultivars of *Sorghum bicolor* (Montllor *et al.*, 1983), *Sitobion fragariae* feeding on *Triticum aestivum* and *Avena sativa* (Ramirez & Niemeyer, 2000), and *Aphis gossypii* feeding on *Gossypium sp., Cucumis sp.* and *Hibiscus syriacus* (Liu *et al.*, 2008). In the pea aphid, an experience effect was observed by McLean *et al.* (2009). Aphid clones originating from the native host plant *Lathyrus pratensis* showed a higher fecundity on a certain host plant (*L. pratense* or *V. faba*) when they already had experience with this species.

Experience effects can be investigated not only via choice assays or fecundity measurements but also by feeding behavior. In a recent study on pea aphid host races (Schwarzkopf *et al.*, 2013), aphids from the Medicago race experienced on *V. faba* fed well on *V. faba*, but not on the native host plant *Medicago sativa*. Such an experience effect may be ascribed to more than one mechanism. 1) Experience might change the time necessary for establishing compatibility between the Medicago race and their host plants, i.e. inexperienced aphids might need more time to reach the phloem and feed compared to experienced ones. 2) Experience might influence the general ability of the Medicago race to establish feeding on a certain host plant.

To test these two possibilities Medicago race aphids were reared either on their native host *M. sativa* or on the universal host plant *V. faba*. Subsequently, aphid penetration and feeding behavior was monitored by the electrical penetration graph (EPG) technique on both the native and the universal host plant. This technique provides accurate information on which specific feeding behavior is occurring and which plant tissue is being penetrated. The results should allow precise inference about how prior experience influences feeding, which stage of feeding is affected and which part of the plant is involved.

3.3 Material and Methods

3.3.1 Plants

Two plant species were used: *Medicago sativa* cv. "Giulia" (Appels Wilde Samen GmbH, Darmstadt, Germany) and *Vicia faba* cv. "The Sutton" (Nickerson-Zwaan, Made, The Netherlands). Plants were reared in 10 cm diameter pots on the substrate "Klasmann Tonsubstrat" (Klasmann-Deilmann GmbH, Geeste, Germany) in a climate chamber under the following conditions: 20 °C, 70 % relative humidity, 16 hours light per day. To ensure a comparable developmental stage of plant species during aphid rearing and the experiments, *M. sativa* plants were used at an age of 34–38 days, and *V. faba* plants at an age of 20–24 days.

3.3.2 Aphids

Two *Acyrthosiphon pisum* clones occurring sympatrically in Western Europe and native to *M. sativa* were used: clone "L1_22" and clone "L84" (called M1 and M2). Both aphid clones were collected in the field from *Medicago sativa* plants (for detailed clone information see Table S1 in (Peccoud *et al.*, 2009b)). Aphid clone stock cultures were maintained on *Vicia faba* cv. "The Sutton". All plants with aphids were covered with air-permeable cellophane bags (Armin Zeller, Nachf. Schütz & Co, Langenthal, Switzerland) to prevent aphid clone cross-contamination. Culture conditions for all aphids in this study were: 20 °C, 70 % relative humidity, 16 hours light per day.

3.3.3 Aphid Rearing

To test for the presence of a putative plant induced experience effect the following rearing procedure was applied to both clones (Figure 1): one single adult apterous aphid was transferred

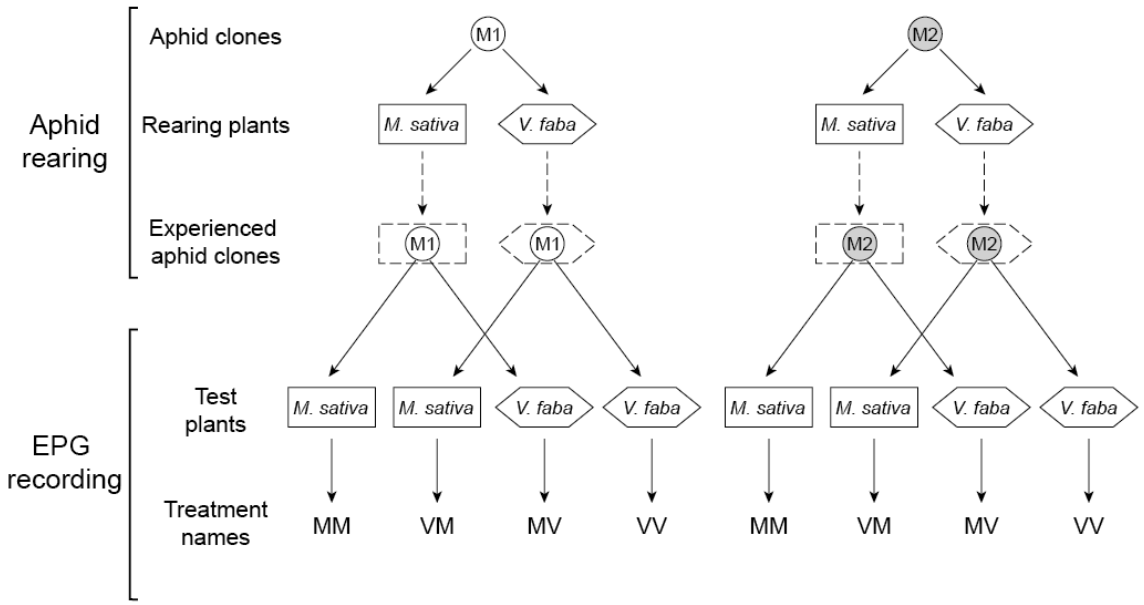

Figure 1 Experimental design showing aphid clones, the plants on which they were reared, the plants on which they were tested while EPGs were recorded and treatment names used in graphs. The first letters refer to the plant that the aphid was reared on; the second letter to the plant that the aphid was feeding on during the EPG recording (e.g. VM – aphid was reared on *Vicia faba*, the EPG was recorded on *Medicago sativa*). Hence, in treatments VM and MV aphids can be considered as "inexperienced"; in treatments MM and VV aphids can be considered as "experienced".

from the clonal stock culture on *V. faba* to a *M. sativa* plant, while another individual was transferred to a *V. faba* plant. The individuals were allowed to reproduce for two days and subsequently removed from the plants. The aphid offspring were kept on the plants until adulthood. To amplify the number of aphids reared on either *M. sativa* or *V. faba* for the EPG recordings, the rearing procedure described above was repeated once, but using three plants per plant species with one or two aphids from the first rearing cycle. For the EPG recordings the offspring of the second rearing cycle were used.

3.3.4 EPG Recordings

EPG recordings were conducted on 9–11 day old adult aphids from both clones. Insect electrodes were prepared from copper pins (length ~ 1.5 cm, diameter 1.3 mm) with a thinner copper wire (length ~ 1.5 cm, diameter 0.18 mm) soldered to the tip of the copper pin. A gold-wire (length 1.5–2.0 cm, diameter 20 µm) was attached to the tip of the copper wire using conductive silver-glue (EPG Systems, Wageningen, The Netherlands). An aphid was immobilized on a pipet tip connected to a vacuum-pump. After application of a small droplet of

conductive silver glue (EPG Systems, Wageningen, The Netherlands) to the aphid's dorsum the gold wire tip was inserted into the glue droplet. After the glue dried completely, the connected electrode was mounted to one port of an 8-channel direct current-EPG device ("GIGA-8", EPG Systems, Wageningen, The Netherlands). The mounting procedure was repeated to equip all 8 channels of the EPG device with aphids connected to electrodes, i.e. with four aphids reared on *M. sativa* and four aphids reared on *V. faba*). The EPG setup was located in a Faraday-cage and the setup completed by placing four *M. sativa* plants and four *V. faba* plants close to the EPG probes equipped with aphids. Aphids were placed on the adaxial side of the uppermost fully developed leaf of a *M. sativa* or *V. faba* plant. Hence, in one EPG run aphids reared on *M. sativa* and aphids reared on *V. faba* were tested on *M. sativa* and *V. faba* (Figure 1). After connecting the EPG device to a computer via an USB analog-digital converter ("DI 710", DATAQ Instruments, Akron OH, USA), aphid probing and feeding behavior was recorded during the following 8 hours using the software Stylet d+ (version v01.00 26.08.2010,EPG Systems, Wageningen, The Netherlands). For every treatment, 18–20 successful (the aphid started probing during the 8 h EPG recording) replicates were recorded.

3.3.5 EPG data processing and statistical analysis

The EPG data processing was performed using the software Stylet a+ (version v01.00 26.08.2010, EPG Systems, Wageningen, The Netherlands). After marking waveform beginnings and endings, 53 EPG parameters (Supporting Information Table S1) were calculated using a Microsoft Office Excel Macro (Schwarzkopf *et al.*, 2013). Statistical analysis was performed using R version 2.12.2 (R Core Team, 2011) as described in Schwarzkopf *et al.* (2013). Additionally, one more EPG parameter was calculated in the current study: the time from the first probe to the first repetitive SE puncture phase (Table 1, #19, Supporting Information Table S1). The influence of the rearing treatments on different feeding behaviors was tested separately for the two clones. To test for the influence of the rearing treatments on EPG parameters the following statistical tests were used: for durations, one-factorial ANOVA; for count data, generalized linear models with poisson/quasipoisson error family; in case of non-normality of the data or inequality of variances, the non-parametric Kruskal-Wallis test; for proportions of individuals showing a certain EPG parameter, the test for equality of proportions; for the proportions of individuals showing repetitive SE punctures without and with subsequent feeding, generalized linear model with binomial/quasibinomial error family. EPG parameters observed in less than five replicates were not included in the analysis. EPG parameters relevant for the study are listed in Table 1. Detailed information about the test statistics are listed in Supporting Information Table S2.

3.4 Results

3.4.1 Effects of prior plant experience on establishment of feeding

Aphids of two different *Medicago sativa* clones reared on either *M. sativa* or the universal host *Vicia faba* were tested on one or the other of these two plants species while feeding was monitored by EPG. For both clones, 75–100 % of individuals started penetration into the plant over 8 h regardless of which plant species they had experience on and which species they were tested on (Table 1, #1).

The total duration of penetration ranged between 13000 and 15000 s for most of the treatments (Figure 2, Table 1, #2). In clone M1, the total penetration times were significantly shorter if aphids were inexperienced (treatments VM and MV, mean values ~ 9000 s), but experience had no effect on the penetration time of clone M2.

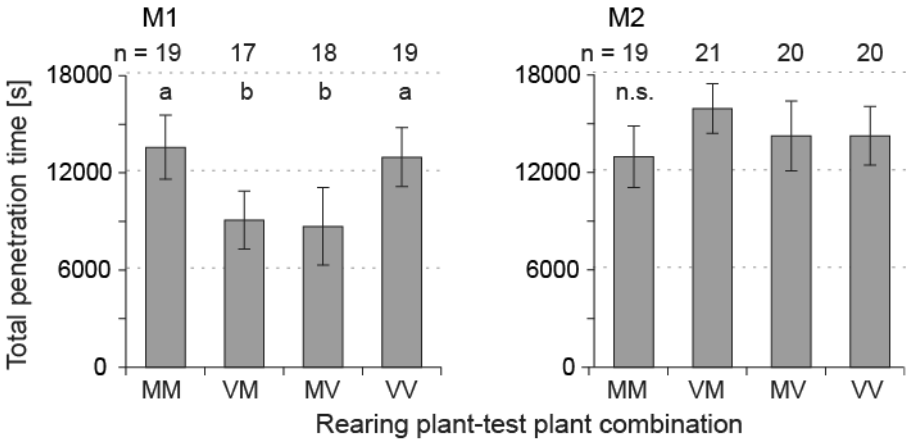

Figure 2 Influence of pea aphid feeding experience on the total duration of plant penetration. Bars represent mean +/- standard error. M1: $P = 0.030$, M2: $P = 0.730$. For details about test statistics please refer to Table 1 and Supporting Information Table S2. Treatments are as described previously. For details and abbreviations, see Figure 1.

If aphids were inexperienced, i.e. reared on a plant other than the test plant, a smaller proportion of individuals were able to establish sustained (> 10 min) feeding on the sieve elements (SEs) of the phloem (dark bars in Figure 3, Table 1, #39). This effect was statistically significant in clone M1; in clone M2 a strong trend towards this could be observed.

Regardless of the length of the feeding period, the proportion of individuals showing any feeding (light bars in Figure 3, Table 1, #36) was significantly higher in both clones for

experienced aphids (treatments MM and VV; M1 ~ 50 %, M2 ~ 30 %) compared to unexperienced aphids (treatments VM and MV: M1 ~ 25 %, M2 ~ 15 %).

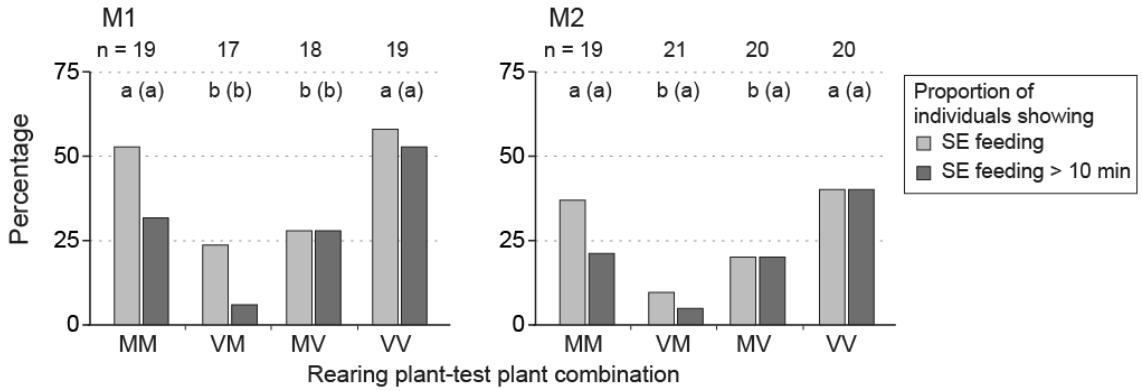

Figure 3 Influence of pea aphid feeding experience on the proportion of individuals showing sieve element (SE) feeding and sustained (> 10 min) SE feeding. Clones M1 and M2 were reared on either *M. sativa* (M) or *V. faba* (V) as host plants for experience and subsequently tested on either one of these two species. Feeding and sustained feeding were analyzed separately. M1: SE feeding $P = 0.020$, SE feeding > 10 min $P = 0.039$. M2: SE feeding $P = 0.030$, SE feeding > 10 min $P = 0.079$. For details about test statistics please refer to Table 1 and Supporting Information Table S2. MM, experienced on *M. sativa* and tested on *M. sativa*; VM, experienced on *V. faba* and tested on *M. sativa*; MV, experienced on *M. sativa* and tested on *V. faba*; VV, experienced on *V. faba* and tested on *V. faba*

Table 1 The influence of pea aphid feeding experience on the selected parameters was analyzed separately for each of the two clones using the following statistical tests: parameters #1, 36, 39, 41: test for equality of proportions; parameters #2, 13, 14, 19, 21, 33: ANOVA; parameter #46 : generalized linear models with poisson/quasipoisson error structure; association of repetitive SE puncture periods with SE feeding periods: generalized linear models with binomial/quasibinomial error structure. For an explanation of abbreviations in column "Treatments", please refer to Figure 1; brackets indicate factor levels which could be merged due to data similarity. *P*-values below significance level (< 0.05) are printed in bold letters. Mean values and standard errors (or proportion data expressed as percentages, respectively) are shown in Figures 2–10. For details about test statistics and corresponding values, see Supporting Information Table S2. Treatments are as described previously. For details and abbreviations, see Figure1.

			M1		M2	
Tissue	**#**	**EPG parameter**	**Treatments**	**P**	**Treatments**	**P**
All tissues	1	Proportion of individuals starting plant penetration	MM VM MV VV	0.505	MM VM MV VV	0.562
	2	Total penetration time	[MM VV] [VM MV]	**0.030**	MM VM MV VV	0.730
Stress Mesophyll	13	Total duration of penetration difficulty periods	MM VM MV VV	0.806	[MM VV] [VM MV]	**<0.001**
Mesophyll	14	Total duration of pathway phase	[MM VM VV] MV	**<0.001**	[MM VM VV] MV	**<0.001**
Mesophyll/SEs	19	Time from first probe to first repetitive SE puncture period	[MM VM MV] VV	**0.038**	MM VM VV	0.759
	21	Time from first probe to first SE feeding	MM MV VV	0.602	MM VV	0.291
SEs	33	Contribution of SE salivation to entire SE phase	MM [MV VV]	**<0.001**	[MM VM] VV	**0.002**
	36	Proportion of individuals showing SE feeding	[MM VV] [VM MV]	**0.020**	[MM VV] [VM MV]	**0.030**
	38	Total duration of SE feeding	[MM VV] MV	**0.002**	MM VV	0.459
	39	Proportion of individuals showing sustained SE feeding	[MM VV] [VM MV]	**0.039**	MM VM MV VV	0.079
	41	Proportion of individuals showing repetitive SE puncture periods	MM VM MV VV	**0.005**	MM VM MV VV	**0.001**
	46	Number of repetitive SE puncture periods	MM VM MV VV	0.323	MM VM VV	0.747
Association of repetitive SE puncture periods with SE feeding periods						
Number of repetitive SE punctures periods without and with subsequent feeding periods			[MM VV] [MV] [VM]	**<0.001**	[MM VV] VM	**<0.001**

3.4.2 Effects of prior plant experience on aphid behavior in epidermis and mesophyll tissue

For experienced aphids of both clones, the mean times from first probing to first SE feeding phase were comparable and ranged between 10000–14400 s (Figure 4 Table 1, # 21). For inexperienced aphids, the mean time to first feeding phase was prolonged (15000–20000 s) for aphids tested on *M. sativa*. However, due to the low replicate number (many of the inexperienced aphids were not able to establish feeding at all), the results have to be interpreted with caution.

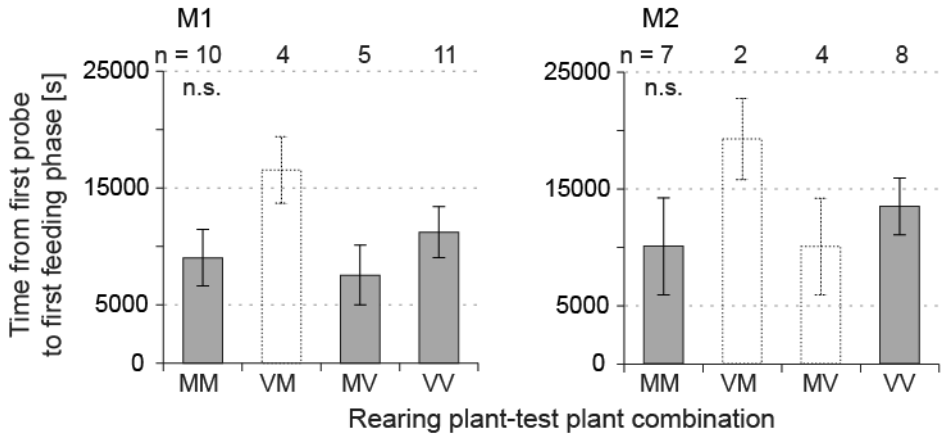

Figure 4 Influence of pea aphid feeding experience on the duration from first probe to first SE feeding phase. Clones M1 and M2 were reared on either *M. sativa* (M) or *V. faba* (V) as host plants for experience and subsequently tested on either one of these two species. Bars represent mean +/- standard error. White bars indicate treatments with replicate numbers below five; these were excluded from statistical analysis. M1: $P = 0.602$, M2: $P = 0.291$. For details about test statistics please refer to Table 1 and Supporting Information Table S2 Treatments are as described previously. For details and abbreviations, see Figure 1.

When pea aphids reach the SEs, they often repetitively puncture the cells and salivate before feeding (Tjallingi and Gabrys, 1999), and this behavior may condition the phloem for subsequent feeding (Schwarzkopf *et al.*, 2013). It took both clones a mean time of 6000-8000 s until the first repetitive SE puncture with one exception: clone M1 needed significantly longer if reared and tested on *V. faba* (~ 10000 s) (Figure 5, Table 1, #19). There was no effect of experience on this parameter.

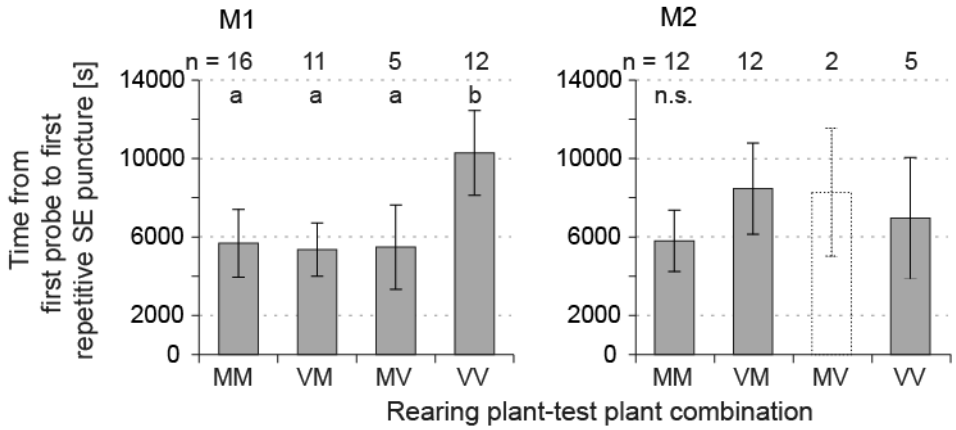

Figure 5 Influence of pea aphid feeding experience on the duration from first probe to the first repetitive SE puncture period). Bars represent mean +/- standard error. The white bar indicates a treatment with replicate numbers below five; this treatment was excluded from the statistical analysis. M1: $P = 0.038$, M2: $P = 0.759$. For details about test statistics please refer to Table 1 and Supporting Information Table S2. Treatments are as described previously. For details and abbreviations, see Figure 1.

After initial penetration of the epidermis, the aphid navigates its stylet bundle through the apoplast towards the phloem, referred to as the pathway phase. Both clones showed the same effects of experience on the duration of the pathway phase (Figure 6, Table 1, 14), with the mean value in treatments MM, VM, and VV between 4000–6000 s. Thus there was no effect of prior experience in tests on *M. sativa* or experienced aphids tested on *V. faba*, but aphids reared on *M. sativa* and tested on *V. faba* (treatment MV) had a significantly shorter pathway phase (~ 2000 s).

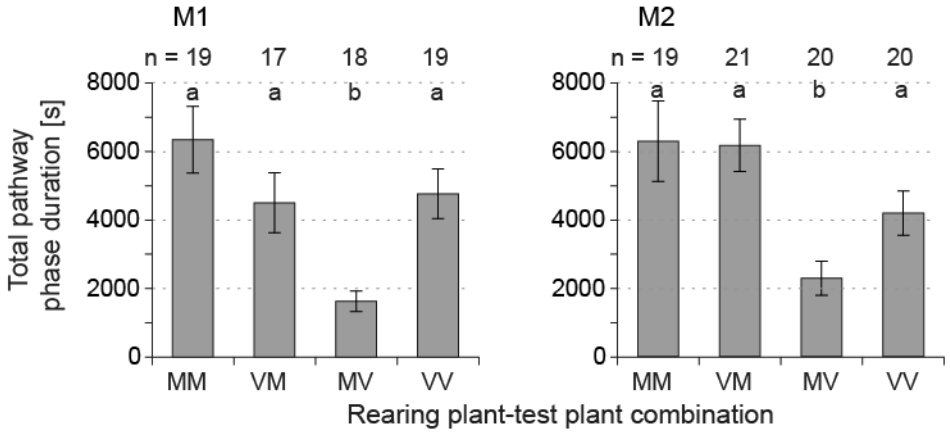

Figure 6 Influence of pea aphid feeding experience on the total duration of pathway phase. Bars represent mean +/- standard error. M1: $P < 0.001$, M2: $P < 0.001$. For details about test statistics please refer to Table 1 and Supporting Information Table S2. Treatments are as described previously. For details and abbreviations, see Figure 1.

During the pathway phase, aphids can encounter so called “penetration difficulties”. During this phase aphids are not able to move their stylet bundle further through the plant tissue towards the phloem. In clone M1 the total time during which the aphid had penetration difficulties was ~ 6000 s regardless whether the aphids were experienced or not (Fig 7, Table 1, # 13). In contrast to this, aphids from clone M2 showed significantly longer periods of penetration difficulty (~ 12000-16000 s) if they were not experienced (treatments VM and MV) compared to experienced aphids (treatment MM and VV, ~ 6000 s).

