A trophic chain as indicator of agricultural practice change in maize

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A trophic chain as indicator of agricultural practice change in maize

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Cover: Jordi Ardanuy. Drawing based on a photograph by B. Lumbierres.

This PhD thesis has been carried out at the Laboratory of Entomology, Department of Crop and Forest Sciences, University of Lleida, Spain. The author was funded by a PhD FPU schlolarship from Ministerio de Educación. The research was funded by Ministerio de Economía y Competitividad (Project AGL2011-23996).

Agraïments

Aquest és l'espai per agrair a totes les persones que m'han acompanyat durant els darrers anys i que m'han donat l'energia per a finalitzar aquest viatge. Aquest camí ha estat tot un aprenentatge de vida.

En primer lloc voldria agrair al meu director Ramon Albajes per la seva confiança i recolzament, i sobretot per obrir-me una porta a l'apassionant món de les relacions planta-insecte.

A en Joan, la Matilde, la Carmen, la Eva i en Xavier que m'heu donat suport tant a Lleida com en la distància. I a la resta d'integrants del grup d'Entomologia de la UdL que durant tant temps heu sigut la meva família científica: Belén, Dolors, Mònica, Jesús, Marcela, Marta, Meritxell, Mª José, Miquel, Rosa, Tere i Aurora... i als molts estudiants que ens han acompanyat. Gràcies.

I'd like to thank Ted Turlings for welcoming me in his lab, it was a great experience... Merci! To FARCE and E-vol lab members Angela, Daniel, Hao, JinWon, Mickaël, Max, Geoffrey, Thomas, Gaylord, Javier, Neil, Diane, Ilham, Gaëtan and Betty thanks for your help: it has been a real pleasure meeting you.

A Victoria Pastor por ser mi madrina científica, compartir cafés y ayudarme a aclarar las ideas. Tu energía positiva es contagiosa, qué gusto trabajar contigo!

Als meus germans de viatge des de l'inici Filipe i Marina: no sé si hauria pogut acabar aquesta tesi sense vosaltres però del que estic segura és... de que no hauria estat tan divertit. I als companys que he trobat pel camí Alberto, Diego, Miguel i Byrappa. Junts no hem trobat problemes sinó contratemps, i els hem superat junts amb alegria (♫ "the bright side of life"). Gràcies per tot.

A la Maison des Liserons, ma colocation du bonheur en Suisse. Merci pour les bonnes moments a Fanny, Michael, Marie, Nico et Lulu. Gracias Fanny por los largos paseos, los baños y por estar siempre a mi lado. Una suerte haberte conocido. Thanks also to my lovely friends "les mafiosi" (Vero et Marco), Filip, Daniela and Alfonso, you made my life significantly better.

A la Spanish mafia de Neuchâtel... Victoria, Raquel, Ruben, Xoaquín, Miguel y Silvia, por todas las fondues, raclettes, migas, pulpos y queimadas que compartimos. Qué placer sentirse uno en casa lejos de ella, qué fácil es arreglar la ciencia y la vida con vosotros.

Als amics de vida lleidatana: Álex, Xavi, Paula, Irene, Eva, César, Pitter, Salomé, Laura, Pere, María, Diana, Alberto, Marcela, Jorge, Addy, Daniela, Albert, Jordi, Marta... Per els bailoteos, confesions, copetes, ploreres i riures... i canvis d'etapa!

A les amigues de vida, que m'entenen com ningú i m'ajuden a ser millor: Núria, Marina, Ari, Irene i Eva. A Paula, por estos cinco años de tesis de fondo: por las filosofadas, tuppers, idas y venidas. Y por tu amistad. Lo logramos! A la Marina, per la teva complicitat i per fer-me veure cada got que em trobo més ple. Sou les millors.

A la meva família, que m'han donat suport incondicional tot aquest temps d'alts i baixos que suposa una tesi doctoral. A la meva mare amb la que puc parlar de tot, al meu pare que em fa veure que totes les coses són més senzilles del que semblen, i a en Jordi que em permet dubtar de tot. Semblava que aquest moment no arribaria mai. Però aqui està. Fet.

Moltes gràcies.

In every walk with nature one receives far more than he seeks.

John Muir

CONTENTS

SUMMARY

Biological control of insect pests through the action of predators and parasitoids is a key component of natural pest management that provides invaluable economic benefits to humans particularly in food production. Invertebrate community composition and dynamics in agro-ecosystems is largely shaped by bottom-up effects which transfer from the plant resource across herbivores to the associated natural enemies of the herbivores. It is not surprising then, that one of the main concerns associated to the change in agricultural practices and in particular of the use of new varieties in agriculture - e.g genetically modified plants - is their potential to adversely affect the community of invertebrates and the ecosystem services they deliver. Given the impossibility to measure all ecological interactions between a plant and its associated insect species, we focused on species that are keystone in the ecological functions herbivory - the leafhopper *Zyginidia scutellaris* -and predation - the complex *Orius* spp.- in maize as indicators of agricultural change.

The first part of the thesis focuses on building knowledge on insect-landscape and plant-insect interactions for our study system to validate their use as indicator species in maize, for that we addressed (i) the occurrence and variation in the populations of the leafhopper *Z. scutellaris* and *Orius* spp. in NE Spain and how they are influenced by landscape characteristics (Chapter 1) and (ii) the specificity in the response of predator *Orius* spp. towards herbivore-induced volatiles (HIPVs) triggered by different herbivore species in maize (Chapter 2). The second part of the thesis deals directly with the evaluation of risk assessment of newly emerging biofortified crops to insects: (i) we addressed the main difficulties in problem formulation, and proposed an ecological approach for the evaluation of Carolight maize-insect interactions (Chapter 3), and lastly (ii) we characterized chemical profiles of Carolight maize and its comparator and evaluated their equivalence in terms of plant-insect interactions in contrasting nitrogen conditions (Chapter 4).

The main outcomes of this PhD thesis are that (1) maize leafhopper *Z. scutellaris* is sensitive to detect differences in plant chemistry at the field scale and may be used as indicator of changes produced by modification of plant metabolism, (2) feeding by *Z. scutellaris* results in the emission of large amount of HIPVs comparable to feeding by a chewing herbivore, and it suppresses the accumulation of the defense hormones JA-Ile and SA, (3) the recruitment of *Orius* spp. by maize fields might be chemically mediated by the release of HIPVs resulting of leafhopper feeding, given that *Orius* spp. have a robust attraction towards *Z. scutellaris* infested plants, (4) the strong positive association between *Orius* spp. and the herbivore *Z. scutellaris* across the three regions reflects a consistent density-dependent process of predator aggregation towards prey (5) temporal, regional and landscape variation greatly influence maize leafhopper and *Orius* spp. populations in NE Spain. All these results provide insights when understanding the plant-herbivore-predator relationships to predict consequences of agricultural management modification on the invertebrate food web and natural pest control.

RESUM

El control biològic és un component clau del control integrat de plagues, que proporciona grans beneficis a la producció alimentaria. La composició i dinàmica de la comunitat d'invertebrats en agroecosistemes es veu fortament influïda per forces anomenades "bottom-up", que es transfereixen del productor primari, la planta, als herbívors i als seus enemics naturals. No sorprèn doncs que una de les majors preocupacions associades al canvi de les pràctiques agrícoles, i en particular a la utilització de noves varietats - entre elles les modificades genèticament - sigui el seu potencial de afectar negativament a la comunitat d'invertebrats i els serveis ecosistèmics que en deriven. Donada la impossibilitat de mesurar totes les interaccions ecològiques entre les plantes i les espècies d'insectes associades, la tesi s'ha focalitzat en aquelles espècies clau en les funcions d'herbivoria - el cicadèlid *Zyginidia scutellaris* - i depredació - el complex *Orius* spp.- en panís com a indicadores de canvis en el maneig agrícola.

La primera part de la tesi està orientada a la creació de coneixement del sistema panís-cicadèlid- *Orius* spp. en relació a les interaccions insecte-paisatge i planta-insecte per tal de validar l'ús d'aquestes espècies com a indicadores en panís. Hem volgut clarificar (i) la presència i variació de es poblacions de *Z. scutellaris* i *Orius* spp. al NE d'Espanya. i com es veuen influïdes per les característiques del paisatge agrícola (Capítol 1), i (ii) la especificitat de la resposta de *Orius* spp. en relació a volàtils induïts per la herbivoria (HIPVs) d'insectes amb estratègies alimentàries diferents (Capítol 2). La segona part de la tesi està dirigida a l'avaluació de riscs de noves varietats biofortificades en relació a insectes: (i) hem avaluat les dificultats principals derivades de la formulació del problema, i hem proposat un enfocament ecològic per a l'avaluació de les interaccions entre la nova varietat de panís Carolight i els insectes (Capítol 3), i finalment (ii) hem caracteritzat els perfils químics de Carolight i la seva varietat isogènica, i hem avaluat la seva equivalència en termes de les relacions planta-insecte (Capítol 4).

