Abstract

MERCHÁN, HECTOR ALEJANDRO. Response to Tobacco Alkaloids and Neonicotinoid Pesticides in Green Peach Aphid (Myzus persicae). (Under the direction of Hannah J. Burrack and Fred Gould.)

In this dissertation I analyzed different aspects about the relationship between the green peach aphid (*Myzus persicae*) and its control in cultivated tobacco (*Nicotiana tabacum*) using the neonicotinoid pesticide imidacloprid. I focused on this system because imidacloprid targets the nicotinic acetylcholine receptor in insects and this is the same target affected by the alkaloid nicotine, present in tobacco. This relationships offers very interesting possibilities in the study of evolution of pesticide tolerance or resistance and adaptation. Also, tobacco is a very important commodity for North Carolina and I was also interested in possible improvements to pest management practices that could come from our experiments and benefit stakeholders.

For the first chapter I studied the effectiveness and longevity of imidacloprid under field conditions using bioassays with *M. persicae*. It is clear that the time period in which a systemic pesticide is effective under field conditions has wide implications for pest management. Pesticide concentration should be lethal when the pest attacks the crop but low when the crop is marketed. Our aim in this study was to determine the duration of insecticidal activity of systemic pesticides commonly used in tobacco, by measuring the levels of insect infestations on field plots, as well as the effect of the pesticide on reproduction and survival of a target pest in controlled bioassays. Plants were treated with different concentration of two systemic, neonicotinoid pesticides, imidacloprid and thiamethoxam, planted and managed using standard agronomic techniques, except for insect control. I used the green peach aphid as the target pest (*Myzus persicae*, Sulzer), because it is a common pest in tobacco in the United States, and is readily reared under laboratory conditions. Our results show that the systemic pesticides used are very effective under field conditions against the green peach aphid for at least 13 weeks after transplant. Pesticides also affected the reproductive output of adult aphids and the survival of small nymphs in bioassays, but these results were not always statistically significant, and some aphids survived on pesticide-treated leaves. Our results also suggest that physical and chemical aspects of leaf maturation seem to affect survival in aphids. I showed that current management techniques using systemic neonicotinoid pesticides are very effective against the green peach aphid, but that pesticide longevity is less important for aphid management in the second half of the growing season.

For the second chapter I analyzed how the systemic concentration of imidacloprid present within the leaves affect establishment and reproduction in *Myzus persicae*. One of our aims in these experiments was to understand how aphids are exposed to the insecticide. Imidacloprid is applied systemically and moves from the root to the leaves through the xylem. It appears that imidacloprid is the stored in the leaf and concentrations can increase with leaf age. Aphids, on the other hand, feed on the phloem sap, so it is not completely clear how they are exposed to imidacloprid. One possibility is that aphids are exposed through the cuticle, as trichomes in tobacco release small amounts of nicotine and other alkaloids in their exudates. To test this hypothesis I put a barrier between treated and untreated leaves and the aphid that prevented contact with the trichomes, but allowed feeding. I demonstrated that feeding is the most likely route of exposure. Another interesting aspect of imidacloprid is that it elicits a feeding deterrence response when present in low concentrations in the leaf. I tested if aphids are able to locate untreated plants in an environment where different concentrations of the insecticide are present. I developed bioassays with four, equidistant leaf discs. In one set of experiments, I tested the response when all discs had the same concentration of insecticide and in another set of experiments all four discs had different concentrations. I used behavioral observations from the no-choice experiments to predict the distribution of live adult aphids in the choice arenas. I found that a proportion of aphids are able to locate the untreated leaf and settle on it. I also found that the leaf disc with an intermediate concentration of insecticide shows higher levels of settled adults than expected, suggesting that aphids eventually stop responding to the insecticide and can feed on treated plants. I also analyzed the role of age and starvation in aphid establishment a found that aphids show signs of senescence and produce a lower number of nymphs when they are older. Low levels of starvation can induce a hormetic response that increases fecundity in *Myzus persicae*.Together these experiments allow us to improve our understanding of the colonization process of tobacco treated with imidacloprid.

For the third chapter I tested levels of tolerance to imidacloprid in different matrilines collected from four research stations in North Carolina. I also compared the level of tolerance to nicotine and imidacloprid between aphid clones that feed on tobacco and clones that do not. I collected 12 lines in each of the four research stations and tested their level of tolerance to imidacloprid by measuring the fecundity of two adults exposed to leaves treated with different concentrations of imidacloprid. I also measured the survival of the nymphs the adults produced in the bioassays. I found evidence of higher tolerance on populations from Oxford and Clayton. I then tested 16 of those lines with more adults per assay and a larger number of insecticide concentrations and did not find any evidence

of significant difference in tolerance between the different populations. I also compared adult responses to different concentrations of technical grade imidacloprid in an artificial diet. These experiments found a relatively higher level of tolerance to imidacloprid in adults from the Oxford populations, but I did not find that difference when looking at survival of the nymphs. Lastly, I compared levels of tolerance between two non-tobacco aphid clones and four tobacco clones. Our results suggest that there is a correlation between high tolerance to nicotine and high tolerance to imidacloprid. Tobacco aphids, which are adapted to nicotine, are able to tolerate higher levels of nicotine when compared to the non-tobacco clones. The results of these experiments show that there was not a high amount of variation in the levels of tolerance to imidacloprid in our sampled populations. While I did find some significant differences in tolerance in aphids coming from Oxford, the results were not consistent in all experiments. I believe that tobacco aphids are more likely to develop resistance to imidacloprid and they are already pre-adapted to tolerate some level of the insecticide. These results suggest that I need to keep monitoring for the possible evolution of resistance to imidacloprid in our tobacco fields.

Response to Tobacco Alkaloids and Neonicotinoid Pesticides in Green Peach Aphid (*Myzus persicae*)

by
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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Entomology

Raleigh, North Carolina

2016

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Dedication

To my family and my lovely wife.

Biography

Hector Alejandro Merchán hails from Cali, Colombia, where he developed a deep love of nature, the outdoors and science in general. He studied Biology at Universidad de Los Andes, one of the top universities in South America, where he obtained his Bachelor's degree. He also did his Masters in Biology in the same university studying the evolution and population genetics of color pattern genes in *Heliconius* butterflies. His work with these butterflies took him to remote locations in Colombia, Peru and Panama, where Alejandro did his field work. He continued to work in genetics at the University of Puerto Rico and NC State and also worked as a lab assistant before developing a keen interest in agricultural science, particularly entomology. He began his PhD in Entomology in fall 2011, working with Dr. Hannah Burrack in tobacco pest management, focusing on the interactions of green peach aphid (*Myzus persicae*) with pesticides and naturally occurring chemicals in tobacco leaves. Alejandro lives in Durham, North Carolina, with his wife and their two cats. In his free time he enjoys both watching and playing sports, especially soccer.

Acknowledgements

I would like to thank my advisor Hannah Burrack for her help and support throughout my PhD. I really cannot thank you enough for taking a chance on me and keeping me focused and enthusiastic about the research.

I also want to thank the rest of my committee, Fred Gould, George Kennedy, Mike Roe and Ramsey Lewis for all their support and helpful comments.

My funding sources, the North Carolina Tobacco Research Commission, the Altria Fellowship and the Department of Entomology Teaching Fellowship were vital in supporting me and my research during all these years.

To Clyde Sorenson, for being there for my oral exam and for all his help and wisdom in the field.

My parents, for everything. And my siblings for all the rest. Love you guys!

My wife Amanda for her love, patience and support. I owe you a lot, Mandy, you know it.

To everybody that spent some time with me in the lab, Katie, Rachel, Aurora, Jesse, Peter, Dylan, Lauren, Doug, Jeremy, Kevin, Anna, Dimitri, Nick, Johanna. Thanks for being fun, interesting, weird and making my experience something unforgettable.

To my entomology friends, Alejandro, Keith, Pete, Amanda, Angela, Ivonne, Andrew, Alex, Emily, Travis, Damon, Forrest, Sally, Jonathan, Gabe and many more, I learned a lot from you guys and I wish you guys all the best!

To all the at the research stations in Kinston, Rocky Mount, Oxford and Clayton, thanks for your during our field seasons and for doing most of the hard work in keeping the tobacco in decent shape while we allowed insects to destroy it. To Wes Watson and Steve Denning, thanks for letting me use the sterile laminar flow hood anytime I needed it.

To Kevin Gross and Tom Chapell for discussions about statistics and modeling.

To all teachers, thanks for making NC State a great university and making me think, work and learn.

To everybody else that helped me go through Grad School, thank you!

Table of Contents

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Longevity and insecticidal activity of two neonicotinoid insecticides using bioassays with the green peach aphid (*Myzus persicae*) on field-grown tobacco

1.1 Introduction

Neonicotinoid insecticides are used worldwide in conventionally-grown annual and perennial cropping systems, including flue-cured tobacco (*Nicotinana tabacum*, L) (Huseth and Groves, 2014; Jeschke and Nauen, 2008; Jeschke et al., 2011). This class of insecticides offers a wide variety of active ingredients, formulations and application methods while providing broad spectrum activity, all of which allows for flexibility of use (Huseth and Groves, 2014). Tobacco growers in the US have widely adopted neonicotinoids, applying them in the greenhouse before transplant (Burrack, 2013). In North Carolina, the major flue-cured tobacco-producing state in the United States, surveys show that more than 91% the acres in production are normally treated with some form of neonicotinoid (Burrack and Chapman, 2012; Burrack, 2013). The active ingredient is taken up by the roots, distributed through the xylem to the leaves and provide systemic protection mainly against piercing-sucking insects like thrips (*Frankliniella fusca*, Hinds and *F. occidentalis*, Pergande), whiteflies (*Bemisia tabaci*, Gennadius) and green peach aphids (*Myzus persicae*, Sulzer), as well as some leaf-chewing pests such as the tobacco flea beetles (*Epitrix*

hirtipennis, Melsheimer) (Cherry and Mila, 2011; Burrack, 2013). Besides their neurotoxicity, these insecticides also affect the pest's behavior, eliciting a feeding deterrent effect that further improves its effectiveness by causing starvation, delays in development and lower population growth (Boina et al., 2009; Fray et al., 2013; Groves et al., 2001; Nauen, 1995). Efficacy trials conducted over several years have shown that neonicotinoids are effective at reducing aphids and other pests in tobacco, particularly early in the season (Burrack and Chapman, 2012; Semtner et al., 2011). I do not have information about persistence of imidacloprid and its metabolites in tobacco leaves throughout the summer, because late in the season the target pests are usually not an economic concern for growers. However, based on studies in other commodities, it is reasonable to expect that the titer of neonicotinoid insecticides and it metabolites decreases throughout the season, raising the potential of exposure to sub-lethal concentrations to the insecticide (Akoijam and Singh, 2014; Karmakar and Kulshrestha, 2009). No study thus far has analyzed the longevity and insecticidal activity of neonicotinoid insecticides in cultivated tobacco and I do not know if aphids or others pests are potentially exposed to sub-lethal concentration of neonicotinoid insecticides at some point during the growing season, thus favoring the evolution of resistant lines (Foster et al., 2003).

The evolution of resistance to imidacloprid and other neonicotinoid insecticides has been a growing concern worldwide, with reports of field failure confirmed in several species (Nauen and Denholm, 2005; Alyokhin et al., 2015). Since imidacloprid is a systemic insecticide, it is no surprise that most of the confirmed cases of resistance involved insects in the suborder Auchenorryncha (Hemiptera), which are all adapted to feeding from plant juices and include key pests such as whiteflies, psyllids, planthoppers, aphids and others (Puinean et al., 2010; Wen et al., 2009; Kim et al., 2015; Karunker et al., 2008; Gorman et al., 2007; Tiwari et al., 2011). The mechanisms involved in resistant populations usually entails over-expression of one or more cytochrome P450 genes, as well as target site mutations and metabolic enzymes, suggesting that the evolution of resistance to these insecticides occur pretty rapidly if they are not properly managed (Bass et al., 2011; Puinean et al., 2010; Karunker et al., 2008; Kim et al., 2015; Liu et al., 2005; Philippou et al., 2010). While tobacco growers in North Carolina, or anywhere else in the US, have never reported consistent field failures of imidacloprid against any of its target pests, there is evidence that at least some populations of the green peach aphid show higher tolerance to the insecticide in laboratory experiments compared to susceptible lines (Srigiriraju et al., 2010). The possible presence of highly tolerant lines in the field requires further analysis to confirm if they express that tolerance consistently, as well as the need for more careful monitoring for possible resistance populations from growers, extension agents and

other stakeholders.

For this study I focused on the green peach aphid (*Myzus persicae*, Sulzer), because it is an important pest of tobacco, can be present from May through August in tobacco fields of North Carolina and can affect leaf quality and reduce yield (Reed and Semtner, 1992). There is also concern regarding the evolution of resistance to neonicotinoids in this pest, particularly in tobacco-feeding populations, because neonicotinoid insecticides target the same receptor as nicotine, the nicotinic acetylcholine receptor (Bass et al., 2013, 2011; Nauen and Elbert, 2003; Srigiriraju et al., 2010). Aphids are suitable for bioassays in the laboratory, because they are easy to maintain in large numbers in a controlled environment, have a short life span and a high reproductive potential (Dedryver et al., 2010; Foster et al., 2003; Srigiriraju et al., 2010; van Emden et al., 1969). The main goal of my study was to determine the longevity and the insecticidal activity of two neonicotinoid insecticides under field conditions in cultivated tobacco (*Nicotiana tabaccum*). While chemical residue analyses of neonicotinoid insecticides are commercially available, they are expensive and thus usually unrealistic for experiments with weekly sampling and standard levels of replication. Due to this economic constraint, another goal of this experiment was to test the convenience of using the green peach aphid in laboratory bioassays to measure insecticidal activity. With this study I expect to improve our knowledge about the dynamics of insecticidal activity in tobacco under field conditions, while also developing a methodology that is easy to adopt and can allow data collection for possible resistance monitoring. Resistance monitoring and management is important, because insect populations exposed to sub-lethal concentrations of insecticides are subjected to lower selective pressures and the rare resistant variants can increase their frequency over time, leading to resistant populations (ffrench Constant, 2013). This information can also help inform pest management decisions and improve current practices, in order to determine if exposure to sub-lethal concentration does indeed happen and, if it does, devise new strategies to mitigate such exposure.

1.2 Methods

1.2.1 Field Experiments

1.2.1.1 2011

Experimental plots were established at the Upper Coastal Plain Research Station (UCPS) near Rocky Mount, NC. Treatments consisted of an untreated control, three rates, which correspond to 0.5X, 1X and 2X of the recommended field rates of imidacloprid

(Admire Pro®, Bayer CropScience, Research Triangle Park, NC - 17.75 mL, 35.5 mL and 71 mL/1000 plants) and thiamethoxam (Platinum 2SC®, Syngenta Crop Protection, Greensboro, NC - 19.2 mL, 35.5 mL and 76.8 mL/1000 plants). All treatments were applied on two month-old tobacco seedlings growing in foam trays (288 plants/tray). Three trays per treatment were taken from the greenhouse and treated with insecticide diluted in 1 L water applied using a CO_2 propelled sprayer fitted with a single nozzle boom and flat fan nozzle (fig. A.1a). The insecticide was then washed down into the root ball with 2 L of water. Plants were returned to the greenhouse until transplant. Insecticide treatments were applied on 19 April, and plants were transplanted 21 April. The field measured 2266.2 m^2 and was divided in 28 plots; six insecticide treatments and one untreated control, replicated four times in a randomized complete block design. Each plot had four rows and each row had between 25 and 30 plants, for an mean of 100 plants per plot. Plots were maintained using standard agricultural practices for tobacco in North Carolina, and no other insecticides were applied (Fisher, 2013). At 11 weeks after transplant, the terminal meristem of the plant was cut, a practice called topping (Qi et al., 2012). This is a common agricultural practice in tobacco that induces the production of nicotine and stimulates leaf maturation(Qi et al., 2012; Fisher, 2013). Topping also promotes the activation of lateral meristems, called suckers, whose growth is prevented using fatty alcohols that burn the fresh plant tissue (Fisher, 2013).

I collected data on natural infestations by counting the number of aphid-infested plants in the middle two rows of each plot. An aphid-infested plant was defined as a plant with more than 50 aphids on any leaf in the upper third of the plant, which is the current economic threshold for the southeastern United States (fig. A.1b). Counts were done weekly, starting at three weeks after transplant for ten weeks (Burrack, 2013). Beginning at eight weeks after transplant I also collected five fresh leaves at random from the upper third of the plants. These leaves were preserved in plastic bags inside a cooler and used in laboratory bioassays as described below.

1.2.1.2 2012

In 2012 I compared an untreated control, two rates (17.75 mL and 35.5 mL/1000 plants; 0.5X and 1X the recommended field rate) of Admire Pro® (Bayer CropScience) and two rates (19.2 mL and 38.4 mL/1000 plants; 0.5X and 1X the recommended field rate) of Platinum 2SC® (Syngenta Crop Protection). I repeated the experiment in two different locations, at the UCPS and at the Lower Coastal Plain Research Station (LCRS) in Kinston, NC. I followed the same procedures as in 2011. Plants at the LCRS were treated 16 April and transplanted on the 20 April. At the UCPS plants were treated

30 April and transplanted 1 May. Each field measured 1456.8 m^2 and was divided into 4 plots each containing four insecticide treatments and one untreated control, arranged in a randomized complete block design. The same plot layout was employed in both locations. Standard agronomic practices were followed, and plants were topped 11 weeks after transplant for both locations (Fisher, 2013). At the UCRS, I started recording natural infestations at two weeks after transplant and collecting leaves for bioassays at five weeks after transplant, while at the LCRS, I started recording natural infestations at two weeks after transplant and collecting leaves for bioassays at seven weeks after transplant. In both locations I collected leaves from the first three blocks (replicates) of the experiment. At UCRS, I collected an additional set of leaves 19 weeks after transplant, but could not replicate this collection at LCRS because plants were already harvested.

1.2.2 Bioassay methods

1.2.2.1 Insect colony

Insects used in bioassays were from a *M. persicae* colony maintained for three years on greenhouse-grown tobacco plants, without any exposure to insecticides. The colony was maintained in mesh cages inside a greenhouse at temperatures between 25℃ and 30℃, and exposed to natural light conditions. Adult, apterous aphids were collected from the colony before the experiments. I chose individuals that were actively reproducing by picking females that were surrounded by small nymphs. The collected females were transferred onto a fresh tobacco leaf and maintained in a closed container, with a wet paper towel, for up to two hours before they were exposed to leaves collected in the fields.