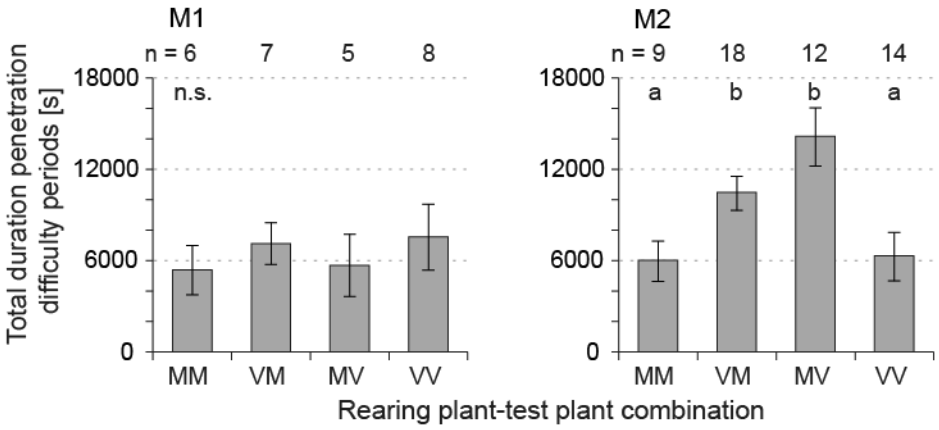

Figure 7 Influence of pea aphid feeding experience on the total duration of penetration difficulties. Bars represent mean +/- standard error. M1: $P = 0.806$, M2: $P < 0.001$. For details about test statistics please refer to Table 1 and Supporting Information Table S2. Treatments are as described previously. For details and abbreviations, see Figure 1.

3.4.3 Effects of prior plant experience on aphid behavior related to sieve elements

Repetitive SE punctures were observed in both clones and all treatments (Figure 8 A, Table 1, #41). For clone M2, aphids tested on *V. faba* (MV, VV) showed significantly fewer repetitive SE punctures compared to aphids tested on *M. sativa* (VM, MM), regardless of experience. The same trend (without statistical significance) was observed in clone M1. Additionally, aphids from both clones showed fewer repetitive SE punctures when reared on *M. sativa* and tested on *V. faba* (MV).

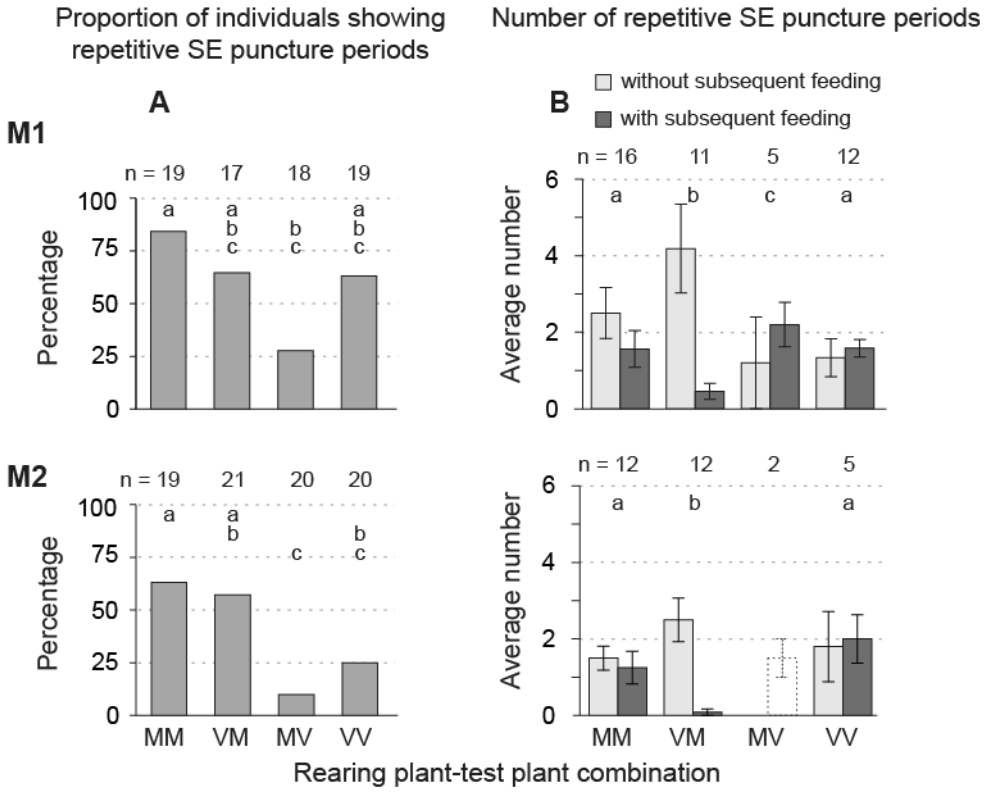

Figure 8 Influence of pea aphid feeding experience on the proportion of individuals showing repetitive SE puncture phase (A), and association of repetitive SE puncture phases with feeding phases (B). Bars represent mean +/- standard error. The white bar with (Figure 9 B) indicates a replicate number below five. This treatment was excluded from statistical analysis. M1: $P = 0.005$, $P < 0.001$; M2: $P = 0.001$, $P < 0.001$. For details about test statistics please refer to Table 1 and Supporting Information Table S2. Treatments are as described previously. For details and abbreviations, see Figure 1.

If the aphids of both clones were experienced on the same host as they were tested, the ratio between the number of repetitive SE punctures without subsequent feeding and with subsequent feeding was similar on both *V. faba* (VV) and *M. sativa* (MM), with about half of all repetitive SE punctures leading to feeding phases (Figure 8 B, Table 1). However, aphids without experience on *M. sativa* (reared on *V. faba* and tested on *M. sativa*, VM), this ratio changed substantially and nearly all repetitive SE punctures ended without a feeding phase.

Independent of experience, the proportion of time aphids from both clones spent salivating into SE before the feeding period to the total time spent in contact with SE (sum of SE salivation and SE feeding) was significantly higher when aphids fed on *M. sativa* (50–75 %) compared to *V. faba* (≤ 10 %) (Figure 9, Table 1, #33).

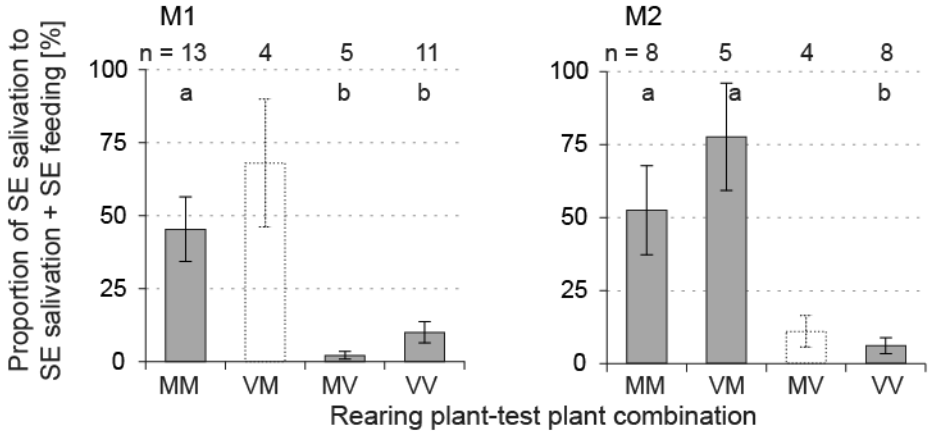

Figure 9 Influence of feeding experience on the proportion of SE salivation to the total time spent in the SE phase (SE salivation + SE feeding). Bars represent mean +/- standard error. White bars indicate replicate numbers below five. Those treatments were excluded from statistical analysis. M1: $P < 0.001$, M2: $P < 0.002$. For details about test statistics please refer to Table 1 and Supporting Information Table S2. Treatments are as described previously. For details and abbreviations, see Figure 1.

3.5 Discussion

3.5.1 Prior host plant experience influences pea aphid ability to feed but not the time required to initiate feeding

Both of the pea aphid clones studied were much more able to feed on a host plant if they had already experience on this species. Fewer inexperienced individuals were able to start feeding on phloem within the 8 h experimental time frame (Figure 3). Inexperienced aphids may simply

have needed more than 8 hours to start feeding. However, this conclusions seems unlikely since most of the individuals who managed to feed needed much less time (usually less than 4 hours = 14400 s, Figure 4) from the beginning of the first probe until their first feeding phase on the sieve elements (SEs) of the phloem regardless of which species they had experience on. Moreover, aphids would most probably leave a plant upon which they could not initiate feeding during such a long time span. Caillaud and Via (2000) found that about 50 % of pea aphid individuals belonging to the Medicago race or the Trifolium race left a plant within 30 min if the plant was not suitable for feeding. This finding is supported by studies on other aphid-plant interactions in which the time needed until the first SE feeding ranges between 0.5 and 4 hours (Vanhelden & Tjallingii, 1993; Caillaud *et al.*, 1995; Gabrys *et al.*, 1997; Sauge *et al.*, 1998; Alvarez *et al.*, 2006; Marchetti *et al.*, 2009). Thus, although prior host plant experience changes the general ability to feed on a certain plant, if feeding takes place, experience does not alter the time required to initiate feeding.

3.5.2 The identity of the prior host plant species influences feeding ability

For pea aphids without experience on a particular host plant, the ability to feed seems to vary with the identity of the original plant on which they fed. In our study, aphids fed least when they were reared on *V. faba* and tested on *M. sativa* (treatment VM). Surprisingly, the prior feeding experience on *V. faba* seems to hinder these Medicago clones from returning to feed on their native host plant. This inability to feed should lead to lower performance as described in other plant-aphid associations. For example, the yellow pecan aphid *Monelliopsis pecanis* exhibits less fecundity, shorter adult life span and lower viability when they are forced to feed on a plant that they are not experienced on (Dickey & Medina, 2011). Pea aphids previously experienced on their native host plant *Lathyrus pratensis* produced fewer offspring on *V. faba* as on *L. pratensis*, an effect that was only detected during the first 24 hours after the aphid encountered the new plant (McLean *et al.*, 2009). However, other studies describe an opposite trend in which aphids with an experience on one host increased their performance on a second host. Thus, Liu *et al.* (2008) reported that feeding experience on *Hibiscus syriacus* enabled an *Aphis gossypii* clone specialized on *Cucumis sativa* to utilize *Gossypium sp.* which was not possible without the previous experience on *H. syriacus*.

3.5.3 Effects of prior host experience are manifested in aphid penetration through the epidermis and mesophyll

To explore the mechanisms underlying feeding performance of experienced vs. inexperienced aphids, EPG recordings were carried out to determine which phase of feeding behavior is affected by experience and in which plant tissue this occurs. The EPG technique is well-suited to characterize the phase and location of aphid activity in the leaf (e.g. Montllor *et al.*, 1983; McCauley *et al.*, 1990; Ramirez & Niemeyer, 2000).

The ability of the aphid to penetrate the epidermis and mesophyll en route to the phloem is reflected in the time elapsed from the first probe until the first repetitive SE puncture. Previous studies showed that prior aphid experience can minimize the time until the first SE contact is established (Montllor *et al.*, 1983; Ramirez *et al.*, 1999). However, this trend could not be detected in the present study. Aphids from clone M2 needed nearly the same time until the first SE puncture regardless of experience, and experienced aphids from clone M1 on *V. faba* (treatment VV) needed even more time from first probe to the first repetitive SE puncture than aphids from all of the other treatments (Figure 5). This increased time might be due to repeated interruptions of the plant penetration process since some *V. faba*-reared aphids showed a high number of very short probes (< 30 sec) when tested on *V. faba* before first SE contact, indicating that the aphid repeatedly stopped and restarted the penetration process (Supporting Information Table S2, #6).

There were no differences in the time from the first probe until the first repetitive SE puncture for most of the treatments. However, this does not mean that aphids with different experience regimes show the same behavior in the epidermis and the mesophyll. Differences can be manifested in how aphids maneuver their stylets through the epidermis and mesophyll and in the extent of penetration difficulties. The duration of the pathway phase (the period from initial epidermal penetration until reaching the phloem or removing the stylet from the plant) was significantly shorter for inexperienced aphids from both clones on *V. faba* (treatment MV) (Figure 6) compared to aphids from all the other treatments. Since these inexperienced aphids rarely reached the SE, they either interrupted the penetration process earlier as in clone M1 (Figure 2), or they encountered more penetration difficulties in epidermis or mesophyll tissue (F-phase) as in clone M2 (Figure 7). Both phenomena were also observed by Ramirez and Niemeyer (2000). Inexperienced *Sitobion fragariae* aphids (reared on oat and tested on wheat) spent significant less time in the pathway phase and had more penetration difficulties compared to aphids which had already experienced wheat. The longer pathway phase for experienced aphids was interpreted as a more selective behavior induced by the previous experience. The

reduced penetration difficulties of experienced aphids might be due to adaptations to plant morphology or chemical compounds during their previous feeding experience. Prior experience might lead to adaptations of the aphid's chemosensitivity due to modulations in the set of chemoreceptors expressed or to modifications in the neural system in terms of habituation or associative learning (Bernays & Chapman, 1994). The sudden encounter with a subsequent "unknown" test plant may then lead to an altered behavior. The reduction of the pathway phase in inexperienced aphids could be due to their reduced ability to cope with plant defense responses locally induced around the stylet pathway (Will, 2008).

3.5.4 Experience effects are also manifested in sieve elements (SEs)

Experience effects could also be detected on the SE level. In the pea aphid, the first SE contact is mainly made via repetitive SE punctures. Repetitively puncturing a SE either reflects a sampling behavior to find a suitable feeding site or it "prepares" the SE for the subsequent feeding phase in terms of delivering salivary effector molecules (Schwarzkopf *et al.*, 2013). The present study shows that for inexperienced aphids tested on *M. sativa* (VM treatment) a very high proportion of repetitive SE puncture periods are not followed by feeding (Figure 8 B) periods.

Since it is known that pea aphids are able to recognize and discriminate among plant species (Caillaud & Via, 2000; Del Campo *et al.*, 2003; Schwarzkopf *et al.*, 2013), it might be that the lack of prior feeding experience on *M. sativa* prevents the recognition of *M. sativa* SEs as feeding sites. This would imply an influence of previous feeding experience on aphid chemoreceptory abilities which could be manifested in terms of altered receptor properties or altered receptor gene expression, as well as in neural changes and learning. Studies on *Drosophila melanogaster* and *Apis mellifera* showed that environmental factors such as CO_2 or odorants can induce changes in the insect nervous system and modulate the responsiveness of individuals to certain stimuli (Sachse *et al.*, 2007; Arenas *et al.*, 2012). Additionally, the idea that chemoreception influences the host plant selection of pea aphid host races is supported by the importance of olfactory genes in host plant adaption by this species (Jaquiery *et al.*, 2012).

Another reason that repetitive SE punctures do not lead to feeding in inexperienced aphids may be that inexperienced individuals are not adapted to the specific metabolites of certain host plants. These ideas are supported by reports of diet-dependent expression of salivary genes and digestive enzyme activity in *Spodoptera exigua* (Afshar *et al.*, 2010; Afshar *et al.*, 2013). In the pea aphid species complex, salivary proteins are likely to be involved in host plant adaptation (Jaquiery *et al.*, 2012). Aphids employ salivary effector molecules (Carolan *et al.*, 2011)

secreted into the plant (Miles, 1959) to suppress phloem-based resistance mechanisms (Will *et al.*, 2007). This behavior seems especially characteristic for pea aphids attempting to feed on *M. sativa,* as there was a higher proportion of SE salivation (waveform E1) during contact with the phloem compared to pea aphids feeding on *V. faba* (Figure 9). The salivary effector molecules are likely secreted not only during the E1 period, but also during repetitive SE puncture periods (Tjallingii & Gabrys, 1999), which were also observed most often in aphids feeding on *M. sativa*. Aphids that were experienced on another plant might not possess the effector molecules necessary for feeding on *M. sativa* which could explain their inability to proceed from repetitive SE puncture periods to feeding.

The prior feeding experiences of insect herbivores are known to influence the suitability of subsequent hosts, but the underlying mechanisms are poorly understood. Our study shows that the effects of prior feeding experience in pea aphids may arise from more than one mechanism operating in different plant tissues depending on the aphid clone and the host plant. Experience effects manifested during penetration of the epidermis and mesophyll were independent of the plant. However, experience effects manifested during contact with phloem were most important for aphids feeding on *M. sativa*. Regardless of the host species and tissue level, inexperienced aphids managed to feed only rarely. Aphids which are unable to use a certain plant would most likely leave it and search for a new host. As a consequence, aphids will have high fidelity to the host they first experience and most aphid-aphid encounters on a plant would take place between experienced aphids. In the case of sexual morphs, this would lead to assortative mating which would reinforce the separation among the different pea aphid host races and thus promote speciation. Hence the effect of feeding experience on host choice could be a major factor contributing to the maintenance of different host races in the pea aphid complex.

3.6 Acknowledgements

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3.7 References

Afshar K, Dube FF, Najafabadi HS, Bonneil E, Thibault P, Salavati R, Bede JC. 2013. Insights into the insect salivary gland proteome: Diet-associated changes in caterpillar labial salivary proteins. *Journal of Insect Physiology* **59**(3): 351-366.

Afshar K, Dufresne PJ, Pan L, Merkx-Jacques M, Bede JC. 2010. Diet-specific salivary gene expression and glucose oxidase activity in Spodoptera exigua (Lepidoptera Noctuidae) larvae. *Journal of Insect Physiology* **56**(12): 1798-1806.

Alvarez AE, Tjallingii WF, Garzo E, Vleeshouwers V, Dicke M, Vosman B. 2006. Location of resistance factors in the leaves of potato and wild tuber-bearing *Solanum* species to the aphid *Myzus persicae*. *Entomologia Experimentalis et Applicata* **121**(2): 145-157.

Arenas A, Giurfa M, Sandoz JC, Hourcade B, Devaud JM, Farina WM. 2012. Early olfactory experience induces structural changes in the primary olfactory center of an insect brain. *European Journal of Neuroscience* **35**(5): 682-690.

Balog A, Schmitz OJ. 2013. Predation Determines Different Selective Pressure on Pea Aphid Host Races in a Complex Agricultural Mosaic. *Plos One* **8**(2).

Bernays EA, Chapman RF 1994. Effects of experience. In: Hall Ca ed. *Host plant selection by phytophagous insects*. New York 206-229.

Bernays EA, Weiss MR. 1996. Induced food preferences in caterpillars: The need to identify mechanisms. *Entomologia Experimentalis et Applicata* **78**(1): 1-8.

Caillaud CM, Pierre JS, Chaubet B, Dipietro JP. 1995. Analysis of wheat resistance to the cereal aphid *Sitobion avenae* using Electrical Penetration Graphs and flow-charts combined with correspondence-analysis. *Entomologia Experimentalis et Applicata* **75**(1): 9-18.

Caillaud MC, Via S. 2000. Specialized feeding behavior influences both ecological specialization and assortative mating in sympatric host races of pea aphids. *American Naturalist* **156**(6): 606-621.

Carolan JC, Caragea D, Reardon KT, Mutti NS, Dittmer N, Pappan K, Cui F, Castaneto M, Poulain J, Dossat C, Tagu D, Reese JC, Reeck GR, Wilkinson TL, Edwards OR. 2011. Predicted effector molecules in the salivary secretome of the pea aphid (*Acyrthosiphon pisum*): A dual transcriptomic/proteomic approach. *Journal of Proteome Research* **10**(4): 1505-1518.

Del Campo ML, Via S, Caillaud MC. 2003. Recognition of host-specific chemical stimulants in two sympatric host races of the pea aphid *Acyrthosiphon pisum*. *Ecological Entomology* **28**(4): 405-412.

Dickey AM, Medina RF. 2011. Immigrant inviability in yellow pecan aphid. *Ecological Entomology* **36**(4): 526-531.

Ferrari J, Via S, Godfray HCJ. 2008. Population differentiation and genetic variation in performance on eight hosts in the pea aphid complex. *Evolution* **62**(10): 2508-2524.

Gabrys B, Tjallingii WF, vanBeek TA. 1997. Analysis of EPG recorded probing by cabbage aphid on host plant parts with different glucosinolate contents. *Journal of Chemical Ecology* **23**(7): 1661-1673.

Jaquiery J, Stoeckel S, Nouhaud P, Mieuzet L, Maheo F, Legeai F, Bernard N, Bonvoisin A, Vitalis R, Simon JC. 2012. Genome scans reveal candidate regions involved in the adaptation to host plant in the pea aphid complex. *Molecular Ecology* **21**(21): 5251-5264.

Liu XD, Zhai BP, Zhang XX. 2008. Specialized host-plant performance of the cotton aphid is altered by experience. *Ecological Research* **23**(5): 919-925.

Marchetti E, Civolani S, Leis M, Chicca M, Tjallingii WF, Pasqualini E, Baronio P. 2009. Tissue location of resistance in apple to the rosy apple aphid established by electrical penetration graphs. *Bulletin of Insectology* **62**(2): 203-208.

McCauley GW, Margolies DC, Reese JC. 1990. Feeding-behavior, fecundity and weight of sorghum-reared and corn-reared greenbugs on corn. *Entomologia Experimentalis et Applicata* **55**(2): 183-190.

McLean AHC, Ferrari J, Godfray HCJ. 2009. Effects of the maternal and pre-adult host plant on adult performance and preference in the pea aphid, Acyrthosiphon pisum. *Ecological Entomology* **34**(3): 330-338.

Miles PW. 1959. Secretion of 2 Types of Saliva by an Aphid. *Nature* **183**(4663): 756-756.

Montllor CB, Campbell BC, Mittler TE. 1983. Natural and induced differences in probing behavior of 2 biotypes of the greenbug, schizaphis-graminum, in relation to resistance in sorghum. *Entomologia Experimentalis et Applicata* **34**(1): 99-106.

Peccoud J, Ollivier A, Plantegenest M, Simon JC. 2009a. A continuum of genetic divergence from sympatric host races to species in the pea aphid complex. *Proceedings of the National Academy of Sciences of the United States of America* **106**(18): 7495-7500.

Peccoud J, Simon JC, McLaughlin HJ, Moran NA. 2009b. Post-Pleistocene radiation of the pea aphid complex revealed by rapidly evolving endosymbionts. *Proceedings of the National Academy of Sciences of the United States of America* **106**(38): 16315-16320.

Peccoud J, Simon JC, von Dohlen C, Coeur d'Acier A, Plantegenest M, Vanlerberghe-Masutti F, Jousselin E. 2010. Evolutionary history of aphid-plant associations and their role in aphid diversification. *Comptes Rendus Biologies* **333**(6-7): 474-487.

R Core Team 2011. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Ramirez CC, Caballero PP, Niemeyer HM. 1999. Effect of previous exposure to hydroxamic acids in probing behavior of aphid Sitobion fragariae on wheat seedlings. *Journal of Chemical Ecology* **25**(4): 771-779.

Ramirez CC, Niemeyer HM. 2000. The influence of previous experience and starvation on aphid feeding behavior. *Journal of Insect Behavior* **13**(5): 699-709.

Sachse S, Rueckert E, Keller A, Okada R, Tanaka NK, Ito K, Vosshall LB. 2007. Activity-dependent plasticity in an olfactory circuit. *Neuron* **56**(5): 838-850.

Sandstrom J, Pettersson J. 1994. Amino acid composition of phloem sap and the relation to intraspecific variation in pea aphid (*Acyrthosiphon pisum*) performance. *Journal of Insect Physiology* **40**(11): 947-955.

Sauge MH, Kervella J, Rahbe Y. 1998. Probing behaviour of the green peach aphid Myzus persicae on resistant Prunus genotypes. *Entomologia Experimentalis et Applicata* **89**(3): 223-232.

Schwarzkopf A, Rosenberger D, Niebergall M, Gershenzon J, Kunert G. 2013. To Feed or Not to Feed: Plant Factors Located in the Epidermis, Mesophyll, and Sieve Elements Influence Pea Aphid's Ability to Feed on Legume Species. *Plos One* **8**(9).

Tjallingii WF, Gabrys B. 1999. Anomalous stylet punctures of phloem sieve elements by aphids. *Entomologia Experimentalis et Applicata* **91**(1): 97-103.

Vanhelden M, Tjallingii WF. 1993. Tissue localization of lettuce resistance to the aphid *Nasonovia ribisnigri* using Electrical penetration graphs. *Entomologia Experimentalis et Applicata* **68**(3): 269-278.

Will T. 2008. Induction as well as suppression: How aphid saliva may exert opposite effects on plant defense. *Plant Signal Behav. 2008 June; 3(6): 427–430.* **3**(6).

Will T, Tjallingii WF, Thonnessen A, van Bel AJE. 2007. Molecular sabotage of plant defense by aphid saliva. *PNAS* **104**(25): 10536-10541.

4 Electrical Penetration Graph (EPG) measurements reveal an association of intracellular punctures and host-plant compatibility in the pea aphid (*Acyrthosiphon pisum*)

4.1 Summary

Pea aphid (*Acyrthosiphon pisum*) clones interact with their host and non-host legumes in various ways before feeding starts. Plant recognition and discrimination, as well as suppression of plant immunity against aphids are all important for the ability of an aphid to establish feeding. It is not yet clear when plant recognition and immunity suppression take place during the complex process by which aphids penetrate the plant. Recognition and immunity suppression could occur during the short and frequent intracellular punctures aphids perform during the penetration process, as those punctures include intracellular salivation (which might be involved in immunity suppression) and ingestion of plant cell content (which might be involved in plant recognition). This study focuses on the question of whether the duration of intracellular salivation and plant content ingestion might contribute to the compatibility of six pea aphid clones with their native host plants. The EPG technique was used to measure the durations of puncture subphases representing intracellular salivation and plant cell content ingestion. The results did not show any connection between the durations of salivation and ingestion and pea aphid-host plant compatibility. However, the duration of another phase of the puncturing process (for which the specific behavior is not yet known) did correlate with the compatibility of some of the pea aphid clones and their host plants.