Les principals conclusions d'aquesta tesi doctoral són que (1) el cicàdelid *Z. scutellaris* és sensible per a detectar diferències en la química de la planta al camp i que pot ser utilitzat com a indicador dels canvis produits per una alteració del metabolisme de la planta, (2) els danys en la planta de panís produïts per l'alimentació del cicadèlid desencadenen la emissió d'una gran quantitat de HIPVs comparable a la emesa per danys produïts per herbívors mastegadors, i suprimeixen l'acummulació de les hormones de defensa JA-Ile i SA, (3) la colonització dels camps de panís per *Orius* ssp. pot ser conseqüència de la orientació cap a HIPVs originats per l'alimentació de Z. scutellaris, ja que *Orius* ssp. presenten una atracció robusta cap a plantes atacades per *Z. scutellaris*, (4) la forta relació entre *Z. scutellaris* i *Orius* spp. en les tres regions d'estudi reflexa un procés densodependent consistent d'agregació del depredador cap a la presa, (5) la variació temporal, regional i paisatjistica influeixen fortament les poblacions del cicàdelid *Z. scutellaris* i d' *Orius* ssp.. Aquests resultats obren via a entendre les relacions planta-insecte en panís, i per a la predicció de les conseqüències de la modificació de les practiques agrícoles en la xarxa tròfica i en el control biològic de plagues.

RESUMEN

El control biológico es un componente clave del control integrado de plagas, que proporciona grandes beneficios a la producción alimentaria. La composición y dinámica de la comunidad de invertebrados en agroecosistemas se ve fuertemente influida por fuerzas llamadas "bottom-up", que se transfieren del productor primario, la planta, los herbívoros y sus enemigos naturales. No es sorprendente entonces que una de las mayores preocupaciones asociadas al cambio de las prácticas agrícolas, y en particular a la utilización de nuevas variedades - entre ellas las modificadas genéticamente - sea su potencial de afectar negativamente a la comunidad de invertebrados y los servicios ecosistémicos que proporcionan. Dada la imposibilidad de medir todas las interacciones ecológicas entre las plantas y sus especies de insectos asociados, la tesis se ha focalizado en aquellas especies clave en las funciones de herbivoría - el cicadélido *Zyginidia scutellaris* - y depredación - el complejo *Orius* spp.- en maíz como indicadoras de cambios en el manejo agrícola.

La primera parte de la tesis está orientada a la ampliación en el conocimiento del sistema maíz-cicadélido- *Orius* spp. en relación a las interacciones insecto-paisaje y planta-insecto para validar el uso de estas especies como indicadoras en maíz. Hemos querido clarificar (i) la presencia y variación de se poblaciones de *Z. scutellaris* y *Orius* spp. al NE de España. y cómo se ven influidas por las características del paisaje agrícola (Capítulo 1), y (ii) la especificidad de la respuesta de *Orius* spp. en relación a volátiles inducidos por la herbivoría (HIPVs) de insectos con estrategias alimentarias diferentes (Capítulo 2). La segunda parte de la tesis está dirigida a la evaluación de riesgos de nuevas variedades biofortificadas en relación a insectos: (i) hemos evaluado las dificultades principales derivadas de la formulación del problema, y hemos propuesto un enfoque ecológico para la evaluación de las interacciones entre la nueva variedad de maíz Carolight y los insectos (Capítulo 3), y finalmente (ii) hemos caracterizado los perfiles químicos de Carolight y su variedad isogénica, y hemos evaluado su equivalencia en términos de las relaciones planta-insecto (Capítulo 4).

Las principales conclusiones de esta tesis doctoral son (1) el cicadélido *Z. scutellaris* es un taxón sensible para detectar diferencias en la química de la planta en el campo y que puede ser utilizado como indicador de los cambios producidos por una alteración del metabolismo de la planta, (2) los daños en la planta de maíz producidos por la alimentación del cicadélido desencadenan la emisión de HIPVs comparable a la emitida por herbívoros masticadores, y suprimen la acumulación de las hormonas de defensa JA-Ile y SA, (3) la colonización de los campos de maíz por *Orius* ssp. puede ser consecuencia de la orientación hacia HIPVs originados por la alimentación de *Z. scutellaris* en maíz, ya que *Orius* ssp. presentan una atracción robusta hacia plantas atacadas por este cicadélido, (4) la fuerte relación entre *Z. scutellaris* y *Orius* spp. en las tres regiones de estudio refleja un proceso densodependiente de agregación del depredador hacia la presa, (5) la variación temporal, regional y paisajística influyen fuertemente las poblaciones del cicadélido *Z. scutellaris* y de *Orius* ssp. Estos resultados abren vía en el conocimiento de las relaciones planta-insecto en maíz, y són una base para la predicción de las consecuencias de la modificación de las prácticas agrícolas en la red trófica y en el control biológico de plagas.

GENERAL INTRODUCTION

Background

Biological control of insect pests through the action of predators and parasitoids is a key component of natural pest management (Kogan, 1998) that provides invaluable economic benefits to humans particularly in food production (Losey & Vaughan, 2006). However, concerns have arisen about the derioration of biological control function as a result of agricultural intensification, the main driver of agricultural change in the last century (Matson et al., 1997). Local scale intesification is driven by farm management practices like shortened rotations, high agrochemical inputs and smaller number of crop species. In turn, invertebrate community composition and dynamics in agro-ecosystems is largely shaped by bottom-up effects which transfer from the plant resources across herbivores to the associated natural enemies of the herbivores. Therefore, plant traits - as chemistry, physiology, and morphology - affect insect community members, shape the interactions between species and consequently greatly determine the composition of the associated food web (Bukovinszky et al., 2008; Van Der Putten et al., 2001). A growing body of evidence suggests that the landscape scale of agricultural intensification adds to the local effects of intensified farming practices (Matson et al., 1997; Tscharntke et al., 2005; Vasseur et al., 2013). It is not surprising then, that one of the main concerns associated to the change in agricultural practices is their potential to adversely affect the community of invertebrates and the ecosystem services they deliver.

The need for keystone indicator species

Given the impossibility to measure all ecological interactions between a plant and its associated insect species in all possible environmental conditions, it is vital to choose the adequate surrogate indicator species for testing the impacts of new agricultural practices both in laboratory and field conditions. Ideally, a relevant indicator species or complex of species (Dale and Beyeler, 2001; Heink and Kowarik, 2010) (i) is representative of a set of species or of an ecological function to protect; (ii) is exposed and sensitive to stresses on the system (e.g. specialists); (iii) responds to stress in a predictable manner; (iv) is easily identified and measured; (v) is abundant in the habitat to monitor and has low variability in response; and (vi) has a known response to natural disturbances, anthropogenic stresses, and changes over time. In the case of selecting species representative of the biological control function we propose selecting species that are keystone species in the ecological functions herbivory and predation in the focal crop (Poelman and Kessler, 2016), that are widely distributed, and that present high statistical power in realistic conditions (Albajes et al., 2013; Prasifka et al., 2008). To address the later conditions of species predictability it is vital to gain further knowledge on the biology and the ecology of the selected species; and to establish baseline variability through multi-year studies at both spatial scales relevant for the analysis of monitoring data: the field scale describing crop and cultivation practices in a region, and at the landscape scale describing regional land use and crop patterns.

Monitoring the impacts of agricultural change

Traditionally, ecological effects arising from changes in on-farm management at a regional scale (e.g. introduction of a new crop or expansion of an existing one, new agri-environmental measures) can then be evaluated by comparing trends of indicator species on treated and control fields before and after implementation of the treatment (a replicated before-after control-impact or BACI approach, e.g. Underwood and Chapman, 2003). However the main constraints in the environmental monitoring are (i) how to distinguish "unusual" changes from "usual" variability in an indicator, and (ii) the difficulty to attribute environmental changes to a particular cause (Sanvido et al., 2011). Baseline data are rarely available for most arthropod species and most times an estimate has to be made when the new management has been in place for a few years (Kleijn et al., 2006). On the other hand it might not be adequate to use the environmental state prior to the introduction of the new practice as a baseline e.g. GM variety or agri-environmental scheme (Sanvido et al., 2011), given that agricultural systems display considerable dynamics in time and space (Dunning et al., 1992; Matson et al., 1997; Tscharntke et al., 2005). Therefore to determine whether an observed change lies within the existing variability of a species or community, the magnitude and sources of the variability should be quantified as precisely as possible (Sanvido et al., 2011).

The model system

An analysis of the historical data of GM field trials assessed their statistical power to detect GM effects on maize arthropods and identified the most representative taxa (Albajes et al., 2013; J. Comas et al., 2013). The analysis highlighted the capacity of the leafhopper *Zyginidia scutellaris* (Herrich-Schäffer) (Hemiptera: Cicadellidae) and the complex of the anthocorid predator *Orius* spp. (Heteroptera: Anthocoridae) to detect differences between the GM maize and its non-GM comparator/s in meta-analysis and single field trials.

Zyginidia scutellaris is a widely distributed species in Europe and is considered a secondary pest of maize in Spain, France and Germany, though it is rarely of economic importance. This leafhopper feeds on the mesophyll of Poaceae producing damage in form of pale stripes on the leaves. *Z. scutellaris* colonizes maize stands at the beginning of the season, and in the

Mediterranean area it is the first herbivore to reach and establish on the aerial part of the plant in significant numbers (Comas et al., 2015). Population densities of *Z. scutellaris* in maize are often high and can exceed those of other taxa (Pons et al. 2005) and they perform an important role as herbivores in maize arthropod communities (Albajes et al., 2011).