1.2.2.2 Bioassays in 2011

A caveat for the use of *M. persicae* in bioassays with neonicotinoid insecticides is that it has been widely shown that besides their toxicity, those materials elicit a strong feeding deterrent response (Boina et al., 2009; Fray et al., 2013; Nauen, 1995; Devine et al., 1996). While this response further improves the effectiveness of the materials by causing delays in development, lower population growth and starvation, it also has to be accounted in the design of the bioassays. One possibility that have been used involves coating the walls of the containers holding the leaves with fluon, which does not allow the aphids to move out of the leaf disc. Aphid mortality is measured after 72 hours in those bioassays (Srigiriraju et al., 2010). One of the goals of this experiment was to develop a bioassay that was simple and fast. In order to accomplish that goal, I decided to focus in measuring the reproductive output of adults (number of nymphs produced) and the mortality of their

nymphs. I believe that I collected meaningful results based on these measurements in 48 hours.

First, I washed the leaves I collected with diluted insecticidal soap at 2.5 fl oz/gal of water (Safe® Insect Killing Soap Concentrate, Woodstream Corp, Lititz, PA) to remove wild aphids and other insects, and I then dipped the petioles in fresh water for about an hour to allow the leaves to recover turgidity. I placed leaves abaxial side up on a cardboard cutting board and cut two 5 cm diameter leaf discs at the distal part of the leaf and at both sides of the midrib, without cutting it. I transferred the disc to a 60 x 15 mm polystyrene petri dish (Falcon®, Benton Dickinson and Company, Franklin Lakes, NJ), layered with wet filter paper (fig. A.2a). I then moved 5 adult aphids to each disc and maintained them in a growth chamber at 20℃and a 16h:8h day:night photoperiod. After 24 h I discarded the adults without counting them and counted the total number of nymphs produced and whether they were dead or alive. I considered this the total reproductive output. Nymphs were left on leaves for another 48 h (72 h total), after which the total number of dead and live nymphs was determined. I analyzed these data as nymph mortality.

1.2.2.3 Bioassays in 2012

Leaves from plants treated with imidacloprid and thiamethoxam at 0.5X and 1X rates, plus an untreated control were collected from the first three blocks of the field experiment. I cut a 5 cm diameter leaf disc and put it in 4-oz GladWare Mini Round plastic container (Glad®, The Clorox Company, Oakland CA), which had been previously filled with 50 mL of a 1% agar solution (fig. A.2b). I transferred three adult aphids to each disc and maintained them in a growth chamber at 20℃and a 16h:8h day:night photoperiod. I counted the number of live and dead adults and nymphs at 24h under a dissecting microscope and discarded the adults. I retained the nymphs and counted them again at 48 h. Nymphs were exposed to leaves for 24 h less than during 2011, because mortality did not significantly differ after this point in previous experiments.

1.2.3 Statistical analysis

To determine if plant age, measured in weeks after transplant (WAT), and the different rates of insecticide treatment affected aphid reproduction and survival of offspring, I used a generalized linear model (GLMM) analysis (Gbur et al., 2012; Stroup, 2012). After visual inspection of q-q plots, I fitted the data on number of aphid-infested plants per plot in the field samples and the data on number of nymphs produced in the bioassays

(i.e. reproductive output) to a Poisson distribution. I also fitted nymph mortality data (number of dead nymphs over total number of nymphs produced) to a binomial distribution in the leaf discs where nymphs were produced and discarded from the analysis the ones where there was no reproduction. I fitted a complete model, with insecticide and rate (imidacloprid and thiamethoxam at 0.5X, 1X and 2X respectively), weeks after transplant (WAT) and its interactions as fixed effects, and replicate and location as a random effects. To analyze the results, I "sliced" the estimates by treatment and by WAT and performed a Tukey HSD pairwise-comparison within each main effect to determine statistical separation between treatments or WAT's. All analysis was performed using the R statistical software v 3.2.2 (R Core Development Team, 2015) with the "lme4" v. 1.1-9 package (Bates et al., 2015). I performed the LS means estimation and Tukey HSD test with the "lsmeans" package v. 2.19 (Lenth and Hervé, 2015). All code is available from the author upon request.

1.3 Results

1.3.1 Field Infestation

Aphid infestations in the field were low, and all treatments resulted in reduced numbers of infested plants throughout the 2011 season. Aphid infestation in the field was clearly affected by the use of both insecticides at all three rates and tthere was no significant interaction with WAT (table 1.1). While statistical analysis does not provide much additional insight into these data and my main goal was not to evaluate the efficacy of insecticides in the field, it is worth noticing that GLMM's can be hard to analyze when data consist mostly of zeroes. Weeks after transplant (WAT) did not appear to affect field infestation in the control plots during the sampled period. Insufficient insect pressure was present during 2012 to compare treatments; both treated and untreated plots at both locations remained virtually insect-free throughout the season.

1.3.2 Reproductive output

I measured nymph production after 24 hours in the bioassays and analyzed the effect of both insecticides at the different application rates for each year separately, because I used a different number of adult aphids each year. In 2011 I used the replicate as a random variable and in 2012 I used replicate nested within locations.

Reproductive output started relatively low early in the season, increased over time, and then decreased in all treatments, with a significant effect of the insecticide treatment,

the WAT and their interaction (fig. 1.1 and table 1.2).

1.3.3 Nymph Mortality

I analyzed the two years separately because the nymphs were exposed to the treated leaves for 72 hours in 2011 and 48 hours in 2012. As with reproductive output, I measured the effect of each material and application rate, with location as a random variable for 2012 (fig. 1.2).

In 2011 both treatment and WAT had a significant interaction and all three rates of imidacloprid were more effective that the untreated control during most weeks. In 2012 I can see that both insecticide treatment and WAT have a significant interaction (table 1.5). As in 2011, leaves coming from the untreated control produce a low nymph mortality early in the season and it increases in subsequent weeks, suggesting that the plant becomes an even worse host for green peach aphids (fig. 1.2b). At WAT 12 in 2011 and WAT 10 it increases and remains high for the rest of the season, including weeks in which the mortality is as high in the untreated control as in the treated leaves (fig. 1.2b).

1.4 Discussion

1.4.1 Longevity of imidacloprid in tobacco

In this study I confirmed that imidacloprid is very effective against *Myzus persicae* in cultivated tobacco, offering excellent control in the field and in the laboratory. Thiamethoxam, on the other hand, does not seem to be as effective, but is as effective as imidacloprid in the field. Imidacloprid affects reproductive output more strongly that thiamethoxam on a consistent basis throughout the season. This can be explained partly by a stronger feeding deterrent effect elicited by imidacloprid, that appears to be stronger and more reliable that the one elicited by thiamethoxam (Fray et al., 2013). Also, imidacloprid seems to have a more durable effect throughout the season, which suggests that it remains longer in the soil and roots can maintain a stable uptake during the season (Goulson, 2013). The bioassays performed in this experiment can be used to quantify a biological effect on the pest and produce results in just 24 hours.

There is also evidence that some environmental variable seems to make tobacco plants more suitable for aphids, this is observed both in the highest reproductive output and the lowest nymph mortality observed at WAT 9 and at WAT 12 for 2011. While I do not know the exact cause of these peaks in reproductive output, it is likely that they are related to changes in chemical composition of leaves or phloem sap, to which aphids

respond. Later in the season, though, there is a clear decrease in reproductive output and an increase in the mortality of nymphs, for all treatments including the control. These observations coincide with what have been documented under field conditions, where aphids are considered an early season pest that has a dramatic reduction in numbers after topping (Lampert, 1989). Taken together, these results suggest that chemical changes within the plant can reduce the suitability of tobacco as a host plant for *M. persicae*, affecting my efforts to detect insecticide residue with my bioassays (Hagel et al., 2012; Divol et al., 2005; Walling, 2008). Under regular agronomic practices, tobacco plants are topped when flower buds start to appear (Fisher, 2013). This practice promotes leaves maturation and produce an increase in nicotine levels within the plant (Wang et al., 2008). This increase in nicotine could affect aphids in untreated plants, which reduce their reproductive output (fig. 1.1) and dramatically increases the mortality of their nymphs (fig. 1.2). This suggests that the suitability of tobacco plants as hosts for *Myzus persicae* changes during the season and aphids are clearly affected by these changes. At WAT 19 tobacco is completely unsuitable for aphid reproduction, even when plants were not treated with insecticides. Another effect of topping in tobacco is that secondary meristems start developing in the main stem and it is standard agronomic practice to spray fatty alcohols to reduce this secondary sprouting (Fisher, 2013; Lampert, 1989). These practices were followed in my plots and while they affect aphids in the field, there is no evidence that those chemicals have systemic activity in the plant, so I do not believe the use of these materials affected my bioassay results. Finally, another aspect that can affect alkaloid titers in older plants is the effect of induced plant defenses following attack by other plant pests, such as tobacco and tomato hormworms which were not controlled in my fields and produced substancial damage (Baldwin, 1988; Qi et al., 2012).

Overall, it seems that while there is a possibility that aphids encounter sub-lethal levels of the insecticide, the changes in the plant's chemical profile due to topping and the use of fatty alcohols for sucker control appear to prevent this from happening under field conditions. This means that while aphid populations with higher tolerance to neonicotinoid insecticides might exist in the field, tobacco might not be an agronomic system that promotes the evolution of resistance. However, I believe that using my bioassay methodology early in the season, when the tobacco leaves are still suitable for aphids, will allow us to monitor effectively for tolerant and resistant populations that might emerge, or to keep a closer watch in locations where high tolerance has already been detected (Srigiriraju et al., 2010).

1.4.2 Resistance monitoring with laboratory bioassays

Monitoring for resistance to neonicotinoids using *Myzus persicae* in bioassays, particularly imidacloprid, is challenging. It is known that both imidacloprid and thiamethoxam elicit a feeding deterrent response in these aphids, reducing their actual exposure to the insecticide. Adult aphids can also survive in the bioassay for couple of days without feeding, obscuring the effects of toxicity (Devine et al., 1996; Nauen, 1995; Nauen and Elbert, 1997). Most authors after 72 h, I believe that measuring reproductive output and nymph mortality can offer an alternative. After an adult aphid is established in the host plant and starts feeding on the phloem, reproduction begins almost immediately and nymph production is very responsive to feeding (Margaritopoulos et al., 2005). On the other hand, I have observed that adult aphids held in empty pretty dishes without access to any food resource do not produce nymphs. This suggests that aphids probably are able to feed and reproduce some before detecting the insecticide, so measuring the differences in reproductive output can be a proxy for the presence of the insecticide and it can be measured after 24 hours.

Also, nymph mortality can be affected both by the anti-feeding effects and by the toxicity of insecticides (Nauen, 1995). And while I do not know the which of the two effects of neocotinoids is the main cause of nymph mortality, I believe that both starvation and chemical toxicity produce rapid nymph death (less than 48 hrs) due to their small size and high metabolism (Randolph et al., 1975). So I believe that measuring reproductive output and the mortality of nymphs together in one experiment can be used effectively in resistance monitoring in aphids. This bioassay also offers the advantage of requiring less adults per cup to obtain results, because it takes advantage of the high fecundity of aphids in the control. Normal bioassay procedures use between 20 and 30 adults per replicate, but I obtain replicable and significant results with as little as three adults per replicate.

1.5 Conclusion

my results also demonstrate that despite the effective control that both imidacloprid and thiamethoxam seemed to have under field conditions, similar high mortality was not necessarily observed in the laboratory. Systemic treatment with neonicotinoid insecticide at prescribed rates are very effective against piercing-sucking insects and consistently reduce population levels below economic injury level for early season pests, such as *M. persicae*. However, aphids that are forced to feed on treated leaves can survive, despite a lower reproductive output and nymph survival, suggesting that other variables are also

important in the control of this pest under field conditions. Another possibility is that the insecticides slow down the growth of the aphid populations in treated plants, making them more susceptible to natural enemies or to abiotic effects such as heavy rains, which can dislodge adult aphids from the plants and affect their survival (Mann et al., 1995). All this suggests that claims about the aphid's resistance observed in the laboratory need to be taken carefully, because bioassays do not necessarily capture the complexity of the insecticide-pest dynamic under field conditions. However, it is important that the monitoring efforts continue and that we keep perfecting our tools to determine insecticide residue under field conditions and its biological activity. An improved understanding about the variables that affect insecticide and plant secondary chemicals, their movement, accumulation and metabolization under field conditions, as well as the combined effects on tobacco pests, would improve management of this crop.

1.6 Tables

Table 1.1 Field infestation rate in 2011. Estimated mean (± SEM) of aphid infested plants in field plots. LS means were estimated using a Generalized Linear Mixed Model (GLMM) for a 'poisson' distribution and a 'log' link function. LS mean separation was accomplished using Tukey's HSD and means followed by the same uppercase letter are not significantly different from one another within each WAT (column). No pest = No insecticide, Imid = imidacloprid and Thia = thiamethoxam.

	Weeks after transplant (WAT)									
	3	4	5	6	7	8	9	10	11	12
No pest.	7.08 B ±3.97	9.67 B ±6.24	10.93 B ±7.04	9.04 B ±5.93	17.81 B ±11.31	18.03 B ±11.50	19.38 B ±12.40	20.66 B ±13.17	18.09 B ±11.54	12.23 B ±7.88
Imid. 0.5X	0.02 A ±0.02	0.06 A ±0.08	0.01 A ±0.02	0.01 A ±0.02	0.23 A ±0.23	0.01 A ±0.03	0.02 A ±0.03	0.02 A ±0.03	0.01 A ±0.03	0.01 A ±0.02
Imid. 1X	0.02 A ±0.02	0.01 A ±0.02	0.01 A ±0.02	0.62 A ±0.52	0.01 A ±0.02	0.01 A ±0.03	0.01 A ±0.03	0.01 A ±0.03	0.01 A ±0.02	0.01 A ±0.02
Imid. 2X	0.01 A ±0.01	0.05 A ±0.07	0.01 A ±0.01	0.01 A ±0.01	0.01 A ±0.02	0.01 A ±0.02	0.01 A ±0.02	0.01 A ±0.02	0.01 A ±0.02	0.01 A ±0.02
Thia. 0.5X	0.01 A ±0.01	0.00 A ±0.01	0.00 A ±0.01	0.00 A ±0.01	0.01 A ±0.01	0.01 A ±0.01	0.01 A ±0.02	0.01 A ±0.02	0.01 A ±0.02	0.01 A ±0.01
Thia. 1X	0.01 A ±0.01	0.00 A ±0.01	0.00 A ±0.01	0.00 A ±0.01	0.01 A ±0.01	0.01 A ±0.01	0.01 A ±0.01	0.01 A ±0.02	0.01 A ±0.01	0.01 A ±0.01
Thia. 2X	0.01 A ±0.01	0.00 A ±0.01	0.00 A ±0.01	0.00 A ±0.01	0.01 A ±0.01	0.01 A ±0.01	0.01 A ±0.01	0.01 A ±0.02	0.01 A ±0.01	0.01 A ±0.01

Table 1.2 Reproductive output in 2011. Estimated mean (± SEM) nymph production by adult aphids in 24 h. LS means were estimated using a Generalized Linear Mixed Model (GLMM) for a 'poisson' distribution and a 'log' link function. LS mean separation was accomplished using Tukey's HSD. Means followed by the same uppercase letter are not significantly different from one another within each WAT (column), while means followed by the same lowercase letters are not significantly different from one another within each insecticide treatment(row) (Tukeys HSD, α= 0.05). No pest = No insecticide, Imid = imidacloprid.

	Weeks after transplant (WAT)				
	8	9	10	11	12
No pest	10.50 A ab 0.81	20.70 B d 1.02	13.65 B bc 0.83	14.45 C c 0.85	8.40 B a 0.65
Imid 0.5X	9.30 A b ±0.68	15.50 A c ±0.88	13.00 B cd ±0.81	12.00 BC bc ± 0.77	6.20 AB a ± 0.56
Imid 1X	8.15 A ab ±0.64	16.55 A c ±0.91	13.75 B c ±0.83	10.25 B b ± 0.71	7.20 AB a ± 0.60
Imid 2X	8.30 A b ±0.64	15.35 A c ±0.87	8.85 A b ±0.66	7.45 A ab ± 0.61	5.65 AB c ± 0.53

Table 1.3 Reproductive output in 2012. Estimated mean (± SEM) mortality of aphid nymphs. LS means were estimated using a Generalized Linear Mixed Model (GLMM) for a 'poisson' distribution and a 'log' link function. LS mean separation was accomplished using Tukey's HSD. Means followed by the same uppercase letter are not significantly different from one another within each WAT (column), while means followed by the same lowercase letters are not significantly different from one another within each insecticide treatment(row) (Tukeys HSD, α= 0.05). No pest = No insecticide, Imid = imidacloprid and Thia = thiamethoxam.

	Weeks after transplant (WAT)												
	5	6	7	8	9	10	11	12	13	14	15	16	19
No pest	6.31 C bcde ±0.67	8.31 B def ±0.78	11.37 C fg ±0.71	8.64 C e ±0.60	12.67 C g ±0.76	8.84 C ef ±0.61	7.74 B cde ±0.56	9.48 B efg ±0.87	5.47 AB b ±0.46	5.60 AB bcd ±0.63	5.60 C bcd ±0.65	4.98 B bc ±0.61	0.90 A a ±0.24
Imid 0.5X	4.06 AB bcd ± 0.53	5.54 A bcde ±0.62	5.07 A bcde ±0.44	6.54 AB de ±0.51	7.07 A e ±0.53	6.44 AB cde ±0.50	5.20 A bcde ±0.45	4.77 A bcde ±0.60	4.87 AB bcd ±0.43	3.99 A bc ±0.52	3.04 A ab ±0.47	5.67 B cde ±0.65	1.48 A a ±0.31
Imid 1X	3.22 A ab ±0.47	7.34 AB e ±0.73	4.63 A bcd ±0.42	5.04 A bcde ±0.44	6.07 A de ±0.49	5.74 A cde ±0.47	4.20 A bcd ±0.40	5.40 A bcde ±0.64	4.94 AB bcde ±0.43	3.54 A abc ±0.49	3.18 AB ab ±0.48	1.80 A a ±0.36	1.87 A a ±0.35
Thia 0.5X	7.08 C bcd ±0.71	8.24 B bcde ±0.78	8.84 B de ±0.61	7.30 BC bcd ±0.54	11.34 BC e ±0.71	9.34 C de ±0.63	5.60 A b ±0.46	8.85 B cde ±0.84	5.77 B b ±0.47	6.76 B bc ±0.70	5.60 C ±0.65	2.77 A a ±0.45	1.35 A a ±0.30
Thia 1X	5.99 BC cd ±0.65	6.18 AB cd ±0.66	7.90 B de ±0.57	7.17 BC d ±0.54	10.17 B e ±0.66	8.00 BC de ±0.57	7.44 B d ±0.55	7.06 AB de ±0.74	3.97 A bc ±0.38	5.34 AB cd ±0.61	5.19 BC bcd ±0.62	2.70 A ab ±0.44	1.67 A a ±0.33

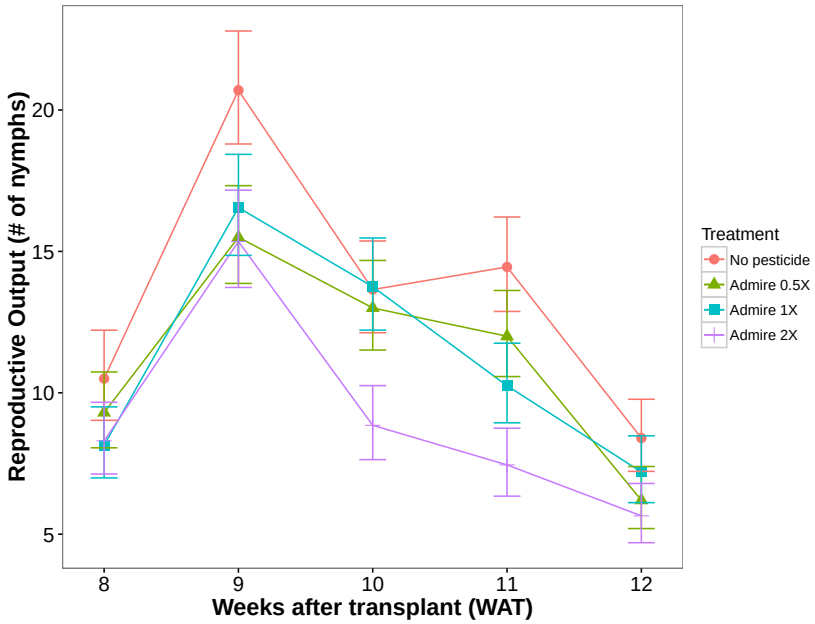
Table 1.4 Mortality of aphid nymphs in 2011. Estimated mean (± SEM) nymph production by adult aphids in 24 h. LS means were estimated using a Generalized Linear Mixed Model (GLMM) for a 'poisson' distribution and a 'log' link function. LS mean separation was accomplished using Tukey's HSD. Means followed by the same uppercase letter are not significantly different from one another within each WAT (column), while means followed by the same lowercase letters are not significantly different from one another within each insecticide treatment(row) (Tukeys HSD, α= 0.05). No pest = No insecticide, Imid = imidacloprid.