4.2 Introduction

The pea aphid *Acyrthosiphon pisum* (Harris) represents a species complex consisting of at least 11 genetically distinct host-races specialized on various plants belonging to the Fabaceae (Peccoud *et al.*, 2009a). Different races can be found in the same habitat. As a consequence, individuals from different races are in principle able to meet and mate. Despite this fact the host races are stable and show a tendency towards further divergence and sympatric speciation (Ferrari *et al.*, 2006). Plant factors likely contribute to host race stability as they influence the aphid's ability to establish feeding. Within the plant, these factors have been shown to be

localized in epidermis, mesophyll, and sieve elements (Schwarzkopf *et al.*, 2013), but their exact nature and the mode of interaction with the aphid are unknown so far. Previous studies imply three different modes of action for plant factors: 1) interaction with aphid salivary effectors to compromise plant immunity and allow successful aphid feeding (Will *et al.*, 2007; Hogenhout & Bos, 2011; Rodriguez & Bos, 2013); 2) discrimination between host and non-host plants (Caillaud & Via, 2000; Del Campo *et al.*, 2003); 3) toxic or xenobiotic effect on aphids (e.g. Powell & Hardie, 2000; Alvarez *et al.*, 2006; Le Roux *et al.*, 2010). Aphid behavioral patterns which are very likely involved in interactions with plant factors include salivation into the plant (mode of action 1) and ingestion of plant material (modes of action 2 and 3). Salivation into the plant and plant material ingestion occur repeatedly during the entire plant penetration process since aphids puncture nearly every cell along the stylet pathway through the plant tissue (Tjallingii & Esch, 1993) including the sieve elements (SEs) before they reach their ultimate goal, SE feeding (Tjallingii & Gabrys, 1999). All intracellular punctures involve a short phase of salivation which gives the possibility to deliver salivary effector molecules to the cell lumen and a short phase of ingestion (i.e. the possibility for plant cell sampling and recognition processes) (Powell *et al.*, 1995; Martin *et al.*, 1997; Tjallingii *et al.*, 2010; Hewer *et al.*, 2011).

Since intracellular punctures represent a key aphid behavior in perception and possibly overcoming the chemistry of its host plant, a more detailed characterization of punctures could help researchers to better understand aphid host selection. An excellent method to measure the total duration of individual intracellular punctures and their subphases is the Electrical Penetration Graph (EPG) technique (Tjallingii, 1978; Tjallingii, 1985; Tjallingii, 1988; Tjallingii & Esch, 1993) which allows real time monitoring and recording of aphid probing and feeding behavior (Figure 1 A, example of a typical EPG trace) including the detailed characterization of intracellular punctures. In EPG terminology, intracellular punctures in the plant epidermis or mesophyll cells are called potential drops ("pds", marked by arrows in Figure 1 A). In the SEs, pds occur usually in a repetitive manner and are called repetitive potential drops ("r-pds", marked with r-pd in Figure 1 A). Both kinds of potential drops exhibit three characteristic phases (Powell *et al.*, 1995; Tjallingii & Gabrys, 1999): the initiation of the intracellular puncture (phase I, Figure 1 B), the phase in which the aphid stylet tip stays inside the intracellular space (phase II), and the stylet withdrawal from the intracellular space back to the apoplast (phase III). Only phase II is relevant for this study, as it represents the intracellular part of the cell puncture (Tjallingii, 1985) and involves three subphases (Powell *et al.*, 1995): salivation during subphase II-1 (Figure 1 B), a period with so far unknown behavioral correlate

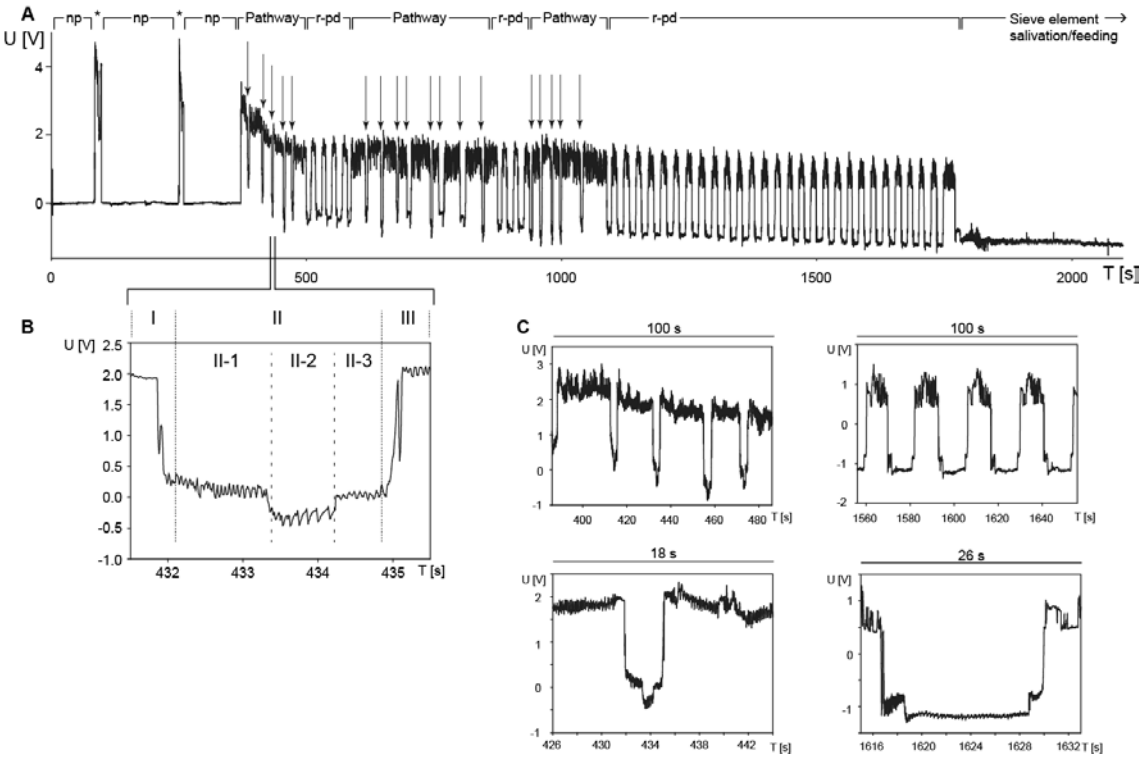

Figure 1 Characteristics of brief intracellular plant cell punctures recorded by the Electrical Penetration Graph technique (EPG). A: Typical pea aphid EPG trace recorded with an individual of clone T1 on its native host plant *T. pratense*. Aphid feeding behavior begins with non-probing phases ("np") and two initial short plant penetrations (*). The following long penetration phase starts with epidermis and mesophyll penetration (known as the "pathway" phase). During this mainly apoplastic pathway phase, many cells are punctured (indicated by arrows) with an irregular frequency. Additionally, sieve elements (SEs) elements are punctured (as shown by repetitive potential drops, "r-pd") with a regular frequency. At the end of this 35 min recording, the SE salivation and SE feeding phase occurs. B: Details of an intracellular puncture showing the various puncture phases. In phase I, cell puncture starts with few very high amplitude voltage fluctuations. Phase II exhibits three subphases: II-1 with high frequency/low amplitude voltage fluctuations reflecting intracellular salivation, II-2 with low frequency/high amplitude voltage fluctuations (behavioral correlate not known), and phase II-3 with high frequency/low amplitude voltage fluctuations reflecting ingestion of plant cell content. Phase III, like phase I, is characterized by few very high amplitude voltage fluctuations and reflects the phase of stylet withdrawal from the intracellular space back to the apoplast. C: Comparison of the durations of intracellular punctures in epidermal and mesophyll cells vs. SEs. Upper panel: Intracellular punctures of mesophyll cells (left graph) last 4–8 s and occur with an irregular frequency. Intracellular punctures of SEs (right graph) last 10–20 s and occur with a very regular frequency. Lower panel: The longer duration of an intracellular SE puncture (right graph) compared with the duration of an intracellular epidermal or mesophyll cell puncture (left graph) is mainly due to prolongation of the puncture-subphase II-2.

during subphase II-2, and plant cell content ingestion during subphase II-3. The behavioral sequences during phase II in pds and r-pds are quite similar, but there are two differences which make it possible to distinguish epidermis/mesophyll and SE punctures in the EPG. The duration of the pds in epidermis and mesophyll cell punctures is very short (duration 4–8 s, left pane in Figure 1 C) (Tjallingii, 1985) and they occur with an irregular frequency (arrows in Figure 1 A, Figure 1 C). The duration of r-pds is longer (duration 10–20 s, right pane in Figure 1 C) and they occur in a repetitive manner (Tjallingii & Gabrys, 1999; Schwarzkopf *et al.*, 2013) with a very regular frequency (Figure 1 A, C). Moreover, r-pd periods are often followed by feeding phases.

If intracellular punctures are involved in influencing pea aphid-host plant compatibility, two scenarios have to be considered: 1) if there are no changes in the intracellular puncture duration of a clone on host- and non-host plants detectable; this would mean that qualitative aspects of plant factor-aphid interaction (e.g. aphid saliva composition or presence/absence of plant factors acting as phagostimulants/deterrents) could be important for the ability of an aphid clone to establish feeding on a plant; 2) if there are significant changes in the intracellular puncture duration of a clone on host- and non-host plants detectable, this would mean that quantitative aspects of plant factor-aphid interaction (e.g. the duration of aphid saliva delivery to the plant or the positive recognition of phagostimulants) could be important for the ability of an aphid clone to establish feeding on a plant.

Hence, the durations of the intracellular puncture phase II and their subphases II-1, II2, and II-3 of clones from three pea aphid host races were compared when aphids were feeding on their native host plants, the universal host plant *Vicia faba*, non-host plants, and legume species on which the performance (survival and growth) and the ability of aphid clones to establish feeding was intermediate (here designated less-suitable plants). The differences in durations of intracellular punctures and their subphases on the different legume species and their relevance for host-plant compatibility of pea aphid clones are discussed.

4.3 Material and Methods

4.3.1 Plants

For this study *Medicago sativa* cv. "Giulia" (Appels Wilde Samen GmbH, Darmstadt, Germany), *Pisum sativum* cv. "Baccara" (S.A.S. Florimond Desprez, Cappelle-en-Pévèle, France), *Trifolium pratense* cv. "Dajana*"* (Appels Wilde Samen GmbH, Darmstadt, Germany), and *Vicia faba* cv. "The Sutton" (Nickerson-Zwaan, Made, The Netherlands) were cultivated in 10 cm diameter pots filled with "Klasmann Tonsubstrat" (Klasmann-Deilmann GmbH, Geeste, Germany) plant substrate. Plants were reared in a climate chamber at 20 °C, 70 % relative humidity, with 16 hours light per day.

4.3.2 Aphids

Six sympatric *Acyrthosiphon pisum* clones were used. They were collected in Western Europe from three legume species: clones "L1_22" and "L84" (called M1 and M2) from *M. sativa*; clones "P136" and "Colmar" (P1 and P2) from *P. sativum*; clones "YR2" and "T3_8V1" (T1 and T2) from *T. pratense* (detailed information available in Table S1 in (Peccoud *et al.*, 2009b)). Stock cultures of the aphid clones were kept on *V. faba* cv. "The Sutton". To prevent aphid cross-contamination, all rearing plants were covered with air-permeable cellophane bags (Armin Zeller, Nachf. Schütz & Co, Langenthal, Switzerland). All aphid cultures in this study were kept in a climate chamber at 20 °C, 70 % relative humidity, and 16 hours light per day.

To obtain enough aphids for the experiment the following rearing scheme was applied and repeated two times: one apterous adult aphid was placed on a *V. faba* plant. After two days of reproduction, the adult aphid was removed and larvae kept on the plant until adulthood. These adults were transferred to three new *V. faba* plants (1 aphid per plant), removed after two days, and the larvae remaining on the plant were kept until adulthood and used for the experiment.

4.3.3 Monitoring aphid probing and feeding behavior by the EPG technique

Each aphid clone was tested on *M. sativa*, *P. sativum*, *T. pratense*, and *V. faba*. To prepare the EPG recordings, young adult aphids were connected to the insect electrodes of the direct current-EPG device ("GIGA-8", EPG Systems, Wageningen, The Netherlands) using conductive silver-glue (EPG Systems, Wageningen, The Netherlands) and placed to the

uppermost fully developed leaf of a 27–32 day-old experimental plant. The entire experimental setup was placed in a Faraday cage. The EPG device was connected to a computer by an USB analog-digital converter device ("DI 710", DATAQ Instruments, Akron OH, USA) and EPG recordings were conducted for 4 hours using the software "Probe 3.5" (EPG Systems, Wageningen, The Netherlands). For each aphid clone-plant combination, six successful EPG recordings were analyzed. The EPG recordings used for the detailed characterization of intracellular punctures were the same as in chapter two of this thesis (Schwarzkopf *et al.*, 2013). For further detailed information about the experimental setup, please refer to this chapter.

4.3.4 EPG data processing

The very first potential drop (pd) after plant penetration started was excluded from the analysis, as this one in most cases represents a puncture of an epidermis cell and looks different from the subsequent pds. In general for both the pds and the r-pds the beginning and the end of phase II and the subphases II-1, II-2 and II-3 were marked using "Stylet a+" software (version v01.00 26.08.2010, EPG Systems, Wageningen, The Netherlands). From each EPG recording 30 pds were analyzed. As the variability in the total phase II duration and the subphase durations is much lower in repetitive pds compared to the pds in epidermis and mesophyll cells, only 10 repetitive pds were analyzed. Mean values of the total pd / r-pd phase II duration and the durations of subphases II-1, II-2 and II-3 in each EPG recording were calculated using an R-based macro and subsequently used for statistical analyses. EPG recordings with less than 30 pds or 10 r-pds were discarded. If the remaining replicate number of an aphid clone-plant combination was lower than two, the respective combination was excluded from the analysis. The effect of each plant species on the total pd / r-pd phase II duration and the subphase durations were tested for every clone separately using one-way ANOVA. Statistical analysis was performed by using R version 2.12.2 (R Core Team, 2011).

4.4 Results

The analysis of the intracellular punctures was focused on the duration of pd- and r-pd-phase II and its subphases. As no clear similarities between the three pea aphid races were detected, each race is discussed separately.

4.4.1 Brief intracellular punctures of Epidermis and Mesophyll cells

4.4.1.1 Medicago race

In both clones, the total pd phase II duration was not influenced by the plant species. On the less-suitable plant *P. sativum*, clone M1 spent significantly less time with intracellular salivation (1.2 s) and ingestion (~ 0.8 s) than on all other plants (salivation ~ 1.4 s, ingestion ~ 0.95 s). Clone M2 showed a significantly longer intracellular ingestion phase on the non-host plant *T. pratense* (~ 1.1 s) compared to this duration on all other plants (~ 0.8 – 0.9 s), but no changes in intracellular salivation. In both clones, the duration of pd subphase II-2 (unknown behavioral correlate/function) was not influenced by the different legume species (Figure 2, Table 1).

4.4.1.2 Pisum race

In clone P1 the total duration of pd-phase II was significantly shorter on the universal host plant *V. faba* (~ 3.6 s) than on the other plant species (~ 4 s) (Figure 2, Table 1). This is due to the fact that both the intracellular salivation (~ 1.4 s) and the ingestion (~ 1 s) on *V. faba* were shorter than on the other plant species (salivation ~ 1.5 s, ingestion ~ 1.2 s). Moreover, the duration clone P1 spent in pd-subphase II-2 (unknown behavioral correlate) was shorter on the native host plant *P. sativum* (~ 1.1 s) compared to all other legume species (~ 1.2 s).

In clone P2, there were no differences in the total pd phase II durations depending on the plant species, and no differences in the subphase durations.

4.4.1.3 Trifolium race

In clones T1 and T2, the total duration of pd-phase II was longer on the native host plant *T. pratense* (T1 ~ 3.5 s; T2 ~4.0 s) than on the non-host plant *M. sativa* and the less-suitable plant *P. sativum* (T1 ~3.3 s; T2 ~ 3.5 s) (Figure 2, Table 1). Moreover, clone T1 showed this prolongation of the pd-phase II additionally on the universal host plant *V. faba*. In both clones, the prolongations of the total durations of pd-phase II were due to a significantly longer pd-subphase II-2 (unknown behavioral correlate). Neither the intracellular salivation phases nor the intracellular ingestion phases were affected by the identity of the plant species in both clones.

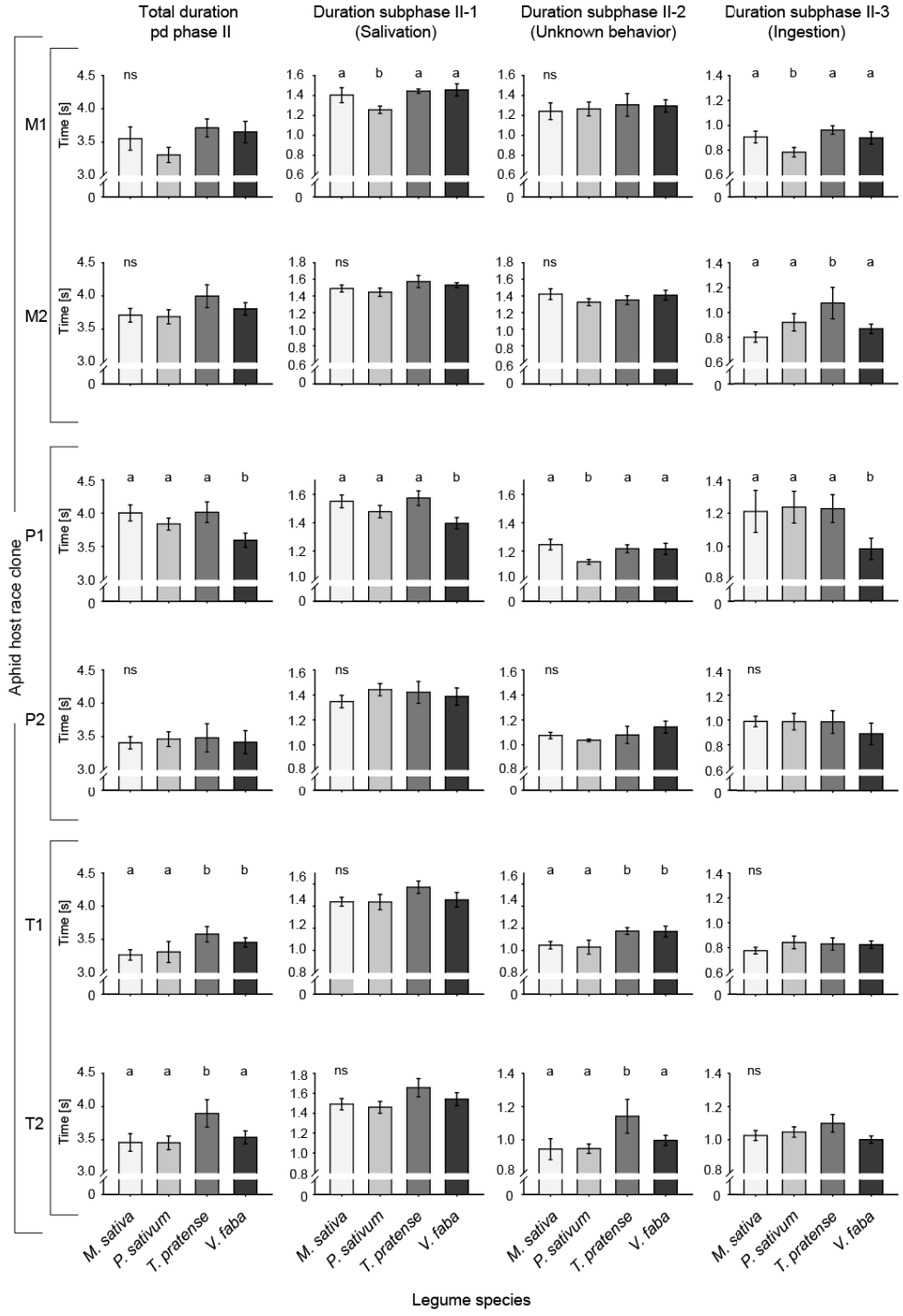

Figure 2 Durations of puncture-subphase II and its subphases in epidermal and mesophyll cells. Depicted are the total duration of epidermal and mesophyll cell punctures (Total duration pd phase II), and the durations of the phase II subphases (II-1, II-2, and II-3) for the six aphid clones on the four legume plant species. Aphid behavioral correlates of the subphases are: salivation (II-1), unknown (II-2), and plant cell content ingestion (II-3). Bars represent mean durations of six replicates, error bars represent standard errors. For details about the statistical analysis please refer to Table 1.

4.4.2 Intracellular punctures of Sieve Elements

4.4.2.1 Medicago race

Neither the durations of the total r-pd phase nor the r-pd-subphase durations of clone M1 and M2 were influenced by the legume species (Figure 3, Table 1).

4.4.2.2 Pisum race

In both Pisum clones, the total r-pd-phase II duration was significantly longer on the native host-plant *P. sativum* (~ 17 s) than on the universal host plant *V. faba* (~ 13 s) (Figure 3, Table 1) mostly due to a significantly longer subphase II-2 (unknown behavioral correlate). Moreover, clone P1 also spent significantly more time salivating in SE (~ 2 s). The intracellular ingestion phases were neither affected in clone P1 nor in clone P2.

4.4.2.3 Trifolium race

Clone T1 showed, like clones P1 and P2, a prolonged total duration of r-pd-phase II on the native host plant (~ 13 s) compared to the universal host *V. faba* and the less-suitable plant *P. sativum* (~ 10 s) (Figure 3, Table 1). Responsible for this prolongation was an extension of r-pd-subphase II-2 (unknown behavioral correlate). This subphase was longest on the native host-plant *T. pratense* (~10 s), shorter on the universal host *V. faba* and the less-suitable plant *P. sativum* (~ 8 s), and shortest on the non-host plant *M. sativa* (~ 6 s). In clone T1, the duration of r-pd-salivation was also significantly longer on the native host *T. pratense* and the universal host *V. faba* than on the less-suitable plant *P. sativum* and the non-host *M. sativa* (Figure 3, Table 1). The duration of the intracellular ingestion phase was not influenced by the plant species.

In clone T2, there were no differences in the total r-pd phase II durations depending on the plant species, and no differences in the subphase durations.

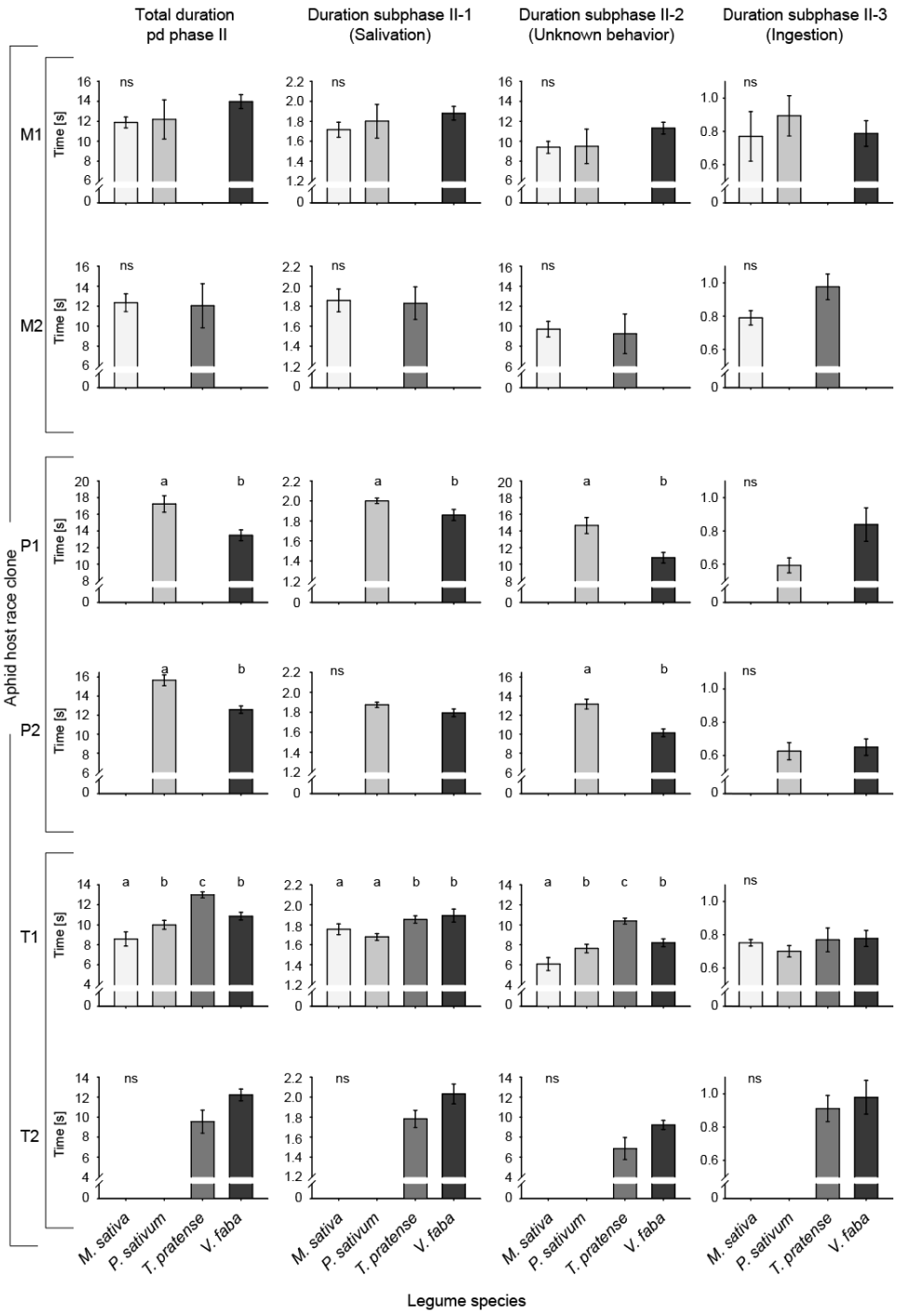

Figure 3 Durations of puncture-subphase II and its subphases in sieve elements (SEs). The illustration shows the total duration of SE punctures (Total duration r-pd phase II), and the durations of the phase II subphases (II-1, II-2, and II-3) for the six aphid clones on the four legume plant species. Aphid behavioral correlates of the subphases are: salivation (II-1), unknown (II-2), and plant cell content ingestion (II-3). Bars represent mean durations of six replicates; missing bars are due to low replicate number < 3. Error bars represent standard errors. For details about the statistical analysis please refer to Table 1.