Early colonization of maize plants by leafhoppers *Z. scutellaris* might also be a key feature for attracting and establishing on-plant generalist predators as *Orius* spp. on maize crops (Albajes et al., 2011). *Orius* spp. are one of the most abundant on-plant predators in arable crops in NE Spain (Albajes et al. 2003; de la Poza et al., 2005) and are regularly found on cereals, maize and alfalfa (Madeira et al., 2014; Pons et al., 2005b), as well as on weeds, depending on plant phenology and crop management. *Orius* spp. prey on small insects (e.g. leafhoppers, thrips, scales, aphids, psyllids, small caterpillars and the eggs of various insects) and mites (Lattin 1999) and are known to respond numerically by aggregation to locations with higher prey density (Albajes et al., 2011; Pons et al., 2005b; Tabic et al., 2010) which makes them ideal for biological control (Symondson et al., 2002).

THESIS OUTLINE

The first part of the thesis focuses on building knowledge on insect-landscape and plant-insect interactions for our study system to validate their use as indicator species in maize for that we addressed (i) their occurrence and variation in NE Spain and how they are influenced by landscape characteristics (Chapter 1) and (ii) the specificity in the response of predator *Orius* spp. towards herbivore-induced volatiles triggered by different herbivore species (Chapter 2). The second part of the thesis focuses directly on the evaluation of risk assessment of newly emerging biofortified GM crops to non target species: (i) we address the difficulties in problem formulation and propose an ecological approach for the evaluation of equivalence in novel maize-insect interactions (Chapter 3) and (ii) characterize chemical profiles of vitamin enriched maize and its near isogenic counterpart and evaluate their equivalence in terms of insect-plant relationships (Chapter 4).

In Chapter 1, we studied the relationships of *Z. scutellaris* and *Orius* spp. with the agricultural landscape in order to gather data on the baseline variation for these species to use in region-wide environmental monitoring schemes. The early detection of an increase/decrease in populations of biological control agents such as predators and parasitoids as a consequence of new agricultural practices (e.g. GM varieties or agro-environmental measures) is only possible through preparation of suitable monitoring plans (Field et al., 2007; Hails, 2002; Lindenmayer and Likens, 2010). For that, it is vital to assess beforehand the magnitude of leafhopper and *Orius spp*. population variability throughout the territory (site-to-site variation) that will be covered by the monitoring program for a number of years (year-to-year variation)

(EFSA, 2010) and how they might be influenced by co-occurring habitats at the landscape scale. The specific objectives of this study were:

- to establish the baseline abundances of *Z. scutellaris* and *Orius* spp. in maize and co-ocurring habitats in three agricultural regions in NE Spain.

-to verify whether relationship between *Z. scutellaris* and *Orius* spp. abundances in maize is maintained across regions and years.

- to determine the influence of compositional and configurational landscape metrics on *Z. scutellaris* and *Orius* spp. abundances.

In Chapter 2, we investigated whether the observations of early season increases in field populations of *Orius spp*. reflect a specific attraction to *Z. scutellaris*-induced maize volatiles, and how the responses of *Orius* predators to herbivore-induced volatiles (HIPVs) might be affected by previous experiences on plants infested by herbivorous prey. We analyzed the volatile profiles emitted by maize plants infested by the three herbivores with different feeding strategies, leafhopper *Z. scutellaris* (mesophyll feeder), lepidopteran *Spodoptera littoralis* (Boisduval) (chewer) and leafhopper *Dalbulus maidis* (Delong y Wolcott) (phloem feeder), and examined the innate and learned preferences of *Orius majusculus* towards volatiles from maize plants attacked by those three herbivores. The specific objectives of this study were:

- to establish the innate preferences of *Orius majusculus* towards maize plants attacked by three herbivores with different feeding strategies, the leafhopper *Z. scutellaris*, the leafhopper *D. maidis*, and the lepidopteran *S. littoralis*.

- to test the potential preference change in the case of previous prey experience on infested maize plants and the nature of this experience (rewarding/no-rewarding).

- to quantify the differences in the volatile profiles emitted by maize plants infested by the three herbivores with different feeding strategies.

Chapter 3 presents an overview of newly emerging biofortified GM crops, identifies the most relevant knowledge gaps to be addressed for problem formulation for Environmental Risk Assessment, and presents vitamin enhanced maize Carolight as a case study. Firstly, we synthesize which are the most common emerging traits and the basis for the current GM regulatory frameworks in the potential countries of adoption of Carolight. Then we present the current hypotheses of change in Carolight phenotype respective of the near isogenic counterpart that may occur due to the genetic modification. Lastly we propose an

Environmental Risk Assessment to evaluate Carolight impacts on insects that follows an ecological tiered approach through trophic relationships focusing on the leafhopper *Z. scutellaris* as the candidate herbivore indicator species of GM maize impacts on the arthropod food web. The specific objectives of this study were:

- to review the current regulatory frameworks in the potential countries of adoption of biofortified crops, mainly the African continent.

- to explore the hypothesis of change in Carolight plants and its potential impacts on herbivore populations.

- to review the family Cicadellidae as a potential indicators to detect differences between GM crops and their isogenic counterparts.

- to develop a tiered approach for Environmental Risk Assessment of Carolight maize through trophic relationships.

In Chapter 4, we investigated the hypothesis that Carolight is similar in terms of plant-NTA interactions to its non-transgenic near isogenic counterpart when compared in a controlled environment and under field conditions. In order to broaden the range of environments and to test the consistency of performance between Carolight and its near isogenic counterpart, we compared both genotypes under different substrate nitrogen availability regimes. The data presented serve as an exploratory scientific study into the use of plant-insect interactions for comparative evaluation of agricultural drivers on arthropod populations (nitrogen, genotypes). The specific objectives of the research were :

- to evaluate the abundance and dynamics of herbivores and natural enemies in Carolight and wild type plots in the field.

- to test the potential effects of both genotypes on a specialist herbivore performance and choice in the lab.

- to test the effects of genotype, nitrogen availability and *Z. scutellaris* feeding on maize plants and characterize the chemical profiles of plant leaves by means of volatile, hormone and metabolite profiling.

REFERENCES

Albajes, R., Lumbierres, B. & Pons, X. (2011). Two heteropteran predators in relation to weed management in herbicide-tolerant corn. Biological Control, 59, 30–36.

Albajes, R., Lumbierres, B., Pons, X. & Comas, J. (2013). Representative taxa in field trials for environmental risk assessment of genetically modified maize. Bulletin of Entomological research, 103, 724–33.

Bukovinszky, T., van Veen, F.J.F., Jongema, Y. & Dicke, M. (2008). Direct and Indirect Effects of Resource. Science, 319, 804–807.

Domínguez, P. C., Ortego, F., Hernández-Crespo, P., Farinós, G. P. et al. (2010). El maíz Bt en España: experiencia tras 12 años de cultivo. Phytoma España: La revista profesional de sanidad vegetal, 219, 64-70.

Comas, J., Lumbierres, B., Comas, C., Pons, X. & Albajes, R. (2015). Optimising the capacity of field trials to detect the effect of genetically modified maize on non-target organisms through longitudinal sampling. Annals of Applied Biology, 166, 183–195.

Comas, J., Lumbierres, B., Pons, X. & Albajes, R. (2013). Ex-ante determination of the capacity of field tests to detect effects of genetically modified corn on nontarget arthropods. Journal of Economic Entomology, 106, 1659–68.

Dale, V.H. & Beyeler, S.C. (2001). Challenges in the development and use of ecological indicators. Ecological Indicators, 1, 3–10.

Dunning, J. B., Danielson, B.J. & Pulliam, H.R. (1992). Ecological processes that affect populations in complex landscapes. Oikos, 65, 169–175.

EFSA Panel on Genetically Modified Organisms (2010). Scientific Opinion on Statistical considerations for the safety evaluation of GMOs. EFSA Journal, 8, 1–59.

Field, S. A., O'Connor, P.J., Tyre, A.J. & Possingham, H.P. (2007). Making monitoring meaningful. Austral Ecology, 32, 485–491.

Hails, R. S. (2002). Assessing the risks associated with new agricultural practices. Nature, 418, 685–688.

Heink, U. & Kowarik, I. (2010). What are indicators? On the definition of indicators in ecology and environmental planning. Ecological Indicators, 10, 584–593.

Kleijn, D., Baquero, R. A., Clough, Y., Díaz, M., De Esteban, J. et al. (2006). Mixed biodiversity benefits of agri-environment schemes in five European countries. Ecology Letters, 9, 243–254.

de la Poza, M., Pons, X., Farinós, G.P., López, C., Ortego, F., Eizaguirre, M., Castañera, P. & Albajes, R. (2005). Impact of farm-scale Bt maize on abundance of predatory arthropods in Spain. Crop Protection, 24, 677–684.

Lattin, J. D. (1999). Bionomics of the Anthocoridae. Annual Review of Entomology, 44, 207-231.

Lindenmayer, D.B. & Likens, G.E. (2010). The science and application of ecological monitoring. Biological Conservation, 143, 1317–1328.

Madeira, F., di Lascio, A., Carlino, P., Costantini, M.L., Rossi, L. & Pons, X. (2014). Stable carbon and nitrogen isotope signatures to determine predator dispersal between alfalfa and maize. Biological Control, 77, 66–75.

Matson, P.A., Parton, W.J., Power, A.G. & Swift, M.J. (1997). Agricultural intensification and ecosystem properties. Science, 277, 504–509.

Poelman, E.H. & Kessler, A. (2016). Keystone Herbivores and the Evolution of Plant Defenses. Trends in Plant Science, 21, 477–485.

Pons, X., Lumbierres, B., López, C. & Albajes, R. (2005a). Abundance of non-target pests in transgenic Bt-maize : A farm scale study. European Journal of Entomology, 102, 73–79.