	Weeks after transplant (WAT)				
	8	9	10	11	12
No pest	0.13 A ab ±0.03	0.19 A b ±0.03	0.09 A a ±0.02	0.08 A a ±0.02	0.63 A c ±0.05
Imid 0.5X	0.44 C b ±0.05	0.38 B b ±0.04	0.17 B a ±0.03	0.46 B b ± 0.05	0.81 B c ± 0.04
Imid 1X	0.30 B a ±0.05	0.54 C b ±0.04	0.35 C a ±0.04	0.58 B b ± 0.05	0.95 C c ± 0.02
Imid 2X	0.73 D b ±0.04	0.48 BC a ±0.04	0.69 D b ±0.05	0.90 C c ± 0.03	0.93 C c ± 0.02

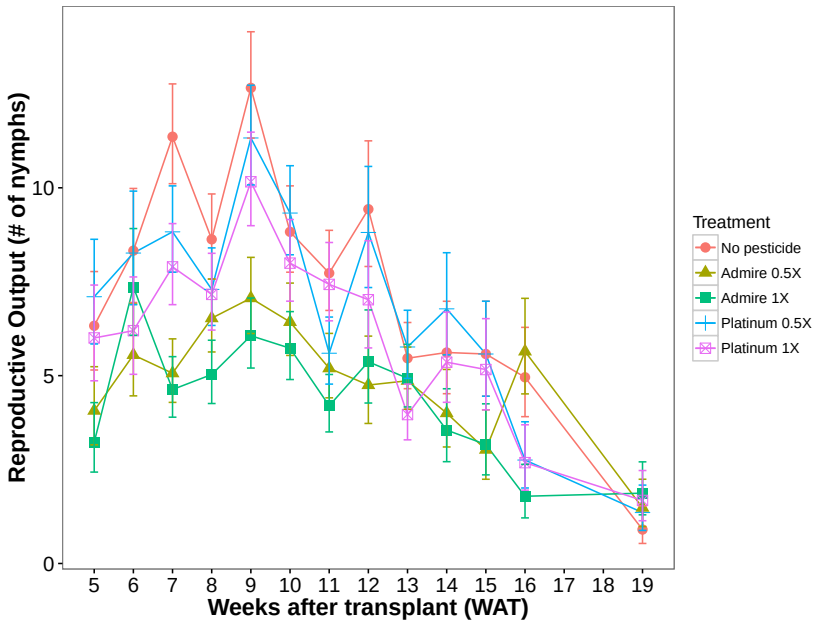
Table 1.5 Mortality of aphid nymphs in 2012. Estimated mean (± SEM) mortality of aphid nymphs. LS means were estimated using a Generalized Linear Mixed Model (GLMM) for a 'binomial' distribution and a 'logit' link function. LS mean separation was accomplished using Tukey's HSD. Means followed by the same uppercase letter are not significantly different from one another within each WAT (column), while means followed by the same lowercase letters are not significantly different from one another within each insecticide treatment(row) (Tukeys HSD, α= 0.05). No pest = No insecticide, Imid = imidacloprid and Thia = thiamethoxam.

	Weeks after transplant (WAT)												
	5	6	7	8	9	10	11	12	13	14	15	16	19
No pest	0.10 A abcd ±0.03	0.18 A cde ±0.04	0.05 A ab ±0.01	0.08 A abc ±0.01	0.04 A a ±0.01	0.21 A de ±0.03	0.31 A ef ±0.03	0.25 A def ±0.04	0.40 A fg ±0.04	0.75 A h ±0.05	0.16 A bcde ±0.04	0.56 A gh ±0.06	0.87 AB gh ±0.09
Imid 0.5X	0.61 C def ± 0.06	0.54 B cde ±0.06	0.35 C abc ±0.04	0.30 C ab ±0.03	0.27 C a ±0.03	0.37 BC abcd ±0.04	0.43 AB abcd ±0.04	0.56 B cde ±0.06	0.64 B ef ±0.04	0.85 A f ±0.04	0.46 B abcde ±0.08	0.66 A ef ±0.05	0.63 AB bcdef ±0.10
Imid 1X	0.85 D f ±0.05	0.30 A a ±0.04	0.61 D bcdef ±0.04	0.48 D abc ±0.04	0.45 D ab ±0.04	0.64 D cdef ±0.04	0.54 B bcde ±0.05	0.49 B abcd ±0.06	0.69 B def ±0.04	0.79 A ef ±0.05	0.44 B abcd ±0.07	0.48 A abcdef ±0.10	0.80 B cdef ±0.07
Thia 0.5X	0.09 A ab ±0.03	0.26 A bcd ±0.04	0.07 A a ±0.02	0.15 AB abc ±0.03	0.13 B abc ±0.02	0.34 B de ±0.03	0.47 B ef ±0.04	0.50 B efg ±0.05	0.68 B gh ±0.04	0.78 A h ±0.04	0.16 A abcd ±0.04	0.66 A fgh ±0.08	0.40 A cdefgh ±0.11
Thia 1X	0.36 B bcd ±0.05	0.57 B de ±0.05	0.16 B a ±0.02	0.23 BC ab ±0.03	0.25 C abc ±0.03	0.49 C d ±0.03	0.50 B d ±0.04	0.37 AB bcd ±0.05	0.78 B ef ±0.04	0.83 A f ±0.04	0.22 AB abc ±0.05	0.62 A def ±0.08	0.56 AB cdef ±0.10

1.7 Figures

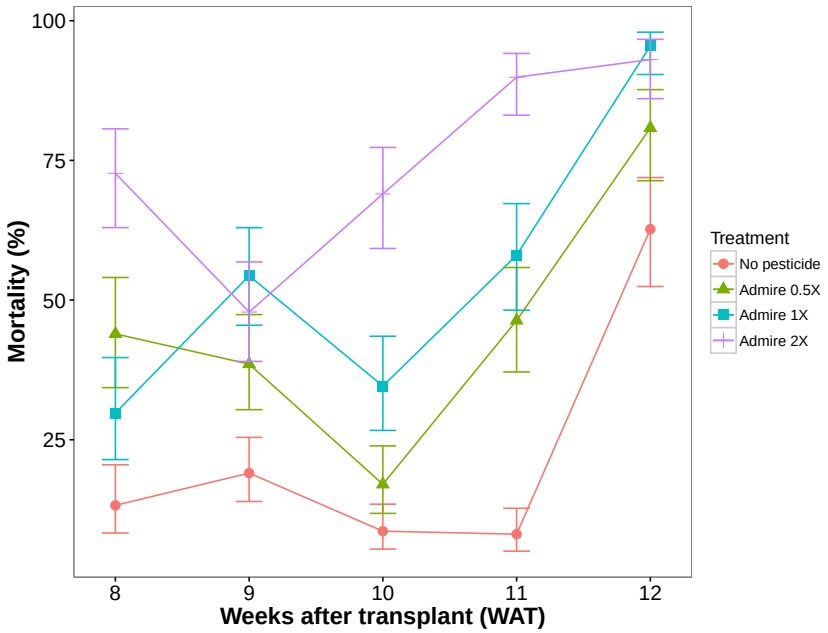


(a) Reproductive output during 2011.

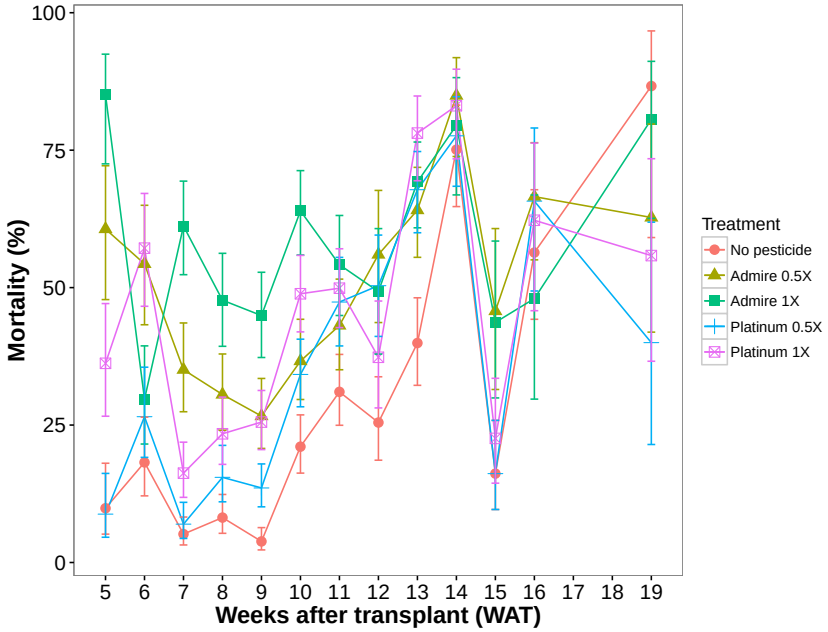


(b) Reproductive output during 2012.

Figure 1.1 Changes in the % mortality of aphid nymphs throughout the season. The lines shows the average mortality measured in leaves from different plots. Bars represent a 95% confidence interval.



(a) Nymph mortality during 2011.



(b) Nymph mortality during 2012.

Figure 1.2 Changes in the % mortality of aphid nymphs throughout the season. Mortality in 2011 was measured after 72h and after 48h in 2012. The lines shows the average mortality measured in leaves from different plots. Bars represent a 95% confidence interval.

Chapter 2

Colonization of cultivated tobacco by the green peach aphid (*Myzus persicae*): The effect systemic imidacloprid applications on colony establishment and growth.

2.1 Introduction

Understanding the processes of establishment of a pest on a crop and growth of its population under field conditions is critical for effective management strategies (Lewis et al., 1997; McCornack et al., 2004; Ragsdale et al., 2011). This is particularly important for pests like aphids, in which one alate female can land on a plant early in the growing season, identify it as suitable host and almost immediately start reproducing parthenogenetically, producing clonal asexual populations (Lampert, 1989; Margaritopoulos et al., 2005; McCornack et al., 2004). The establishment and growth of aphid populations is influenced by both biotic and abiotic factors, such as predators, parasitoids, other herbivores and pathogens, and weather and plant suitability (Awmack and Leather, 2002; Dedryver et al., 2010; Powell et al., 2006; Webster, 2012; Zhang et al., 2015). Aphids are also strongly constrained by host plant quality due to their sedentary lifestyle, so they are expected to respond rapidly to changes to plant chemistry due to natural causes, such as plant age, environmental conditions, and human action e.g.pesticides or management practices, without the benefit of genetic variation within the population (Awmack and

Leather, 2002; Dedryver et al., 2010).

Neonicotinoids are the most widely use insecticide class in North American flue cured tobacco (*Nicotinana tabacum* production, L). The active ingredients in neonicotinoid insecticides have a similar mode of action to nicotine, and act by blocking nicotinic acetylcholine receptors in insects, which produces uncontrollable muscle activity and eventually death (Gavilano et al., 2007; Nabeshima et al., 2003; Ramsey et al., 2014; Steppuhn et al., 2004; Yang and Guthrie, 1969). Shortly after the commercial release of this class of insecticides, it was shown that the mechanisms that allow insects to detoxify nicotine in cultivated tobacco also gave them higher levels of tolerance to the synthetic chemiclas (Devine et al., 1996). However, those levels of tolerance were not enough to produce field failures in crop protection and neonicotinoids are as effective against piercing-sucking pests in tobacco as in other crops (Bass et al., 2014, 2013). In North Carolina, the major flue cured tobacco-producing state in the United States, more than 91% the acres in production are treated annually with some form of neonicotinoid, based on replies to an annual cooperative extension agent survey covering 47% of the 165,000 acres in the state (Burrack 2013 and https://tobacco.ces.ncsu.edu/2016/03/2015-flue-cured-tobacco-survey-entomology-survey-data/). Efficacy trials conducted over several years show that neonicotinoids applied before transplant are effective at reducing aphids and other pests in tobacco, particularly early in the season (Burrack and Chapman, 2012; Semtner et al., 2011). The vast majority of neonicotinoids, primarily imidacloprid and thiamethoxam, are applied to tobacco plants in the greenhouse before they are transplanted to the field (Burrack and Chapman, 2012; Cherry and Mila, 2011). These chemicals are taken up by the roots, distributed by the xylem throughout the plant and provide systemic protection against insects including thrips (*Frankliniella fusca*, Hinds), Pergande), green peach aphids (*Myzus persicae*, Sulzer), and tobacco flea beetles (*Epitrix hirtipennis*, Melsheimer), reducing virus transmission and leaf damage (Cherry and Mila, 2011; Burrack and Chapman, 2012; Semtner et al., 2011). In addition to their neurotoxicity, these insecticides also affect pest behavior, causing a feeding deterrent response that results in starvation, delays in development and lower population growth (Boina et al., 2009; Fray et al., 2013; Groves et al., 2001; Nauen, 1995).

The green peach aphid (*Myzus persicae*, Sulzer) is an important pest of tobacco that has been demonstrated to have a strong feeding deterrence to neonicotinoid insecticides (Devine et al., 1996; Nauen, 1995; Reed and Semtner, 1992). Aphids move to tobacco shortly after transplant, as adult alate females that migrate from winter hosts. These alates land on leaves and conduct feeding probes in order to recognize a suitable host and start reproducing (Bruce and Pickett, 2011; Powell et al., 2006). For these colonizers,

possible routes of insecticide exposure include: contact with exudates from trichomes, which can contain nicotine, hence could potentially also have imidacloprid (Thurston et al., 1966); and ingestion by tapping the xylem, either by accident during exploration or purposefully for water consumption (Pompon et al., 2010, 2011; Walling, 2008). The main goal of my study was to determine how systemic insecticides affect establishment, reproductive output and nymph survival of *M. persicae*, important components of population growth. I conducted a series of controlled experiments to identify the effects of neonicotinoids on *M. persicae* establishment that have implications for both pest control and resistance management (Rattan, 2010).

2.2 Methods

2.2.1 Insect colony

Insects used in bioassays were from a laboratory maintained *M. persicae* colony reared on greenhouse-grown tobacco plants, without any exposure to insecticides, for three years. The colony was maintained in mesh cages at ambient greenhouse temperatures between 25 ℃ and 30 ℃ and exposed to natural light conditions. For experiments involving Alates used in experiments were aspirated from the walls and ceiling of the cage every day at midday. Since the plants were surrounded by moats, any alates collected from the walls and ceiling were assumed to have flown there during the preceding 24 hours, as alates have a very narrow window during which they are able to fly (Pompon et al., 2010).

2.2.2 Leaf treatments

My goal was to expose aphids to imidacloprid in a biologically relevant manner while also controlling insecticide concentration. This has normally been done either by dipping the leaf in a insecticide solution and then letting it air dry, or by dipping the leaf's petiole in a insecticide solution and allowing the uptake and systemic spread of the material throughout the whole leaf. The second approach most closely resembles the way that imidacloprid (Admire Pro®, Bayer Crop Sciences) is used under field conditions and was employed in my experiments (fig. A.3a). I prepared 400 mL of an 8.725 ppb solution of Admire Pro® (equivalent to a 3.7342 ppb solution of imidacloprid) and then used it as a stock solution to produce 4.3625 ppb and 2.18125 ppb solutions. For experiments at higher rates, I prepared 400 mL of a 872.5 ppb solution of Admire Pro® (equivalent to a 373.42 ppb solution of imidacloprid) which was then diluted to produce 436.25 ppb, and 218.125 ppb solutions. Dilutions were prepared independently for each experiment.

Tobacco plants were grown without any application of synthetic insecticides, but were treated with diluted insecticidal soap at 2.5 fl oz/gal of water (Safer® Insect Killing Soap Concentrate, Woodstream Corp, Lititz, PA) if needed to control aphids and whiteflies. Fresh leaves used in experiments were collected from these greenhouse-grown plants and washed thoroughly with insecticidal soap to ensure that they did not harbor insects from the greenhouse. I then cut the ca. 5 cm of the petiole and immediately dipped leaves in 100 mL of each of the three Admire Pro® solutions or 100 mL of distilled water in the case of untreated control (fig. A.3b) where they remained under room temperature and continous light for 24 h. Five leaves of each treatment were then dried on an oven for 48 h at $\backsim$ 50 ℃ and ground to a fine powder. Five grams of leaf powder from each leaf were sent for residue analysis by the United States Department of Agriculture Agricultural Marketing Service (USDA AMS) Laboratory in Gastonia, NC.

2.2.3 Bioassays

2.2.3.1 Aphid establishment

I conducted bioassays with wingless aphids to assess effects of systemic neonicotinoid insecticides on *M. persicae* establishment. I caged ten adult aphids per replicate over leaf discs treated with the three concentrations of imidacloprid and with or without a Parafilm® M (Bemis NA, WI, USA) barrier preventing direct leaf contact by body parts other than stylets. Aphids were held in cages consisting of 50 ml centrifuge tubes (Falcon®, Corning Life Sciences, NY, USA) cut in half with the cut end covered with nylon netting. Leaf discs, 2.54 cm in diameter, were placed on top of a layer of Parafilm® stretched over the centrifuge tube lid and covered with a second layer of Parafilm® for barrier treatments (fig. A.4). To ensure that the leaf disc was in close contact with the barrier, I put a round lead 85 g weight on top of the setting. Live and dead adults and the number of nymphs produced were counted after 24 h, and the number live and dead nymphs were counted at 48 h. Each treatment was replicated four times.