Table 1 *P*-values of the statistical tests for differences in durations of puncture phase II and its subphases for the six pea aphid clones feeding on all four legume species tested. The left half of the table shows the *P*-values for epidermal and mesophyll cells ("Cell punctures, pd"), and the right half of the table shows the *P*-values for the SEs ("Repetitive SE punctures, r-pd"). Statistical test: one-way ANOVA.

	Cell punctures (pd)		Repetitive SE punctures (r-pd)	
Aphid clone	Parameter	Pr (>F)	Parameter	Pr (>F)
M1	pd II total	0.064 .	r-pd II total	0.110
	pd II-1	**0.009** **	r-pd II-1	0.260
	pd II-2	0.944	r-pd II-2	0.090 .
	pd II-3	**0.014** *	r-pd II-3	0.449
M2	pd II total	0.069 .	r-pd II total	0.887
	pd II-1	0.164	r-pd II-1	0.890
	pd II-2	0.152	r-pd II-2	0.809
	pd II-3	**0.025** *	r-pd II-3	0.052 .
P1	pd II total	**0.011** *	r-pd II total	**0.010** *
	pd II-1	**0.013** *	r-pd II-1	**0.045** *
	pd II-2	**0.014** *	r-pd II-2	**0.007** **
	pd II-3	**0.024** *	r-pd II-3	0.050 .
P2	pd II total	0.985	r-pd II total	**0.001** **
	pd II-1	0.799	r-pd II-1	0.118
	pd II-2	0.262	r-pd II-2	**0.001** ***
	pd II-3	0.257	r-pd II-3	0.742
T1	pd II total	**0.047** *	r-pd II total	**< 0.001** ***
	pd II-1	0.064 .	r-pd II-1	**0.008** **
	pd II-2	**0.006** **	r-pd II-2	0.000 ***
	pd II-3	0.681	r-pd II-3	0.785
T2	pd II total	**0.015** *	r-pd II total	0.065 .
	pd II-1	0.088 .	r-pd II-1	0.086 .
	pd II-2	**0.019** *	r-pd II-2	0.076 .
	pd II-3	0.077 .	r-pd II-3	0.608

4.5 Discussion

In four (M1, M2, P1, and P2) out of the six clones tested, the total duration of the intracellular puncture phase II is not significantly different in host vs. non-host plants. Thus it is unlikely that the length of the puncture contributes to host-plant compatibility in those clones. In a previous study (Wilkinson and Douglas (1998), the total durations of intracellular punctures of a range of

six pea aphid clones belonging to the Medicago, Pisum and Trifolium races were determined on *P. sativum* and *V. faba*, but here also no correlation of the total duration of intracellular punctures and host-plant compatibility could be detected. Unfortunately, there were no data on the durations of the subphases of the intracellular punctures representing aphid salivation and ingestion. In contrast, the present study revealed significant plant dependent changes in the durations of salivation and ingestion in clones M1, M2 and P1. However, the differences were specific for every clone-plant combination, and no uniform host plant-associated patterns among these clones were visible. Due to this heterogeneous pattern, the duration of intracellular salivation or ingestion does not seem relevant for host plant compatibility. But these observations do not exclude a relevance of intracellular punctures for host-plant compatibility completely. Qualitative aspects of the intracellular punctures, such as the presence of specific effectors in the saliva of various aphid clones or the ability of a clone to detect plant compounds mediating host recognition during puncturing may be important for compatibility. There is increasing evidence from some studies that those aspects could be responsible for aphid-plant compatibility differences (Jaquiery *et al.*, 2012; Smadja *et al.*, 2012; Pitino & Hogenhout, 2013). Further studies describing aphid salivary effector composition and chemoreceptors, as well as the critical plant factors should shed more light on the importance of intracellular punctures.

In contrast to the lack of change in intracellular punctures with respect to plant species in most aphid clones or the heterogeneous patterns observed, both clones from the Trifolium race showed a longer total duration of the intracellular puncture phase II on their native host plant *T. pratense* compared to the duration on non-host plants. This finding supports the idea that the length of certain phases of intracellular punctures might be relevant for the compatibility of the Trifolium clones with their native host plant. That a prolongation of intracellular punctures might be involved in aphid-plant compatibility was already implied by Sauge *et al.* (1998). In this study on *Myzus persicae,* the duration of intracellular punctures (or aphid behavioral patterns during the punctures) was longer on a susceptible *Prunus persica* cultivar compared to the duration on a resistant *P. persica* cultivar. This was interpreted as a positive recognition of the plant during the intracellular punctures. But, as in the study by Wilkinson and Douglas (1998), data on the duration of the puncture subphases are not available. Thus it is not possible to determine which subphase might be responsible for the prolongation. In the present study, neither the duration of the intracellular salivation nor the duration of intracellular ingestion phase in clones T1 and T2 were influenced by the plant species (Figure 2). The longer total duration of the intracellular puncture phase II in the Trifolium clones was caused instead by a prolongation of subphase II-2 of the intracellular puncture (Figure 2). While the subphases II-1

and II-3 are relatively well understood to represent salivation and ingestion, respectively, the behavioral correlate of the puncture subphase II-2 is still unclear. However, it might have a role in plant recognition if aphids ingest cell content not only during the subphase II-3 of the intracellular puncture, as postulated before (Powell *et al.*, 1995; Martin *et al.*, 1997), but also in subphase II-2 (Hewer *et al.*, 2011).

Interestingly, the duration of cell puncture subphase II-2 in the compatible interactions of the Trifolium clones with their native host plant was longer than on non-host plants, while it was shorter in the Pisum clone P1. That the intracellular puncture subphase is shorter on a compatible host plant was already described by Marchetti *et al.* (2009). *Dysaphis plantaginea* showed a shorter duration of the puncture subphase II-2 on a susceptible cultivar of *Malus domesticus* compared to a resistant cultivar. Maybe the brevity of subphase II-2 in this case reflects a strategy to avoid inducing a plant response which will prevent the further penetration process and subsequent establishment of phloem feeding. The differences between the Pisum clone and both clones of the Trifolium race in the duration of subphase II-2 on their native host plants highlight again the behavioral divergence within the pea aphid species complex which may represent adaptations to different host plants.

That a prolonged intracellular puncture subphase II-2 is likely to be relevant for aphid-host plant compatibility is additionally supported by the fact that this subphase is also prolonged on the native host plant during repetitive SE punctures (Figure 3). In clones P1, P2, and T1, the total duration of the repetitive puncture subphase II-2 is longer on the native host plants *P. sativum* or *T. pratense*, respectively, compared to the duration on *V. faba*. Pea aphids (especially the Trifolium clones tested in Schwarzkopf *et al.* 2013) might be able to positively recognize their native host plant by tasting SEs as well as by making punctures earlier in the feeding pathway while passing epidermal and mesophyll cells (Caillaud & Via, 2000; Del Campo *et al.*, 2003; Schwarzkopf *et al.*). However, the likelihood of host plant recognition by pea aphid clones in early feeding stages is supported by the fact that most pea aphid individuals never reach the SEs on less suitable and non-host plants and do not show repetitive SE punctures (missing bars in Figure 3).

In conclusion, the total duration of intracellular punctures in epidermis, mesophyll, and SEs are not likely to be crucial in host plant-compatibility of pea aphid clones, nor are the durations of intracellular salivation and plant cell content ingestion. However, the prolonged intracellular puncture subphase II-2 in clones of the Trifolium race and Pisum race clone P2 on their native host plants might contribute to host plant-compatibility. For subphase II-2, there is as yet no evidence for the behavior it represents. Further studies are necessary to test the suggestion made

in the present study and by Hewer *et al.* (2011) that behavior during subphase II-2 might be associated with ingestion of plant cell content. The present study also revealed interclonal and interracial divergence in pea aphid clones in the duration of intracellular puncture subphases which may contribute to their host range diversification.

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4.7 References

Alvarez AE, Tjallingii WF, Garzo E, Vleeshouwers V, Dicke M, Vosman B. 2006. Location of resistance factors in the leaves of potato and wild tuber-bearing *Solanum* species to the aphid *Myzus persicae*. *Entomologia Experimentalis et Applicata* **121**(2): 145-157.

Caillaud MC, Via S. 2000. Specialized feeding behavior influences both ecological specialization and assortative mating in sympatric host races of pea aphids. *American Naturalist* **156**(6): 606-621.

Del Campo ML, Via S, Caillaud MC. 2003. Recognition of host-specific chemical stimulants in two sympatric host races of the pea aphid *Acyrthosiphon pisum*. *Ecological Entomology* **28**(4): 405-412.

Ferrari J, Godfray HCJ, Faulconbridge AS, Prior K, Via S. 2006. Population differentiation and genetic variation in host choice among pea aphids from eight host plant genera. *Evolution* **60**(8): 1574-1584.

Hewer A, Becker A, van Bel AJE. 2011. An aphid's Odyssey - the cortical quest for the vascular bundle. *Journal of Experimental Biology* **214**(22): 3868-3879.

Hogenhout SA, Bos JIB. 2011. Effector proteins that modulate plant-insect interactions. *Current Opinion in Plant Biology* **14**(4): 422-428.

Jaquiery J, Stoeckel S, Nouhaud P, Mieuzet L, Maheo F, Legeai F, Bernard N, Bonvoisin A, Vitalis R, Simon JC. 2012. Genome scans reveal candidate regions involved in the adaptation to host plant in the pea aphid complex. *Molecular Ecology* **21**(21): 5251-5264.

Le Roux V, Dugravot S, Brunissen L, Vincent C, Pelletier Y, Giordanengo P. 2010. Antixenosis phloem-based resistance to aphids: is it the rule? *Ecological Entomology* **35**(4): 407-416.

Marchetti E, Civolani S, Leis M, Chicca M, Tjallingii WF, Pasqualini E, Baronio P. 2009. Tissue location of resistance in apple to the rosy apple aphid established by electrical penetration graphs. *Bulletin of Insectology* **62**(2): 203-208.

Martin B, Collar JL, Tjallingii WF, Fereres A. 1997. Intracellular ingestion and salivation by aphids may cause the acquisition and inoculation of non-persistently transmitted plant viruses. *Journal of General Virology* **78**: 2701-2705.

Peccoud J, Ollivier A, Plantegenest M, Simon JC. 2009a. A continuum of genetic divergence from sympatric host races to species in the pea aphid complex. *Proceedings of the National Academy of Sciences of the United States of America* **106**(18): 7495-7500.

Peccoud J, Simon JC, McLaughlin HJ, Moran NA. 2009b. Post-Pleistocene radiation of the pea aphid complex revealed by rapidly evolving endosymbionts. *Proceedings of the National Academy of Sciences of the United States of America* **106**(38): 16315-16320.

Pitino M, Hogenhout SA. 2013. Aphid Protein Effectors Promote Aphid Colonization in a Plant Species-Specific Manner. *Molecular Plant-Microbe Interactions* **26**(1): 130-139.

Powell G, Hardie J. 2000. Host-selection behaviour by genetically identical aphids with different plant preferences. *Physiological Entomology* **25**(1): 54-62.

Powell G, Pirone T, Hardie J. 1995. Aphid stylet activities during potyvirus acquisition from plants and an *in vitro* system that correlate with subsequent transmission. *European Journal of Plant Pathology* **101**(4): 411-420.

R Core Team 2011. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Rodriguez PA, Bos JIB. 2013. Toward Understanding the Role of Aphid Effectors in Plant Infestation. *Molecular Plant-Microbe Interactions* **26**(1): 25-30.

Sauge MH, Kervella J, Rahbe Y. 1998. Probing behaviour of the green peach aphid Myzus persicae on resistant Prunus genotypes. *Entomologia Experimentalis et Applicata* **89**(3): 223-232.

Schwarzkopf A, Rosenberger D, Niebergall M, Gershenzon J, Kunert G. 2013. To Feed or Not to Feed: Plant Factors Located in the Epidermis, Mesophyll, and Sieve Elements Influence Pea Aphid's Ability to Feed on Legume Species. *Plos One* **8**(9).

Smadja CM, Canback B, Vitalis R, Gautier M, Ferrari J, Zhou JJ, Butlin RK. 2012. Large-Scale Candidate Gene Scan Reveals the Role of Chemoreceptor Genes in Host Plant Specialization and Speciation in the Pea Aphid. *Evolution* **66**(9): 2723-2738.

Tjallingii WF. 1978. Electronic recording of penetration behavior by aphids. *Entomologia Experimentalis et Applicata* **24**(3): 721-730.

Tjallingii WF. 1985. Membrane potentials as an indication for plant-cell penetration by aphid stylets. *Entomologia Experimentalis et Applicata* **38**(2): 187-193.

Tjallingii WF 1988. Electrical recording of stylet penetration activities. In: Minks AK, Harrewijn P eds. *Aphids - Their Biology, Natural Enemies and Control*. Amsterdam: Elsevier, 95-108.

Tjallingii WF, Esch TH. 1993. Fine-structure of aphid stylet routes in plant tissues in correlation with EPG signals. *Physiological Entomology* **18**(3): 317-328.

Tjallingii WF, Gabrys B. 1999. Anomalous stylet punctures of phloem sieve elements by aphids. *Entomologia Experimentalis et Applicata* **91**(1): 97-103.

Tjallingii WF, Garzo E, Fereres A. 2010. New structure in cell puncture activities by aphid stylets: a dual-mode EPG study. *Entomologia Experimentalis et Applicata* **135**(2): 193-207.

Wilkinson TL, Douglas AE. 1998. Plant penetration by pea aphids (*Acyrthosiphon pisum*) of different plant range. *Entomologia Experimentalis et Applicata* **87**(1): 43-50.

Will T, Tjallingii WF, Thonnessen A, van Bel AJE. 2007. Molecular sabotage of plant defense by aphid saliva. *PNAS* **104**(25): 10536-10541.

5 General Discussion

The interaction of an aphid with a plant involves a sequence of behavioral patterns essential for aphid feeding and its survival on the plant. This sequence begins when the aphid inserts its mouthparts, a straw-like structure called "stylet bundle", through the plant cuticle between two epidermal cells into the plant apoplast (Tjallingii & Esch, 1993). The aphid navigates its stylet on an extracellular pathway through the plant apoplast (Chapter 2, Figure 1), and punctures nearly every cell along its way. Thus the stylet tip briefly contacts the cellular lumen (Tjallingii & Esch, 1993) (Chapter 4, Figure 1).The sequence can be considered as completed when the aphid reaches a plant sieve element and successfully feeds on the nutritious phloem sap. During this behavioral sequence the aphid contacts different plant tissues and cell types. Hence, it encounters various plant factors including structural components, chemical compounds, and immune factors mediating resistance against aphids. All these factors may influence the success or failure of the aphid in reaching a sieve element and establishing feeding. The central aim of the present study was to localize with the help of the Electrical Penetration Graph Technique (EPG) (McLean & Kinsey, 1964; McLean & Weigt, 1968) the plant factors (in terms of tissue or cellular localization) which enable pea aphid biotypes to feed and survive on certain legume plant species and deter them from feeding and surviving on other legume species. Adopted from the terminology used to describe the ability of phytopathogens to infest a plant or not (e.g. Nomura *et al.*, 2005), the two types of plant–aphid interactions (plant resistance or susceptibility against aphids) are here called "compatible" and "incompatible". With the knowledge about plant factor localization responsible for compatibility or incompatibility, it is possible to infer the nature of the plant factors and the mechanisms which influence the compatibility of pea aphid biotypes and legume species.

5.1 Plant–aphid compatibility in the pea aphid species complex

To infer plant factors which are responsible for compatibility or incompatibility of a pea aphid biotypes to legume plant species, it was vital to determine the degree of compatibility of each aphid biotype-plant species combination (by measuring biotype performance by measuring aphid survival and weight) on all plant species (Chapter 2, Figure 2 & 3). Table 1 summarizes the results in a simplified way.

Table 1. Simplified illustration of the degree of pea aphid biotypes' with four legume species. There are compatible interactions (+), interactions with intermediate compatibility (±), and incompatible interactions (-).

		Plant species			
Biotype	Clone	*M. sativa*	*P. sativum*	*T. pratense*	*V. faba*
M	M1	+	±	–	+
	M2	+	±	±	+
P	P1	–	+	±	+
	P2	–	+	+	+
T	T1	–	±	+	+
	T2	–	±	+	+

The highest compatibility of each aphid biotype was observed on the legume species the biotype was collected from in the field (Peccoud *et al.*, 2009b Supporting Information Table S1) (Table 1) Using microsatellite markers, the identity and host-plant affiliation of the biotypes used in the present study have been shown to match the biotypes from Medicago, Pisum and Trifolium described by Peccoud *et al.* (2009b). Hence, the plants on which the biotypes showed the highest compatibility were classified as their native host plants.

Notably, all biotypes showed as high a compatibility to *V. faba* as to their native host plants. Hence, *V. faba* can be considered as a universal host plant. Previous studies also agree with this conclusion (Sandstrom & Pettersson, 1994; Ferrari *et al.*, 2006) which is supported also by the fact that nearly all research groups using pea aphids raise them on plants of *V. faba*. Additionally, the present study revealed that not only performance, but also many behavioral parameters measured during the localization experiment are similar on *V. faba* compared to their host plants. An explanation for the similarities of pea aphid biotype performance and behavior on the native host and the universal host plant might be that ancestors of the *V. faba* cultivars growing at present represented the plant from which the adaptive radiation of the pea aphid started about 9000 years ago (Peccoud *et al.*, 2009b). However, neither the plant on which the adaptive radiation started, nor the ancestor of the present biotypes has yet been identified.

Legume plant species other than the native and universal host plants can be classified as less-suitable plants or as non-host plants (Table 1). On these plants, biotypes showed reduced compatibility or incompatibility, respectively. Of the four tested plant species *M. sativa* was the most unsuitable plant for all non-Medicago biotype pea aphids. The assumption that *M. sativa* represents a non-host plant for many pea aphid biotypes is suggested by similar performance results from previous studies (Via, 1991; Peccoud & Simon, 2010). In contrast to *M. sativa*,

most of the tested biotypes survived and grew at least to a certain extent on *P. sativum* and *T. pratense* (Table 1). Taken together, the results of previous studies and the present study depict well the continuum of biotype-plant compatibility within the pea aphid species complex described by Peccoud *et al.* (2009a). Interestingly, this study revealed that the continuum of biotype-plant compatibility is mirrored by a continuum of genetic divergence within the pea aphid species complex. This raises the question if a continuum of aphid behavioral divergence can also be detected. If so, this could be interpreted as a clear sign for pea aphid biotype diversification in response to plant specialization.

The present study included all the degrees of pea aphid-plant compatibility defined above. Hence our experiments could detect the full range of behavioral divergence among the pea aphid biotypes in response to plant factor localization. But before discussing the impact of plant factors which might trigger pea aphid diversification, I will illustrate which plant factors and interaction mechanisms could be responsible for compatibility and incompatibility of pea aphid biotypes on legumes.

5.2 Putative mechanisms influencing the plant-compatibility of pea aphid biotypes and legume plant species

As observed in chapter 2 plant, factors influencing success or failure in establishing feeding are located on multiple tissue levels – in plant epidermal cells, mesophyll cells, and the sieve elements. From investigations of behavioral patterns, two main mechanisms were associated with the failure to feed. Firstly, the plant penetration process was stopped between the initial penetration and the beginning of sieve element feeding. Secondly, the aphids reached the sieve elements but were not able to start feeding after they have reached a sieve element – a process which likely involves aphid salivary effectors interacting with plant factors.

5.2.1 Plant factors influencing the continuation of aphid plant penetration

Pea aphid biotypes which tried to feed on less suitable and non-host plants often failed to establish feeding (Chapter 2, Figure 7) and consequently had low survival rates (Chapter 2, Figure 2). Thus, one central reason for pea aphid biotype–legume incompatibility is the inability to establish feeding on a plant. However, nearly all individuals of the biotypes tested in this study started to penetrate the plant whether it was compatible or not (Chapter 2, Figure 3), a

trend seen in previous studies (Wilkinson & Douglas, 1998; Caillaud & Via, 2000). Our study showed that perception of plant factors, encountered by aphids before reaching a sieve element, influenced compatibility. Biotypes on non-host and less suitable plants often stopped penetration soon after beginning (Chapter 2, Figure 4 A and 5). Whether the penetration process is continued or disrupted might be due to plant factors which are perceived by aphids. Such factors can function in three ways: (1) Deterrent factors might lead to a disruption of the penetration process. (2) The absence of stimulating or recognition factors might have the same result. (3) The perception of recognition factors might lead to a continued penetration and successful feeding.

Deterrent plant factors in the upper plant tissue layers might be the reason for the very brief plant probing attempts of the Trifolium biotype aphids on incompatible plant species, which were also described by Caillaud and Via (2000).The recognition of factors acting as inhibitors of aphid penetration are known from other plant-aphid interactions, e.g. from wild *Solanum* species which are resistant to *M. persicae* (Alvarez *et al.*, 2006). Often the relevant plant factors are not known, but chemical compounds might be involved. In the interaction of legumes and aphids, saponins, flavonoids and alkaloids seem to be important. For example, saponins occurring in *M. sativa* have been shown to inhibit pea aphid penetration behavior in artificial diet assays (Golawska *et al.*, 2014). In another study, the flavonoids luteolin and genistein were identified to have similar effects on pea aphid penetration behavior (Golawska & Lukasik, 2012). From the results of the present study, it is not possible to disentangle which legume chemical compounds might contribute to the interruption of the plant penetration by pea aphids on less-suitable and non-host plants. To elucidate this, compounds affecting pea aphid biotype-plant compatibility need to be selected. Then choice experiments might be conducted involving the dissolution of compounds in artificial aphid diet and EPG analyses to give evidence about the relevance of certain compounds in pea aphid-plant compatibility.

Like the recognition of deterrent or toxic plant factors, the lack of stimulating plant factors in less-suitable and non-host plants might also lead to an early disruption of the plant penetration process. Such a phenomenon has been described much earlier by Wensler (1962) in a study on *Brevicoryne brassicae*. Usually, *B. brassicae* individuals left the non-host plant *V. faba* shortly after a brief penetration attempt. But if the plant leaves were fed before the experiment with sinigrin, a key component of one host plant of *B. brassicae*, *Brassica rapa*, the aphids remained on the leaves and started plant penetration. Interestingly *B. brassicae* could live healthy on sinigrin-treated *V. faba* leaves for several generations, which demonstrates that only the lack of a certain stimulus might be responsible for the rejection of an otherwise suitable plant.

The presence of plant factors can also be used to recognize a host plant. This was implied by results of a study by Del Campo *et al.* (2003), which showed that host plant extracts applied to a non-host plant stimulate pea aphid penetration on the non-host plant. As Del Campo *et al.* (2003) used aqueous extracts of plant tissue, water soluble plant factors should be responsible. Water-soluble plant factors might be either present in the inside the epidermal cells or in the polysaccharide matrix of the cell wall. In the present study, both Trifolium clones showed very short penetration attempts involving an epidermal cell puncture and including the ingestion of some cell content before these were aborted (Chapter 2, Figure 4 B). Hence, an intracellular location of plant factors is likely. The exact location of stimulating plant factors inside a cell is not known. It is possible that factors might be located in the vacuoles, as there is some evidence for an obligate tonoplast puncturing during intracellular punctures in epidermis and mesophyll Hewer *et al.* (2011). However, it cannot be excluded that the substances are located in the epidermal cytoplasm, as aphids are likely to contact the cytoplasm during intracellular punctures. Further studies are necessary to assess exactly the localization of plant factors serving as key stimulants for the pea aphid penetration process in the epidermis. To find out if penetration stimulating plant factors are located in vacuole or cytoplasm, vacuolar extracts (Robert *et al.*, 2007) of host plants could be used. Experiments with the vacuolar extracts dissolved in artificial diet or applied to plant leaves (as described by Del Campo *et al.*, 2003) could give the causal link to subcellular localization of the plant factors and their function. As during intracellular punctures, the aphid has to penetrate the plant cell wall in order to enter the cell lumen, so it is also worth trying to test if cell wall compounds such as polysaccharides have an effect on pea aphid probing behavior. In artificial diet experiments, it has been shown that plant matrix polysaccharides evoke host discrimination behavior in the pea aphid (Campbell *et al.*, 1986). Hence, artificial diet experiments with host plant-specific polysaccharides may give further insight into a putative role of these macromolecular compounds in the compatibility of pea aphid biotypes with host plants.