Pons, X., Núñez, E., Lumbierres, B. & Albajes, R. (2005b). Epigeal aphidophagous predators and the role of alfalfa as a reservoir of aphid predators for arable crops. European Journal of Entomology, 102, 519–525.

Prasifka, J.R., Hellmich, R.L., Dively, G.P., Higgins, L.S., Dixon, P.M. & Duan, J.J. (2008). Selection of nontarget arthropod taxa for field research on transgenic insecticidal crops: using empirical data and statistical power. Environmental entomology, 37, 1–10.

Van Der Putten, W.H., Vet, L.E.M., Harvey, J.A. & Wäckers, F.L. (2001). Linking above- and belowground multitrophic interactions of plants, herbivores, pathogens, and their antagonists. Trends in Ecology and Evolution, 16, 547–554.

Sanvido, O., Romeis, J. & Bigler, F. (2011). Environmental change challenges decision-making during post-market environmental monitoring of transgenic crops. Transgenic Research, 20, 1191–201.

Symondson, W.O.C., Sunderland, K.D. & Greenstone, M.H. (2002). Can generalist predators be effective biocontrol agents? Annual Review of Entomology, 47, 561–594.

Tabic, A., Yonah, R., & Coll, M. (2010). Association between omnivorous *Orius* bugs and their thrips prey at different spatial scales of *Verbesina encelioides* flowers. Israel Journal of Plant Sciences, 58, 131-141.

Tscharntke, T., Klein, A.M., Kruess, A., Steffan-Dewenter, I. & Thies, C. (2005). Landscape perspectives on agricultural intensification and biodiversity - ecosystem service management. Ecology Letters, 8, 857–874.

Underwood, A. J. & Chapman, M.G. (2003). Power, precaution, Type II error and sampling design in assessment of environmental impacts. Journal of Experimental Marine Biology and Ecology, 296, 49–70.

Vasseur, C., Joannon, A., Aviron, S., Burel, F., Meynard, J.M. & Baudry, J. (2013). The cropping systems mosaic: How does the hidden heterogeneity of agricultural landscapes drive arthropod populations? Agriculture, Ecosystems and Environment, 166, 3–14.

CHAPTER 1

Landscape context influences leafhopper and predatory *Orius* spp. abundances in maize fields

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ABSTRACT

1- Biological control relies on periodical colonization of crops by natural enemies from surrounding habitats. In NE Spain predatory *Orius* spp. disperse among cereal, maize and alfalfa according to crop phenology and management.

2- In this study we examined (1) the variation of *Orius* spp. and its leafhopper prey *Zyginidia scutellaris* populations in co-occurring habitats (maize, alfalfa and semi-natural) for two years in three regions; and (2) the effects of agricultural landscape context on their abundance in maize.

3- Variance partitioning revealed that inter-annual variation accounted for the largest proportion of variation for *Orius* spp. and its prey. Maize leafhopper abundance was positively related to winter cereal cover in the landscape and negatively related to semi-natural habitat across the three regions. *Orius* spp. were unresponsive to shifts in habitat composition despite being present in maize and associated habitats; however they were positively related to edge density. Larger-scale variation in *Orius* spp. abundance was best explained by changes in *Z. scutellaris* abundance in maize.

4- Leafhopper colonization is responsible for the recruitment of *Orius* spp. in maize. *Orius* spp. conservation in intensive agricultural landscapes might require permanent field margins and complementary crops -like alfalfa- that ensure resource continuity in time.

Key words: *Zyginidia scutellaris*; *Orius* spp.; aggregation; landscape; biological control

The content of this chapter has been accepted for publication in Agricultural and Forest Entomology in April 2017.

INTRODUCTION

Biological control of pests provided by predators and parasitoids is a key ecosystem service supporting agricultural production (Losey & Vaughan 2006). Biological control relies on periodical natural colonization of a crop by natural enemies from surrounding habitats, their survival and reproduction through the crop season and their return to more permanent habitats that act as reservoirs (Wissinger, 1997). However, concerns have arisen about the derioration of biological control function as a result of agricultural intensification. A growing body of evidence suggests that simplified agricultural landscapes - based on bigger field sizes, smaller number of crop species, high agrochemical inputs, and very little natural or semi-natural areas - lead to disturbances in the community composition of herbivorous insects (Kennedy and Storer, 2000), their natural enemies (predators and parasitoids) and ultimately to an erosion of natural pest regulation (Matson et al., 1997; Tscharntke et al., 2005). On the other hand heterogenous or complex landscapes would strongly benefit biological control by supporting more diverse and abundant communities of natural enemies in permanent habitats that offer shelter from crop disturbances, overwintering refuges, alternative hosts and prey, and additional floral resources (Bianchi et al., 2006; Chaplin-Kramer et al., 2011; Veres et al., 2013).

Landscape heterogeneity is based on two components: compositional heterogeneity -that is related to the variety of different cover types- and configurational heterogeneity -related to the complex spatial pattern of those cover types (Fahrig et al., 2011). Both components can account for the abundance of semi-natural habitats in the landscape: the first by describing the extension of forest, schrubland or other natural habitats and the second by describing the potential ecotones between habitats. These semi-natural habitats can represent important sources of natural enemies colonizing crop fields, and proximity to such habitats may result in increased control of agricultural pests (e.g. spill over hypothesis, Rand et al. 2006) . To disentangle the effects of landscape context on pests as well as on their natural enemies, it is necessary to identify those landscape elements necessary for the species life cycle (Fahrig et al., 2011), such as overwintering hosts outside the focal crop or alternative host crops (Kennedy and Storer, 2000), and to consider resource availability and continuity over the species life history (Schellhorn et al., 2015; Vasseur et al., 2013) together with the spatial scales and time periods to be considered in the study (Kremen et al., 2007; Chaplin-Kramer et al., 2013).

Early colonization of maize plants by leafhoppers is a key feature for attracting and establishing on-plant generalist predators such as *Orius* spp. on maize crops in NE Spain (Albajes et al., 2011). In this area, arable crops predominate the agricultural landscape, where winter cereals, maize and alfalfa co-exist, and form a mosaic that also includes field margins and other semi-natural areas. These arable crops partially overlap throughout the year: alfalfa and winter cereals in spring, alfalfa and maize in summer, and in some cases all three crops in late spring and autumn. *Orius* spp. are one of the most abundant in-plant predators in arable crops in Spain (Albajes et al. 2003; de la Poza et al., 2005)and move among at least cereal, maize and alfalfa according to crop phenology and management (Madeira et al., 2014; Pons et al., 2005b). *Orius* spp. feed on small insects (e.g., thrips, scales, aphids, psyllids, small caterpillars, and the eggs of various insects) and mites (Lattin 1999) and are known to respond numerically by aggregation to locations with higher prey density (Albajes et al., 2011; Pons et al., 2005b; Tabic et al., 2010) which makes them ideal for biological control (Symondson et al., 2002). Maize leafhopper *Z. scutellaris* is a polyphagous herbivore that can feed on multiple Poaceae, it develops the first generation in field margins or cereal crops (Della Giustina, 2002; Nickel, 2003); and afterwards colonizes maize fields early in the season reaching up to 100 individuals per plant before pollen shed (Pons and Albajes, 2002). Previous work indicated a strong positive relationship between yearly cumulative numbers of *Orius* spp. and *Z. scutellaris* in maize plots (Albajes et al., 2011)*,* and coincidental population peaks around maize anthesis (Albajes et al., 2009).

In this context, the big challenge in explaining the variation of *Orius* spp. abundance lies in differentiating the role of the landscape context and that of available prey on focal crop (Chaplin-Kramer et al., 2011; Fahrig et al., 2011; Vasseur et al., 2013; Veres et al., 2013). For this, we monitored for two years the abundance of *Orius* spp. and *Z. scutellaris* in commercial maize fields in three agricultural regions in NE Spain to account for year-to-year and region-to-region variation of populations. We also monitored two typically co-occurring habitats in maize production systems in NE Spain, alfalfa and associated semi-natural patches, to account for the *Orius* spp. presence in alternative hosts plants/habitats. We then tested species specific hypotheses on the influence of the landscape context on *Orius* spp. and *Z. scutellaris* abundances in commercial maize fields, considering that both the herbivore and the predator are influenced by resource availability over time at the landscape scale. Firstly, provided the importance of Poaceae for *Z. scutellaris* leafhoppers, we hypothesized that the area of spring cereal and the extent of winter Poaceae plants present in other habitats (mainly field edges) in the landscape are key in sustaining hibernating females and boosting leafhopper populations earlier in the season. Secondly, taking into consideration the potential effect of semi-natural areas on natural enemies, we hypothesized that an increased area of semi-natural habitats and edge density leads to increased predation by *Orius* spp. and to a decreased maize leafhopper abundance. We also expected that the amount of permanent habitats in the landscape -like semi-natural habitat and field margins - would provide overwintering habitats and thus favor *Orius* spp. populations at the landscape scale. Lastly we hypothesized that an increase in spring crop area would enhance *Orius* spp. populations by providing habitat and prey where predator populations could increase their numbers during spring time.

METHODS

Study area

The study was conducted during years 2012 and 2013. The study sites were located in three maize-growing regions in the NE Iberian Peninsula surrounding the municipalities of Bujaraloz, Almacelles and La Seu d'Urgell. We selected these three regions because they are representative of the management of maize in NE Spain.