A second series of experiments comparing the response of alate aphids to only the 4.3625 ppb imidacloprid solution with or without a barrier was conducted using a modified cage. Briefly, I placed leaf discs over unset 2% agar poured in petri dishes of the same diameter. I then allowed the agar to set and stretched a piece of Parafilm® on top. I then put the cage on top of the leaf disc and introduced ten adult alate aphids. I counted the total number of live and dead adults and nymphs after 120 h. Each treatment was replicated four times.

2.2.3.2 Aphid host selection

Next, I conducted two experiments to assess the reaction of wingless aphids when presented with plants treated with imidacloprid at different concentrations: a) choice experiments in which arenas were provisioned with four randomly arranged leaf discs, one with each different insecticide concentration (fig. A.5a), and b) a no-choice experiments in which all four discs had the same insecticide concentration (fig. A.5b). To prepare arenas, I cut 20 leaf discs, 2.54 cm in diameter, from each leaf and placed each disc, adaxial surface up, in a small petri dish filled with a warm 1% agar solution. After the agar gelled I put four petri dishes with their respective leaf discs inside a square plastic container (Up & Up 0.74 L Entre Containers®, Target Corporation, Minneapolis MA) with each dish touching the middle of one of the container's walls. I then poured 50 ml of warm 1% agar solution in the containers which was enough to cover the petri dishes but not the leaf discs. I allowed the agar to cool and placed a round piece of filter paper in the middle of the arena. Finally, I transferred 20 adult aphids to the center of the filter paper and sealed the arena. Each lid had a two-inch vent covered with fine netting to reduce humidity. Treatments in the choice experiment were replicated 4 times, and each no-choice treatment was replicated four times (16 total arenas). Arenas were held at 20℃ 1℃ under a 16:8 light:dark cycle for three days (72 hours). At the end of the experiment, I counted live and dead adults and nymphs and noted the location where they were found, either on a leaf disc or on the agar. Usually, live aphids on the agar were mobile and counted first, while aphids on top of the leaf disc were more stationary and counted last. Aphids physically present on top of a leaf disc were recorded as on the disc regardless of whether they were settled or walking. In general, aphids in arenas with insecticide were more mobile and active. The entire experiment was repeated two times using an untreated control (distilled water) and three insecticide concentrations (2.18125 ppb, 4.3625 ppb and 8.725 ppb). I also performed the same experiment using higher insecticide concentrations, namely 218.125 ppb, 436.25 ppb and 872.5 ppb.

2.2.3.3 Effect of starvation

Among the challenges for alate aphids moving to crop plants from winter hosts is starvation (Pompon et al., 2011). To measure the effect of starvation on susceptibility to imidacloprid, alate and wingless aphids were held in groups of ten individuals within empty petri dishes for 24 hours without access to food or water. Mortality during this period was quite low, and I had previously observed that wingless adult *M. persicae* can survive for at least 48 hours without access to food or water (Merchán, unpublished data, Nauen1997).

I treated leaves with a 218.125 ppb insecticide concentration as previously described, but instead of cutting leaf discs, I left the petioles in the insecticide solution or distilled water and affixed two clip cages to each leaf. Clip cages were constructed from 50 ml plastic centrifuge vials (Falcon®, Corning Life Sciences, NY, USA) glued to a bent hair clip and sealed with organdy mesh (fig. A.6). Ten adult aphids were placed in each cage, and held for 24 hours. Cages were then removed and adult survival and fertility were scored. Each treatment was replicated three times, and the entire experiment was repeated three times for apterous aphids and two times for alates.

2.2.3.4 Effect of age

Finally, I measured the effect of age on alate susceptibility to imidacloprid. I divided alate adults into two age groups: old and early-reproductive. Early-reproductive alates were individuals approximately two-three days after collection. These individuals could, in some cases still fly when disturbed, but had already begun to reproduce, indicating that they had re-hydrated after their teneral period and flight. Old alates were collected more than five days prior to the start of the experiment. As a general rule, alates experienced high mortality when kept in culture past seven days. These experiments were performed with the same concentrations (control and 218.125 ppb, each replicated three time) as the starvation experiments and were repeated two times.

2.2.4 Statistical analysis

For establishment and host selection experiments, I analyzed three parameters, adult survival, nymph survival, and fertility. In age and starvation experiments, I only compared adult survival and fertility. Fertility was defined as the number of nymphs produced by the adults over the course of the experiment. I consider fertility to be a good proxy for feeding, because aphids start reproducing after settling on a leaf, even before they reach the phloem sieve elements (Powell et al., 2006). I also analyzed nymph survival, with the caveat that insecticide exposure varies between nymphs depending on the day they were born. However, I believe that this variability is small, because nymphs respond faster to insecticides and because adults affected by insecticide have a significant reduction in their fertility (Devine et al., 1996).

Nymph and adult survival estimates were fitted to a binomial distribution, and fertility was fitted to a Poisson distribution. All data were analyzed via a generalized linear mixed model, with date of the experiment and replicate within each experiment as random variables.

To determine if adult insects avoid treated leaf discs in host selection experiments, I fitted the number of live adults that were found on the leaf discs versus in the agar for each treatment. Counts were fitted to a negative binomial distribution, with experiment and replicates as random effects, as above, and treatment and location, and their interaction, as fixed effects. I next used the results from the no choice experiment to determine the expected distribution of live adults on and outside leaf discs, for a total of five categories (table 2.3). For the expected number of aphids outside of the leaf discs, I added all the estimates calculated in the no choice experiment. All five averages were transformed into probabilities and multiplied by the total number of live adult aphids counted in the choice experiments to obtain an expected number for each category. I compared this expectation with the observed number using a chi-square goodness of fit test.

All statistical analyses were performed using the R statistical software v 3.2.2 (R Core Development Team, 2015), using "dplyr" to manipulate and organize data (Wickham and Francois, 2015). The generalized linear models were fitted using the "glmmADMB" package v. 0.8.1 (Fournier et al., 2011) and the LS means estimation and Tukey HSD test with the "lsmeans" package v. 2.19 (Lenth and Hervé, 2015). All code is available from the author upon request.

2.3 Results

2.3.1 Imidacloprid residue on leaves

I detected a linear correlation between the concentration of insecticide in the solution and the amount of insecticide residue, both as imidacloprid (*Imidacloprid residue* = $9464.7 + 2158.6$ *imidacloprid concentration*, Adjusted R^2: 0.7222) and its main metabolite olefin (*Olefin residue* = $41500 + 280.64$ *imidacloprid concentration*, Adjusted R^2: 0.5415), within treated leaves. While there is variability in the residue found within the leaves for each concentration, about 72.2% of the variation in imidacloprid residue (fig. 2.1a) and 54.5 % of olefin residue (fig. 2.1b), is explained by the amount of insecticide in the solution. This shows that my setting results in movement of imidacloprid and olefin into the leaves to a degree that reflects the insecticide concentration in the solution and it allowed us to expose the aphids to the neonicotinoid in a way that is biologically relevant and controlled.

2.3.2 Aphid establishment

Neither insecticide concentration (F = 2.75, df = 3, p >0.05) nor surface contact (F = 5.73, df = 1, p >0.05) affected wingless adult survival table 2.1. Both fertility (F = 4.62, df = 3, p <0.05) and mortality of nymphs (F = 3.80, df = 3, p <0.05) showed a significant interaction between the effect of insecticide concentration and contact. There were significantly more nymphs on untreated leaves relative to treated leaves, which suggests that the barrier protected nymphs from insecticide exposure. Alate adult survival ($F_{Concentration}$ = 46.28, $F_{Contact}$ = 59.93 df = 1, p <0.05), reproductive output ($F_{Concentration}$ = 373.22, $F_{Contact}$ = 297.46 df = 1, p <0.05 were all significantly affected by insecticide concentration and the barrier table 2.1. And the effect on nymph survival was so strong for both concentration and barrier that I did not observed any live nymph in out treated leaves with the Parafilm ® and I could not obtain reliable estimates with my analysis method.

2.3.3 Aphid host selection

In no-choice arenas there was an effect of imidacloprid concentration on adult mortality (F = 13.09, df = 3, p <0.05), but there were no significant differences between the three insecticide concentrations (table 2.2).

The effect of imidacloprid concentration on fertility was also significant (F = 214.03, df = 3, p <0.05). The number of nymphs produces was highest in the no insecticide arenas, between 7.44 and 9.15 juveniles produced in 72 hours per adult. These results are consistent with my previous estimates of an average of 3 nymphs per day per adult (Merchán, personal observation). Fecundity in the control was also significantly higher than in all three insecticide concentrations (table 2.2).

The effect of imidacloprid on nymph survival in no choice experiments was also significant (F = 243.06, df = 3, p <0.05) and similar to those on adult survival, but their magnitude was much stronger (table 2.2, third column). Nymphs were very likely to survive in the control and very likely to die in all of the concentrations, which did not differ significantly between them (table 2.2).

The results obtained in the experiments using higher concentrations of imidacloprid produced similar results and the effect of the insecticide was also significant for all three parameters. As expected, I obtained a stronger effect on mortality of both adults and nymphs when using higher concentrations (table 2.2).

In choice experiments I counted the number of adults, both live and dead, found on top of each leaf disc or out in the agar. I first analyzed the number of live adult aphids

and found a significant effect of location ($F = 11.134$, df = 4, $p < 0.05$). Live aphids were more likely to be found on top of the control disc or walking in the agar and lees likely to be on top of treated leaves(table 2.3, first column). I did the same analysis for dead aphids and found an significant effect of location ($F = 20.04$, df = 4, $p < 0.05$), but in this case dead aphids were more likely to be found on the agar and there was no significant difference between the different imidacloprid concentrations (table 2.3, second column). I then analyzed the pooled data and found a significant effect of location ($F = 24.994$, df = 4, $p < 0.05$). Adults aphids, both live and dead, were more likely to be found outside of the agar than on any of the leaf discs. The number of adult aphids was also significantly different between the untreated leaf discs and all other concentrations (table 2.3, thid column). The results were similar in the experiments using higher concentration of imidacloprid, except for the number of total adults found on the 4.3625 ppb leaf disc, which was not significantly different from the control in those experiments (table 2.3).

2.3.4 Effect of starvation

For apterous adult aphids there was a significant effect of imdacloprid concentration in the survival of adults ($F_{Concentration} = 48.01$, df = 1, $p < 0.05$), but no effect from the starvation treatment ($F_{Starvation} = 092$, df = 1, $p > 0.05$). Fertility of apterous adult aphids was affected by the presence of imidacloprid and the starvation treatment, with a significant interaction between them ($F_{Interaction} = 16.33$, df = 1, $p < 0.05$). Starved apterous aphids appear to increase their fertility, even when exposed to a leaf disc treated with imidacloprid (table 2.5). There was no effect from imidacloprid concentration or starvation ($F_{Concentration} = 3.53$, $F_{Starvation} = 6.20$ df = 1, $p > 0.05$) on survival of winged adult aphids, but they both affected the fertility of those aphids, with a significant interaction between the two variables ($F_{Interaction} = 7.61$, df = 1, $p < 0.05$). As with apterous aphids, the starvation treatments appear to increase their fertility in both imidacloprid concentrations (table 2.5).

2.3.5 Effect of age

Both age and insecticide concentration had a significant effect on the survival probability of winged adult aphids ($F_{Concentration} = 6.09$, $F_{Age} = 26.47$ df = 1, $p < 0.05$) and on fertility ($F_{Concentration} = 25.52$, $F_{Age} = 22.35$ df = 1, $p < 0.05$), with no significant interaction between the two variables. Older adults were more likely to die overall, but were also more susceptible to the insecticide concentration. Fertility was affected by the insecticide concentration at both ages and older alate aphids had a lower reproductive output than

the younger ones (table 2.6).

2.4 Discussion

In order to establish a population, an aphid must first identify the plant as acceptable, next it must survive in contact with the plant long enough to reproduce, and finally its progeny must then survive to reproductive maturity. I conducted a series of experiments to measure the effects of systemic imidacloprid applications on each of these step for *M. persicae* in cultivated tobacco. my results are in line with previous observations that adult aphids are capable of detecting leaves treated systemically with imidacloprid and that they will reject these leaves if not exposed to a lethal dose (Nauen, 1995; Fray et al., 2013). While this behavior does not necessarily allow all individuals to find the leaf discs without insecticide, it does significantly increase their chance of survival in a variable environment. This wandering capacity has been shown before in adult aphids (Nauen, 1995; Fray et al., 2013), but these experiments only tested the capacity of the aphid to move from a leaf (or piece of leaf) that was treated to an adjacent untreated one. In my experiments I subjected my aphids to a more complex environments with four insecticide concentrations present. Aphids were more likely to settle on treated leaf discs, so the fact that they are able to locate the untreated leaf is more significant, because it represented only 25% of the available options. Also, the complexity of the environment allowed the detection of a behavior that had not been observed before. While I do not understand how aphids decide to stay or move out of a leaf disc, my results show that the process is not random and some aphids stay in the mid-range concentration. Adult aphids were more likely to settle on leaves treated with the mid-range concentrations of imidacloprid (436.25 ppb and 4.3625 ppb), suggesting that the insects might be capable of some basic behavioral decision making. These experiments do not test how aphids decide and experiments focusing more on behavior of individual aphids when exposed to treated leaves need to be done, the implications of this behavior is that even though an feeding deterrent effect is a desirable characteristic of an insecticide (Griffiths et al., 1989; Jermy, 1990), its effectiveness can be reduced if there is a high level of variability in the distribution of the insecticide within a plant. If a material's concentration is uneven and the insect manages to detect it a move away without damage, there is a likelihood that the insects can locate those spots with lower concentration, survive, and reproduce to a certain extent (Fenton et al., 2010; Fray et al., 2013).

When measuring adult survival in my contact experiments, I did not find a significant effect from imidacloprid concentration or from the barrier treatment, but fertility was

reduced on imidacloprid treated leaf discs with and without the Parafilm® barrier. This suggest that aphids are exposed mainly through feeding and that any contact exposure through the trichome exudates is not significant. The Parafilm® barrier also caused an increase in the fecundity of the aphids on untreated leaf discs. It has been shown that aphids exposed to low levels of imidacloprid can increase their reproductive output, a process known as hormetic response (Yu et al., 2010; Janmaat et al., 2011; Ayyanath et al., 2014). I did not detect a hormetic response with the imidacloprid concentrations used in these experiments. In this case, the barrier and the starvation treatment elicited a similar response, as the aphid reacts to low levels of stress in the environment (Guedes and Cutler, 2014). Another interesting aspect of the contact experiment is the fact that nymphs appear more likely to survive on treated leaf discs when they did not have the protective barrier. This result was unexpected and I believe it might have been due to the fact that water loss could have differed between both barrier treatments. Leaf discs with the barrier were more protected against water loss because they were surrounded by a Parafilm® on both sides, while the non-barrier treatment was only covered on one side, and this allowed imidacloprid to remain active longer in leaf discs with the barrier. When I tried to correct for this in the experiments with winged adults, I did not observe live nymphs on treated leaves and reproduction was too low to compare treatments. It is clear that both experiment setups have caveats, but I believe they allow us to determine that feeding was a more likely route of exposure than contact with trichomes.

As I observed, three of the main aspects of the establishment of green peach aphid *Myzus persicae* in tobacco are significantly affected by the presence of the systemic insecticide imidacloprid. Survival of adults, its reproductive capacity, and the survival of the newborn nymphs are all significantly affected by the tested levels of insecticide in this study. However, I also showed that despite the toxic effect from the insecticide, adult aphids can attain low levels of reproduction in the presence of systemic imidacloprid in the leaf. This strongly suggests that the aphid does not detect the insecticide during the early probes and the antifeeding responses is only elicited after the ingestion of phloem sap, which shows a reduction in duration on plants treated with imidacloprid using electrical penetration graphs (EPG's) (Tosh et al., 2002; Garzo et al., 2016). Those studies have also shown that aphids exposed to imidacloprid in artificial diet take longer to start salivation and ingestion, and also perform more probings than when exposed to untreated diets (Nauen, 1995; Garzo et al., 2016). It is clear that aphids use chemical clues from the apoplast to accept the host plant and can start parturition before reaching the sieve elements of the phloem (Powell et al., 2006; Walling, 2008; Tosh et al., 2002; Sauge et al., 2006).

Fertility seems to be a good measure of insecticide concentration, which suggests that at higher insecticide concentration, adult aphids could detect the insecticide faster and spend less time feeding. feeding deterrent responses to imidacloprid have also been seen in other insects, like thrips, psyllids and whiteflies, which are also a very important virus vectors in many plants (Boina et al., 2009; Joost and Riley, 2005).

Establishment of the pest in the crop is also affected by other variables like age and starvation of the founder. Imidacloprid affected the fertility of starved aphids, both apterous and winged, eliciting a hormetic response. This hormetic response can be caused by differential resource allocation (Guedes and Cutler, 2014). Under this hypothesis, aphids under stress due to starvation redirect most of their resources towards reproduction. This response could be an evolutionary strategy for rapid colonization after landing on the host plant. It also has crop management implications, because aphid populations exposed to low, sub-lethal levels of insecticide could potentially grow faster, and in systemic pesticides such a imidacloprid this a scenario that can occur later in the season, when management options are more limited. I also expected that starved aphids showed a stronger response to a low concentration of imidacloprid, because they are more likely to tap directly into the xylem for water, but my results do not support that hypothesis and they responded in a similar manner as non-starved ones (Pompon et al., 2010; Spiller et al., 1990). I also determined that aphids show signs of senescence after 5 days and their reproduction is reduced compared to younger aphids. All this information shows that alate aphids are evolved to disperse and colonize rapidly and constitute a very important link in the growth of aphid populations in the field.

2.5 Conclusion

I confirmed that aphids are able to move to areas with lower concentrations of insecticide (Nauen, 1995; Fray et al., 2013). In this study I used a more complex environment and documented that aphids might be able to make "decisions" about where they settle and that the feeding deterrence that has been documented for imidacloprid can produce interesting behaviors in these insects. I also demonstrated that while contact with trichomes may affect aphids, most of the exposure to insecticides occurs during feeding. Aphids are phloem-feeders, but both imidacloprid and nicotine in cultivated tobacco are xylem-mobile. It was thought that aphids colonized tobacco because they were not exposed to nicotine in the phloem (Guthrie et al., 1962). It is clear now that *M. persicae* clones adapted to feed on tobacco require a genetic machinery that allows them to process the alkaloid, and that the evolution of this adaptation was a key aspect in the colonization of

Nicotiana tabacum (Bass et al., 2013; Ramsey et al., 2014; Tapia et al., 2008). While I still do not know exactly at what point of the feeding process the aphid encounters nicotine or imidacloprid, I do know that these insects obtain information about host quality during probing and can react to secondary chemicals and insecticides, and that the plants can also react to the attack from the insect (Thompson and Goggin, 2006; Hagel et al., 2012; Walling, 2008; Divol et al., 2005). I need to continue studying the aphid-plant interaction in order to understand how plant can evolve resistance mechanisms against these pests. And finally, I also began to study the role that other aspect of the pest, such as age and starvation play on the establishment of the pest. Most experiments rely on aphids in the same physiological state, well-fed or starved, for example, but there is little research comparing these different states. I can expect variation in this aspect in nature and understanding how aphids behave when subjected to different stresses will improve our understanding of the colonization process.