Plant factors which stimulate the penetration of a certain plant can also be located in subepidermal tissues, i.e. mesophyll cells or phloem parenchyma cells. This is indicated by the duration of the “pathway phases” which reflect the stylet bundle movement between epidermis and sieve elements. In cases of high compatibility to the plant, most aphid biotypes showed a significantly longer “pathway phase” (Chapter 2, Figure 5). As in epidermal cells, intracellular punctures including ingestion of cell content are an obligate feature of the aphid behavior during the pathway phase. Thus, the same mechanisms which work in the epidermis might also be present in mesophyll cells.

In addition to what is known from various studies which emphasize a key role of plant factor recognition by aphids in epidermal or subepidermal tissues (Wensler, 1962; Caillaud & Via, 2000; Powell & Hardie, 2000; Del Campo *et al.*, 2003), the present study raised evidence for location of plant factors inside sieve elements. Immediately before the potential establishment of a feeding period, aphids punctured sieve elements repeatedly in a way very similar to intracellular punctures of epidermis and mesophyll cells (Tjallingii & Gabrys, 1999) (Chapter 4). Compared to epidermal and mesophyll cell punctures, the sieve element punctures were longer and occured in a repeated manner with a constant frequency (Chapter 4, Figure 1). Many pea aphid biotypes on less-suitable and non-host plants showed significantly higher number of repeated punctures of sieve elements that ended without subsequent feeding (i.e. the stylet bundle is withdrawn from the sieve element) compared to repeated punctures that ended with subsequent feeding (Chapter 2, Figure 6). The lack of stimulating plant factors or the presence of deterrent factors in sieve elements of less-suitable and non-host plants might explain the more frequent occurrence of repeated sieve element punctures that ended without subsequent feeding. In contrast, stimulating plant factors in host plants might have triggered ongoing sieve element penetration with subsequent feeding. Water soluble plant compounds that are transported through the phloem possibly serve as such stimulating or deterrent factors. For instance, quinolizidine alkaloids in lupines have been shown to be transported through the phloem and to have a deterrent or toxic effect (Wink *et al.*, 1982). Thus, stylet bundle withdrawal on less-suitable and non-host plants could be part of an avoidance strategy against toxic plant factors present in the sieve elements. One plant factor which might be sensed as a positive signal stimulating aphid feeding is a suitable amino acid composition in a sieve element. As there is variation in the amino acid composition amongst different sieve elements (Gattolin *et al.*, 2008), it would be beneficial for pea aphid biotypes to recognize the presence of a suitable amino acid composition in a sieve element to be targeted for feeding. This could explain why on native host plants and the universal host more repeated sieve element punctures resulted in feeding than on less-suitable and non-host plants (Chapter 2, Figure 6). That plant chemical compounds are used by the pea aphid biotypes to sense their host plants is supported by recent genomic studies which identified large families of chemoreceptor genes (i.e. gustatory receptor genes (*Gr* gene family), and olfactory receptor genes (*Or* gene family) (Jaquiery *et al.*, 2012; Smadja *et al.*, 2012; Duvaux *et al.*, 2015). These findings strengthen the hypothesis that pea aphid-legume compatibility is highly influenced by recognition and plant discrimination processes involving chemoreception. However, the identity of the plant factors which interact with olfactory and especially gustatory receptors and serve as positive signal for biotype-plant compatibility remains unclear so far.

Regardless which plant factors can be sensed by pea aphids in order to make a decision to further penetrate a plant or not, the pea aphid penetration and feeding behavior could also be influenced by interplay of aphid salivary proteins and legume plant factors mediating plant defence mechanisms. Aphid saliva is omnipresent at the interface between aphid and salivary proteins have been shown to influence plant-aphid compatibility in different ways.

5.2.2 Aphid salivary effectors interacting with plant factors as putative determinants of aphid-plant-compatibility

5.2.2.1 Herbivore associated molecular patterns (HAMPs) trigger plant immunity

During the entire plant penetration process aphid saliva is secreted into the plant. There are two types of aphid saliva, "gelling saliva" and "watery saliva" (Miles, 1959; Miles, 1999). Even before an aphid inserts its stylet tip into the plant tissue, a droplet of gelling saliva is applied to the plant cuticle (Miles, 1999). The aphid's stylet bundle, surrounded by gelling saliva, is then inserted into the plant apoplast between two epidermal cells (Will *et al.*, 2012). The continuously secreted gelling saliva forms a canal or sheath which envelops and protects the stylet bundle during the entire stylet pathway from plant surface to the sieve elements (Cherqui & Tjallingii, 2000; Will *et al.*, 2012). The saliva sheath facilitates the penetration process by protecting the aphid's mouthparts from direct contact to plant compounds. The second type of saliva, the watery saliva (Miles, 1999), is less viscous and is mainly delivered directly into the cell lumen during the intracellular punctures (Powell, 1991; Powell *et al.*, 1995), but also during the period in which the aphid's stylet remains in a sieve element in order to feed on the phloem sap. Molecules in the aphid saliva are able to induce plant defense mechanisms against aphids resulting in "herbivore associated molecular pattern (HAMP)-triggered immunity". HAMP-triggered immunity is based on the recognition of an aphid specific molecular pattern, i.e. aphid saliva proteins, by a plant receptor. Also plant derived breakdown products of salivary enzymes were shown to be able to activate the HAMP-triggered immunity. The recognition mechanism results in an array of plant responses resulting in specifically tailored defense against the invader: increase of intracellular concentration of Ca^{2+} ions, release of reactive oxygen species (ROS), local cell death and necrotic lesions (hypersensitive response, HR), and downstream defense reactions mediated by salicylic acid, jasmonic acid, and ethylene signaling (reviewed in Wu & Baldwin, 2010; Hogenhout & Bos, 2011). As described above, the contact of aphid saliva with the plant cells is an constant feature of the plant penetration process. This makes it a difficult task to localize plant factors targeted by aphid salivary effectors in order to determine if certain aphid behavioral patterns or periods of the penetration and salivation process play a particular role in inducing plant defense.

5.2.2.2 Interaction of salivary effectors and plant target factors in epidermis and mesophyll before reaching a sieve element

In the present study, several aphid behavioral phenomena which led to an interruption of the plant penetration can be interpreted as response to HAMP-triggered immunity in incompatible biotype-legume interactions. (1) Three out of six aphid clones spent less time penetrating non-host plants compared to host plants, and the remaining three aphid clones showed a trend towards this (Chapter 2, Table 2). (2) The aphid clones from the Trifolium biotype showed repeated stylet withdrawal from epidermal cells on non-host plants very shortly after the penetration attempt started (Chapter 2, Figure 4). (3) The duration pea aphid clones navigated their stylet bundle between epidermis and the sieve element on less suitable and non-host plants was significantly shorter in five out of the six tested clones and most of the individuals were not even able to reach the sieve elements and to establish feeding (Chapter 2, Figure 5). As known from former studies on other plant-aphid interactions, the plant hypersensitive response (HR) is a defense reaction which can be activated very soon after the penetration started and likely contributes to aphid resistance in epidermal and subepidermal tissues (Klingler *et al.*, 2009). However, a potential role of HR for pea aphid-plant incompatibility seems unlikely. One study aiming at elucidating legume resistance mechanisms against pea aphids implied that defense principles independent from the HR are involved in stopping the plant penetration process. The plant resistance gene involved (*RAP1*) is not associated with HR, but is responsible for the resistance of *Medicago truncatula* to the pea aphid (Stewart *et al.*, 2009). A recent study also implied that defense mechanisms of legumes against pea aphids are not dependent on HR. Naessens *et al.* (2015) reported that a cytokine (MIF1) triggers plant resistance against the pea aphid. The cytokine is secreted into the plant tissue by the aphid and its effect is a nice example which shows that salivary proteins called salivary effectors can suppress the initial HAMP-triggered immunity. That secretion of salivary effectors might represent a key step for modulating aphid-plant compatibility is supported by additional examples of so called "effector-triggered susceptibility". In the interaction of *Myzus persicae* with *Arabidopsis thaliana* the salivary effectors C002, PIntO1 and PIntO2 promoted insect infestation when overexpressed *in planta* (Bos *et al.*, 2010; Pitino & Hogenhout, 2013). The salivary effector C002 was also found in the pea aphid (Mutti *et al.*, 2006), and silencing of the effector gene in the pea aphid by the RNAi technique led to inability to feed on the universal host plant *V. faba* (Mutti *et al.*, 2008). The individuals lacking the effector protein started plant penetration, but the penetration process was stopped during the pathway phase in accordance with the results of the present study. Although it is known that the salivary effector C002 is responsible for the compatible interaction of the pea aphid and its universal host plant *V. faba* it is still unknown whether the effector also enables the different pea aphid biotypes to feed on their respective native host

plants. Taken together, the compatibility of pea aphid biotypes with legumes might be dependent on sufficient effector delivery to the plant. The question remains if the amount of watery saliva secreted directly into the lumina of epidermis and mesophyll cells during the intracellular punctures contributes to compatibility of pea aphid biotype-legume interactions. To investigate this, the duration of the salivation phase during the intracellular punctures of epidermis and mesophyll cells was measured on native host plants, universal host plant, less-suitable and non-host plants. A longer salivation into the cell could give additional evidence for the putative relevance of intracellular salivation for pea aphid biotype-legume compatibility. However, the intracellular salivation time of the pea aphid biotypes did not vary in a meaningful way on compatible and incompatible plant species (Chapter 4, Figure 2). Hence, instead of the duration (which might correlate with the quantity) of intracellular salivation and effector delivery, the quality of the effector composition in the aphid saliva might be essential for the aphid-plant compatibility. This might be true not only for the saliva secreted during the intracellular punctures, but also for the aphid salivary sheath, as both types of aphid saliva contain partly overlapping sets of putative salivary effectors (Will *et al.*, 2012).

5.2.2.3 Interaction of salivary effectors and plant target factors in sieve elements

The repetitive intracellular punctures include salivation into the sieve element. Hence, the puncturing period could be relevant for conditioning the sieve element for subsequent feeding (Tjallingii & Gabrys, 1999). There are two results involving repetitive sieve element punctures which clearly emphasize that repetitive sieve element punctures are essential for establishing feeding. Firstly, nearly all successful cases of establishing aphid feeding in compatible biotype-plant interactions involved repetitive sieve element punctures (Chapter 2, Figure 6 C). This implies that this period is important for conditioning the sieve element for the subsequent feeding period. Secondly, in most cases repetitive sieve element punctures did not lead to feeding periods in incompatible biotype-plant interactions (Chapter 2, Figure 6 B). This can be interpreted as the failure of sieve element conditioning to enable subsequent feeding. A comparable pattern was found for *Brevicoryne brassicae* on *Sinapis alba* for which most feeding periods followed repetitive sieve element punctures (Tjallingii & Gabrys, 1999). The results from other studies also point to a relevance of repetitive sieve element punctures as a prerequisite for sieve element feeding, as the total durations of the repetitive sieve element punctures were lower on non-host plants than on host plants (Caillaud & Via, 2000; Gao *et al.*, 2008).

Repetitive sieve element punctures are not the only period involving salivation into the sieve element. The so called “sieve element salivation period” is considered to be important for

successful aphid feeding and common to all studied aphid species within the family of Aphididae (Miles, 1999). Sieve element salivation has been shown to prevent the calcium-dependent shut-down of the phloem sap flow mediated by sieve element located proteins (Furch *et al.*, 2007; Will *et al.*, 2007; Will *et al.*, 2009). Salivary effectors that have the potential to regulate intracellular calcium levels, and hence be able to control the calcium dependent sealing of sieve elements (Will *et al.*, 2007) may be responsible for keeping the phloem open. To investigate if sieve element salivation influences the compatibility of pea aphid biotypes to the legumes, several parameters associated with this behavior were investigated. For example, extended sieve element salivation could be interpreted as important intracellular delivery phase for salivary effectors to the sieve elements preventing phloem sap shut-down. However, none of the six aphid clones in the present study showed any compatibility-specific prolongation of sieve element salivation. The absence of any compatibility-specific prolongation of sieve element salivation can be interpreted in two ways. A sieve element shut-down by protein-plugging might be not relevant for the compatibility of the pea aphid biotypes with legume plants. Altenatively, the amount of saliva/effectors could be less important than the specificity of the salivary effectors.

The comparison of aphid behavioral patterns on native host plants, the universal host plant, less-suitable and non-host plants allowed localizing plant factors and interaction mechanisms that influence the compatibility of pea aphid biotypes and their respective host plants. Similar behavioral patterns amongst the clones point to a general importance of chemosensory perception of plant factors by the aphid, and to plant immunity factors interacting with aphid salivary effectors. Those interactions are likely to take place in epidermis and mesophyll cells as well as in the sieve elements. Remarkably, in some parameters the biotypes behaved significantly different than other biotypes on the same plants.

5.3 The diversity of legume plant factors promotes behavioral divergence among pea aphid biotypes

In Table 2 several examples of behavioral patterns are listed, that occurred exclusively in certain aphid biotype or clone-legume combinations tested in the present study.

Table 2. Behavioral patterns which occurred exclusively in certain aphid biotype or clone-legume combinations tested in the present study.

Clones	Behavioral pattern	Reference
T1, T2	Very short probes occur on less-suitable and non-host plants	Ch. 2, Fig. 4
M1, M2	Experience effect / ability to feed on test plant depends on rearing plant species	Ch. 2, Fig. 7; Ch. 3, Fig. 3
M1, M2, P1	Duration of intracellular salivation and ingestion depends on plant species	Ch. 4, Fig. 2
T1, T2	Intracellular behavioral pattern (epidermis/mesophyll) differs on native host and other plants	Ch. 4, Fig. 2
P1, P2, T1	Intracellular behavioral pattern (sieve elements) differs on native host and other plants	Ch. 4, Fig. 3

A behavior exclusive to the Trifolium clones on less-suitable and non-host plants was an increased number of very short probes which involved repeated stylet bundle withdrawal from the plant tissue soon after the penetration was started. Both Medicago clones showed an experience effect, i.e. the plant species on which the biotypes were reared/amplified for the localization experiment (*V. faba*) influenced the subsequent probing and feeding performance on their host plant *M. sativa* in a negative way. This was connected to a very high incidence of single repetitive sieve element puncture phases. These repetitive sieve element punctures ended without subsequent feeding and were restricted to individuals reared on *V. faba* and tested on *M. sativa*. However, if reared on their native host plant *M. sativa*, the clones showed the typical ability to establish feeding. Additionally, intracellular behavioral patterns were influenced in some biotypes by the plant species, e.g. a significantly longer duration of intracellular behavioral patterns in mesophyll cells on a certain plant species. Due to these results, it is clear that the continuum of biotype-plant compatibility is mirrored not only by a continuum of genetic divergence within the pea aphid species complex (as detected by Peccoud *et al.* (2009a)), but also by behavioral divergence. The presence of diverging behavioral patterns amongst the different pea aphid clones on different plants points to diverse factors influencing the biotype-plant compatibility. This provides a basis for behavioral biotype diversification and further plant specialization. Such diversification could explain why we can observe stable pea aphid biotypes despite the presence of the universal host plant *V. faba* which enables gene-flow since in nature this plant can serve as a platform where the different biotypes survive, meet and mate.

The pea aphid is not the only aphid species considered to represent a species complex (Shaposhnikov, 1987). However, within the Aphididae, pea aphids represent the best and most widely studied species complex, based on numerous reports shedding light on the population genetics, molecular plant-aphid interactions, or ecology of pea aphids. Moreover, the elucidation of the pea aphid genome provides a very strong base for detailed studies using genetically modified pea aphids (Richards *et al.*, 2010). These techniques will contribute further to determining which plant defense mechanisms are involved and drove the evolution to the presence of early recognition of host and universal host and rejection of less-suitable and non-

host plants. Finally, transfer of this knowledge to other aphid species complexes will help to elucidate the mechanisms behind the compatibility of many different aphid biotypes with their associated plant species.

6 Summary/Zusammenfassung

6.1 English summary

The pea aphid *Acyrthosiphon pisum* (Harris) species complex consists of at least 15 genetically distinct host races, also called biotypes. While all pea aphid biotypes live on plant species belonging to the Fabaceae, each host race shows distinct preferences for certain legume species. However, it is not yet known which plant factors contribute to the ability of different biotypes to feed on different legumes. In this dissertation, a selection of different pea aphid biotypes and legume species were used to study the localization of plant factors affecting pea aphid ability to feed on host and non-host plants. Mechanisms that influence the ability of aphids to feed on a certain plant are discussed in the light of the behavioral data.

First, the performance of six pea aphid clones belonging to three different biotypes was determined on four legume species – *Medicago sativa*, *Pisum sativum*, *Trifolium pratense*, and *Vicia faba*. The ability to survive and grow was highest on the native host plant from which the aphid clones were collected in the field. However, aphid clones performed less well or were not able to survive at all on other legumes, which were denoted as less-suitable or non-host plants, respectively. On *V. faba*, the universal host, all clones performed as well as on their native host plant.

Based on knowledge of the performance of the clones on the different plants, the probing and feeding behavior of each clone on each of the legume species was recorded using the electrical penetration graph technique (EPG). This technique enables real-time monitoring of aphid probing and feeding behavior by measuring the fluctuations in the electrical resistance generated in a circuit set up to include both the aphid and the plant. The obtained waveforms can be assigned to the various activities of the aphid. By comparing the activities of the biotypes on native versus less suitable or non-host plants, it was possible to detect in which tissues aphid probing and feeding behavior was altered. The results showed that the low survival on less-suitable or non-host plants was due to an impaired ability to feed, with probing behavior being affected during several stages of the plant penetration process. Since all clones started to penetrate the plants, the impaired ability to feed was either due to interruption after initial penetration or disruption after the aphid successfully reached and punctured the sieve element.

The disruption in the initial probing phases could be due to deterrents, or the lack of stimulating factors in the upper tissue layers of less-suitable and non-host plants. Since nearly every cell is punctured while the aphid navigates its stylet bundle towards the sieve elements, plant factors might be located in the intracellular spaces of the epidermal and subepidermal tissue layers.

The interruption in later phases occurred prior to phloem feeding. Before aphids were able to feed they usually punctured their target sieve element repeatedly, ingested their content and salivated into them. On host plants, these repeated sieve element punctures often led to feeding, while on non-host plants, repeated sieve element punctures mostly ended without subsequent feeding. Again, the lack of stimulating factors or the presence of deterrent factors might be one reason.

When aphids puncture cells, they not only ingest cell content, but also inject saliva containing effector molecules, which may interfere with plant immunity to aphids. Thus, a longer duration of intracellular salivation on host plants could explain a higher performance, since a higher amount of salivary effectors could be delivered. But there was no significant association between the duration of salivation into epidermis, mesophyll or the sieve elements and subsequent feeding. However, nearly all successful cases of feeding were preceded by repetitive sieve element punctures including salivation. This can be interpreted as successful conditioning of sieve elements for feeding by suppression of defence mechanisms. In contrast, the repetitive sieve element punctures did not usually lead to feeding on less suitable or non-host plants perhaps because of a failure of sieve-element conditioning for feeding.

For all aphid biotypes tested, the ability to feed appears to depend on differential chemosensory perception of plant factors and differences in the interaction of aphid salivary effectors with plant immune factors. Biotype-specific differences were found for the Medicago biotype, which after being reared on *V. faba* exhibited altered probing behavior on its native host plant, *M. sativa*, and was unable to feed. The duration of intracellular salivation or intracellular ingestion on different plant species were altered in a clone-specific way.

The presence of diverging probing and feeding behavior among the pea aphid biotypes can be interpreted as part of host specialization in response to the plant. Ongoing specialization might explain why pea aphid biotypes are maintained despite the presence of the universal host *V. faba* which could enable gene flow between the biotypes.

6.2 Deutsche Zusammenfassung

Der Artkomplex der Erbsenblattlaus *Acyrthosiphon pisum* (Harris) besteht aus mindestens 15 genetisch unterscheidbaren Wirtsrassen, auch Biotypen genannt. Alle Biotypen leben auf Leguminosen (Fabaceae), wobei jeder Biotyp eine klare Präferenz für eine oder wenige Pflanzenarten aufweist. Bisher sind die Pflanzenfaktoren, die es den Biotypen ermöglichen oder verhindern an bestimmten Leguminosen zu fressen, nicht bekannt. Im Rahmen dieser Dissertation wurde die Lokalisierung entsprechender pflanzlicher Faktoren im Gewebe von Wirts- und Nichtwirtspflanzen untersucht. Dazu wurden verschiedene Biotypen der Erbsenblattlaus in Kombination mit verschiedenen Leguminosen eingesetzt. Die möglichen Mechanismen, welche die Fähigkeit der Biotypen beeinflussen sich von einer Pflanze zu ernähren oder nicht, werden vor dem Hintergrund der in den Experimenten gewonnen verhaltensbiologischen Daten diskutiert.

Von sechs Blattlausklonen, zugehörig zu drei Biotypen, wurde die Fähigkeit zu wachsen und zu überleben auf vier Leguminosenarten (*Medicago sativa*, *Pisum sativum*, *Trifolium pratense* und *Vicia faba*) untersucht. Am besten entwickelten sich die Blattlausklone auf den natürlichen Wirtspflanzenarten, von welchen die Blattlausklone im Feld gesammelt wurden. Auf anderen Pflanzenarten überlebten nur wenige oder keine Blattläuse. Diese Pflanzenarten wurden als weniger geeignete, beziehungsweise als Nicht-Wirtspflanzen bezeichnet. Auf der universellen Wirtspflanze *Vicia faba* überlebten alle sechs Blattlausklone genauso gut wie auf ihren natürlichen Wirtspflanzen.

Nach der Charakterisierung der Überlebensfähigkeiten der Klone auf den verschiedenen Pflanzen wurde das Penetrations- und Fressverhalten aller Klone auf allen Pflanzenarten untersucht. Dies geschah mittels der *Electrical Penetration Graph*-Technik (EPG). Diese Technik ermöglicht es, in Echtzeit das Verhalten einer Blattlaus während der Penetration des pflanzlichen Gewebes zu überwachen und aufzuzeichnen. Bei dieser Technik sind Blattlaus und Pflanze Bestandteile eines Stromkreises. In Abhängigkeit vom Verhalten der Blattlaus kommt es zu distinkten Änderungen des elektrischen Widerstands, welche in unterschiedlichen Wellenmustern sichtbar werden. Durch den Vergleich der Verhaltensmuster der Biotypen auf ihren natürlichen Wirtspflanzen und auf den weniger geeigneten bzw. Nicht-Wirtspflanzen war es möglich, Rückschlüsse auf die Lokalisierung von Pflanzenfaktoren zu ziehen, welche zur Änderung der Verhaltensmuster beitragen. Die Ergebnisse zeigten, dass die schlechte Überlebensfähigkeit auf weniger geeigneten Pflanzen und Nicht-Wirtspflanzen durch die Unfähigkeit der Blattläuse zu Fressen begründet war. Die Blattläuse unterbrachen während

verschiedener Phasen des Penetrationsvorganges die weitere Penetration – entweder recht bald nach Penetrationsbeginn oder erst nach erfolgreicher Punktion eines Siebelementes.

Die Unterbrechung der Penetration der Pflanze während der initialen Phase könnte von abschreckenden pflanzlichen Faktoren verursacht werden. Aber genauso ist das Fehlen von die Penetration stimulierenden Faktoren in der Epidermis oder dem Mesophyll von wenig geeigneten oder Nicht-Wirts-Pflanzen denkbar. Da während des Penetrationsvorganges nahezu jede Pflanzenzelle von der Blattlaus punktiert wird, ist eine intrazelluläre Lokalisation beteiligter pflanzlicher Faktoren in Epidermis oder Mesophyll wahrscheinlich.

Die Unterbrechungen während späterer Penetrationsphasen auf weniger geeigneten und Nicht-Wirtspflanzen wurden unmittelbar vor dem normalerweise erwarteten Beginn einer Fressphase in einem Siebelement beobachtet. Vor dem Beginn einer Fressphase punktierten die Blattläuse wiederholt das erreichte Siebelement. Dabei nahmen sie Zellinhalt auf und sekretierten Speichel in das punktierte Siebelement. Auf Wirtspflanzen mündeten die Phasen wiederholter Punktion zumeist in Fressphasen – auf Nicht-Wirtspflanzen endeten diese Phasen wiederholter Punktion in der Regel mit dem Beenden der Punktion und dem Rückziehen des Stechborstenbündels aus dem Siebelement. Wiederum können sowohl abschreckend wirkende Faktoren, als auch fehlende die Penetration stimulierende pflanzliche Faktoren Auslöser der Penetrationsabbrüche sein.