Bujaraloz (41°29′50″N 0°9′13″O; 300-400m altitude; 200-400 mm average rainfall) is part of the Monegros badlands; its transformation to irrigation from the 1970s to the 2000s modified the agriculture of the area from basically rainfed cereals to maize, alfalfa and sporadically non-maize annuals. Almacelles (41°43′57″N 0°26′25″E; 200-300m altitude; 200-400 mm rainfall) and its surrounding municipalities were converted to irrigation in the 1910s, creating an agricultural mosaic that alternates the common rotation in the area -winter cereals, alfalfa, and maize - with orchards and other annuals. Climate and cultivation practices are similar in Bujaraloz and Almacelles where maize is cultivated for grain: intensive soil cultivation, applications of pre-emergence herbicides and deployment of Bt maize (event MON810) for cornborer control are common practice. La Seu d'Urgell (42°21′32″N 1°27′43″E) is an inter-mountain municipality in the Pyrenees (700–800 m altitude), with a no-till crop rotation of winter cereal and silage maize surrounded by pastures, and an average rainfall of 650 mm per year. In all three regions insecticide treatment of maize is rare.

Study plots

We selected plots consisting of commercial maize fields with a similar phenology paired with an adjacent alfalfa field and with semi-natural vegetation fragments (year 2012 and 2013). The alfalfa and semi-natural vegetation habitats were selected to detect the use of alternative habitats by our focal species. This group of three habitats (maize, alfalfa and semi-natural vegetation) constituted a site within a region (Fig. 1). New sites were selected each year with a total of 31 unique sites (Bujaraloz n=5 fields in 2012, n=6 in 2013; Almacelles, n=5 in 2012, n=7 in 2013; La Seu n=4 in 2012 and n=4 in 2013). Maize fields within each region were at least 2 km apart in order to avoid potential spatial autocorrelation, with the exception of La Seu, where two sites were closer as a result the of landscape constraints, as the valley is narrow and the crop area limited.

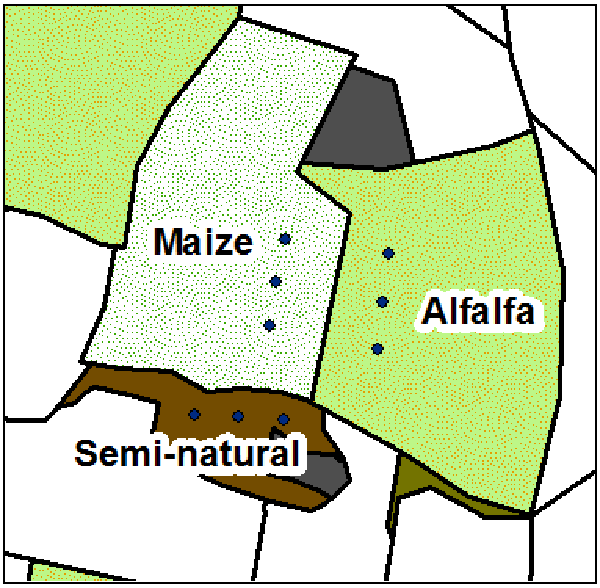

Fig.1 Sampling design of a site consisting of a commercial maize field paired with an alfalfa field and with semi-natural vegetation fragments. Dots indicate the position of yellow sticky traps.

Insect sampling and processing

We sampled the epigeal insects in maize, alfalfa and natural habitats with yellow sticky traps (Pherocon ® AM, TRÉCÉ, Adair, OK, USA). Three sticky traps were used per site and habitat (maize, alfalfa and semi-natural). Each trap was mounted on a metal bar and placed inside maize and alfalfa fields at 30 m from the border, with a distance of 25 m between traps. When the natural habitat area was reduced, we sampled the center of the patch and kept a minimum separation of 3 m between sticky traps. The sampling height was the cob height in maize and the vegetation height in alfalfa and natural habitats.

Study plots were sampled twice around the *Orius* spp. and maize leafhopper population peaks, that occur around maize anthesis, as reported in previous field studies conducted by our team (Albajes et al., 2009). Yellow traps were left in the plots for 1week; afterwards, they were collected and conserved at 6ºC-8ºC before insect identification. *Orius* spp. individuals were counted and identified to genus level. All leafhopper specimens were counted and identified to species level in the case of *Z.scutellaris*.

Quantifying and selecting landscape features

Landscape structure was quantified using ArcGIS 9.3 (ESRI 2005). For the years 2012 and 2013, we characterized the landscape surrounding each site in a circular buffer area at two scales of 0.5 km and 1km radius. The radius was chosen according to the results reported by previous biological control studies conducted at the landscape scale (reviewed by Veres et al. 2013; Chaplin-Kramer et al. 2011) and dispersal capacity of insects studied. During field inspections landscape patches were classified as crop (including the cover classes: maize, alfalfa, cereals, orchards, pastures and other non-cereal annuals) or non-crop (including semi-natural habitats such as steppe vegetation and forests, and fallow, urban and water-associated areas). We later mapped the land use in these landscapes on the basis of geo-referenced aerial photographs (ICC, 2008). Land use changes between the image date and the study period were verified in the terrain annually, and corrections were made during the digitalization process. The proportion of all cover classes in each landscape buffer was calculated for all sites. We considered all interfaces between differentiated cover classes or crop fields managed by different farmers as edges. Then, the landscape metrics Shannon diversity index (SDI), Richness (sum of cover types present in each landscape buffer, excluding urban cover) and edge density (ED, total length of patch edges divided by total area) were calculated. The class areas and the other landscape metrics for each region are summarized in Table 1.

As is often found in landscape studies, the amount of natural/semi-natural habitat was inversely correlated with the proportion of cultivated land ($\varrho = -0.90$, $P < 0.0001$) as well as with the proportion of cereal ($\varrho = -0.63$, $P < 0.0002$) in our study (Landscape variable correlations to be found in Supporting Information, Tables S.1 and S.2) . We used Spearman rank correlations <0.70 (according to Dormann et al. 2013), and the functional relevance of each landscape cover for our focal species life cycle (hypotheses, see Introduction) to select landscape variables that are ecologically meaningful (Fahrig et al., 2011). The potential Poaceae species that occur in the different cover types is reflected in Tables S.3 and S.4 (Supporting Information). As a result we chose the compositional landscape metrics Cereal cover (C), Alfalfa cover (A) and Semi-Natural vegetation cover (SN), and the configurational metrics edge density (ED), to be used in data analysis.

Data analysis

Leafhopper and *Orius* spp. abundance was obtained by summing the means of the two sampling dates per habitat and site, and then transformed [log10(x + 1)].We used linear models for abundance data analysis because log-transformation yields a better controlled type I error than generalized linear models assuming a Poisson distribution (Ives, 2015). All statistical analyses were performed using R (R Development Core Team 2005).

Estimation of baseline variation of Orius spp. *and maize leafhopper in maize based agroecosystems* - We assessed the effects of the main factors habitat, region and year and their interactions on Z. scutellaris and *Orius* species by means of the variation partitioning method (Boccard et al., 1992) to obtain information on the sources of population variability. We estimated eight variances for *Z. scutellaris* and *Orius* species abundance by fitting linear models in which the response variable was species abundance and the variance components estimated were among-region, among-year, among-habitat, those of their interactions, and residual variance. We calculated the percent variance corresponding to each of the factors with the function varPart (package modEvA; Barbosa et al., 2014) using the coefficients of determination (R-squared values) of the linear regressions; and obtaining as outputs the amounts of variance explained exclusively by each factor, the amounts explained exclusively by the overlapping effects of each pair of factors, the amount explained by the overlap of the 3 factors and the residual variation. Negative R2 can be ignored (considered as null) for the ecological interpretation of the results (Legendre and Legendre, 1998).

Table 1. Mean and standard error of landscape compositional and configurational variables (0.5 km and 1 km radii) in the three study regions Bujaraloz, Almacelles and La Seu. In bold the four predominant cover types in each region.

	Bujaraloz (n=11)				**Almacelles (n=12)**				**La Seu (n=8)**			
	0.5 km		1 km		0.5 km		1 km		0.5 km		1 km	
	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
Maize (%)	**49.88**	**7.67**	**45.51**	**6.11**	**25.50**	**4.45**	**19.43**	**3.03**	**19.56**	**3.47**	**13.38**	**1.85**
Alfalfa (%)	**18.89**	**4.28**	**11.60**	**1.89**	**29.02**	**3.53**	**25.24**	**2.01**	4.14	0.99	3.95	0.56
Cereal (%)	**14.47**	**3.01**	**24.34**	**3.48**	**20.94**	**3.96**	**25.20**	**2.88**	4.26	1.54	4.03	1.14
Orchard (%)	0.32	0.32	0.32	0.21	**10.70**	**4.57**	**12.69**	**3.31**	1.06	1.00	0.50	0.34
Fallow (%)	3.21	1.06	3.60	1.33	4.34	1.33	4.25	1.04	4.16	1.04	3.76	0.79
Pasture (%)	0.00	0.00	0.11	0.11	0.46	0.34	0.69	0.38	**21.88**	**4.11**	**14.99**	**1.99**
Semi-Natural (%)	**9.36**	**2.52**	**7.52**	**2.26**	2.63	0.98	4.51	1.07	**30.27**	**7.90**	**44.17**	**5.33**
Urban (%)	1.37	0.58	3.40	1.43	3.86	0.74	4.82	0.88	**10.53**	**2.73**	**12.55**	**2.98**
Crop (%)	86.04	3.14	85.46	3.29	88.63	1.86	84.23	2.87	50.89	6.91	36.84	4.21
Non-Crop (%)	12.56	3.06	11.13	4.21	6.98	1.61	8.76	1.54	34.43	7.55	47.93	5.51
Edge Density (x100)	2.32	0.16	2.01	0. 14	2.87	0.12	2.68	0. 13	3.94	0.30	2.89	0.27
SDI	1.16	0.12	1.31	0.09	1.47	0.04	1.67	0.03	1.59	0.10	1.54	0.10
Richness	4.40	0.31	5.10	0.25	5.60	0.23	6.13	0.23	5.88	0.23	6.60	0.15

Effects of landscape composition and configuration on species populations- We analyzed landscape composition influence on *Orius* spp. and Z. scutellaris abundance in maize with linear mixed-effects models (Pinheiro and Bates, 2000; nlme package), which accounted for non-independent errors of the hierarchically nested sampling design. We fitted two different sets of models at each spatial scale (radii of 0.5 and 1 km). In the first set (single species models), all the combinations of standardized landscape metrics ED, C, A and SN and their interactions, were included as fixed factors, whereas region and year were used as random factors to account for the nested design. Years were nested to the regions. In the second set (multispecies models), we fitted the models in the same manner as in the first set and added the log-abundance of *Orius* spp. or *Z. scutellaris* as a covariate.