2.6 Tables

Table 2.1 Contact experiments. Estimated mean probability of survival of adult aphids (± SEM), number of nymphs produced (± SEM) in 72 h (wingless aphids) or 120 h (alate aphids), and probability of nymph survival (± SEM). Means followed by the same uppercase letters are not significantly different from one another within insecticide concentrations, while means followed by the same lowercase letters are not significantly different from one another within barrier treatments via Tukey HSD (α= 0.05).

	Concentration (ppb)	Barrier	Probability of survival Adults (mean ± Std. error)	Fertility (mean ± Std. error)	Probability of survival Nymphs (mean ± Std. error)
Apterous aphids	No insecticide	No	0.914 ± 0.039	9.00 ± 1.060 B a	0.960 ± 0.024
	218.125	No	0.852 ± 0.045	5.50 ± 0.829 A	0.822 ± 0.065 b
	436.25	No	0.890 ± 0.039	4.00 ± 0.707 B	0.943 ± 0.041 b
	872.5	No	0.902 ± 0.036	6.50 ± 0.901 AB	0.960 ± 0.029 b
	No insecticide	Yes	0.902 ± 0.036	14.625 ± 1.352 B b	0.967 ± 0.017 B
	218.125	Yes	0.765 ± 0.056	4.375 ± 0.739 A	0.451 ± 0.101A a
	436.25	Yes	0.790 ± 0.053	4.50 ± 0.750 A	0.384 ± 0.095 A a
	872.5	Yes	0.852 ± 0.045	4.375 0.739 A	0.587 ± 0.098 A a
Alate aphids	No insecticide	No	0.791 ± 0.404 B a	73.295 ± 3.878 B b	–
	4.3625	No	0.020 ± 0.140 A	2.996 ± 0.558 A b	–
	No insecticide	Yes	0.190 ± 0.039 B b	17.874 ± 1.498 B a	–
	4.3625	Yes	0.010 ± 0.009 A	0.799 ± 0.284 A a	–

Table 2.2 No choice experiments. Estimated mean probability of survival of adult aphids (± SEM), number of nymphs produced in 72 h (± SEM) and probability of nymph survival (± SEM). Means followed by the same uppercase letters are not significantly different from one another within insecticide concentrations via Tukey HSD (α= 0.05) for each set of concentrations (low and high).

	Concentration (ppb)	Adult mortality (mean ± Std. error)	Reproductive output (mean ± Std. error)	Nymph mortality (mean ± Std. error)
Low conc.	No insecticide	0.192 ± 0.05 A	30.081 ± 5.59 A	0.171 ± 0.01 A
	2.18125	0.467 ± 0.08 B	12.864 ± 2.43 B	0.780 ± 0.10 B
	4.3625	0.399 ± 0.07 B	11.65 ± 2.21 B	0.797 ± 0.09 BC
	8.725	0.519 ± 0.08 B	9.43 ± 1.80 C	0.863 ± 0.07 C
High conc.	No insecticide	0.113 ± 0.07 A	32.820 ± 4.17 A	0.014 ± 0.006 A
	218.125	0.469 ± 0.16 B	8.346 ± 1.13 A	0.927 ± 0.03 B
	436.25	0.792 ± 0.11 C	2.782 ± 0.43 B	0.939 ± 0.03 B
	872.5	0.758 ± 0.12 C	2.511 ± 0.40 C	0.944 ± 0.03 B

Table 2.3 Choice experiments. Estimated mean number of live adult aphids (± SEM), dead adult aphids (± SEM) and total aphids (± SEM) by position in choice cages. Means followed by the same uppercase letters are not significantly different from one another within insecticide concentrations via Tukey HSD (α= 0.05) for each set of concentrations (low and high).

	Location	Live adults (mean ± Std. error)	Dead adults (mean ± Std. error)	Total adults (mean ± Std. error)
Low conc.	No insecticide	5.00 ± 0.79 A	0.50 ± 0.25 B	5.50 ± 0.83 B
	2.18125 ppb	0.50 ± 0.25 B	0.50 ± 0.25 B	1.00 ± 0.35 A
	4.3625 ppb	1.625 ± 0.45 B	0.625 ± 0.28 B	2.25 ± 0.53 A
	8.725 ppb	0.375 ± 0.22 B	0.375 ± 0.22 B	0.75 ± 0.31 A
	Outside	4.375 ± 0.74 A	6.50 ± 0.90 A	10.875 ± 1.16 C
High conc.	No insecticide	4.375 ± 0.74 A	0.125 ± 0.12 B	4.50 ± 0.75 B
	218.125 ppb	0.625 ± 0.28 B	0.75 ± 0.31 B	1.375 ± 0.41 A
	436.25 ppb	1.250 ± 0.39 B	1.375 ± 0.41 B	2.625 ± 0.57 AB
	872.5 ppb	0.250 ± 0.18 B	0.625 ± 0.28 B	0.875 ± 0.33 A
	Outside	6.375 ± 0.89 A	4.25 ± 0.73 A	10.625 ± 1.15 C

Table 2.4 Observed and expected number of adult aphids in low and high concentration choice experiments. The expected probability of aphids for each concentration was estimated using the results observed in non-choice experiments. Deviation from expectations for each of the experiments was calculated using a Chi-Square Goodness of fit test ($\alpha = 0.05$).

(a) Low concentration

Concentration (ppb)	Live adults			Chi-Square sum	*d.f*	Prob.
	Observed number of live adults	Expected probability	Expected number of live adults			
Not on leaf disc	35	0.410	38.96	11.061	4	<0.0001
No insecticide	40	0.242	23.045			
2.18125	4	0.129	12.25			
4.3625	13	0.137	13.00			
8.725	3	0.081	7.71			
Total	**95**	**1.00**	**95.00**			

(b) High concentration

Concentration (ppb)	Live adults			Chi-Square sum	*d.f*	Prob.
	Observed number of live adults	Expected probability	Expected number of live adults			
Not on leaf disc	51	0.604	57.43	4.423	4	0.3517
No insecticide	35	0.239	22.71			
218.125	5	0.076	7.26			
436.25	10	0.058	5.48			
872.5	2	0.022	2.11			
Total	**103**	**1.00**	**103.00**			

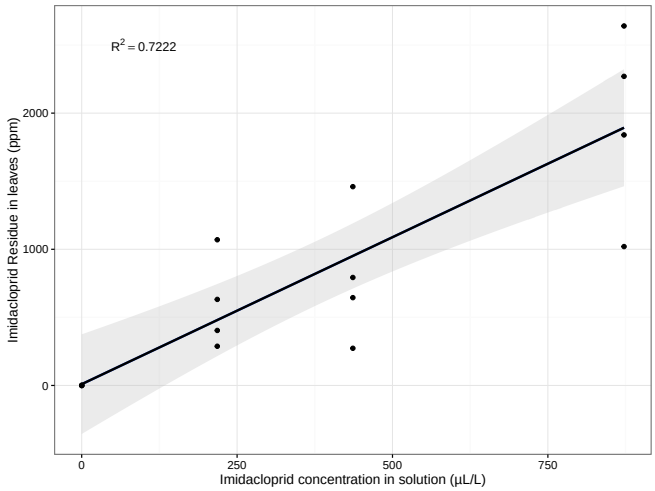
Table 2.5 Starvation experiments. Estimated mean probability of survival of adult aphids (± SEM), number of nymphs produced (± SEM) in 24 h, and probability of nymph survival (± SEM). Means followed by the same uppercase letters are not significantly different from one another within insecticide concentrations, while means followed by the same lowercase letters are not significantly different from one another within starvation treatments via Tukey HSD (α= 0.05).

	Concentration (ppb)	Starvation	Probability of survival Adults (mean ± Std. error)	Fertility (mean ± Std. error)
Apterous aphids	No insecticide	Non starved	0.900 ± 0.052 A	16.276 ± 1.257 A b
	218.125	Non starved	0.632 ± 0.127 B	1.789 ± 0.402 B b
	No insecticide	Starved	0.923 ± 0.424 A	30.405 ± 1.776 A a
	218.125	Starved	0.675 ± 0.120 B	9.569 ± 0.948 B a
Alate aphids	No insecticide	Non starved	0.788 ± 0.059	18.759 ± 3.153 A
	218.125	Non starved	0.671 ± 0.069	5.882 ± 1.272 B b
	No insecticide	Starved	0.967 ± 0.023	23.846 ± 3.878 A
	218.125	Starved	0.788 ± 0.586	14.149 ± 2.491 B a

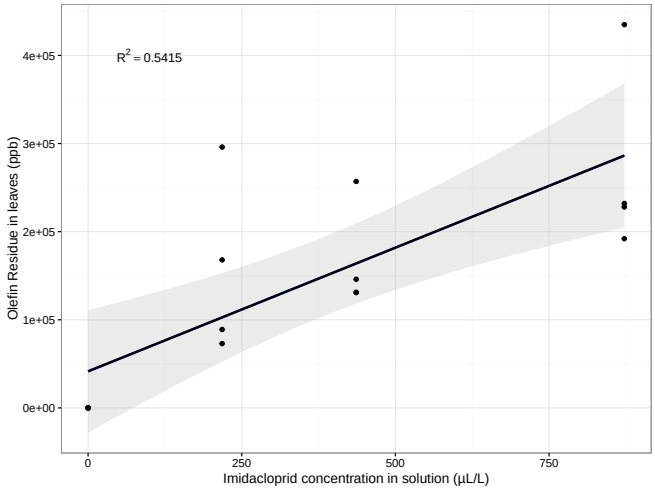
Table 2.6 Age experiments. Estimated mean probability of survival of alate adult aphids (± SEM), number of nymphs produced (± SEM) in 24 h, and probability of nymph survival (± SEM). Means followed by the same uppercase letters are not significantly different from one another within insecticide concentrations, while means followed by the same lowercase letters are not significantly different from one another within age treatments via Tukey HSD (α= 0.05).

Concentration (ppb)	Age	Probability of survival Adults (mean ± Std. error)	Fertility (mean ± Std. error)
No insecticide	2 -3 days	0.938 ± 0.030 a	11.740 ± 1.587 B a
218.125	2 -3 days	0.825 ± 0.050 a	4.566 ± 0.914 A a
No insecticide	5 + days	0.693 ± 0.063 B b	5.381 ± 1.002 B b
218.125	5 + days	0.447 ± 0.067 A b	1.304 ± 0.469 A b

2.7 Figures



(a) Imidacloprid. Residue concentration in ppm (parts per million)



(b) Olefin.Residue concentration in ppb (parts per billion)

Figure 2.1 Relationship between insecticide concentration in the solution and insecticide residue within the leaves and the adjusted R^2. The line represents the best adjustment to the the data and the grey area represents a 95% confidence interval.

Chapter 3

Nicotine and imidacloprid tolerance in tobacco-feeding green peach aphid (*Myzus persicae*)

3.1 Introduction

Plants have been dealing with insects herbivores for millions of years in a coevolutionary arms race using an array of physical and chemical defenses to avoid, reduce or recover from insect damage (Cornell and Hawkins, 2003; Mithöfer and Boland, 2012; Walling, 2000; Wu and Baldwin, 2010). Among these adaptations, secondary metabolites play a prominent role in plant protection (Chen, 2008; Kaplan et al., 2008). Plant secondary metabolites are organic molecules usually produced as by-products during the synthesis of primary metabolic products and do not play an essential metabolic function for the organism (Chen, 2008; Walling, 2000). They have evolved mainly to defend the plant when attacked by herbivores, either directly by affecting or poisoning the insects or indirectly by attracting natural enemies that feed on or attack the pest (Mithöfer and Boland, 2012; Wu and Baldwin, 2010). Insect herbivores have been subjected to strong natural selection to overcome these plant defenses and have evolved adaptations that allow them to circumvent them, exploiting the plants for food and shelter (Agrawal and Konno, 2009; Karban and Agrawal, 2002).

Cultivated tobacco (*Nicotiana tabacum*) is an ideal system to study defensive chemicals in plants and their effect on herbivores, because its chemistry and genetics are well studied due to their economic importance (Dewey and Xie, 2013; Morita et al., 2009; Vandenborre et al., 2009). The plant produces pyridine alkaloids, such as nicotine, which have a known

toxic effect on insects and other organisms and plays an important role in protecting the plant against herbivores (Steppuhn et al., 2004). It has also been suggested that alkaloid composition and herbivore pressure played an important role in the evolution of the whole Nicotiana genus and in the domestication of *N. tabacum* (Clarkson et al., 2004; Gavilano et al., 2007). Typically, the dominant pyridine alkaloid in cultivated tobacco is nicotine, which comprises about 2-4% of the total dry weight and about 90% of the total alkaloid content, followed by nornicotine (a derivative of nicotine), anatabine and anabasine (Dewey and Xie, 2013). The dynamics of alkaloids in tobacco have been studied both in the laboratory and in the field, and we have a good understanding of the mechanisms that control the production of alkaloids (Dewey and Xie, 2013). Nicotine is synthesized in the roots by two main pathways, one called the pyridine-nucleotide cycle and another one called the methylpyrroline pathway and each one produces a ring molecule that are joined to form nicotine. This alkaloid is produced constitutively at low levels and moves through the xylem to the leaves where it is stored in the vacuoles of the mesophyll cells (Morita et al., 2009; Saunders, 1979). However, injury due to an herbivore attack in the leaves or the stalk of the plant increases the production of nicotine and its concentration in the leaves (Steppuhn and Baldwin, 2007; Steppuhn et al., 2004). It has been shown that nicotine has a negative effect on insects by acting as an agonist of the nicotinic acetylcholine receptor, causing nervous over-excitation and death (Rattan, 2010). When offered the choice, individuals prefer diets or plants with lower nicotine levels (Glendinning, 2002; Kliot et al., 2014; Steppuhn et al., 2004). Nicotine in cultivated tobacco offers an opportunity to study how insect herbivores evolved the genetic architecture to cope with a noxious secondary chemical in a well-defined system, with practical implications for humans.

Despite the toxic effect of nicotine, some insects can effectively utilize *N. tabacum* as a hostplant and most can reach pestiferous levels. In tobacco grown in North Carolina, the main insect pests are the tobacco hornworm, *Manduca sexta* (Lepidoptera), the tobacco budworm, *Heliothis virescens* (Lepidoptera) and the green peach aphid, *Myzus persicae* (Hemiptera) (Burrack, 2013). The *M. persicae* taps the plant's phloem with piercing sucking mouthparts that act like a needle and ingests phloem sap (Peccoud et al., 2010). This sap is rich in carbohydrates but lacks nicotine, and presumably aphids in tobacco are not exposed to the alkaloid during feeding. Some have suggested that aphids are exposed to the alkaloid through contact with toxic secretions released by glandular trichomes on the leaf (Guthrie et al., 1962) while others have suggested that they are exposed when piercing plant cells and their vacuoles on their way to the phloem (Powell et al., 2006; Ramsey et al., 2014; Walling, 2008). Regardless of the exposure route *en planta*,

experiments using artificial diets have shown that aphids are indeed heavily affected by nicotine (Ramsey et al., 2014). Researchers have recently suggested that tobacco-adapted populations in Europe, Africa and Asia are able to metabolize the nicotine due to an overexpression of a cytochrome P450 monooxygenase and that this gene, called *CYP6CY3*, could have played a role in the colonization of *Nicotiana tabacum* as a host plant (Bass et al., 2011, 2013). There is also genetic evidence that suggests that at least some of the tobacco-feeding lines of *Myzus persicae* have become at least partially isolated and morphologically distinct, and it should be considered a subspecies or a variety, with incomplete reproductive isolation (Clements et al., 2000b,a; Margaritopoulos et al., 2009, 2007).

Defensive secondary compounds have also been used as a blueprint to find and create new synthetic chemicals that attack different targets in the insects (Rattan, 2010). The neonicotinoid insecticide class was developed based on the relationship between nicotine and the nicotinic acetylcholine receptor in insects, and several very effective pesticides affecting that receptor have been synthetized (Tomizawa and Casida, 2005). Shortly after the commercial release of the first neonicotinoid insecticide, imidacloprid, if was shown that aphids adapted to tobacco showed higher levels of tolerance to it when compared with susceptible lines (Devine et al., 1996; Nauen and Elbert, 1997). It has also been shown that the overexpression of the cytochrome P450 *CYP6CY3* gene is responsible for conferring the tolerance to nicotine in *M. persicae* and that the gene also plays a role in resistance to imidacloprid (Bass et al., 2011, 2013). However, other mechanisms such as target site mutation and changes in the the cuticle that reduce the penetration of the insecticide have also shown to be produce tolerance in certain clones (Bass et al., 2011, 2013; Puinean et al., 2010; Slater et al., 2012; Bass et al., 2015). I use the tobacco-aphid system to test if resistance to nicotine, a natural plant secondary chemical, is associated with similar resistance to synthetic pesticides.

M. persicae has evolved resistance mechanisms to a wide variety of insecticides, including pyrethroids, DDT, pirimicarb and neonicotinoids, and all this variation seems to be present in both color morphs and in every continent (Bass et al., 2011; Martinez-Torres et al., 1999; Nabeshima et al., 2003). In North Carolina as in most regions with fairly mild winters, tobacco-feeding *M. persicae* clones are anholocyclic, producing only parthenogenetic females (Clements et al., 2000b). Research has shown that *M. persicae* populations from tobacco fields in the United States have medium levels of genetic variability in microsatellite loci when compared to populations in Europe (*Allelic diversity (A)*: 4.29 for the US, 6.29 for France and 5.71 for Greece; *Observed heterozygosity (H_O)*: 0.476 for the US, 0.842 for France and 0.686 for Greece) (Zepeda-Paulo et al.,

2010). There has been no research measuring the haplotype composition of *M. persicae* populations in tobacco farms in North Carolina, but a previous detailed study suggested that there is genetic diversity in the state (Harlow et al., 1991). Another interesting aspect *M. persicae* biology in tobacco is color polymorphism between red and green morphs. Both colors are found on tobacco plants, but red morphs seem more common, particularly at the end of the season and to be better adapted to heat (Harlow and Lampert, 1990; Harlow et al., 1991). Also, the different mechanisms of resistance to insecticides are present in both color morphs and, in the particular case of tolerance to imidacloprid, clones of both colors have been reported to have higher tolerance to that insecticide in laboratory assays (Abdel-Aal et al., 1992; Srigiriraju et al., 2010). Using tobacco-adapted and non-tobacco lines of *M. persicae*, I set out to address an important evolutionary question: is resistance or tolerance to plant defensive chemicals associated with resistance or tolerance to pesticides? To answer this question I first surveyed levels of resistance to imidacloprid in different populations of tobacco-adapted *M. persicae* in North Carolina. I conducted a series of experiments comparing the response of geographically distinct populations of tobacco-feeding *M. persicae* to imidacloprid, in order to measure base levels of tolerance in the state. I also compared response to imidacloprid and nicotine between tobacco-adapted and non-tobacco lines of the aphid. The non-tobacco lines were the G006 and the G006 clones, collected in Geneva, New York on pepper plants in 2003 (Ramsey et al., 2007). Both lines are green and have been maintained in the laboratory on cabbage plants since their collection (Alex Wilson, *pers. comm.*) The rationale behind these experiments is that if responses are similar to both chemicals within populations that have the same feeding preference, but different between those same populations, there is a possibility that similar genetic and physiological mechanisms underlie the adaptation to natural and synthetic toxic chemicals that target the same receptor in insects.