Wie oben bereits erwähnt, nehmen Blattläuse während der intrazellulären Phasen der Penetration der Pflanze nicht nur Zellinhalt auf, sondern geben auch Speichel in die pflanzlichen Zellen ab. Es ist bekannt, dass der Speichel der Blattläuse Effektormoleküle enthält, welche mit pflanzlichen Abwehrmechanismen gegen Blattläuse interferieren können. Daher stellte sich im Verlauf der Studien die Frage, ob das Injizieren einer größeren Menge an Speichel in die Pflanzenzellen das gute Wachstum und Überleben der Blattläuse auf den Wirtspflanzen erklären könnte. Dies war jedoch nicht der Fall. Die Dauer der Speichelabgabe während der intrazellulären Punktion von Epidermis- oder Mesophyllzellen, und von Siebelementen stand nicht im Zusammenhang mit der Fähigkeit eine Fressphase auf einer Pflanze einzuleiten. Jedoch wurden nahezu alle erfolgreichen Fressphasen von einer wiederholten Punktion des entsprechenden Siebelementes eingeleitet. Da diese wiederholten Punktionen immer von einer Speichelabgabe in das Siebelement begleitet waren, ist davon auszugehen, dass die Siebelmente für die nachfolgende Fressphase konditioniert wurden, möglicherweise indem Verteidigungsmechanismen erfolgreich unterdrückt werden. Im Gegensatz dazu führten die wiederholten Punktionen von Siebelementen von weniger geeigneten bzw. Nicht-Wirtspflanzen

in keinem Fall zu einer Fressphase. Dies könnte durch eine nicht ausreichende Vorbereitung des Siebelementes für eine nachfolgende Fressphase begründet sein.

Bei allen getesteten Biotypen scheint die Fähigkeit an einer Pflanze zu fressen, von der chemosensorischer Erkennung von Pflanzenfaktoren und vom Zusammenspiel der im Blattlausspeichel enthaltenen Effektoren mit Komponenten der pflanzlichen Abwehr abzuhängen. Neben diesen für alle Biotypen zutreffenden Beobachtungen wurden jedoch auch für einzelne Biotypen oder sogar einzelne Blattlausklone spezifische Verhaltensunterschiede auf den verschiedenen Pflanzen entdeckt. Ein für die Medicago-Rasse spezifisches Phänomen war die Unfähigkeit beider Klone auf ihrer natürlichen Wirtspflanze *M. sativa* zu fressen, nachdem sie auf der universellen Wirtspflanze *V. faba* vermehrt wurden. Klonspezifische Unterschiede zeigten sich in der Dauer der Aufnahme von Zellinhalt oder der Abgabe von Speichel in die Pflanzenzellen.

Das Vorhandensein divergenter Verhaltensweisen zwischen verschiedenen Biotypen kann als Teil der Spezialisierung einzelner Biotypen interpretiert werden. Eine andauernde Spezialisierung von Biotypen könnte erklären, warum die Biotypen der Erbsenblattlaus fortbestehen, obwohl ein Genfluss zwischen den Biotypen durch die universelle Wirtspflanze *V. faba* theoretisch möglich ist.

7 References

Afshar K, Dube FF, Najafabadi HS, Bonneil E, Thibault P, Salavati R, Bede JC. 2013. Insights into the insect salivary gland proteome: Diet-associated changes in caterpillar labial salivary proteins. *Journal of Insect Physiology* **59**(3): 351-366.

Afshar K, Dufresne PJ, Pan L, Merkx-Jacques M, Bede JC. 2010. Diet-specific salivary gene expression and glucose oxidase activity in Spodoptera exigua (Lepidoptera Noctuidae) larvae. *Journal of Insect Physiology* **56**(12): 1798-1806.

Alvarez AE, Tjallingii WF, Garzo E, Vleeshouwers V, Dicke M, Vosman B. 2006. Location of resistance factors in the leaves of potato and wild tuber-bearing *Solanum* species to the aphid *Myzus persicae*. *Entomologia Experimentalis et Applicata* **121**(2): 145-157.

Arenas A, Giurfa M, Sandoz JC, Hourcade B, Devaud JM, Farina WM. 2012. Early olfactory experience induces structural changes in the primary olfactory center of an insect brain. *European Journal of Neuroscience* **35**(5): 682-690.

Backus EA, McLean DL. 1985. Behavioral evidence that the precibarial sensilla of leafhoppers are chemosensory and function in host discrimination. *Entomologia Experimentalis et Applicata* **37**(3): 219-228.

Balog A, Schmitz OJ. 2013. Predation Determines Different Selective Pressure on Pea Aphid Host Races in a Complex Agricultural Mosaic. *Plos One* **8**(2).

Berlocher SH, Feder JL. 2002. Sympatric speciation in phytophagous insects: Moving beyond controversy? *Annual Review of Entomology* **47**: 773-815.

Bernays EA, Chapman RF 1994. Effects of experience. In: Hall Ca ed. *Host plant selection by phytophagous insects*. New York 206-229.

Bernays EA, Weiss MR. 1996. Induced food preferences in caterpillars: The need to identify mechanisms. *Entomologia Experimentalis et Applicata* **78**(1): 1-8.

Bos JIB, Prince D, Pitino M, Maffei ME, Win J, Hogenhout SA. 2010. A functional genomics approach identifies candidate effectors from the aphid species *Myzus persicae* (Green Peach Aphid). *PLoS Genetics* **6**(11).

Caillaud CM, Pierre JS, Chaubet B, Dipietro JP. 1995. Analysis of wheat resistance to the cereal aphid *Sitobion avenae* using Electrical Penetration Graphs and flow-charts combined with correspondence-analysis. *Entomologia Experimentalis et Applicata* **75**(1): 9-18.

Caillaud MC. 1999. Behavioural correlates of genetic divergence due to host specialization in the pea aphid, *Acyrthosiphon pisum*. *Entomologia Experimentalis et Applicata* **91**(1): 227-232.

Caillaud MC, Via S. 2000. Specialized feeding behavior influences both ecological specialization and assortative mating in sympatric host races of pea aphids. *American Naturalist* **156**(6): 606-621.

Campbell BC, Jones KC, Dreyer DL. 1986. Discriminative behavioral responses by aphids to various plant matrix polysaccharides. *Entomologia Experimentalis et Applicata* **41**(1): 17-24.

Carolan JC, Caragea D, Reardon KT, Mutti NS, Dittmer N, Pappan K, Cui F, Castaneto M, Poulain J, Dossat C, Tagu D, Reese JC, Reeck GR, Wilkinson TL, Edwards OR. 2011. Predicted effector molecules in the salivary secretome of the pea aphid (*Acyrthosiphon pisum*): A dual transcriptomic/proteomic approach. *Journal of Proteome Research* **10**(4): 1505-1518.

Carolan JC, Fitzroy CIJ, Ashton PD, Douglas AE, Wilkinson TL. 2009. The secreted salivary proteome of the pea aphid *Acyrthosiphon pisum* characterised by mass spectrometry. *Proteomics* **9**(9): 2457-2467.

Cherqui A, Tjallingii WF. 2000. Salivary proteins of aphids, a pilot study on identification, separation and immunolocalisation. *Journal of Insect Physiology* **46**(8): 1177-1186.

Del Campo ML, Via S, Caillaud MC. 2003. Recognition of host-specific chemical stimulants in two sympatric host races of the pea aphid *Acyrthosiphon pisum*. *Ecological Entomology* **28**(4): 405-412.

Dickey AM, Medina RF. 2011. Immigrant inviability in yellow pecan aphid. *Ecological Entomology* **36**(4): 526-531.

Dres M, Mallet J. 2002. Host races in plant-feeding insects and their importance in sympatric speciation. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* **357**(1420): 471-492.

Dreyer DL, Jones KC, Jurd L, Campbell BC. 1987. Feeding deterrency of some 4-hydroxycoumarins and related compounds relationship to host-plant resistance of alfalfa towards pea aphid *Acyrthosiphon pisum*. *Journal of Chemical Ecology* **13**(4): 925-930.

Duvaux L, Geissmann Q, Gharbi K, Zhou JJ, Ferrari J, Smadja CM, Butlin RK. 2015. Dynamics of Copy Number Variation in Host Races of the Pea Aphid. *Molecular Biology and Evolution* **32**(1): 63-80.

Ferrari J, Godfray HCJ, Faulconbridge AS, Prior K, Via S. 2006. Population differentiation and genetic variation in host choice among pea aphids from eight host plant genera. *Evolution* **60**(8): 1574-1584.

Ferrari J, Via S, Godfray HCJ. 2008. Population differentiation and genetic variation in performance on eight hosts in the pea aphid complex. *Evolution* **62**(10): 2508-2524.

Furch ACU, Hafke JB, Schulz A, van Bel AJE. 2007. Ca2+-mediated remote control of reversible sieve tube occlusion in Vicia faba. *Journal of Experimental Botany* **58**(11): 2827-2838.

Gabrys B, Tjallingii WF, vanBeek TA. 1997. Analysis of EPG recorded probing by cabbage aphid on host plant parts with different glucosinolate contents. *Journal of Chemical Ecology* **23**(7): 1661-1673.

Gao LL, Klingler JP, Anderson JP, Edwards OR, Singh KB. 2008. Characterization of pea aphid resistance in *Medicago truncatula*. *Plant Physiology* **146**(3): 996-1009.

Gaston KJ, Reavey D, Valladares GR. 1991. Changes in feeding habit as caterpillars grow. *Ecological Entomology* **16**(3): 339-344.

Gattolin S, Newbury HJ, Bale JS, Tseng HM, Barrett DA, Pritchard J. 2008. A diurnal component to the variation in sieve tube amino acid content in wheat. *Plant Physiology* **147**(2): 912-921.

Gibson RW, Pickett JA. 1983. Wild potato repels aphids by release of aphid alarm pheromone. *Nature* **302**(5909): 608-609.

Goggin FL. 2007. Plant-aphid interactions: molecular and ecological perspectives. *Current Opinion in Plant Biology* **10**(4): 399-408.

Golawska S, Leszczyznski B, Oleszek W. 2006. Effect of low and high-saponin lines of alfalfa on pea aphid. *Journal of Insect Physiology* **52**(7): 737-743.

Golawska S, Lukasik I. 2009. Acceptance of low-saponin lines of alfalfa with varied phenolic concentrations by pea aphid (Homoptera: Aphididae). *Biologia* **64**(2): 377-382.

Golawska S, Lukasik I. 2012. Antifeedant activity of luteolin and genistein against the pea aphid, Acyrthosiphon pisum. *Journal of Pest Science* **85**(4): 443-450.

Golawska S, Sprawka I, Lukasik I. 2014. Effect of saponins and apigenin mixtures on feeding behavior of the pea aphid, Acyrthosiphon pisum Harris. *Biochemical Systematics and Ecology* **55**: 137-144.

Gururani MA, Venkatesh J, Upadhyaya CP, Nookaraju A, Pandey SK, Park SW. 2012. Plant disease resistance genes: Current status and future directions. *Physiological and Molecular Plant Pathology* **78**: 51-65.

Hewer A, Becker A, van Bel AJE. 2011. An aphid's Odyssey - the cortical quest for the vascular bundle. *Journal of Experimental Biology* **214**(22): 3868-3879.

Hogenhout SA, Bos JIB. 2011. Effector proteins that modulate plant-insect interactions. *Current Opinion in Plant Biology* **14**(4): 422-428.

Jaquiery J, Stoeckel S, Nouhaud P, Mieuzet L, Maheo F, Legeai F, Bernard N, Bonvoisin A, Vitalis R, Simon JC. 2012. Genome scans reveal candidate regions involved in the adaptation to host plant in the pea aphid complex. *Molecular Ecology* **21**(21): 5251-5264.

Kaloshian I, Kinsey MG, Williamson VM, Ullman DE. 2000. *Mi*-mediated resistance against the potato aphid *Macrosiphum euphorbiae* (Hemiptera : Aphididae) limits sieve element ingestion. *Environmental Entomology* **29**(4): 690-695.

Karley AJ, Douglas AE, Parker WE. 2002. Amino acid composition and nutritional quality of potato leaf phloem sap for aphids. *Journal of Experimental Biology* **205**(19): 3009-3018.

Klingler J, Creasy R, Gao LL, Nair RM, Calix AS, Jacob HS, Edwards OR, Singh KB. 2005. Aphid resistance in *Medicago truncatula* involves antixenosis and phloem-specific, inducible antibiosis, and maps to a single locus flanked by NBS-LRR resistance gene analogs. *Plant Physiology* **137**(4): 1445-1455.

Klingler J, Powell G, Thompson GA, Isaacs R. 1998. Phloem specific aphid resistance in *Cucumis melo* line AR 5: effects on feeding behaviour and performance of *Aphis gossypii*. *Entomologia Experimentalis et Applicata* **86**(1): 79-88.

Klingler JP, Nair RM, Edwards OR, Singh KB. 2009. A single gene, AIN, in Medicago truncatula mediates a hypersensitive response to both bluegreen aphid and pea aphid, but confers resistance only to bluegreen aphid. *Journal of Experimental Botany* **60**(14): 4115-4127.

Le Roux V, Dugravot S, Brunissen L, Vincent C, Pelletier Y, Giordanengo P. 2010. Antixenosis phloem-based resistance to aphids: is it the rule? *Ecological Entomology* **35**(4): 407-416.

Liu XD, Zhai BP, Zhang XX. 2008. Specialized host-plant performance of the cotton aphid is altered by experience. *Ecological Research* **23**(5): 919-925.

Marchetti E, Civolani S, Leis M, Chicca M, Tjallingii WF, Pasqualini E, Baronio P. 2009. Tissue location of resistance in apple to the rosy apple aphid established by electrical penetration graphs. *Bulletin of Insectology* **62**(2): 203-208.

Martin B, Collar JL, Tjallingii WF, Fereres A. 1997. Intracellular ingestion and salivation by aphids may cause the acquisition and inoculation of non-persistently transmitted plant viruses. *Journal of General Virology* **78**: 2701-2705.

Mayr E. 1982. Speciation and Macroevolution. *Evolution* **36**(6): 1119-1132.

McCauley GW, Margolies DC, Reese JC. 1990. Feeding-behavior, fecundity and weight of sorghum-reared and corn-reared greenbugs on corn. *Entomologia Experimentalis et Applicata* **55**(2): 183-190.

McLean AHC, Ferrari J, Godfray HCJ. 2009. Effects of the maternal and pre-adult host plant on adult performance and preference in the pea aphid, Acyrthosiphon pisum. *Ecological Entomology* **34**(3): 330-338.

McLean DL, Kinsey MG. 1964. Technique for Electronically Recording Aphid Feeding + Salivation. *Nature* **202**(493): 1358-&.

McLean DL, Kinsey MG. 1968. Probing behavior of pea aphid *Acyrthosiphon pisum*. 3. Effect of temperature differences on certain probing activities. *Annals of the Entomological Society of America* **61**(4): 927-&.

McLean DL, Weigt WA. 1968. An electronic measuring system to record aphid salivation and ingestion. *Annals of the Entomological Society of America* **61**(1): 180-&.

Miles PW. 1959. Secretion of 2 Types of Saliva by an Aphid. *Nature* **183**(4663): 756-756.

Miles PW. 1999. Aphid saliva. *Biological Reviews of the Cambridge Philosophical Society* **74**(1): 41-85.

Montllor CB, Campbell BC, Mittler TE. 1983. Natural and induced differences in probing behavior of 2 biotypes of the greenbug, schizaphis-graminum, in relation to resistance in sorghum. *Entomologia Experimentalis et Applicata* **34**(1): 99-106.

Mutti NS, Louis J, Pappan LK, Pappan K, Begum K, Chen MS, Park Y, Dittmer N, Marshall J, Reese JC, Reeck GR. 2008. A protein from the salivary glands of the pea aphid, *Acyrthosiphon pisum*, is essential in feeding on a host plant. *Proceedings of the National Academy of Sciences of the United States of America* **105**(29): 9965-9969.

Mutti NS, Park Y, Reese JC, Reeck GR. 2006. RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, *Acyrthosiphon pisum*. *Journal of Insect Science* **6**.

Myazaki M 1987. Morphology of aphids. In: Minks AK, Harrewijn P eds. *Aphids - Their Biology, Natural Enemies and Control*. Amsterdam: Elsevier, 1-25.

Naessens E, Dubreuil G, Giordanengo P, Baron OL, Minet-Kebdani N, Keller H, Coustau C. 2015. A Secreted MIF Cytokine Enables Aphid Feeding and Represses Plant Immune Responses. *Current Biology* **25**(14): 1898-1903.

Nomura K, Melotto M, He SY. 2005. Suppression of host defense in compatible plant-Pseudomonas syringae interactions. *Current Opinion in Plant Biology* **8**(4): 361-368.

Nosil P. 2012. *Ecological Speciation*. Oxford: Oxford University Press.

Pallipparambil GR, Reese JC, Avila CA, Louis JM, Goggin FL. 2010. *Mi*-mediated aphid resistance in tomato: tissue localization and impact on the feeding behavior of two potato aphid clones with differing levels of virulence. *Entomologia Experimentalis et Applicata* **135**(3): 295-307.

Peccoud J, Maheo F, De La Huerta M, Laurence C, Simon JC. 2015. Genetic characterisation of new host-specialised biotypes and novel associations with bacterial symbionts in the pea aphid complex. *Insect Conservation and Diversity* **8**(5): 484-492.

Peccoud J, Ollivier A, Plantegenest M, Simon JC. 2009a. A continuum of genetic divergence from sympatric host races to species in the pea aphid complex. *Proceedings of the National Academy of Sciences of the United States of America* **106**(18): 7495-7500.

Peccoud J, Simon JC. 2010. The pea aphid complex as a model of ecological speciation. *Ecological Entomology* **35**: 119-130.

Peccoud J, Simon JC, McLaughlin HJ, Moran NA. 2009b. Post-Pleistocene radiation of the pea aphid complex revealed by rapidly evolving endosymbionts. *Proceedings of the National Academy of Sciences of the United States of America* **106**(38): 16315-16320.

Peccoud J, Simon JC, von Dohlen C, Coeur d'Acier A, Plantegenest M, Vanlerberghe-Masutti F, Jousselin E. 2010. Evolutionary history of aphid-plant associations and their role in aphid diversification. *Comptes Rendus Biologies* **333**(6-7): 474-487.

Pitino M, Hogenhout SA. 2013. Aphid Protein Effectors Promote Aphid Colonization in a Plant Species-Specific Manner. *Molecular Plant-Microbe Interactions* **26**(1): 130-139.

Powell G. 1991. Cell-membrane punctures during epidermal penetrations by aphids - consequences for the transmission of 2 potyviruses. *Annals of Applied Biology* **119**(2): 313-321.

Powell G, Hardie J. 2000. Host-selection behaviour by genetically identical aphids with different plant preferences. *Physiological Entomology* **25**(1): 54-62.

Powell G, Maniar SP, Pickett JA, Hardie J. 1999. Aphid responses to non-host epicuticular lipids. *Entomologia Experimentalis et Applicata* **91**(1): 115-123.

Powell G, Pirone T, Hardie J. 1995. Aphid stylet activities during potyvirus acquisition from plants and an *in vitro* system that correlate with subsequent transmission. *European Journal of Plant Pathology* **101**(4): 411-420.

Powell G, Tosh CR, Hardie J. 2006. Host plant selection by aphids: Behavioral, evolutionary, and applied perspectives. *Annual Review of Entomology* **51**: 309-330.

R Core Team 2011. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Ramirez CC, Caballero PP, Niemeyer HM. 1999. Effect of previous exposure to hydroxamic acids in probing behavior of aphid Sitobion fragariae on wheat seedlings. *Journal of Chemical Ecology* **25**(4): 771-779.

Ramirez CC, Niemeyer HM. 2000. The influence of previous experience and starvation on aphid feeding behavior. *Journal of Insect Behavior* **13**(5): 699-709.

Richards S, Gibbs RA, Gerardo NM, Moran N, Nakabachi A, Stern D, Tagu D, Wilson ACC, Muzny D, Kovar C, Cree A, Chacko J, Chandrabose MN, Dao MD, Dinh HH, Gabisi RA, Hines S, Hume J, Jhangian SN, Joshi V, Lewis LR, Liu YS, Lopez J, Morgan MB, Nguyen NB, Okwuonu GO, Ruiz S, Santibanez J, Wright RA, Fowler GR, Hitchens ME, Lozado RJ, Moen C, Steffen D, Warren JT, Zhang JK, Nazareth LV, Chavez D, Davis C, Lee SL, Patel BM, Pu LL, Bell SN, Johnson AJ, Vattathil S, Williams RL, Shigenobu S, Dang PM, Morioka M, Fukatsu T, Kudo T, Miyagishima SY, Jiang HY, Worley KC, Legeai F, Gauthier JP, Collin O, Zhang L, Chen HC, Ermolaeva O, Hlavina W, Kapustin Y, Kiryutin B, Kitts P, Maglott D, Murphy T, Pruitt K, Sapojnikov V, Souvorov A, Thibaud-Nissen F, Camara F, Guigo R, Stanke M, Solovyev V, Kosarev P, Gilbert D, Gabaldon T, Huerta-Cepas J, Marcet-Houben M, Pignatelli M, Moya A, Rispe C, Ollivier M, Quesneville H, Permal E, Llorens C, Futami R, Hedges D, Robertson HM, Alioto T, Mariotti M, Nikoh N, McCutcheon JP, Burke G, Kamins A, Latorre A, Moran NA, Ashton P, Calevro F, Charles H, Colella S, Douglas A, Jander G, Jones DH, Febvay G, Kamphuis LG, Kushlan PF, Macdonald S, Ramsey J, Schwartz J, Seah S, Thomas G, Vellozo A, Cass B, Degnan P, Hurwitz B, Leonardo T, Koga R, Altincicek B, Anselme C, Atamian H, Barribeau SM, de Vos M, Duncan EJ, Evans J, Ghanim M, Heddi A, Kaloshian I, Vincent-Monegat C, Parker BJ, Perez-Brocal V, Rahbe Y, Spragg CJ, Tamames J, Tamarit D, Tamborindeguy C, Vilcinskas A, Bickel RD, Brisson JA, Butts T, Chang CC, Christiaens O, Davis GK, Duncan E, Ferrier D, Iga M, Janssen R, Lu HL, McGregor A, Miura T, Smagghe G, Smith J, van der Zee M, Velarde R, Wilson M, Dearden P, Edwards OR, Gordon K, Hilgarth RS, Rider SD, Srinivasan D, Walsh TK, Ishikawa A, Jaubert-Possamai S, Fenton B, Huang WT, Rizk G, Lavenier D, Nicolas J, Smadja C, Zhou JJ, Vieira FG, He XL, Liu RH, Rozas J, Ashton PD, Campbell P, Carolan JC, Douglas AE, Fitzroy CIJ, Reardon KT, Reeck GR, Singh K, Wilkinson TL, Huybrechts J, Abdel-latief M, Robichon A, Veenstra JA, Hauser F, Cazzamali G, Schneider M, Williamson M, Stafflinger E, Hansen KK, Grimmelikhuijzen CJP, Price DRG, Caillaud M, van Fleet E, Ren QH, Gatehouse JA, Brault V, Monsion B, Diaz J, Hunnicutt L, Ju HJ, Pechuan X, Aguilar J, Cortes T, Ortiz-Rivas B, Martinez-Torres D, Dombrovsky A, Dale RP, Davies TGE, Field LM, Williamson MS, Jones A, Sattelle D, Williamson S, Wolstenholme A, Cottret L, Sagot MF, Heckel DG, Hunter W, Int Aphid Genomics C. 2010. Genome Sequence of the Pea Aphid Acyrthosiphon pisum. *PLoS Biology* **8**(2).

Robert S, Zouhar J, Carter C, Raikhel N. 2007. Isolation of intact vacuoles from Arabidopsis rosette leaf-derived protoplasts. *Nature Protocols* **2**(2): 259-262.

Rodriguez PA, Bos JIB. 2013. Toward Understanding the Role of Aphid Effectors in Plant Infestation. *Molecular Plant-Microbe Interactions* **26**(1): 25-30.

Sachse S, Rueckert E, Keller A, Okada R, Tanaka NK, Ito K, Vosshall LB. 2007. Activity-dependent plasticity in an olfactory circuit. *Neuron* **56**(5): 838-850.

Sandstrom J. 1994. Performance of pea aphid (*Acyrthosiphon pisum*) clones on host plants and synthetic diets mimicking the same plants phloem amino acid composition. *Journal of Insect Physiology* **40**(12): 1051-1057.

Sandstrom J, Pettersson J. 1994. Amino acid composition of phloem sap and the relation to intraspecific variation in pea aphid (*Acyrthosiphon pisum*) performance. *Journal of Insect Physiology* **40**(11): 947-955.

Sarria E, Cid M, Garzo E, Fereres A. 2009. Excel Workbook for automatic parameter calculation of EPG data. *Computers and Electronics in Agriculture* **67**(1-2): 35-42.

Sauge MH, Kervella J, Rahbe Y. 1998. Probing behaviour of the green peach aphid Myzus persicae on resistant Prunus genotypes. *Entomologia Experimentalis et Applicata* **89**(3): 223-232.