The fact that in La Seu region semi-natural area covers an area six to ten times larger than at the other two regions could potentially bias results towards this predominant landscape variable (see Table 1). For this reason, in a second analysis we fitted the same landscape models excluding the region La Seu with the intention to corroborate the first analysis, and only considered the more intensive agricultural regions with similar agricultural practices and more homogenous agricultural landscape.

We performed a model selection procedure based on the information-theoretic approach and Akaike's information criterion corrected for small sample sizes (AICc) (Burnham & Anderson, 2002). We present the best-fit model, defined as the model with the minimum AICc value, i.e. with the best support for the data, and any competing models at the spatial scale of the best-fit model that had an AICc difference of less than two. We used the multi-model inference method for calculating the parameter estimates and standard errors as this method is advantageous in reaching a robust inference when data supports more than one model (Burnham & Anderson, 2002). The estimates and standard errors of the parameters that are not conditional on only the best model but instead derive from weighted averages of these values across multiple models (thus they are referred as unconditional estimates and standard errors). We also calculated the direction and the 95% confidence interval of the effect size of the variables in the models with ΔAICc <2 to determine the relationship between the response and the explanatory variables. All models were examined at the 0.5 and 1 km scales using the R package AICcmodavg (Mazerolle, 2016). We checked diagnostic plots of the best models for homoscedasticity, normality of residuals, and presence of outliers. As the variable Alfalfa cover was not included in any of the best fit models we excluded it to reduce the number of models used in the model selection procedure. We represented in partial residual plots the relationships between leafhopper and predator abundances and landscape metrics for the best-fit models using the Effects package (Fox, 2003).

Even though we minimized the overlapping of landscape buffers between sites, prior to interpreting the results of the model selection we examined potential spatial autocorrelation of the residuals of the best-fit model for each response variable and spatial scale between each site and the nearest neighboring site using Moran's I statistic (Ape package, Paradis et al., 2014) . We did not find evidence of spatial autocorrelation for the best fitted models at either scale for *Orius* spp. abundance (at 0.5 km and 1 km Moran's I statistic = 0.060, P = 0.597) and Z. scutellaris abundance (at 0.5 km scale Moran's I statistic < -0.080, P = 0.418; at 1 km Moran's I statistic = -0.057, P = 0.734).

RESULTS

Contribution of habitat, region and year to overall variability of Orius spp. and Z. scutellaris abundance

Variance partitioning explained 36.5% of the variation in *Orius* spp. populations and 63.4% in the case of *Z. scutellaris*; and revealed that inter-annual variation accounted for the largest proportion of explained variation for both taxa (Fig. 2a), followed by habitat identity in the case of the predator and regional variation for the leafhopper. Overall there was a great differences in insect population abundances between years 2012 and 2013, with 2013 characterized by much lower *Orius* spp. and maize leafhopper densities than those of 2012 (Fig 2b).

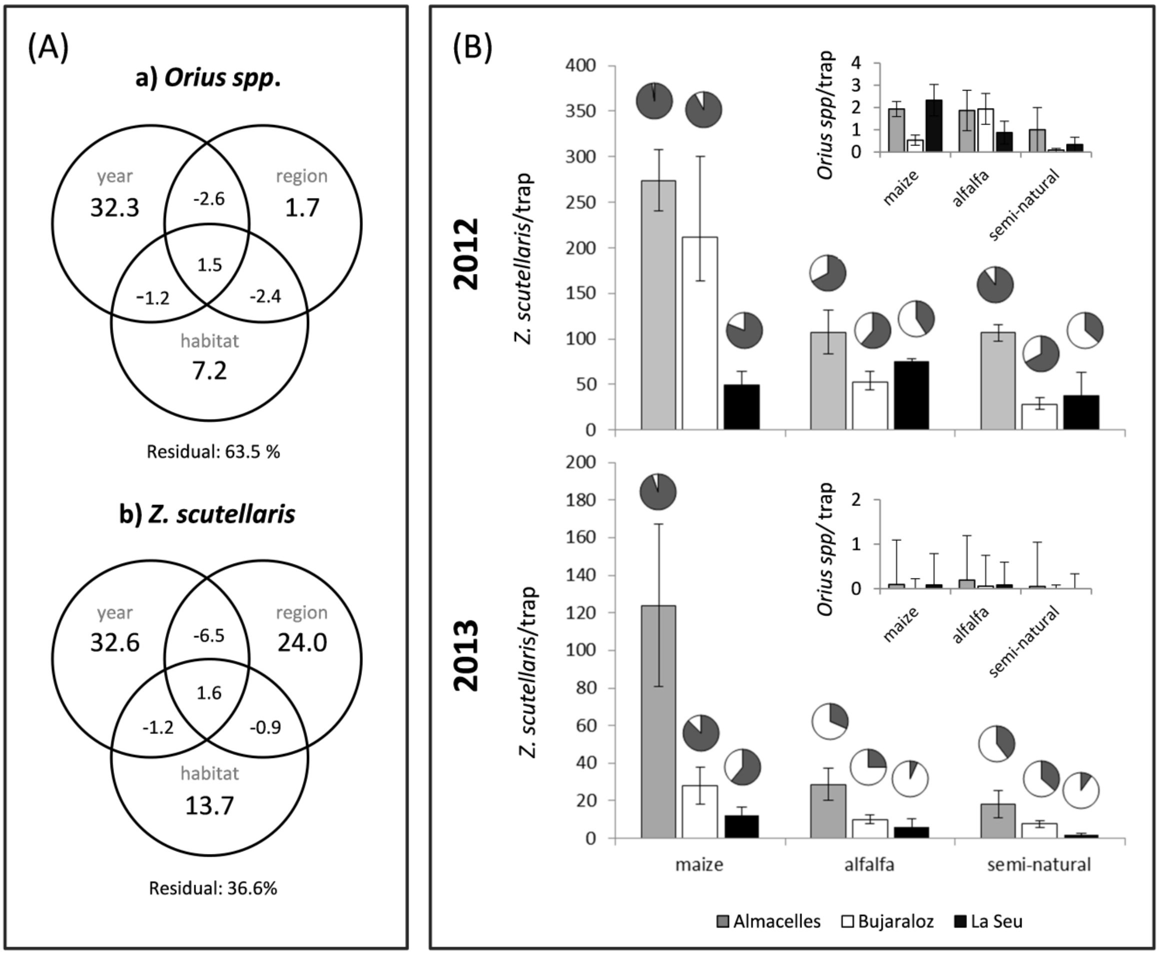

Fig. 2 a) Variation partitioning of *Z. scutellaris* and *Orius spp.* populations to among-region, among-year, among-habitat, their interactions, and residual variation. b) Abundances of *Z. scutellaris* adults per season (mean±SE) in yellow traps in three co-occurring habitats: maize, alfalfa and semi-natural (bar chart). Proportion of *Z. scutellaris* (gray) to total leafhoppers captured in yellow traps (pie chart). Note different scales on the y axis.

Predatory *Orius* spp. was more abundant in alfalfa and maize than in the semi-natural habitat in 2012 (Fig 2.) (habitat $F_{2,32} = 4.69$, $P = 0.016$), no such effect was detected in 2013 probably as a result of low *Orius* spp. populations that year (Fig 2a). Abundances of *Orius* spp. per site in maize and co-occurring habitats presented strong correlations (maize-alfalfa: Spearman $\varrho = 0.58$, $P = 0.0008$; maize-semi-natural: Supporting Information, Table S.5), as we initially predicted on the basis of the continuous movement of *O. majusculus* between alfalfa and maize during the season reported by Madeira et al. (2014) in the area.

Abundance of leafhopper *Z. scutellaris* in maize was 2 to 4 times higher than those in the other habitats (Fig.2b, $F_{2,82} = 16.53$, $P < 0.001$), with the exception of alfalfa in 2012 at La Seu, which had more captures than the adjacent maize. However, when alfalfa fields were sampled in 2012 with a sweeping net for other purposes, no *Z. scutellaris* nymphs were found whereas they were abundant on maize (A. Ardanuy and F. Madeira, personal observation), so we assumed that alfalfa is not a source habitat for *Z. scutellaris* at least in summer in spite it can harbor adults occasionally.