3.2 Methods

3.2.1 Response of field collected aphids to imidacloprid

I collected three fresh tobacco leaves from greenhouse grown, pesticide-free plants. Leaves were washed with diluted insecticidal soap at 2.5 fl oz/gal of water (Safer® Insect Killing Soap Concentrate, Woodstream Corp, Lititz, PA) to remove wild aphids and whiteflies and thoroughly rinsed. Then, I dipped the petiole for 24 hours into 100 mL of distilled water (untreated control) or 100 mL of one of two different imidacloprid solutions, 436.25 nL Admire Pro® (Bayer CropScience, Research Triangle Park, NC) /ml distilled water

(0.01 times the recommended field rate of 35.5 mL/1000 plants) and 218.125 nL Admire Pro®/distilled water mL (0.005 times the field rate). After the treatment, I took 1-inch leaf discs and put them on top of warm 1% agar in a 0.5 oz plastic cup, making 36 cups from each leaf. All 108 cups were divided into 12 groups, with nine cups each (three untreated controls, three 0.01X and three 0.005X). I then visited four North Carolina Department of Agriculture & Consumer Services (NCDA & CS) and North Carolina State University Research Stations, located at Kinston (35.297 ºN, 77.577 ºW), Rocky Mount (35.894 ºN, 77.682 ºW), Oxford (36.305 ºN, 78.611 ºW) and Clayton (35.668 ºN, 78.503 ºW). At each research station, I collected aphids from aphid-infested tobacco plants. For each plant, I collected 24 aphids and put two in each cup, recorded the research station where it was collected, its color morph and assigned it an identification number. I repeated this protocol for a total of 12 collecting points and points were separated by at least 50 m between them (See example in fig. B.1). Red morphs were more common in all research stations, but I collected three green lines in Oxford, two in Clayton, three in Kinston and two in Rocky Mount. Collected aphids were held in growth chamber and maintained at 20℃and a 12h:12h day:night cycle and total number of nymphs produced in each cup was counted after six days, as well as the number of dead nymphs.

3.2.2 Colony maintenance

After the experiments, I created single female lines from four different subpopulations coming from each research station, for a total of 16 different lines. These lines were maintained in plastic 0.5 oz cups that had a tobacco leaf disc dipped in 1% agar. The cups were maintained in a growth chamber and maintained at 20℃and a 12h:12h day:night cycle and renewed every week. On every change, ten adult aphids were transferred to the new leaf disc, and populations were grown according to need. Later, when I obtained the Non-tobacco lines G002 and G006 from the University of Miami, courtesy of Dr. Alex Wilson, I switched all tobacco lines to collard greens leaf discs and maintained both the tobacco and non-tobacco lines on collard discs, under the same conditions. I also maintained stock population of the non-tobacco clones on cabbage seedlings and every five generations a subsample of each tobacco line was grown on tobacco discs to ensure that they remained tolerant to nicotine.

3.2.3 Laboratory tolerance to imidacloprid treated leaf discs

I also tested the 16 tobacco-adapted lines for tolerance to imidacloprid in a controlled experiment, using leaf discs dipped in a solution of technical grade imidacloprid diluted

in distilled water. I diluted 100 mg imidacloprid in 1 mL of acetone which was added to a liter of water to produce a 100 g/L solution. I then made serial dilutions to produce solutions at 50 mg/L, 25 mg/L, 10 mg/L, 5 mg/L, 1 mg/L and 0.5 mg/L. I then cut 1-inch leaf discs from greenhouse grown, pesticide free tobacco leaves and dipped them for 20 seconds in a pesticide solution or distilled water control. I also included a control treatment without a leaf disc to account for mortality due to starvation, a potential side effect of antifeedant responses to imidacloprid (Nauen, 1995). Discs were allowed to air dry and placed on warm 1% agar until. Ten apterous adult aphids (+/- 1) each from 16 different single-female matrilines were placed in each cup. Bioassay arenas were in a controlled environment at 20 ℃and a 16H:8H day:night cycle. Live and dead adults, and live and dead nymphs, were counted after 72 h. Each pesticide concentration was replicated four times for each population.

3.2.4 Laboratory to imidacloprid in artificial diet

To isolate the effect of imidacloprid on aphids without nicotine from tobacco leaves, I conducted a series of experiments using a complete artificial diet consisting of 15% sucrose and 20 amino acids, vitamins and minerals on seven of the tobacco lines from the previous experiment that were selected at random (Douglas, 2007). This diet has been used in previous experiments and allows aphid survival and reproduction for up to 7 days (van Emden, 2009) and has been previously used to assess nicotine dose-response in the whitefly *Bemisia tabaci* and *M. persicae* (Kliot et al., 2014; Ramsey et al., 2014). I prepared 1 L of diet in sterile conditions, to prevent bacterial growth, which was used for all experiments. I dissolved 10 mg of technical grade imidacloprid in 1 mL of acetone which was added to 200 mL of diet to create a 50 mg/L stock solution. Out of this solution I made logarithmic serial dilutions to obtain solutions at 5 mg/L, 0.5 mg/L, 0.05 mg/L and 0.005 mg/L. A pesticide free control consisted of the artificial diet without imidacloprid.

To construct cages, I cut 50 mL Falcon® tubes (Corning Life Sciences, NY, USA) at about 10 cm from the upper lip, glued a fine mesh to the bottom to allow aeration, and made a small hole on the side. I then cut a round hole in the middle of the lid and stretched two pieces of Parafilm® over the opening. I dispensed 200 L of the test solution between the two Parafilm® layers to form a satchel, put the lid on the tube, placed aphids inside the cage through the lateral hole, and sealed it with stretched Parafilm®. Aphids were maintained in collard greens (*Brassica oleraceae* Acephala group) for at least one full generation before the beginning of the experiment. The number of adult aphids per

replicate was variable due differential mortality between matrilines, cages contained a maximum of 10 adult aphids each. Due to this imbalance, I did not analyze reproductive output for these experiments. Each treatment was replicated four times

3.2.5 Tolerance to nicotine vs. tolerance to imidacloprid

I conducted a simultaneous comparison of the response of *M. persicae* populations to nicotine and imidacloprid diet solutions. A 10% nicotine stock solution was made by mixing 2 ml of nicotine with 18 ml of artificial diet. Nicotine has a density of 1.010 g/mL at 20 ℃, so the 10% solution amounts to a solution with about 101000 mg/L. At high nicotine concentrations (>5%) the diet lost viscosity and required a double layer of Parafilm® to contain the diet within the cage. At 10% it was very challenging to maintain the diet for the 72 h of the experiment. Hence these concentrations were tested only once. I also had variable aphid production between stock colonies, and adults per cage ranged from 20 to 4, with a mean number of adults of 11, these differences between matrilines were observed consistently during all experiments and were difficult to control. Cages were prepared in the same way as the previous experiments and I also performed the counts after 72 h.

3.2.6 Statistical analysis

To determine if pesticide treatment, matriline, locality of origin or color had any effect on aphid survival and reproduction, I used a Bayesian generalized linear mixed model (GLMM) analysis (Gbur et al., 2012; Stroup, 2012). After visual inspection of q-q plots to test for the distribution probability of the response variable, I fitted survival data (number of live aphids divided by total number of aphids) to a binomial distribution. In experiment for which were I were able to analyze the reproductive output of the adults (field tolerance), I fitted those data to a Poisson distribution. I fitted a complete model, with pesticide treatment, matriline identity and its interactions as fixed effects, and experimental replicate as a random effect. I used flat priors,meaining I had no previous information regarding the system. The use of flat priors generally produces comparable estimates to maximum likelihood estimation of GLMM's, I tested this hypothesis with several of my models and did indeed obtained similar results, except that Bayesian estimates tend to be more conservative due to wider credible intervals. I then fitted different models that allowed testing different biologically relevant hypotheses. For example, I tested the effect of research station, by grouping all the lines that were collected on each research station and analyzing that effect. I did the same with color when possible (See

a more detailed example on appendix C). I then compared all the different models, and selected the one with the lowest AIC. To further analyze the results, I sliced estimates by the significant effects I obtained after selecting the model, and performed a Tukey HSD pairwise-comparison within each main effect to determine statistical separation between levels of the different variables. I ran all Bayesian models with three Monte Carlo Markov chains, each with a total number of 100000 iterations and a "burn-in" period of 10000 iterations, with thinning every 10 iterations. All analyses were performed using the R statistical software v 3.2.2 (R Core Development Team, 2015), I fitted the Bayesian GLMM's using the "MCMCglmm" v. 2.21 package (Hadfield, 2010) and performed the model comparison using "MuMIn" v.1.15.1 (Barton, 2014). I performed the LS means estimation and Tukey HSD test with the "lsmeans" package v. 2.19 (Lenth and Hervé, 2015). Code is available from the author upon request.

3.3 Results

3.3.1 Imidacloprid response in field collected clones of *Myzus persicae*

There was no significant effect of pesticide concentration, matriline identity, locality of origin, or color on nymph production at six days after exposure on reproductive output of adult aphids; of the 11 models tested, the one with lowest AIC had only the intercept (table C.1a). However, the model including locality where aphids were collected, pesticide concentration, and their interaction had the lowest AIC for nymph mortality (table C.1b). Nymph survival differed between all three concentrations, and the highest the concentration had the lowest the survival (table 3.1). The effect of locality on nymph mortality was more complicated and varied according to pesticide concentration. However, lines from Clayton had higher survivorship in every treatment, suggesting a higher overall tolerance to the pesticide.

3.3.2 Leaf disc tolerance

I tested 11 different models for the probability of survival for both adults and nymphs and only pesticide concentration had a significant effect on these two variables (table C.1a and table C.1b). Adult mortality was similarly low in the no pesticide control and the two lowest pesticide concentrations. Nymphs, on the other hand, responded more strongly to the insecticide at lower concentrations and their mortality plateaus at about 25 µg/L

(table 3.2).

3.3.3 Tolerance in Artificial diet

I tested eight different models, and for adult survival, the best fit model included only insecticide concentration and research station (table C.2a). Survival was higher in the control and at low pesticide concentrations. Unlike the leaf dip experiments, though, the highest concentration used did not cause close to 100% mortality (table 3.2a). With respect to location, aphids from Oxford had a significant higher survival probability than those from other research stations. This differed from the previous analyses that suggested that individuals collected at Clayton research station had overall higher tolerance to imidacloprid (Srigiriraju et al., 2010).

Nymph survival was not affected by location and only differed significant between pesticide concentrations (table C.2b). As with adults, the highest concentration did not produce close to 100% mortality (table 3.2b).

3.3.4 Comparison between tobacco adapted and non-tobacco lines

For this experiment I included four of my tobacco-adapted lines selected because they showed high survival in experiments with artificial diet and two lines that were originally collected in Geneva, New York from crops other than tobacco and that had been maintained under laboratory conditions since then on cabbage plants (Ramsey et al., 2007). I tested 13 different models and the best model for both adult and nymph survival included pesticide concentration (nicotine and imidacloprid) and phenotype (tobacco-adapted vs. non-tobacco), with no interaction between them (table 3.4 and section 3.7). Nymphs seem to be more sensitive to both materials in general, but the difference between phenotypes is still maintained between both stages (table 3.5 and section 3.7).

3.4 Discussion

3.4.1 Variation in tolerance levels to imidacloprid was detected in North Carolina tobacco feeding *M. persicae*

Imidacloprid is widely used by tobacco growers in North Carolina and about 91% of the acres in production are normally treated with some form of neonicotinoid, based on replies

annual cooperative extension agent surveys (https://tobacco.ces.ncsu.edu/2016/03/2015-flue-cured-tobacco-survey-entomology-survey-data/). While there are no reports that suggested the emergence of imidacloprid resistance in the state, I have seen that aphids can indeed colonize plants that have been treated but these infestations are typically small and disappear after topping. I did not find consistent differences in the tolerance levels for imidacloprid in different matrilines from different localities throughout the state, although I detected some variability in survival at different levels. *M. persicae* from Clayton, for example, showed evidence of higher tolerance when feeding on tobacco leaves, but was not different from the other three localities when exposed to imidacloprid in artificial diet. On the other hand, nymphs produced by adults collected directly from the field in Oxford and Clayton also showed a higher survival than nymphs from Rocky Mount and Clayton. However, experiments with artificial diet, which expose the aphids directly with the insecticide did not show differences at the population level in North Carolina. Tobacco leaf dip expose aphids simultaneously to both pesticide and plant defensive chemicals. While these are more biologically relevant because they are more akin to the way aphids feed on a plant, experiments with artificial diets allow us to isolate responses to single chemicals, either imidacloprid and nicotine. In both experimental conditions, all the sampled populations showed very similar levels of tolerance to imidacloprid and that these levels do not constitute resistant populations when growers follow the standard agronomical practices for tobacco. These findings do not agree with previous experiments that showed higher levels of variation in tolerance levels to imidacloprid in tobacco-feeding populations of M. persicae in North Carolina (Srigiriraju et al., 2010).

3.4.2 Tobacco-adapted aphids appear to be more tolerant to imidacloprid than non-tobacco ones

My experiments demonstrates that tobacco-adapted lines of *M. persicae* are indeed more tolerant to imidacloprid than lines that feed on other plants. This difference in tolerance was observed shortly after neonicotinoids hit the market (Devine et al., 1996; Nauen and Elbert, 1997). Recent findings in *M. persicae* suggest that the same gene involved in the adaptation to nicotine may be associated with imidacloprid resistance observed in Europe (Bass et al., 2013). It is possible the same process happened in the US, but my results have limitations relative to their ability to inform conclusions regarding this hypothesis. In particular, non-tobacco feeding *M. persicae* had high mortality when fed pesticide free diet; higher, in fact, than mortality in the lowest concentration of imidacloprid (0.005 mg/L). None of the aphids populations used in these experiments

were adapted to artificial diet, so I assume that this is not a plausible explanation. It is possible that tobacco aphids are more robust and could pierce through the Parafilm® with more ease than the non-tobacco lines. Several authors have contended that tobacco-adapted and non-tobacco lines of *Myzus persicae* can be distinguished phenotipically from non-tobacco lines (Margaritopoulos et al., 1998). Also tobacco leaves are usually covered with trichomes and might be harder to penetrate than other host plants, making those lines better adapted to my experimental setup (Tapia et al., 2008; Goundoudaki et al., 2003). Another possible explanation is that generalist lines are more sensitive to transfers from an optimal diet to a suboptimal one, while specialists lines are not as sensitive (Nikolakakis et al., 2003; Olivares-Donoso et al., 2007). My experiments suggest that clones of *M. persicae* that feed on tobacco have a higher tolerance to imidacloprid. This also implies that tobacco-adapted lines might be more likely to develop resistance to imidacloprid, although there is no evidence of resistance development in the tobacco system in North Carolina.

Another important aspect regarding resistance management is the possibility that tobacco-adapted aphids move to different crops. Cultivated tobacco is not widely grown in the US or Europe, so there few places where this changes in host plant can be significant. I know that tobacco-adapted lines can survive and reproduce on other host plants, such as Brassicas and other solanaceous crops (Cabrera-Brandt et al., 2014). While there is evidence that there is competitive exclusion between tobacco-adapted and non-tobacco lines in non-tobacco host plants so that tobacco-adapted lines will not survive very long in the absence of their preferred host plant (Tapia et al., 2008), this hypothesis has not been analyzed under field conditions and in parts of the world where cultivated tobacco is one of the most important crops. In North Carolina, it is reasonably expected that most of the *M. persicae* lines presented in the landscape are tolerant to nicotine and may have a higher tolerance to imidacloprid.

3.4.3 Adult mortality vs. nymph mortality

I compared adult mortality and nymph mortality across my experiments, and in general, both life stages were similarly affected by imidacloprid or nicotine. Nymphs are not commonly used in experiments analyzing the toxicity of systemic pesticides in aphids (Nauen, 1995; Srigiriraju et al., 2010). While nymph data may be complicated by lack of controlled exposure time, they produce a faster response to pesticide than adults, and their abundance can also be used a measurement for toxicity. In the particular case of imidacloprid, it is clear that number of nymphs produced is correlated with pesticide

toxicity, due in part to the anti-feeding effect elicited by this material, but also due to adult mortality. Nymph abundance might provide a more complete measure of toxic effects under low pesticide concentrations.. Protocols including daily nymph counts to keep track of fecundity and mortality may be ideal, but, even my more simple protocol, with just one nymph count, produced comparable results to adult counts.

3.4.4 The use of Bayesian statistics in toxicological and ecological studies

I analyzed my data using a Bayesian framework because my experiments were not necessarily balanced, and in some cases regular Maximum Likelihood estimation did not converge to a solution. For these cases Bayesian statistics offer a powerful and reliable alternative (Che and Xu, 2010; Takakura, 2012). Bayesian analysis has advanced at a rapid pace in the last decade, thanks to powerful and cheaper computers, giving users enough power to run the required simulations. Also, the development of tools such as R (R Core Development Team, 2015) and associated packages provide an adequate platform to run the analyses in parallel, facilitating the comparisons. This type of approach is common in fields like phylogenetics, where Bayesian and Likelihood models can produce different phylogenetic trees, but is also becoming common in other fields due to the flexibility and power of Bayesian tests (Che and Xu, 2010). While the type of Bayesian analysis performed here is still not part of the mainstream in agricultural research, I believe it should be embraced and used more widely. The power from Bayesian analysis comes from the ability to produce a posterior distribution that can be sampled through Monte Carlo algorithms, while still using the familiar setup of a Generalized Linear Mixed Model (GLMM). One of the main criticisms of Bayesian statistics is the idea that there is a prior distribution, selected by the researcher, which captures any kind of previous knowledge the researcher might have about the system. To avoid that criticism, I used very weak, flat priors in my models which means I had no preconceived expectations about my results. This approach is common in Bayesian statistics when researchers want to obtain estimates from the data, or when there is no relevant previous information that can inform the results. In cases where flat priors are used, Bayesian estimation should produce very similar results as Maximum Likelihood estimation, as was the case in my experiments. Bayesian analysis can be more flexible than maximum likelihood estimation, where solutions are not always possible analytically and there is not a fast and reliable equivalent to the Monte Carlo method. Bayesian statistics is also very powerful in highly unbalanced experiments, which I had in some cases. Because of all this, I believe that

Bayesian statistics should become an integral part of agricultural research, especially for new, more powerful analytic tools like GLMMs.