Schwarzkopf A, Rosenberger D, Niebergall M, Gershenzon J, Kunert G. 2013. To Feed or Not to Feed: Plant Factors Located in the Epidermis, Mesophyll, and Sieve Elements Influence Pea Aphid's Ability to Feed on Legume Species. *Plos One* **8**(9).

Shaposhnikov GC 1987. Organization (Structure) of Populations and Species and Speciation. In: Minks AK, Harrewijn P eds. *Aphids - Their Biology, Natural Enemies and Control*. Amsterdam: Elsevier, 415-430.

Smadja CM, Canback B, Vitalis R, Gautier M, Ferrari J, Zhou JJ, Butlin RK. 2012. Large-Scale Candidate Gene Scan Reveals the Role of Chemoreceptor Genes in Host Plant Specialization and Speciation in the Pea Aphid. *Evolution* **66**(9): 2723-2738.

Srivastava PN 1987. Nutritional Physiology. In: Minks AK, Harrewijn P eds. *Aphids - Their Biology, Natural Enemies and Control*. Amsterdam: Elsevier, 99-122.

Stewart SA, Hodge S, Ismail N, Mansfield JW, Feys BJ, Prosperi JM, Huguet T, Ben C, Gentzbittel L, Powell G. 2009. The RAP1 Gene Confers Effective, Race-Specific Resistance to the Pea Aphid in Medicago truncatula Independent of the Hypersensitive Reaction. *Molecular Plant-Microbe Interactions* **22**(12): 1645-1655.

Tjallingii WF. 1978. Electronic recording of penetration behavior by aphids. *Entomologia Experimentalis et Applicata* **24**(3): 721-730.

Tjallingii WF. 1985a. Electrical nature of recorded signals during stylet penetration by aphids. *Entomologia Experimentalis et Applicata* **38**(2): 177-186.

Tjallingii WF. 1985b. Membrane potentials as an indication for plant-cell penetration by aphid stylets. *Entomologia Experimentalis et Applicata* **38**(2): 187-193.

Tjallingii WF. 1986. Wire Effects on Aphids During Electrical Recording of Stylet Penetration. *Entomologia Experimentalis et Applicata* **40**(1): 89-98.

Tjallingii WF 1988. Electrical recording of stylet penetration activities. In: Minks AK, Harrewijn P eds. *Aphids - Their Biology, Natural Enemies and Control*. Amsterdam: Elsevier, 95-108.

Tjallingii WF. 1995. Aphid-plant interactions: What goes on in the depth of the tissues? *Proceedings of the Section Experimental and Applied Entomology of the Netherlands Entomological Society* **6**: 163-169.

Tjallingii WF, Esch TH. 1993. Fine-structure of aphid stylet routes in plant tissues in correlation with EPG signals. *Physiological Entomology* **18**(3): 317-328.

Tjallingii WF, Gabrys B. 1999. Anomalous stylet punctures of phloem sieve elements by aphids. *Entomologia Experimentalis et Applicata* **91**(1): 97-103.

Tjallingii WF, Garzo E, Fereres A. 2010. New structure in cell puncture activities by aphid stylets: a dual-mode EPG study. *Entomologia Experimentalis et Applicata* **135**(2): 193-207.

van Emden H, Kilbane J, Pettersson J. 2009. Changing the host selection responses of aphids through a short experience of a novel secondary plant compound. *Redia* **92**: 183-186.

Van Hoof HA. 1958. *An investigation of the biological transmission of a nonpersistent virus.* PhD thesis, The Agricultural University Wageningen, The Netherlands.

Vanhelden M, Tjallingii WF. 1993. Tissue localization of lettuce resistance to the aphid *Nasonovia ribisnigri* using Electrical penetration graphs. *Entomologia Experimentalis et Applicata* **68**(3): 269-278.

Via S. 1991. Specialized host plant performance of pea aphid clones is not altered by experience. *Ecology* **72**(4): 1420-1427.

Via S. 1999. Reproductive isolation between sympatric races of pea aphids. I. Gene flow restriction and habitat choice. *Evolution* **53**(5): 1446-1457.

Walling LL. 2008. Avoiding effective defenses: Strategies employed by phloem-feeding insects. *Plant Physiology* **146**(3): 859-866.

Webster B, Bruce T, Dufour S, Birkemeyer C, Birkett M, Hardie J, Pickett J. 2008. Identification of volatile compounds used in host location by the black bean aphid, Aphis fabae. *Journal of Chemical Ecology* **34**(9): 1153-1161.

Wensler RJ, Filshie BK. 1969. Gustatory sense organs in food canal of aphids. *Journal of Morphology* **129**(4): 473-&.

Wensler RJD. 1962. Mode of host selection by an aphid. *Nature* **195**(4843): 830-&.

Wilkinson TL, Douglas AE. 1998. Plant penetration by pea aphids (*Acyrthosiphon pisum*) of different plant range. *Entomologia Experimentalis et Applicata* **87**(1): 43-50.

Will T. 2008. Induction as well as suppression: How aphid saliva may exert opposite effects on plant defense. *Plant Signal Behav. 2008 June; 3(6): 427–430.* **3**(6).

Will T, Kornemann SR, Furch ACU, Tjallingii WF, van Bel AJE. 2009. Aphid watery saliva counteracts sieve-tube occlusion: a universal phenomenon? *Journal of Experimental Biology* **212**(20): 3305-3312.

Will T, Steckbauer K, Hardt M, van Bel AJE. 2012. Aphid Gel Saliva: Sheath Structure, Protein Composition and Secretory Dependence on Stylet-Tip Milieu. *Plos One* **7**(10).

Will T, Tjallingii WF, Thonnessen A, van Bel AJE. 2007. Molecular sabotage of plant defense by aphid saliva. *PNAS* **104**(25): 10536-10541.

Will T, van Bel AJE. 2006. Physical and chemical interactions between aphids and plants. *Journal of Experimental Botany* **57**(4): 729-737.

Wink M, Hartmann T, Witte L, Rheinheimer J. 1982. Interrelationship between quinolizidine alkaloid producing legumes and infesting insects - exploitation of the alkaloid-containing phloem sap of Cytisus scoparius by the broom aphid Aphis cytisorum. *Zeitschrift Fur Naturforschung C-a Journal of Biosciences* **37**(11-1): 1081-1086.

Wu JQ, Baldwin IT 2010. New Insights into Plant Responses to the Attack from Insect Herbivores. In: Campbell A, Lichten M, Schupbach G eds. *Annual Review of Genetics, Vol 44*, 1-24.

A Appendix

A.1 Supporting Information Chapter 2

Table S 1. Parameters derived from EPG recordings used to indicate the location of plant factors affecting pea aphid penetration and feeding. For detailed information about EPG waveform standard terms and corresponding aphid behavioral correlates, please refer to Tjallingii and Esch (1993), Tjallingii and Gabrys (1999), and Tjallingii (2006). Additional abbreviations in column "EPG waveform": r-pdsg = single repetitive potential drop period, E1sg = single sieve element salivation period, E1fr = fraction SE salivation period (SE salivation associated with SE feeding period).

Tissue	#	EPG parameter	EPG waveform
Global	1	Proportion of individuals starting penetration	Pathway
	2	Total penetration time	All
Volatile/Surface	3	Time from start of experiment to first probe	All
Surface/Epidermis/Mesophyll	4	First probe duration	All
Epidermis	5	Number of probes shorter than 30 sec	Pathway
	6	Proportion of probes shorter than 30 sec with cell puncture and without cell puncture	Pathway/pd
	7	Number of probes shorter than 30 sec before first SE salivation	Pathway
Epidermis/Mesophyll	8	Number of probes shorter than 3 min	Pathway
	9	Number of probes shorter than 3 min before first SE salivation	Pathway
	10	Number of probes	All
	11	Number of occurrences of probes before first SE salivation or feeding	All
Stress Mesophyll	12	Proportion of individuals showing penetration difficulties	F
	13	Number of penetration difficulties	F
	14	Total duration of penetration difficulties	F
Mesophyll	15	Total duration of pathway phase	Pathway
	16	Cell puncture frequency per min pathway phase	Pathway/pd
	17	Proportion of individuals showing unknown waveform	Unknown waveform
	18	Number of unknown waveforms	Unknown waveform
	19	Total duration of unknown waveform	Unknown waveform
Mesophyll/SEs	20	Time from first probe to first SE salivation	All but E
	21	Time from first probe to first SE feeding	All but E2
	22	Time from first probe to first sustained SE feeding	All but E2 > 10 min
	23	Time from start of successful probe to first SE salivation	All but np, E2
	24	Time from experiment start to first sustained SE feeding	All but E1, E2 < 10 min
SEs	25	Proportion of individuals showing SE salivation	E1
	26	Number of SE salivations	E1
	27	Total duration of SE salivation	E1
	28	Proportion of individuals showing SE salivation without subsequent feeding	E1sg
	29	Number of SE salivations without subsequent feeding	E1sg
	30	Total duration of SE salivations without subsequent feeding	E1sg
	31	Number of SE salivations with subsequent feeding	E1fr
	32	Total duration of SE salivation with subsequent feeding	E1fr
	33	Contribution of SE salivation to SE phase	E1/E2
	34	Duration of first SE salivation with subsequent feeding	E1/E2
	35	Total duration of SE salivation with subsequent feeding	E1/E2
	36	Proportion of individuals showing SE feeding	E2
	37	Number of SE feedings	E2
	38	Total duration of SE feeding	E2
	39	Proportion of individuals showing sustained SE feeding	E2 > 10 min
	40	Number of sustained SE feedings	E2 > 10 min
	41	Proportion of individuals showing repetitive SE punctures	r-pd
	42	Proportion of individuals showing single repetitive SE punctures	r-pdsg
	43	Proportion of individuals showing repetitive SE punctures with subsequent SE salivation	r-pdE1
	44	Proportion of individuals showing repetitive SE punctures with subsequent SE salivation/feeding	r-pdE1E2
	45	Proportion of individuals showing repetitive SE punctures with subsequent SE salivation/sustainedfeeding	r-pdE1E2 > 10 min
	46	Number of repetitive SE puncture periods	r-pd
	47	Total duration of repetitive SE puncture periods	r-pd
	48	Number of single repetitive SE puncture periods	r-pdsg
	49	Number of repetitive SE puncture periods with subsequent SE salivation	r-pdE1
	50	Number of repetitive SE puncture periods with subsequent SE salivation/feeding	r-pdE1E2
	51	Number of repetitive SE puncture periods with subsequent SE salivation/sustainedfeeding	r-pdE1E2 > 10 min
Xylem	52	Proportion of individuals showing xylem ingestion	G
	53	Number of xylem ingestions	G
	54	Total duration of xylem ingestions	G

Table S2. Test statistics for comparing the proportions of repetitive SE puncture periods without and with subsequent feeding period and the proportion of feeding periods without and with preceding repetitive SE puncture periods. KW = Kruskal-Wallis test; GLM B = Generalized linear model with binomial error structure (P-values calculated by $\chi 2$ -test, deviance values printed in regular letters); GLM Q = Generalized linear model with quasibinomial error structure (P-values calculated by F-test, F-values printed in italic letters).

	Medicago race						Pisum race						Trifolium race					
	M1			M2			P1			P2			T1			T2		
	Stat. test	χ^2 or *F*	*P*	Stat. test	χ^2 or *F*	*P*	Stat. test	χ^2 or *F*	*P*	Stat. test	χ^2 or *F*	*P*	Stat. test	χ^2 or *F*	*P*	Stat. test	χ^2 or *F*	*P*
Repetitve SE punctures without vs. repetitive SE punctures with subsequent feeding	KW	17.846	< **0.001**	KW	14.327	< **0.001**	GLM Q	*1.275*	0.273	KW	9.790	**0.007**	KW	21.375	< **0.001**	GLM B	-0.946	0.331
Feeding without vs. feeding with preceding repetitive SE punctures	-	-	-	-	-	-	GLM B	-4.881	**0.027**	GLM B	-5.099	**0.024**	GLM B	-21.936	< **0.001**	GLM B	-1.293	0.256

Table S3. Test statistics of all EPG parameters. Statistical tests: ANOVA = Analysis of variance; KW = Kruskal-Wallis test; GLM QB = generalized linear model with quasibinomial error structure; GLM QP = generalized linear model with quasipoisson error structure; GLM P = generalized linear model with poisson error structure; EQP = test for equality of proportions. Transformations: (-) no transformation; log = logarithmic; sqrt = square root; 1/y = reciprocal transformation; asinsqrt = arcsine square root transformation. $\chi 2$ and F-values: $\chi 2$ printed in regular letters, F-values printed in italic letters. P-values: P-values < 0.05 are printed in bold letters. Further signs/abbreviations: (-) = not analyzed as replicate number < five or no contrasts available. no = parameter not observed. na = parameter not analyzed.

			M1				M2				P1			
		EPG parameter	Stat. test	Transf.	χ^2 or *F*	*P*	Stat. test	Transf.	χ^2 or *F*	*P*	Stat. test	Transf.	χ^2 or *F*	*P*
Global	1	Proportion of individuals starting penetration	EQP	-	2.292	0.514	EQP	-	1.158	0.763	EQP	-	0.246	0.970
	2	Total penetration time	AOV	-	*2.439*	0.074	KW	-	6.038	0.110	AOV	-	*7.162*	**<0.001**
Volatile/Surface	3	Time from start of experiment to first probe	KW	-	1.382	0.710	AOV	log	*3.312*	**0.025**	KW	-	8.910	**0.031**
Surface/Epidermis/Mesophyll	4	First probe duration	KW	-	6.029	0.110	AOV	log	*2.587*	0.060	KW	-	1.886	0.596
Epidermis	5	Number of probes shorter than 30 sec	GLM QP	-	*0.538*	0.659	GLM QP	-	*3.586*	**0.021**	GLM QP	-	*0.550*	0.651
	6	Proportion of probes shorter than 30 sec without/with pd	na	-	-	-	na	-	-	-	na	-	-	-
	7	Number of probes shorter than 30 sec before first E1	-	-	-	-	-	-	-	-	GLM QP	-	*< 0.001*	0.990
Epidermis/Mesophyll	8	Number of probes shorter than 3 min	GLM QP	-	*1.048*	0.389	GLM QP	-	*1.748*	0.171	GLM QP	-	*1.795*	0.162
	9	Number of probes shorter than 3 min before first E1	-	-	-	-	-	-	-	-	-	-	-	-
	10	Number of probes	GLM QP	-	*0.589*	0.625	GLM QP	-	*0.160*	0.923	GLM QP	-	*2.039*	0.118
	11	Number of occurrences of probes before first E	-	-	-	-	-	-	-	-	GLM QP	-	*0.583*	0.455
Stress Mesophyll	12	Proportion of individuals showing F	EQP	-	4.509	0.212	EQP	-	3.332	0.343	EQP	-	4.070	0.254
	13	Number of F	AOV	-	*1.072*	0.369	KW	-	2.947	0.400	AOV	-	*0.019*	0.893
	14	Total duration of F	KW	-	1.746	0.627	GLM P	-	15.923	0.546	GLM P	-	7.489	0.069
Mesophyll	15	Total duration of pathway phase	AOV	sqrt	*5.874*	**0.001**	AOV	sqrt	*7.280*	**<0.001**	AOV	sqrt	*4.245*	**0.009**
	16	Potential drop frequency per min pathway phase	KW	-	4.440	0.218	AOV	log	*2.422*	0.073	AOV	-	*1.740*	0.169
	17	Proportion of individuals showing unknown waveform	EQP	-	5.017	0.171	EQP	-	11.692	**0.009**	EQP	-	6.403	0.094
	18	Number of unknown waveforms	KW	-	5.017	0.398	KW	-	6.229	0.313	GLM QP	-	*1.203*	0.318
	19	Total duration of unknown waveform	AOV	log	*0.138*	0.872	AOV	log	*1.914*	0.165	AOV	log	*0.831*	0.448
Mesophyll/SEs	20	Time from first probe to first E1	-	-	-	-	-	-	-	-	KW	-	0.013	0.909
	21	Time from first probe to first E2	-	-	-	-	-	-	-	-	KW	-	0.071	0.790
	22	Time from first probe to first E2 > 10 min	-	-	-	-	-	-	-	-	KW	-	0.387	0.534
	23	Time from start of successful probe to first E1	-	-	-	-	-	-	-	-	AOV	log	*1.196*	0.289
	24	Time from experiment start to first E2 > 10 min	-	-	-	-	-	-	-	-	KW	-	0.640	0.424
SEs	25	Proportion of individuals showing E1	EQP	-	21.542	**<0.001**	EQP	-	10.553	**0.014**	EQP	-	20.349	**<0.001**
	26	Number of E1	-	-	-	-	-	-	-	-	GLM P	-	9.310	**0.009**
	27	Total duration of E1	-	-	-	-	-	-	-	-	AOV	1/y	*0.960*	0.340
	28	Proportion of individuals showing single E1	EQP		0.988	0.804	EQP	-	1.905	0.592	EQP	-	0.889	0.828
	29	Number of single E1	-	-	-	-	-	-	-	-	-	-	-	-
	30	Total duration of single E1	-	-	-	-	-	-	-	-	-	-	-	-
	31	Number of SE salivation fractions	-	-	-	-	-	-	-	-	GLM P	-	13.866	0.096
	32	Total duration of SE salivation fractions	-	-	-	-	-	-	-	-	AOV	log	*0.003*	0.954
	33	Contribution of SE salivation to SE phase	-	-	-	-	-	-	-	-	AOV	asinsqrt	*0.189*	0.669
	34	Total duration of first E1->E2	-	-	-	-	-	-	-	-	KW	-	1.052	0.305
	35	Total duration E1->E2	-	-	-	-	-	-	-	-	AOV	-	*0.071*	0.793
	36	Proportion of individuals showing E2	EQP	-	24.881	**<0.001**	EQP	-	11.618	**0.009**	EQP	-	29.673	**<0.001**
	37	Number of E2	-	-	-	-	-	-	-	-	GLM P	-	12.857	0.063
	38	Total duration of E2	-	-	-	-	-	-	-	-	AOV	-	*0.076*	0.786
	39	Proportion of individuals showing sustained E2	EQP	-	21.598	**<0.001**	EQP	-	13.742	**0.003**	EQP	-	25.662	**<0.001**
	40	Number of sustained E2	-	-	-	-	-	-	-	-	GLM P	-	4.112	0.308
	41	Proportion of individuals showing r-pd	EQP	-	14.226	**0.003**	EQP	-	25.989	**<0.001**	EQP	-	15.268	**0.002**
	42	Proportion of individuals showing single r-pd	EQP	-	20.780	**<0.001**	EQP		31.126	**<0.001**	EQP	-	7.819	**0.050**
	43	Proportion of individuals showing r-pd->E1	EQP	-	1.973	0.578	EQP	-	1.905	0.592	EQP	-	2.204	0.531
	44	Proportion of individuals showing r-pd->E1->E2	EQP	-	4.967	0.174	EQP	-	9.560	**0.023**	EQP	-	12.506	**0.006**
	45	Proportion of individuals showing r-pd->E1->E2 sustained	EQP	-	22.786	**<0.001**	EQP	-	10.756	**0.013**	EQP	-	25.662	**<0.001**
	46	Number of r-pd	GLM QP	-	*0.252*	0.622	GLM P	-	25.232	0.335	GLM P	-	15.201	0.273
	47	Total duration of r-pd	KW	-	4.053	**0.044**	KW	-	0.315	0.854	AOV	sqrt	*1.642*	0.216
	48	Number of single r-pd	-	-	-	-	KW	-	3.426	0.635	GLM P	-	5.700	0.303
	49	Number r-pd->E1	-	-	-	-	-	-	-	-	-	-	-	-
	50	Number r-pd->E1->E2	-	-	-	-	-	-	-	-	-	-	-	-
	51	Number r-pd->E1->E2 sustained	-	-	-	-	-	-	-	-	KW	-	1.063	0.588
Xylem	52	Proportion of individuals showing xylem ingestion	EQP	-	21.676	**<0.001**	EQP	-	-	0.342	no	-	-	-
	53	Number of xylem ingestions	-	-	-	-	-	-	-	-	no	-	-	-
	54	Total duration of xylem ingestions	-	-	-	-	-	-	-	-	no	-	-	-

		EPG parameter	P2				T1				T2			
			Stat. test	Transf.	χ^2 or F	P	Stat. test	Transf.	χ^2 or F	P	Stat. test	Transf.	χ^2 or F	P
Global	1	Proportion of individuals starting penetration	EQP	-	1.198	0.754	EQP	-	2.264	0.520	EQP	-	0.248	0.970
	2	Total penetration time	AOV	sqrt	*6.747*	**<0.001**	KW	-	17.289	**<0.001**	KW	-	7.023	0.071
Volatile/Surface	3	Time from start of experiment to first probe	KW	-	5.067	0.167	KW	-	5.394	0.145	KW	-	2.358	0.501
Surface/Epidermis/Mesophyll	4	First probe duration	KW	-	5.554	0.135	KW	-	15.367	**0.002**	KW	-	3.833	0.280
Epidermis	5	Number of probes shorter than 30 sec	GLM QP	-	*1.315*	0.278	GLM QP	-	*3.662*	**0.017**	GLM QP	-	*4.716*	**0.005**
	6	Proportion of probes shorter than 30 sec without/with pd	na	-	-	-	GLM QB	-	*7.4434*	**0.008**	GLM QB	-	*17.004*	**<0.001**
	7	Number of probes shorter than 30 sec before first E1	-	-	-	-	GLM QP	-	*0.990*	0.384	GLM QP	-	*2.498*	0.132
Epidermis/Mesophyll	8	Number of probes shorter than 3 min	GLM QP	-	*1.525*	0.218	GLM QP	-	*0.689*	0.563	GLM QP	-	*1.307*	0.283
	9	Number of probes shorter than 3 min before first E1	GLM QP	-	*1.816*	0.199	GLM QP	-	*0.335*	0.569	-	-	-	-
	10	Number of probes	GLM QP	-	*0.872*	0.460	GLM QP	-	*1.285*	0.286	GLM QP	-	*1.695*	0.176
	11	Number of occurrences of probes before first E	GLM QP	-	*0.583*	0.455	GLM QP	-	*1.080*	0.351	GLM QP	-	*4.248*	0.055
Stress Mesophyll	12	Proportion of individuals showing F	EQP	-	9.293	**0.026**	EQP	-	9.473	**0.024**	EQP	-		0.156
	13	Number of F	AOV	sqrt	*0.754*	0.461	KW	-	0.388	0.533	AOV	log	*1.889*	0.152
	14	Total duration of F	GLM P	-	*7.627*	0.490	GLM P	-	4.438	0.714	GLM P	-	8.747	0.197
Mesophyll	15	Total duration of pathway phase	AOV	-	*1.892*	0.138	AOV	sqrt	*2.882*	**0.042**	KW	-	13.473	**0.004**
	16	Potential drop frequency per min pathway phase	AOV	-	*4.854*	**0.004**	AOV	1/y	*1.525*	0.216	AOV	-	*2.006*	0.121
	17	Proportion of individuals showing unknown waveform	EQP	-	11.277	**0.010**	EQP	-	8.432	**0.038**	EQP	-	6.291	0.098
	18	Number of unknown waveforms	KW	-	4.697	0.319	KW	-	2.190	0.701	GLM P	-	9.175	0.831
	19	Total duration of unknown waveform	KW	-	2.890	0.236	KW	-	2.539	0.281	KW	-	0.417	0.519
Mesophyll/SEs	20	Time from first probe to first E1	AOV	log	*0.472*	0.500	KW	-	1.011	0.603	KW	-	**0.007**	0.933
	21	Time from first probe to first E2	AOV	log	*0.789*	0.384	KW	-	1.613	0.446	KW	-	0.064	0.800
	22	Time from first probe to first E2 > 10 min	AOV	log	*1.106*	0.305	KW	-	0.094	0.759	KW	-	0.278	0.598
	23	Time from start of successful probe to first E1	KW	-	0.123	0.725	KW	-	3.312	0.191	KW	-	0.864	0.353
	24	Time from experiment start to first E2 > 10 min	AOV	-	*5.773*	**0.025**	KW	-	**0.048**	0.826	KW	-	**0.000**	1.000
SEs	25	Proportion of individuals showing E1	EQP	-	19.261	**<0.001**	EQP	-	38.482	**<0.001**	EQP	-	17.188	**<0.001**
	26	Number of E1	GLM P	-	21.529	0.498	GLM QP	-	*52.865*	**0.028**	GLM P	-	5.635	0.076
	27	Total duration of E1	KW	-	0.343	0.558	KW	-	*7.491*	**0.024**	AOV	log	-	**0.003**
	28	Proportion of individuals showing single E1	EQP	-	3.699	0.296	EQP	-	3.771	0.287	EQP	-	1.657	0.647
	29	Number of single E1	-	-	-	-	-	-	-	-	-	-	-	-
	30	Total duration of single E1	-	-	-	-	-	-	-	-	-	-	-	-
	31	Number of SE salivation fractions	GLM P	-	14.715	0.808	GLM QP	-	0.341	0.713	GLM P	-	9.106	0.169
	32	Total duration of SE salivation fractions	KW	-	0.219	0.640	AOV	log	*1.394*	0.262	AOV	log	*5.028*	**0.039**
	33	Contribution of SE salivation to SE phase	AOV	asinsqrt	*0.009*	0.287	AOV	asinsqrt	*1.489*	0.240	AOV	asinsqrt	*15.344*	**0.001**
	34	Total duration of first E1->E2	AOV	-	*2.029*	0.168	AOV	log	*2.007*	0.151	AOV	sqrt	*2.129*	0.163
	35	Total duration E1->E2	AOV	-	*1.675*	0.209	AOV	log	*0.561*	0.576	AOV	-	*3.254*	0.089
	36	Proportion of individuals showing E2	EQP	-	30.472	**<0.001**	EQP	-	39.448	**<0.001**	EQP	-	17.188	**<0.001**
	37	Number of E2	GLM P	-	10.014	0.736	GLM P	-	26.134	0.882	GLM P	-	9.106	0.169
	38	Total duration of E2	AOV	-	-	0.210	KW	-	1.375	0.503	AOV	-	*5.479*	0.080
	39	Proportion of individuals showing sustained E2	EQP	-	33.856	**<0.001**	EQP	-	36.077	**<0.001**	EQP	-	22.533	**<0.001**
	40	Number of sustained E2	GLM P	-	1.272	0.739	GLM P	-	10.060	0.542	KW	-	2.516	0.284
	41	Proportion of individuals showing r-pd	EQP	-	14.292	**0.003**	EQP	-	27.000	**<0.001**	EQP	-	24.450	**<0.001**
	42	Proportion of individuals showing single r-pd	EQP	-	6.856	0.077	EQP	-	24.965	**<0.001**	EQP	-	19.808	**<0.001**
	43	Proportion of individuals showing r-pd->E1	EQP	-	6.772	0.080	EQP	-	0.796	0.851	EQP	-	0.731	0.866
	44	Proportion of individuals showing r-pd->E1->E2	EQP	-	6.207	0.102	EQP	-	9.016	**0.029**	EQP	-	9.976	**0.019**
	45	Proportion of individuals showing r-pd->E1->E2 sustained	EQP	-	29.211	**<0.001**	EQP	-	44.117	**<0.001**	EQP	-	22.346	**<0.001**
	46	Number of r-pd	GLM QP	-	*0.974*	0.390	GLM QP	-	*0.608*	0.614	GLM QP	-	*8.917*	**0.006**
	47	Total duration of r-pd	KW	-	7.508	**0.023**	KW	-	22.620	**<0.001**	AOV	log	*0.189*	0.668
	48	Number of single r-pd	GLM QP	-	*0.451*	0.643	GLM QP	-	*0.972*	0.417	GLM P	-	17.326	**0.035**
	49	Number r-pd->E1	-	-	-	-	-	-	-	-	-	-	-	-
	50	Number r-pd->E1->E2	-	-	-	-	GLM P	-	1.881	0.466	-	-	-	-
	51	Number r-pd->E1->E2 sustained	GLM P	-	0.000	1.000	GLM P	-	10.124	0.406	GLM P	-	3.014	0.533
Xylem	52	Proportion of individuals showing xylem ingestion	EQP	-	3.996	0.262	EQP	-	1.847	0.605	no	-	-	-
	53	Number of xylem ingestions	-	-	-	-	-	-	-	-	no	-	-	-
	54	Total duration of xylem ingestions	-	-	-	-	-	-	-	-	no	-	-	-

A.2 Supporting Information Chapter 3

Table S1. Parameters derived from EPG recordings used to indicate the tissue localization of plant factors affecting pea aphid penetration and feeding behavior. For detailed information about EPG waveform standard terms and corresponding aphid behavioral correlates, please refer to Tjallingii and Esch (1993), Tjallingii and Gabrys (1999), and Tjallingii (2006). Additional abbreviations in colums "EPG waveform": r-pdsg = single repetitive potential drop period, E1sg = single sieve element salivation period, E1fr = fraction SE salivation period (SE salivation associated with SE feeding period).