Effects of landscape characteristics on Orius spp. and Z. scutellaris

Sets of models fitted to *Orius* species and *Z. scutellaris* are summarized in Table 3 for models including the three study regions and Table 4 for models including the two intensive agricultural regions (Bujaraloz and Almacelles). Variance inflation factors (VIF) for each landscape predictor included in our initial statistical models were ≤2.2, well below the level considered to be problematic from the perspective of multicollinearity.

When considering the three regions, the selected compositional (SN, C) and configurational (ED) landscape variables had no influence on *Orius* spp. populations at either scale (0.5 and 1 km radii), being the null model the one with lower AICc (Table 2 and Table 4). On the other hand when considering Bujaraloz and Almacelles, the configurational metrics ED was consistently and significantly positively related to *Orius* spp. abundances in maize fields (Table 3 and Table 5).

A second goal of the analysis was to estimate how the importance of the landscape composition compared with that of prey levels. *Orius* spp. populations increased as the abundance of the herbivore *Z. scutellaris* increased in maize fields across the three regions, as indicated by the magnitude of its effect (unconditional standard error) and its confidence intervals, which did not include the value 0 (Table 4). Overall for the predators, multispecies models performed better than single species models when considering the three regions, but the opposite was found when considering exclusively the two most agricultural intensive regions, as shown by differences on their AICc values (Δi), Table 2 and Table 4.

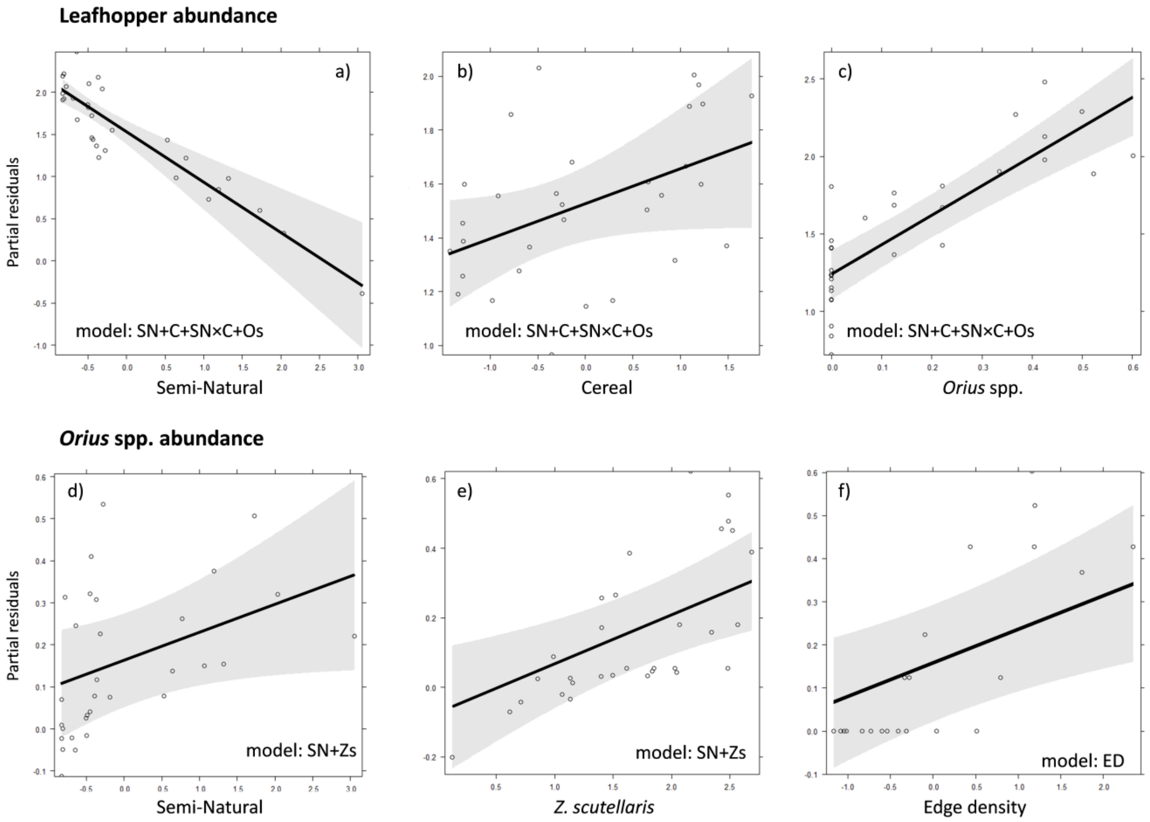

Fig. 3 Partial residual plots illustrate the relationships of *Z. scutellaris* (a, b and c) and *Orius* spp. (d, e and f) and landscape metrics and predator/prey co-variable for the best-fit models. Leafhopper abundance was best explained by a model containing Semi-Natural cover, Cereal cover, their interaction and *Orius* spp. abundance as a co-variable at a radius of 1km for the three study regions. *Orius* spp. was best explained by a model containing Semi-Natural cover and leafhopper abundance at a radius of 1km for the three study regions; and a model containing the predictor Edge density at a radius of 0.5 km for two intensive agricultural regions. Best-fit models estimates are presented in Table 4 and 5.

Table 2. Landscape model selections for leafhopper *Z. scutellaris* and predatory *Orius* spp. abundance for the three study regions (Almacelles, Bujaraloz and La Seu). The metrics Semi-Natural, Cereal, Edge Density and their interactions were included as fixed factors. In multispecies models the log-abundance of *Orius* spp. and *Z. scutellaris* was used as a co-variable. The full balanced sets of models tested can be seen in the online supporting information. Only models with Δi < 2 are shown.

	A) Single-species					**B) Multi-species**				
	Models	K	AICc	Δi	Wi	Models	K	AICc	Δi	Wi
Z. scutellaris										
0.5 km	SN*	5	51.52	0.00	0.30	SN*+Os	6	35.14	0	0.46
	SN*+C·	6	52.24	0.72	0.21					
1 km	SN**+C**	6	44.31	0.00	0.37	C***+SN***+C×SN*+Os***	7	23.94	0.00	0.72
	SN**+C**+ SN×C	7	45.74	1.43	0.18					
***Orius* spp.**										
0.5 km	1	4	-34.01	0	0.38	Zs*	6	-36.52	0	0.27
	ED	5	-33.12	0.90	0.21	SN+Zs**	8	-35.95	0.56	0.20
						SN+C+Zs**	5	-35.57	0.95	0.17
1 km	1	4	-34.01	0	0.38	SN+Zs*	6	-37.18	0	0.17
	ED	5	-33.12	0.90	0.21	C+ED+ED:C*+Zs*	8	-36.87	0.31	0.14
						Zs*	5	-36.52	0.66	0.12
						SN+C+Zs*	7	-36.24	0.93	0.10
						SN+ED+Zs*	7	-35.79	1.39	0.08
						SN+C+ED+ED:C+Zs*	9	-35.73	1.45	0.08

*** K , number of parameters; AICc, Akaike's information criterion corrected for small sample size; **Δ**i, the AICc differences compared with the most parsimonious model; Wi, Akaike weights; ED, edge density in a landscape; SN, percentage of semi-natural habitat in a landscape; C, percentage of cereal crop in a landscape; Zs, *Z. scutellaris*; Os, *Orius* spp.. * $p<0.05$ **$p<0.01$ ***$p<0.001$

Table 3. Landscape model selections for leafhopper *Z. scutellaris* and predatory *Orius* spp. abundance for the two agricultural intensive regions (Almacelles and Bujaraloz). The metrics Semi-Natural, Cereal, Edge Density and their interactions were included as fixed factors. In multispecies models the log-abundance of *Orius* spp. and *Z. scutellaris* was used as a co-variable. The full balanced sets of models tested can be seen in the online supporting information. Only models with Δi < 2 are shown ***

	A) Single-species					**B) Multi-species**				
	Models	K	AICc	Δi	Wi	Models	K	AICc	Δi	Wi
Z. scutellaris										
0.5 km	ED*+SN**	6	35.91	0.00	0.56	SN*+Os	6	35.14	0	0.46
1 km	ED*+SN**+C	7	36.36	0.00	0.23	C**+SN**+Os**	7	26.38	0.00	0.75
	ED*+SN**	6	36.49	0.13	0.21					
	ED**+SN**+ED×SN	7	36.58	0.22	0.20					
	SN*+C*	6	37.74	1.39	0.11					
***Orius* spp.**										
0.5 km	ED**	5	-27.51	0.00	0.68	ED**+Zs	6	-24.99	0	0.51
1 km	ED*	5	-25.33	0.00	0.61	ED*+Zs	6	-23.51	0.00	0.22
						Zs	5	-23.12	0.39	0.18
						ED*+Zs+ED×Zs	7	-22.05	1.47	0.11
						SN+Zs*+SN×Zs·	7	- 21.95	1.57	0.10

*** K , number of parameters; AICc, Akaike's information criterion corrected for small sample size; **Δ**i , the AICc differences compared with the most parsimonious model; Wi, Akaike weights; ED, edge density in a landscape; SN, percentage of semi-natural habitat in a landscape; C, percentage of cereal crop in a landscape; Zs, *Z. scutellaris*; Os, *Orius* spp.. * p<0.05 **p<0.01 ***p<0.001

Table 4. Model-averaged estimate, unconditional standard error of the estimate (UnSE) and confidence interval (CI 5% and 95%) of the predictor variables and their interaction for the leafhopper *Z. scutellaris* and the predator *Orius* spp. in the three study regions (Almacelles, Bujaraloz and La Seu). In bold, predictors with substantial support.***