3.5 Conclusion

In this study I did not find the same levels of tolerance to imidacloprid in tobacco-feeding clones of *Myzus persicae* that had been reported in previous studies (Srigiriraju et al., 2010). By testing different aphid lines in bioassays using leaf dips, or imidacloprid diluted in an artificial diet, I showed that there is no clear signal for the presence of clones with high levels of tolerance to imidacloprid in North Carolina. I also showed that the estimates of adult survival and nymph survival produced similar results in most cases, but nymphs show a stronger reaction to imidacloprid and reach higher levels of mortality faster than adults. Counting nymphs appears to be a very effective tool to detect an effect from imidacloprid in experiments analyzing sub-lethal concentrations of the insecticide. I also found that *M. persicae* clones that feed on tobacco appear to have higher levels of tolerance to imidacloprid when compared to clones that do not feed on tobacco. I believe that this difference can be a starting point for the evolution of resistance to imidacloprid in aphid clones of the United States and efforts are needed to keep monitoring these clones carefully.

3.6 Tables

Table 3.1 Field tolerance to imidacloprid. Estimated mean probability of survival of aphid nymphs (± SEM). LS means were estimated using a Bayesian Generalized Linear Mixed Model and LS mean separation was accomplished using Tukey HSD. Means followed by the same uppercase letters are not significantly different from one another for the different pesticide treatments within each locality, while means followed by the same lowercase letters are not significantly different from one another for the different localities within each pesticide treatment (Tukey HSD α= 0.05)

Treatment	Probability of survival Nymphs (mean ± Std. error)			
	Kinston	**Clayton**	**Rocky Mount**	**Oxford**
No pesticide	0.496 ± 0.253 A b	0.918 ± 0.483 A a	0.534 ± 0.278 A b	0.929 ± 0.493 A a
0.005 X	0.111 ± 0.058 B b	0.209 ± 0.109 B a	0.022 ± 0.013 B c	0.048 ± 0.026 B c
0.01 X	0.010 ± 0.006 C b	0.039 ± 0.022 C a	0.003 ± 0.002 C b	0.017 ± 0.010 C ab

Table 3.2 Laboratory tolerance to imidacloprid: leaf discs. Estimated mean probability of survival of aphid adults and nymphs (± SEM). LS means were estimated using a Bayesian Generalized Linear Mixed Model and LS mean separation was accomplished using Tukey HSD. Means followed by the same uppercase letters are not significantly different from one another for the different pesticide treatments (Tukey HSD α= 0.05)

Treatment	Probability of survival Adults (mean ± Std. error)	Probability of survival Nymphs (mean ± Std. error)
No Pesticide	0.670 ± 0.537 A	0.964 ± 1.199 A
0.5 mg/L	0.664 ± 0.536 A	0.892 ± 1.120 B
1 mg/L	0.549 ± 0.442 A	0.746 ± 0.936 C
5 mg/L	0.189 ± 0.153 B	0.171 ± 0.216 D
10 mg/L	0.107 ± 0.087 B	0.044 ± 0.056 E
25 mg/L	0.031 ± 0.025 C	0.015 ± 0.021 E
50 mg/L	0.035 ± 0.029 C	0.013 ± 0.018 E
100 mg/L	0.032 ± 0.026 C	0.014 ± 0.021 E
No leaf disc	0.028 ± 0.023 C	0.029 ± 0.045 DE

Table 3.3 Laboratory tolerance to imidacloprid: artificial diet. Estimated mean probability of survival of aphid adults and nymphs (± SEM). LS means were estimated using a Bayesian Generalized Linear Mixed Model and LS mean separation was accomplished using Tukey HSD. Means followed by the same uppercase letters are not significantly different from one another for the different pesticide treatments (Tukey HSD α= 0.05)

(a) Adults

Treatment	Probability of survival Adults (mean ± Std. error)			
	Kinston	**Clayton**	**Rocky Mount**	**Oxford**
No Pesticide	0.852 ± 0.379 A b	0.813 ± 0.388 A b	0.816 ± 0.372 A b	0.893 ± 0.395 A a
0.005 mg/L	0.846 ± 0.377 A b	0.805 ± 0.385 A b	0.808 ± 0.367 A b	0.888 ± 0.392 A a
0.05 mg/L	0.810 ± 0.361 A b	0.763 ± 0.364 A b	0.766 ± 0.349 A b	0.861 ± 0.381 A a
0.5 mg/L	0.609 ± 0.270 B b	0.540 ± 0.257 B b	0.545 ± 0.247 B b	0.693 ± 0.305 B a
5 mg/L	0.376 ± 0.165 C b	0.313 ± 0.147 C b	0.317 ± 0.142 C b	0.467 ± 0.203 C a
50 mg/L	0.300 ± 0.132 C b	0.245 ± 0.147 C b	0.248 ± 0.111 C b	0.384 ± 0.167 C a
No diet	0.293 ± 0.135 C b	0.238 ± 0.117 C b	0.241 ± 0.112 C b	0.375 ± 0.171 C a

(b) Nymphs

Treatment	Probability of survival Nymphs (mean ± Std. error)
No Pesticide	0.971 ± 0.407 A
0.005 mg/L	0.974 ± 0.406 A
0.05 mg/L	0.929 ± 0.377 B
0.5 mg/L	0.293 ± 0.119 C
5 mg/L	0.165 ± 0.068 D
50 mg/L	0.186 ± 0.077 CD
No diet	0.241 ± 0.114 CD

Table 3.4 Adults. Estimated mean probability of survival of aphid adults (± SEM). LS means were estimated using a Bayesian Generalized Linear Mixed Model and LS mean separation was accomplished using Tukey HSD. Means followed by the same uppercase letters are not significantly different from one another for the different pesticide treatments (Tukey HSD α= 0.05)

Treatment	Probability of survival Adults (mean ± Std. error)	
	Tobacco	Non-tobacco
No Pesticide	0.742 0.614 AB a	0.318 0.265 AB b
Imid 0.005 mg/L	0.864 0.769 A a	0.507 0.458 A b
Imid 0.05 mg/L	0.850 0.753 AB a	0.479 0.430 AB b
Imid 0.5 mg/L	0.757 0.667 ABC a	0.334 0.300 ABC b
Imid 1 mg/L	0.589 0.511 ABCDE a	0.188 0.165 ABCDE b
Imid 5 mg/L	0.440 0.373 CDE a	0.113 0.097 CDE b
Imid 10 mg/L	0.547 0.473 ABCDE a	0.164 0.143 ABCDE b
Imid 50 mg/L	0.428 0.360 DE a	0.108 0.092 DE b
Imid 100 mg/L	0.323 0.282 DE a	0.072 0.063 DE b
Nic 0.1 %	0.407 0.356 CDE a	0.100 0.088 CDE b
Nic 0.5 %	0.576 0.486 BCD a	0.180 0.153 BCD b
Nic 1 %	0.219 0.193 E a	0.043 0.038 E b
Nic 2 %	0.038 0.033 F a	0.006 0.006 F b
Nic 3 %	0.011 0.013 F a	0.002 0.002 F b
Nic 5 %	0.004 0.005 F a	0.001 0.001 F b
Nic 7 %	0.004 0.005 F a	0.001 0.001 F b
Nic 10 %	0.004 0.005 F a	0.001 0.001 F b

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Table 3.5 Nymphs. Estimated mean probability of survival of aphid nymphs (± SEM). LS means were estimated using a Bayesian Generalized Linear Mixed Model and LS mean separation was accomplished using Tukey HSD. Means followed by the same uppercase letters are not significantly different from one another for the different pesticide treatments (Tukey HSD $\alpha = 0.05$)

Treatment	Probability of survival Adults (mean ± Std. error)	
	Tobacco	Non-tobacco
No Pesticide	0.784 0.597 B a	0.394 0.315 B b
Imid 0.005 mg/L	0.965 0.814 A a	0.831 0.741 A b
Imid 0.05 mg/L	0.745 0.610 B a	0.343 0.299 B b
Imid 0.5 mg/L	0.266 0.219 C a	0.061 0.053 C b
Imid 1 mg/L	0.240 0.206 C a	0.053 0.048 C b
Imid 5 mg/L	0.222 0.178 C a	0.048 0.041 C b
Imid 10 mg/L	0.163 0.143 C a	0.034 0.031 C b
Imid 50 mg/L	0.198 0.161 C a	0.042 0.036 C b
Imid 100 mg/L	0.200 0.176 C a	0.043 0.040 C b
Nic 0.1 %	0.125 0.107 C a	0.025 0.022 C b
Nic 0.5 %	0.230 0.187 C a	0.050 0.043 C b
Nic 1 %	0.133 0.145 C a	0.027 0.030 C b
Nic 2 %	0.093 0.106 C a	0.018 0.021 C b
Nic 3 %	0.211 0.337 C a	0.046 0.073 C b
Nic 5 %	0.290 0.480 ABC a	0.068 0.113 ABC b
Nic 7 %	0.331 0.565 ABC a	0.081 0.139 ABC b
Nic 10 %	0.555 1.039 ABC a	0.183 0.342 ABC b

3.7 Figures

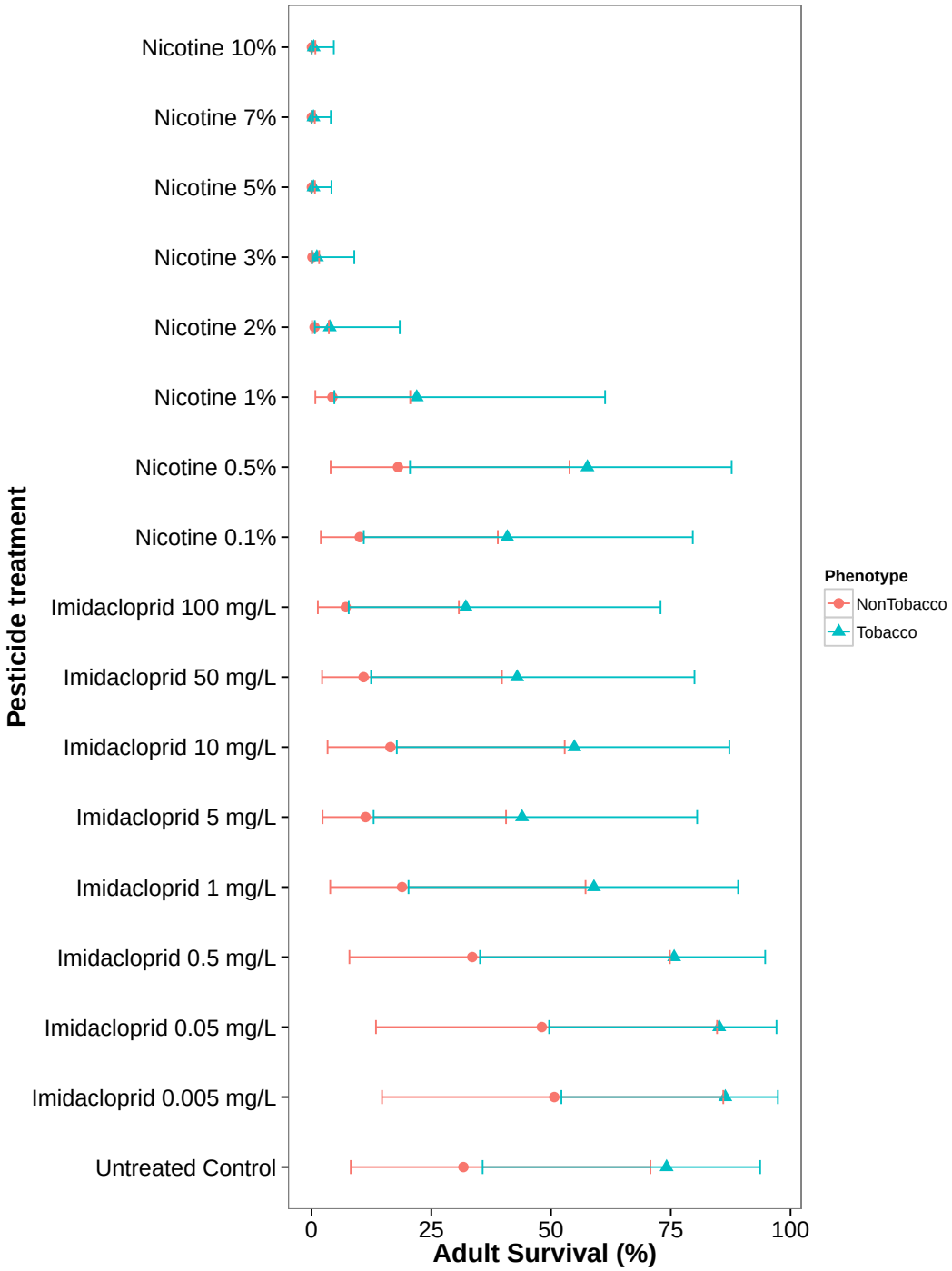

Figure 3.1 Relationship between insecticide treatment and the survival of aphid nymphs. Lines represents a 95% Bayesian credible interval. Red dots represent non-tobacco clones and blue dots represent tobacco clones.

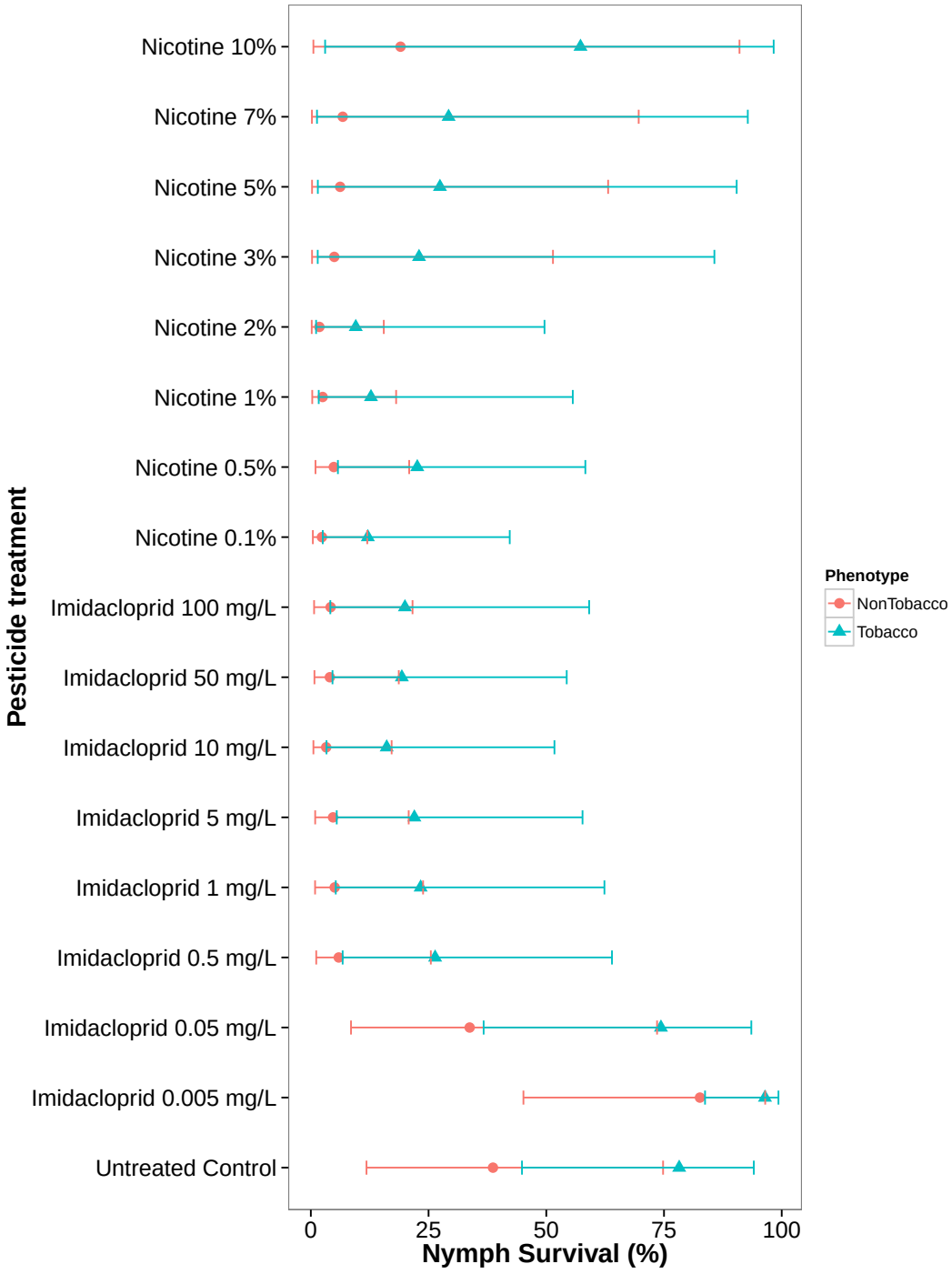

Figure 3.2 Relationship between insecticide treatment and the survival of aphid nymphs. Lines represents a 95% Bayesian credible interval. Red dots represent non-tobacco clones and blue dots represent tobacco clones.

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APPENDICES

Appendix A

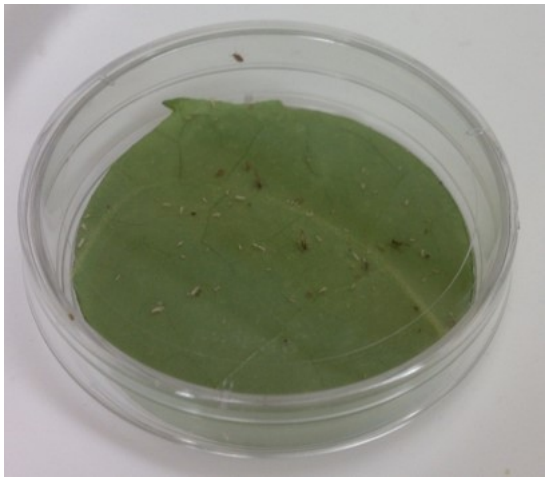
Methodology explained

A.1 Imidacloprid longevity

(a) Tobacco being treated in the greenhouses. Trays with two month old seedlings are treated two weeks before transplant to allow for the uptake of the pesticide. © Hannah Burrack

(b) Field example of an infested plant. Numbers are clearly above the current threshold in North Carolina of 50 or more aphids on upper leaves.© Hannah Burrack

Figure A.1 Examples of tobacco being treated in the greenhouses and a tobacco plant infested with aphids.