Tissue	#	EPG parameter	EPG waveform
Global	1	Proportion of individuals starting plant penetration	Pathway
	2	Total penetration time	All
Surface	3	Time from start of experiment to first probe	All
Surface/Epidermis/Mesophyll	4	First probe duration	All
Epidermis	5	Number of probes shorter than 30 sec	Pathway
	6	Number of probes shorter than 30 sec before first SE salivation	Pathway
Epidermis/Mesophyll	7	Number of probes shorter than 3 min	Pathway
	8	Number of probes shorter than 3 min before first SE salivation	Pathway
	9	Number of probes	All
	10	Number of occurrences of probes before first SE salivation	All
Stress Mesophyll	11	Proportion of individuals showing penetration difficulties	F
	12	Number of penetration difficulty periods	F
	13	Total duration of penetration difficulty periods	F
Mesophyll	14	Total duration of pathway phase (C)	Pathway
	15	Potential drop frequency per min pathway phase	pd/pathway
?	16	Proportion of individuals showing unknown waveform periods	Unknown waveform
	17	Number of unknown waveform periods	Unknown waveform
	18	Total duration of unknown waveform periods	Unknown waveform
Mesophyll/SEs	19	Time from first probe to first repetitive SE puncture period	All but r-pd and E
	20	Time from first probe to first SE salivation	All but E
	21	Time from first probe to first SE feeding	All but E2
	22	Time from first probe to first sustained SE feeding	All but E2 > 10 min
	23	Time from start of successful probe to first SE salivation	All but np and E
	24	Time from start of experiment to first sustained SE feeding	All but E1and E2 < 10 min
SEs	25	Proportion of individuals showing SE salivation	E1
	26	Number of SE salivation periods	E1
	27	Total duration SE salivation periods	E1
	28	Proportion of individuals showing SE salivation without subsequent feeding	E1sg
	29	Number of SE salivation periods without subsequent feeding	E1sg
	30	Total duration of SE salivation periods without subsequent feeding	E1sg
	31	Number of SE salivation periods with subsequent feeding	E1fr
	32	Total duration of SE salivation periods with subsequent feeding	E1fr
	33	Proportion of SE salivation period to total time in SE phase	E1/E2
	34	Duration of first SE salivation with subsequent feeding	E1and E2
	35	Total duration of SE salivation with subsequent feeding	E1 and E2
	36	Proportion of individuals showing SE feeding	E2
	37	Number of SE feeding periods	E2
	38	Total duration of SE feeding periods	E2
	39	Proportion of individuals showing sustained SE feeding	E2 > 10 min
	40	Number of sustained SE feeding periods	E2 > 10 min
	41	Proportion of individuals showing repetitive SE punctures	r-pd
	42	Proportion of individuals showing single repetitive SE punctures	r-pdsg
	43	Prop. of individuals showing rep. SE punctures with subsequent SE salivation	r-pdE1
	44	Prop. of individuals showing rep. SE punctures with subsequent SE salivation/feeding	r-pdE1E2
	45	Prop. of individuals showing rep. SE punctures with subsequent SE salivation/sustained feeding	r-pdE1E2 > 10 min
	46	Number of rep. SE puncture periods	r-pd
	47	Total duration of rep. SE puncture periods	r-pd
	48	Number of single rep. SE puncture periods	r-pdsg
	49	Number of rep. SE punctures with subsequent SE salivation	r-pdE1
	50	Number of rep. SE punctures with subsequent SE salivation/feeding	r-pdE1E2
	51	Number of rep. SE punctures with subsequent SE salivation/sustained feeding	r-pdE1E2 > 10 min
Xylem	52	Proportion of individuals showing xylem ingestion periods	G
	53	Number of xylem ingestion periods	G
	54	Total duration of xylem ingestion periods	G

Table S2. Test statistics of all EPG parameters. Statistical tests: ANOVA = Analysis of variance; KW = Kruskal-Wallis test; GLM B = generalized linear model with binomial error structure; GLM QB = generalized linear model with quasibinomial error structure; GLM P = generalized linear model with poisson error structure; GLM QP = generalized linear model with quasipoisson error structure; EQP = test for equality of proportions. Transformations: (-) no transformation; log = logarithmic; sqrt = square root; 1/y = reciprocal transformation; asinsqrt = arcsine square root transformation. $\chi 2$ and F-values: $\chi 2$ printed in regular letters, F-values printed in italic letters.P-values: P-values < 0.05 are printed in bold letters. Further signs/abbreviations: (-) = not analyzed as replicate number < five or no contrasts available.

				M1					
Tissue	#	EPG parameter	Acronym	Treatments	Statistical test	Transformation	df	X^2 or *F*	P
Global	1	Proportion of individuals starting penetration	prop_pr	MM VM MV VV	EQP	-	3	2.338	0.505
	2	Total penetration time	t_pr	[MM VV] [VM MV]	AOV	-	1	*4.878*	**0.030**
Surface	3	Time from start of experiment to first probe	t_start_1pr	MM VM MV VV	AOV	sqrt	3	*1.311*	0.278
Surface/Epidermis/Mesophyll	4	First probe duration	t_1Pr	MM VM MV VV	KW	-	3	2.450	0.484
Epidermis	5	Number of probes shorter than 30 sec	nr_Prshort05	MM VM MV VV	GLM QP	-	3	*1.152*	0.336
	6	Number of probes shorter than 30 sec before first E1	nr_Prshort05_1E1	MM VV	GLM QP	-	1	*11.056*	**0.005**
Epidermis/Mesophyll	7	Number of probes shorter than 3 min	nr_prshort3	MM VM MV VV	GLM QP	-	3	*2.776*	0.053
	8	Number of probes shorter than 3 min before first E1	nr_prshort3_1E1	MM VV	GLM QP	-	1	*0.531*	0.480
	9	Number of probes	nr_Pr	MM VM MV VV	GLM QP	-	3	*1.062*	0.371
	10	Number of occurrences of probes before first E	nr_Pr_1E	MM VV	GLM QP	-	1	*1.260*	0.275
Stress Mesophyll	11	Proportion of individuals showing F	prop_F	MM VM MV VV	EQP	-	3	1.195	0.754
	12	Number of F periods	nr_F	MM VM MV VV	GLM P	-	3	*2.977*	0.395
	13	Total duration of F periods	t_F	MM VM MV VV	AOV	sqrt	3	*0.328*	0.806
Mesophyll	14	Total duration of pathway phase (C)	t_C	[MM VM VV] MV	AOV	sqrt	1	*18.000*	**<0.001**
	15	Potential drop frequency per min pathway phase	freq_pd	MM VM MV VV	AOV	sqrt	3	*1.996*	0.123
	16	Proportion of individuals showing unknown waveform periods	prop_11	MM VM MV VV	EQP	-	3	3.780	0.286
?	17	Number of unknown waveform periods	nr_11	VM MV VV	GLM QP	-	2	*3.674*	**0.042**
	18	Total duration of unknown waveform periods	t_11	VM MV VV	AOV	log	2	*5.622*	**0.011**
Mesophyll/SEs	19	Time from first probe to first repetitive SE puncture period	t_1pr_1rpd	[MM VM MV] VV	AOV	log	1	*4.579*	**0.038**
	20	Time from first probe to first SE salivation	t_1pr_1E1	MM MV VV	AOV	-	2	*0.317*	0.731
	21	Time from first probe to first phloem ingestion	t_1pr_1E2	MM MV VV	AOV	-	2	*0.518*	0.602
	22	Time from first probe to first sustained phloem ingestion	t_1pr_1E2sust	MM MV VV	AOV	-	2	*3.243*	0.063
	23	Time from start of successful probe to first SE salivation	t_sucPr_1E	MM MV VV	AOV	log	2	*0.106*	0.900
	24	Time from start of experiment to first sustained SE feeding	t_start_1E2sust	MM MV VV	AOV	sqrt	2	*5.912*	**0.011**
SEs	25	Proportion of individuals showing SE salivation	prop_E1	MM VM MV VV	EQP	-	3	10.801	**0.013**
	26	Number of SE salivation periods	nr_E1	MM MV VV	GLM QP	-	2	*1.766*	0.191
	27	Total duration SE salivation periods	t_E1	MM MV VV	AOV	log	2	*2.598*	0.094
	28	Proportion of individuals showing single SE salivation	prop_sgE1	MM VM MV VV	EQP	-	3	17.286	**0.001**
	29	Number of single SE salivation periods	nr_sgE1	MM	GLM P/QP	-	-	-	-
	30	Total duration of single SE salivation periods	t_sgE1	MM	AOV	-	-	-	-
	31	Number of SE salivation fractions	nr_frE1	MM MV VV	GLM QP	-	2	*1.243*	0.307
	32	Total duration of SE salivation fractions	t_frE1	MM MV VV	AOV	log	2	*1.214*	0.315
	33	Contribution of SE salivation to entire SE phase	frac_E1/E	MM [MV VV]	AOV	asinsqrt	1	*16.120*	**<0.001**
	34	Duration of first E1->E2	t_1E1E2	MM MV VV	KW	-	2	3.958	0.138
	35	Total duration E1->E2	t_E1E2	[MM VV] MV	AOV	log	1	*7.938*	**0.010**
	36	Proportion of individuals showing E2	prop_E2	[MM VV] [VM MV]	EQP	-	1	5.408	**0.020**
	37	Number of E2	nr_E2	MM VM VV	GLM QP	-	2	*1.154*	0.333
	38	Total duration E2	t_E2	[MM VV] MV	AOV	sqrt	1	*12.340*	**0.002**
	39	Proportion of individuals showing sustained E2	prop_E2sust	[MM VV] [VM MV]	EQP	-	1	4.272	**0.039**
	40	Number of sustained E2 periods	nr_E2sust	MM MV VV	GLM P	-	2	*1.781*	0.411
	41	Proportion of individuals showing r-pd	prop_12	MM VM MV VV	EQP	-	3	12.690	**0.005**
	42	Proportion of individuals showing single r-pd periods	prop_sg12	MM VM MV VV	EQP	-	3	22.450	**<0.001**
	43	Proportion of individuals showing r-pd->E1 periods	prop_12E1	MM VM MV VV	EQP	-	3	14.399	**0.002**
	44	Proportion of individuals showing r-pd->E1->E2 periods	prop_12E1E2	MM VM MV VV	EQP	-	3	9.288	**0.026**
	45	Proportion of individuals showing r-pd->E1->E2 sustained periods	prop_12E1E2sust	MM VM MV VV	EQP	-	3	8.351	**0.039**
	46	Number of r-pd periods	nr_12	MM VM MV VV	GLM QP	-	3	*1.197*	0.323
	47	Total duration of r-pd periods	t_12	MM VM MV VV	AOV	log	3	*0.504*	0.682
	48	Number of single r-pd periods	nr_sg12	MM VM VV	GLM QP	-	2	*1.514*	0.236
	49	Number r-pd->E1 periods	nr_12E1	MM	GLM P/QP	-	-	-	-
	50	Number r-pd->E1->E2 periods	nr_12E1E2	MM VV	GLM P	-	1	0.462	0.490
	51	Number r-pd->E1->E2 sustained periods	nr_12E1E2sust	MV VV	GLM P	-	1	0.880	0.348
Xylem	52	Proportion of individuals showing xylem ingestion periods	prop_G	MM VM MV VV	EQP	-	3	7.949	**0.047**
	53	Number of xylem ingestion periods	nr_G	MM	GLM P/QP	-	-	-	-
	54	Total duration of xylem ingestion periods	t_G	MM	AOV	-	-	-	-
		R-pd analysis							
		Number of r-pd periods without and with subsequent feeding periods		[MM VV] [MV] [VM]	GLM QB	-	2	*16.516*	**<0.001**
		Number of feeding periods without and with r-pd periods		MM MV VV	GLM QB	-	2	*0.462*	0.636

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				M2					
Tissue	#	EPG parameter	Acronym	Treatments	Statistical test	Transformation	df	X^2 or *F*	P
Global	1	Proportion of individuals starting penetration	prop_pr	MM VM MV VV	EQP	-	3	2.052	0.562
	2	Total penetration time	t_pr	MM VM MV VV	AOV	-	3	*0.434*	0.730
Surface	3	Time from start of experiment to first probe	t_start_1pr	[MM VM] [MV VV]	AOV	log	1	*4.864*	**0.030**
Surface/Epidermis/Mesophyll	4	First probe duration	t_1Pr	MM VM MV VV	AOV	log	3	*2.125*	0.104
Epidermis	5	Number of probes shorter than 30 sec	nr_Prshort05	MM VM MV VV	GLM QP	-	3	*2.153*	0.102
	6	Number of probes shorter than 30 sec before first E1	nr_Prshort05_1E1	MM VV	GLM QP	-	1	*0.088*	0.772
Epidermis/Mesophyll	7	Number of probes shorter than 3 min	nr_prshort3	MM VM MV VV	GLM QP	-	3	*0.651*	0.585
	8	Number of probes shorter than 3 min before first E1	nr_prshort3_1E1	VV	GLM P/QP	-	-	-	-
	9	Number of probes	nr_Pr	MM VM MV VV	GLM QP	-	3	*1.808*	0.153
	10	Number of occurrences of probes before first E	nr_Pr_1E	MM VV	GLM QP	-	1	*1.093*	0.314
Stress Mesophyll	11	Proportion of individuals showing F	prop_F	MM VM MV VV	EQP	-	3	7.063	0.070
	12	Number of F periods	nr_F	MM VM MV VV	GLM P	-	3	2.890	0.409
	13	Total duration of F periods	t_F	[MM VV] [VM MV]	AOV	-	1	*14.200*	**<0.001**
Mesophyll	14	Total duration of pathway phase (C)	t_C	[MM VM VV] MV	AOV	sqrt	1	*17.160*	**<0.001**
	15	Potential drop frequency per min pathway phase	freq_pd	MM VM MV VV	AOV	sqrt	3	*6.574*	**0.001**
?	16	Proportion of individuals showing unknown waveform periods	prop_11	MM VM MV VV	EQP	-	3	5.128	0.163
	17	Number of unknown waveform periods	nr_11	VM MV VV	GLM QP	-	2	*0.058*	0.943
	18	Total duration of unknown waveform periods	t_11	VM MV VV	AOV	sqrt	2	*2.190*	0.139
Mesophyll/SEs	19	Time from first probe to first repetitive SE puncture period	t_1pr_1rpd	MM VM VV	AOV	log	2	*0.279*	0.759
	20	Time from first probe to first SE salivation	t_1pr_1E1	MM VM VV	AOV	sqrt	2	*3.265*	0.062
	21	Time from first probe to first phloem ingestion	t_1pr_1E2	MM VV	AOV	sqrt	1	*1.214*	0.291
	22	Time from first probe to first sustained phloem ingestion	t_1pr_1E2sust	VV	AOV	-	-	-	-
	23	Time from start of successful probe to first SE salivation	t_sucPr_1E	MM VM VV	AOV	1/y	2	*0.900*	0.424
	24	Time from start of experiment to first sustained SE feeding	t_start_1E2sust	VV	AOV	-	-	-	-
SEs	25	Proportion of individuals showing SE salivation	prop_E1	MM VM MV VV	EQP	-	3	3.474	0.324
	26	Number of SE salivation periods	nr_E1	MM VM VV	GLM QP	-	2	*2.831*	0.085
	27	Total duration SE salivation periods	t_E1	MM VM VV	AOV	sqrt	2	*1.538*	0.242
	28	Proportion of individuals showing single SE salivation	prop_sgE1	MM VM MV VV	EQP	-	3	10.669	**0.014**
	29	Number of single SE salivation periods	nr_sgE1	MM	GLM P/QP	-	-	-	-
	30	Total duration of single SE salivation periods	t_sgE1	MM	AOV	-	-	-	-
	31	Number of SE salivation fractions	nr_frE1	MM VV	GLM P	-	1	0.092	0.761
	32	Total duration of SE salivation fractions	t_frE1	MM VV	AOV	none	1	*2.996*	0.107
	33	Contribution of SE salivation to entire SE phase	frac_E1/E	[MM VM] VV	AOV	asinsqrt	1	*12.510*	**0.002**
	34	Duration of first E1->E2	t_1E1E2	MM VV	AOV	log	1	*0.091*	0.768
	35	Total duration E1->E2	t_E1E2	MM VV	AOV	sqrt	1	*0.418*	0.529
	36	Proportion of individuals showing E2	prop_E2	[MM VV] [VM MV]	EQP	-	1	4.696	**0.030**
	37	Number of E2	nr_E2	MM VV	GLM P	-	1	0.287	0.592
	38	Total duration E2	t_E2	MM VV	AOV	sqrt	1	*0.584*	0.459
	39	Proportion of individuals showing sustained E2	prop_E2sust	MM VM MV VV	EQP	-	1	3.085	0.079
	40	Number of sustained E2 periods	nr_E2sust	VV	GLM P/QP	-	-	-	-
	41	Proportion of individuals showing r-pd	prop_12	MM VM MV VV	EQP	-	3	16.321	**0.001**
	42	Proportion of individuals showing single r-pd periods	prop_sg12	MM VM MV VV	EQP	-	3	22.145	**<0.001**
	43	Proportion of individuals showing r-pd->E1 periods	prop_12E1	MM VM MV VV	EQP	-	3	8.243	**0.041**
	44	Proportion of individuals showing r-pd->E1->E2 periods	prop_12E1E2	MM VM MV VV	EQP	-	3	13.275	**0.004**
	45	Proportion of individuals showing r-pd->E1->E2 sustained periods	prop_12E1E2sust	MM VM MV VV	EQP	-	3	4.233	0.237
	46	Number of r-pd periods	nr_12	MM VM VV	GLM QP	-	2	*0.295*	0.747
	47	Total duration of r-pd periods	t_12	MM VM VV	AOV	log	2	*0.837*	0.445
	48	Number of single r-pd periods	nr_sg12	MM MV	GLM P	-	1	1.243	0.265
	49	Number r-pd->E1 periods	nr_12E1	None	GLM P/QP	-	-	-	-
	50	Number r-pd->E1->E2 periods	nr_12E1E2	MM	GLM P/QP	-	-	-	-
	51	Number r-pd->E1->E2 sustained periods	nr_12E1E2sust	VV	GLM P/QP	-	-	-	-
Xylem	52	Proportion of individuals showing xylem ingestion periods	prop_G	MM VM MV VV	EQP	-	3	4.232	0.238
	53	Number of xylem ingestion periods	nr_G	None	GLM P/QP	-	-	-	-
	54	Total duration of xylem ingestion periods	t_G	None	AOV	-	-	-	-
		R-pd analysis							
		Number of r-pd periods without and with subsequent feeding periods		[MM VV] VM	GLM B	-	1	-14.462	**<0.001**
		Number of feeding periods without and with r-pd periods		MM VV	GLM QB	-	1	*3.315*	0.092

List of publications and oral presentations

Publications

Schwarzkopf A, Rosenberger D, Niebergall M, Gershenzon J, Kunert G.(2013). To feed or not to feed: plant factors located in epidermis, mesophyll, and sieve elements infuencing pea aphid's ability to feed on legume species.

Markovich O, Kafle D, Elbaz M, Malitsky S, Aharoni A, **Schwarzkopf A**, Gershenzon J, Morin S.(2013). Arabidopsis thaliana plants with different levels of aliphatic- and indolyl-glucosinolates affect host selection and performance of *Bemisia tabaci*. Journal of Chemical Ecology.

Schwarzkopf A.(2006). Beteiligung von Transportproteinen bei Stressantworten von *Arabidopsis thaliana*. Diplomarbeit, Eberhard-Karls-Universität, Tübingen.

Schmidt A, Baumann N, **Schwarzkopf A**, Frommer WB, Desimone M.(2006). Comparative studies on Ureide Permeases in *Arabidopsis thaliana* and analysis of two alternative splice variants of AtUPS5. Planta.

Oral Presentations

Schwarzkopf A, Gershenzon J, Kunert G. Localizing plant factors infuencing feeding behavior of *Acyrthosiphon pisum* clones on host and non-host plant species. Hemipteran-Plant Interactions Symposium, Piracicaba, BR, Jul 2011.

Schwarzkopf A, Gershenzon J, Kunert G. Plant factors forcing sympatric speciation in the *Acyrthosiphon pisum* species complex. 9th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE, Feb 2010.

Schwarzkopf A, Gershenzon J, Kunert G. Plant amino acid transporters – Gateway for aphid nutrition or plant defence?. International Conference Plant interactions with aphids and other insects with piercing mouthparts, Wageningen, NL, Aug 2008.

Schwarzkopf A, Gershenzon J, Kunert G. Plant transport proteins – Gate to parasite nutrition or plant defence?. 6th Biannual IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE, Mar 2007.

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Erklärung

Hiermit erkläre ich, dass mir die zur Zeit gültige Fassung der Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller Universität bekannt ist und dass ich die vorliegende Arbeit selbst angefertigt habe. Ich habe keine Textabschnitte von Dritten oder eigene Prüfungsarbeiten ohne Kennzeichnung übernommen und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in der vorliegenden Arbeit angegeben. Welche Personen mich bei der Auswahl und Auswertung unterstützt haben, ist in der Übersicht der Manuskripte nach der allgemeinen Einleitung ausführlich dargelegt. Hilfe eines Promotionsberaters habe ich nicht in Anspruch genommen und Dritte haben weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten. Die Arbeit wurde weder als Prüfungsarbeit, noch für andere staatliche oder andere wissenschaftliche Tätigkeiten eingereicht. Eine Einreichung dieser oder in wesentlichen Teilen ähnlichen Arbeit oder Abhandlung an anderen Hochschulen hat nicht stattgefunden.

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