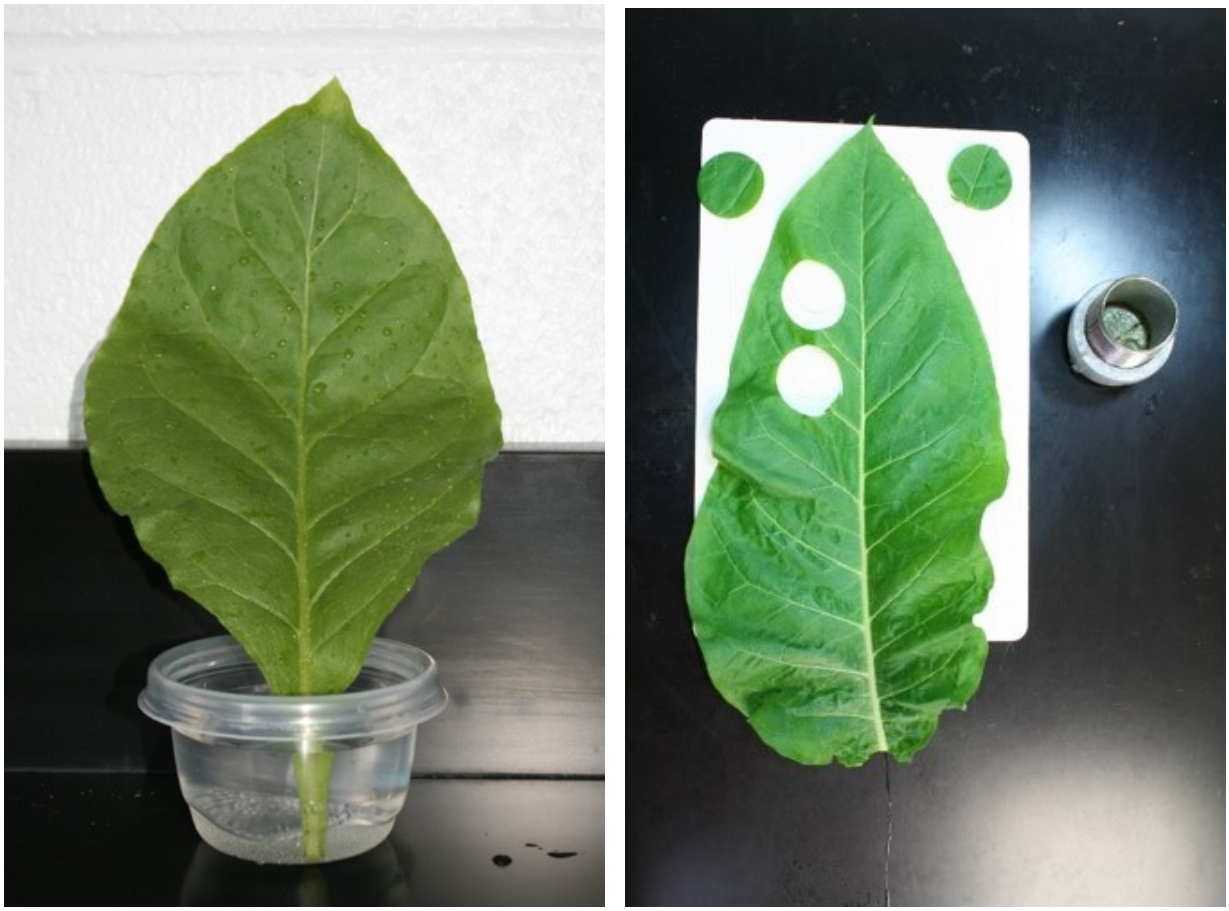


(a) Container used for bioassays in 2011. Petri dishes were layered with wet filter paper with leaf disc resting on top. © H.Alejandro Merchán

(b) Container used for bioassays in 2012. Containers we layered with 1% agar with leaf disc resting on top. © H.Alejandro Merchán

Figure A.2 Examples of the containers used for bioassays.

A.2 Aphid establishment

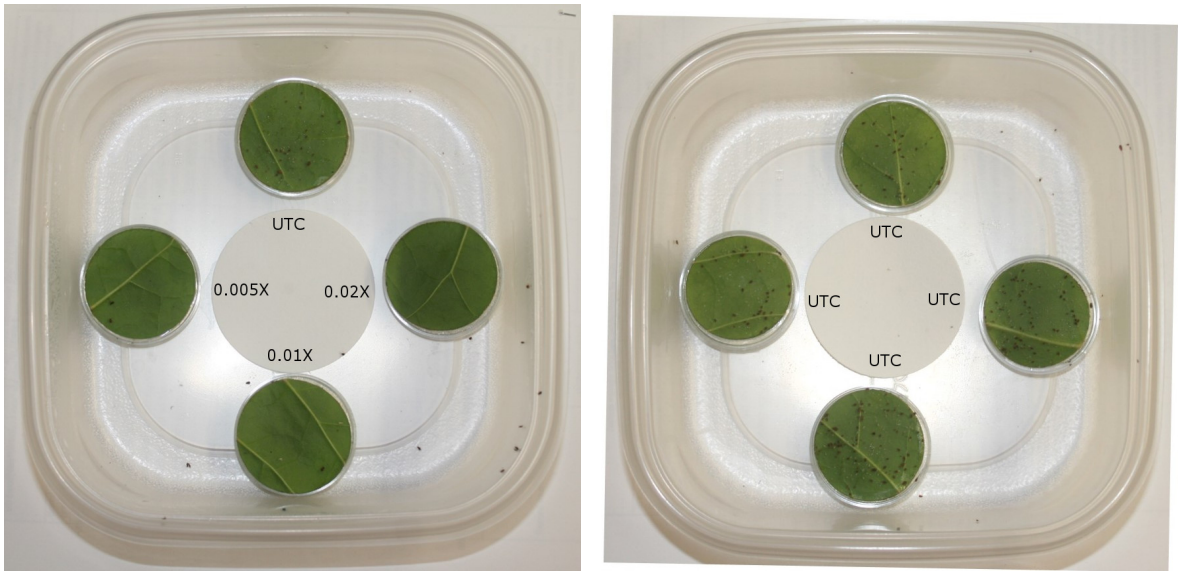


(a) Leaves were dipped in different pesticides solutions or in distilled water for 24h. © H.Alejandro Merchán

(b) After allowing the uptake of the pesticide, leaves discs were cut and used for the experiments. © H.Alejandro Merchán

Figure A.3 Method of exposure to the pesticide solution and subsequent extraction of leave discs.

Figure A.4 Cages were constructed cutting the bottom half of Falcon® tubes. Nylon netting was glued to the bottom and the lid was cut to allow the leaf disc to be put on top. The right lid has an extra Parafilm® layer to prevent contact, while the left lid does not have it.



(a) Example of a choice cage. © H.Alejandro Merchán **(b)** Example of a no choice cage. © H.Alejandro Merchán

Figure A.5 Cages used for choice (left) and no choice (right) experiments. 20 aphids were placed on the filter paper and counts were done 72h later. (UTC = Untreated control)

Figure A.6 Example of an experiment setup. Cages were constructed cutting about 5 cm from Falcon® tubes, sealing one end with a metal washer and organdy mesh. Each cage was glued to one arm of a bent hair clip while another metal washer with a round piece of foam glued to it was affixed to the other arm. Extra foam was also glued to the open end of the cage to produce a tighter seal and to avoid damage to the leaf.

Appendix B

Example of collection points at Oxford Tobacco Research Station

Figure B.1 Location of different collection points in the Oxford Tobacco Research Station in North Carolina. When possible, collection points were separated by more than 50 m, but smaller distances were allowed if needed. Not all 12 locations points are shown on this map.

Appendix C

Worked example on model selection based on AIC

The following methodology is based on the book *Model Selection and Multimodal Inference: A Practical Information-Theoretic Approach* (Burnham and Anderson, 2002). In summary, we fitted the full model using all variables that we measured and interactions between them. After that, we fitted models that were simplified versions of the full model, but made biological sense. For example, the full model had survival estimates for each of the 48 aphids lines that we collected, but a simplified model had four estimates because we joined all lines coming from the same research station (location). We did the same with color morph, phenotype, and insecticide material. We also included a null model that consisted only of an intercept and it serves as a control to make sure any effect we measure is really significant. After fitting all the models, we ranked them based on the Akaike Information Criterion (AIC) and chose the one with the lowest value. There are several packages in the R statistical software that perform the ranking, but we chose the "MuMIn" v. 1.15.1 (Barton, 2014) because we can use it to rank the Bayesian models we produced. The estimates from the "best" model were then used for the analysis and discussion.

In the following pages we present the tables summarizing all the models we ran for each of the experiments and the one on top of each table was selected as the "best" for that particular experiment.

Table C.1 Ranking of the different models tested for tolerance to imidacloprid in field populations. The ranking was done based on the Akaike Information Criterion (AIC). The first model on the list was used for data analysis as it provides the best fit to the data. **Population**: each of the 48 sampled lines. **Treatment**: insecticide concentrations. **Location**: The four research stations where we sampled the populations (Oxford, Rocky Mount, Kinston and Clayton). **Color**: the color morph of the population (green or red). Combinations of two parameters in the table represent interactions if those parameters in the model.

(a) Fecundity

	(Intercept)	Population	Treatment	Population:Treatment	Location1	Location1:Treatment	Color	Color:Treatment	family	df	logLik	AIC	delta	weight
5	2.73									3.00	-1239.27	2484.55	0.00	0.64
11	2.66						+			4.00	-1239.43	2486.86	2.31	0.20
3	3.07		+							5.00	-1239.26	2488.53	3.98	0.09
10	3.00		+				+			6.00	-1239.23	2490.46	5.91	0.03
4	2.81	+								6.00	-1239.30	2490.60	6.05	0.03
9	2.82		+				+	+		8.00	-1238.90	2493.80	9.26	0.01
2	3.14	+	+							8.00	-1239.81	2495.62	11.07	0.00
1	3.09	+	+	+						14.00	-1239.98	2507.97	23.42	0.00
8	2.50				+					50.00	-1236.95	2573.90	89.35	0.00
7	2.83		+		+					52.00	-1237.19	2578.39	93.84	0.00
6	2.82		+		+	+				146.00	-1236.96	2765.92	281.38	0.00

(b) Nymph survival

	(Intercept)	Population	Treatment	Population:Treatment	Location1	Location1:Treatment	Color	Color:Treatment	df	logLik	AICc	delta	weight
1	-0.02	+	+	+					14	-2744.0	5517.0	0.0	1.00
2	0.62	+	+						8	-2756.2	5528.7	11.6	0.00
3	1.06		+						5	-2782.5	5575.1	58.1	0.00
10	0.83		+				+		6	-2782.7	5577.6	60.6	0.00
9	0.56		+				+	+	8	-2781.9	5580.1	63.1	0.00
7	1.02		+		+				52	-2742.8	5604.1	87.1	0.00
6	1.11		+		+	+			146	-2712.6	5867.9	350.9	0.00
4	-1.55	+							6	-2988.0	5988.2	471.2	0.00
5	-1.14								3	-3004.0	6014.0	497.0	0.00
11	-1.36						+		4	-3003.6	6015.2	498.2	0.00
8	-1.32				+				50	-2978.6	6070.6	553.6	0.00

Table C.2 Ranking of the different models tested for tolerance using leaf dip analysis. The ranking was done based on the Akaike Information Criterion (AIC). The first model on the list was used for data analysis as it provides the best fit to the data. **Population**: each of the 16 matrilines. **Treatment**: insecticide concentrations. **Location**: The four research stations where we sampled the populations (Oxford, Rocky Mount, Kinston and Clayton). **Color**: the color morph of the population (green or red). Combinations of two parameters in the table represent interactions if those parameters in the model.

(a) Adult survival

	(Intercept)	Population	Treatment	Population:Treatment	Location	Location:Treatment	Color	Color:Treatment	family	df	logLik	AICc	delta	weight
3	0.75		+							11.00	-2103.44	4229.36	0.00	0.62
11	0.63		+				+			12.00	-2103.46	4231.48	2.12	0.22
7	0.64		+		+					14.00	-2102.15	4233.06	3.71	0.10
2	0.58	+	+							26.00	-2089.68	4233.94	4.59	0.06
10	0.43		+				+	+		20.00	-2103.36	4248.25	18.89	0.00
6	0.38		+		+	+				38.00	-2100.05	4281.68	52.33	0.00
1	0.63	+	+	+						146.00	-2075.50	4544.47	315.11	0.00
5	-1.03									3.00	-2303.34	4612.72	383.36	0.00
9	-1.14						+			4.00	-2303.77	4615.61	386.26	0.00
8	-1.11				+					6.00	-2303.64	4619.44	390.08	0.00
4	-1.15	+								18.00	-2297.64	4632.52	403.16	0.00

(b) Nymph survival

	(Intercept)	Population	Treatment	Population:Treatment	Location	Location:Treatment	Color	Color:Treatment	family	df	logLik	AICc	delta	weight
11	3.10		+				+			12.00	-834.88	1694.32	0.00	0.52
3	3.26		+							11.00	-836.03	1694.53	0.22	0.46
7	3.29		+		+					14.00	-836.18	1701.13	6.81	0.02
10	2.78		+				+	+		20.00	-832.41	1706.35	12.03	0.00
2	3.32	+	+							26.00	-834.48	1723.55	29.23	0.00
6	3.07		+		+	+				38.00	-834.36	1750.31	56.00	0.00
1	3.22	+	+	+						146.00	-826.66	2047.05	352.73	0.00
5	0.84									3.00	-1119.29	2244.62	550.31	0.00
9	0.96						+			4.00	-1119.69	2247.45	553.13	0.00
8	0.87				+					6.00	-1119.36	2250.88	556.56	0.00
4	1.12	+								18.00	-1118.51	2274.26	579.94	0.00

Table C.3 Ranking of the different models tested for tolerance using artificial diet. The ranking was done based on the Akaike Information Criterion (AIC). The first model on the list was used for data analysis as it provides the best fit to the data. **Population**: each of the 6 matrilines. **Treatment**: insecticide concentrations. **Location**: The four research stations where we sampled the populations (Oxford, Rocky Mount, Kinston and Clayton). Combinations of two parameters in the table represent interactions if those parameters in the model.

(a) Adult survival

	(Intercept)	Population	Treatment	Population:Treatment	Location	Location:Treatment	family	df	logLik	AICc	delta	weight
7	1.79		+		+			12.00	-2988.72	6001.96	0.00	0.57
3	1.85		+					9.00	-2992.41	6003.11	1.15	0.32
2	1.56	+	+					15.00	-2987.16	6005.13	3.17	0.12
6	1.52		+		+	+		30.00	-2987.39	6038.01	36.04	0.00
1	1.37	+	+	+				51.00	-2985.79	6083.12	81.16	0.00
8	0.44				+			6.00	-3083.48	6179.09	177.13	0.00
5	0.49							3.00	-3086.68	6179.40	177.44	0.00
4	0.24	+						9.00	-3082.62	6183.53	181.57	0.00

(b) Nymph survival

	(Intercept)	Population	Treatment	Population:Treatment	Location	Location:Treatment	family	df	logLik	AICc	delta	weight
3	-1.14		+					9.00	-1869.28	3756.87	0.00	0.86
7	-1.10		+		+			12.00	-1868.18	3760.88	4.02	0.12
2	-1.50	+	+					15.00	-1866.47	3763.76	6.89	0.03
6	-1.14		+		+	+		30.00	-1870.21	3803.66	46.80	0.00
1	-1.35	+	+	+				51.00	-1867.35	3846.28	89.41	0.00
5	0.86							3.00	-2159.47	4324.99	568.12	0.00
8	0.95				+			6.00	-2157.17	4326.48	569.61	0.00
4	0.37	+						9.00	-2157.23	4332.76	575.89	0.00

Table C.4 Ranking of the different models tested for tolerance of imidacloprid and nicotine using artificial diet. The ranking was done based on the Akaike Information Criterion (AIC). The first model on the list was used for data analysis as it provides the best fit to the data. **Population**: each of the 7 sampled matrilines. **Treatment**: insecticide concentrations. **Material**: chemical used in the artifical diet (nicotine, imidacloprid or none). **Phenotype**: the feeding habit of the line (tobacco and non-tobacco). Combinations of two parameters in the table represent interactions if those parameters in the model.

(a) Adult survival

	(Intercept)	Population	Treatment	Population:Treatment	Material	Material:Population	Phenotype	Phenotype:Treatment	Material:Phenotype	family	df	logLik	AICc	delta	weight
6	-0.77		+				+				20.00	-1811.35	3664.95	0.00	0.95
2	-0.84	+	+								24.00	-1809.72	3670.70	5.75	0.05
5	-1.26		+				+	+			36.00	-1809.65	3698.77	33.82	0.00
9	0.43		+								19.00	-1831.73	3703.48	38.53	0.00
8	-0.51				+		+				6.00	-1889.89	3792.00	127.06	0.00
7	-1.09				+		+		+		8.00	-1887.89	3792.16	127.21	0.00
4	-0.61	+			+						10.00	-1888.09	3796.76	131.81	0.00
3	-0.87	+			+	+					20.00	-1883.65	3809.54	144.59	0.00
10	0.52				+						5.00	-1904.28	3818.71	153.76	0.00
1	-0.99	+	+	+							104.00	-1804.68	3892.93	227.98	0.00
12	-2.00						+				4.00	-1966.93	3941.96	277.01	0.00
11	-2.09	+									8.00	-1965.61	3947.59	282.64	0.00
13	-0.80										3.00	-1991.42	3988.90	323.95	0.00

(b) Nymph survival

	(Intercept)	Population	Treatment	Population:Treatment	Material	Material:Population	Phenotype	Phenotype:Treatment	Material:Phenotype	family	df	logLik	AICc	delta	weight
6	-0.49		+				+				20.00	-857.44	1757.14	0.00	0.96
2	0.07	+	+								24.00	-856.16	1763.58	6.44	0.04
9	0.81		+								19.00	-863.87	1767.78	10.64	0.00
7	-1.11				+		+		+		8.00	-882.10	1780.58	23.44	0.00
8	-0.44				+		+				6.00	-884.18	1780.58	23.44	0.00
5	-1.17		+				+	+			36.00	-851.92	1783.33	26.19	0.00
4	0.07	+			+						10.00	-883.39	1787.36	30.22	0.00
10	0.76				+						5.00	-893.01	1796.17	39.04	0.00
3	-0.19	+			+	+					20.00	-882.23	1806.71	49.57	0.00
12	-1.79						+				4.00	-911.43	1830.96	73.82	0.00
11	-1.33	+									8.00	-911.01	1838.39	81.25	0.00
13	-0.50										3.00	-921.83	1849.72	92.58	0.00
1	-0.20	+	+	+							104.00	-850.55	1984.94	227.80	0.00