			Z. scutellaris				***Orius*** **spp**			
			Estimate	**UnSE**	**CI**		**Estimate**	**UnSE**	**CI**	
a) Single-species										
	0.5 km	Intercept	**1.65**	**0.22**	**1.23**	**2.07**	**0.16**	**0.07**	**0.02**	**0.30**
		SN	**-0.23**	**0.10**	**-0.43**	**-0.04**				
		C	0.12	0.08	-0.04	0.28				
		ED					0.03	0.03	-0.02	0.08
	1km	Intercept	**1.66**	**0.16**	**1.34**	**1.98**	0.16	0.08	0.00	0.32
		SN	**-0.34**	**0.11**	**-0.56**	**-0.13**				
		C	**0.21**	**0.08**	**0.04**	**0.37**				
		ED					0.03	0.02	-0.01	0.07
		SN:C	-0.19	0.12	-0.43	0.05				
b) Multi-species										
	0.5 km	Intercept	**1.36**	**0.15**	**1.07**	**1.65**	0.00	0.10	-0.21	0.20
		Os	**1.90**	**0.46**	**1.00**	**2.81**				
		Zs					**0.11**	**0.04**	**0.03**	**0.19**
		SN	**-0.31**	**0.09**	**-0.51**	**-0.12**	0.04	0.02	0.00	0.09
		C	0.10	0.08	-0.06	0.26				
		ED	-0.15	0.09	-0.32	0.01	0.04	0.02	-0.01	0.08
	1km	Intercept	**1.39**	**0.07**	**1.26**	**1.53**	-0.06	0.11	-0.28	0.15
		Os	**1.87**	**0.28**	**1.31**	**2.43**				
		Zs					**0.14**	**0.05**	**0.04**	**0.24**
		SN	**-0.35**	**0.08**	**-0.5**	**-0.20**	0.07	0.03	0.00	0.13
		C	**0.22**	**0.07**	**0.09**	**0.36**	-0.03	0.02	-0.08	0.01
		ED					0.03	0.02	-0.01	0.06
		C:ED					**0.04**	**0.01**	**0.01**	**0.06**

***ED, edge density in a landscape; SN, percentage semi-natural habitat in a landscape; C, percentage of cereal crop in a landscape; Zs, *Z. scutellaris*; Os, *Orius* spp..

Table 5. Model-averaged estimated, unconditional standard error of the estimate (UnSE) and confidence interval (CI, α = 0.95) of the predictor variables and their interaction for the leafhopper Z. scutellaris and the predator *Orius* spp. in the two agricultural intensive regions (Almacelles and Bujaraloz). In bold, predictors with substantial support. ***

		Z. scutellaris				***Orius* spp.**			
		Estimate	**UnSE**	**CI**		**Estimate**	**UnSE**	**CI**	
***a)* Single-species**									
0.5 km	Intercept	**1.92**	**0.09**	**1.75**	**2.10**	**0.16**	**0.07**	**0.03**	**0.29**
	ED	**0.30**	**0.08**	**0.14**	**0.46**	**0.08**	**0.03**	**0.03**	**0.13**
	SN	**-0.26**	**0.08**	**-0.43**	**-0.10**				
1 km	Intercept	**1.93**	**0.12**	**1.69**	**2.16**	0.16	0.08	0.00	0.32
	ED	**0.29**	**0.09**	**0.10**	**0.47**	**0.05**	**0.02**	**0.01**	**0.10**
	SN	**-0.23**	**0.08**	**-0.39**	**-0.07**				
	C	**0.16**	**0.08**	**0.01**	**0.31**				
	ED×SN	**0.25**	**0.12**	**0.01**	**0.49**				
***a)* Multi-species**									
0.5 km	Intercept	**1.70**	**0.11**	**1.49**	**1.92**	0.02	0.11	-0.18	0.23
	Os	**1.48**	**0.49**	**0.53**	**2.43**				
	Zs					0.07	0.04	-0.02	0.16
	SN	**-0.23**	**0.08**	**-0.38**	**-0.08**				
	ED					0.07	0.04	-0.02	0.15
1 km	Intercept	**1.66**	**0.07**	**1.52**	**1.80**	**0.16**	**0.07**	**0.03**	**0.29**
	Os	**1.80**	**0.30**	**1.20**	**2.39**				
	Zs					0.09	0.05	-0.01	0.19
	SN	**-0.25**	**0.06**	**-0.37**	**-0.14**	-0.02	0.07	-0.16	0.12
	C	**0.23**	**0.06**	**0.11**	**0.35**				
	ED					0.01	0.10	-0.18	0.2

***ED, edge density in a landscape; SN, percentage semi-natural habitat in a landscape; C, percentage of cereal crop in a landscape; Zs, *Z. scutellaris*; Os, *Orius* spp..

DISCUSSION

The main goal of this study was to estimate how landscape variables influenced predator *Orius* spp. and its prey *Z. scutellaris* abundances in maize fields; and how the importance of the landscape context compared to that of prey levels and habitat complementation for *Orius* spp.. Overall our results suggest that the predatory *Orius* spp. are highly unresponsive to shifts in landscape-scale habitat composition despite being readily available in maize and associated habitats across the three maize producing regions included in our study. Larger-scale variation in *Orius* spp. abundance in maize fields was best explained by changes in leafhopper *Z. scutellaris* abundance despite regional and inter-annual variation in population densities. Nevertheless the configurational variable edge density appears in agricultural intensive regions as the best predictor of *Orius* spp. abundance, whereas *Z. scutellaris* availability had only a minor influence.

In contrast we detected consistent and significant effects of landscape variables on maize leafhoppper *Z. scutellaris* populations: increasing areas of semi-natural habitat cover in a landscape decreased maize leafhopper population while, on the contrary, increasing winter cereal cover earlier in the year boosted leafhopper abundance in maize. Specifically, these findings suggest that (1) there is a direct positive response of maize leafhopper *Z. scutellaris* to increasing cover of a highly suitable crop earlier in the year and that its populations may be maintained in NE Spain by means of Poaceae resource continuity over time and space, and (2) that *Z. scutellaris,* as an early season colonizer of maize stands, is largely responsible for the subsequent colonization by predatory *Orius* spp., which is readily present in perennial crops, like alfalfa, and semi-natural habitats.

Pest response to landscape composition

We showed that maize leafhopper abundance in maize fields was positively related to the proportion of a previous suitable crop, cereal crop cover, across the three maize producing regions in NE Spain. Since *Z. scutellaris* is restricted to feeding on grasses, we considered that the proportion of area of winter cereal crop in the landscape -ranging from 1% to 40% at 1 km radius buffer- is an appropriate measure of potential available habitat during winter and spring. This finding agrees with the prediction of increased pest density with an increase of a suitable crop in arable systems (reviewed by Veres et al., 2013); and with what we know of *Z. scutellaris* biology in Central Europe where it depends on boundaries or grasslands for overwintering (Frey and Mahnhart, (1992) in Nentwig et al., 1998), and develops the first generation in cereal crops (Della Giustina, 2002; Nickel, 2003). However, in farming systems of NE Spain maize leafhopper populations rely mainly on cereal crops both for overwintering and likely for the development of first generations. Cereal and maize crops partially overlap in late spring and early autumn, as part of the traditional crop rotation in the area, providing a continuum of Poaceae availability that is ideal for sustaining *Z. scutellaris* leafhoppers throughout the year. In general the direct response of maize leafhopper to increasing cover of a highly suitable crop(s) may be an important component of landscape simplification on crop pest dynamics (Rand et al., 2014); nevertheless, we consider that the temporal overlapping of host crops can be the key element in sustaining its populations throughout the year.

In contrast, maize leafhopper populations decreased as the proportion of semi-natural habitat in the landscape increased, despite the presence of Poaceae in this kind of habitats. A suppressive effect of semi-natural areas on agricultural pests would be expected because semi-natural areas (i) increase in-field populations of natural enemies, and consequently tend to increase conservation biological control, and (ii) may be unsuitable habitats for pests reducing the crop source pool in the landscape (Veres et al., 2013). In this respect, the reduction of *Z. scutellaris* populations associated with higher semi-natural cover and lower cereal cover was not driven by changes in *Orius* spp. effectiveness as we found no effect of landscape habitat composition variables on their abundance. Our results suggest that this reduction in pest density is more likely a direct result of reductions in the area, and therefore abundance, of a highly suitable Poaceae crops in the landscape (cereal and maize). Similar results were reported for the sawfly *Cephus cinctus* a pest of wheat in the Great Plains of North America, where significant increases in levels of C. *cinctus* infestation were observed with increasing wheat cover at the landscape scale but no effects on conservation biological control were detected (Rand et al., 2014).

Lack of predator response to landscape composition

We found that *Orius* spp. predators were not significantly influenced by either Cereal, Alfalfa or Semi-Natural cover at the landscape scale despite being present in maize and associated habitats across the three maize producing regions included in our study (for alfalfa and *Orius* spp. in the area see Pons et al. 2005). The lack of landscape composition effects on *Orius* spp. could be due to several causes. The low number of the predator recorded in 2013 could lead to low statistical power in the analysis and therefore effects could be not detected even if they occurred (see in Comas et al., 2013 that statistical power of field records in maize in the area are strongly affected by predator densities). On the other hand, the experimental design used in the study could be the cause of not detecting the influence of landscape alfalfa cover on maize *Orius* spp.. We selected maize fields paired with adjacent alfalfa field, minimizing thus the variation in the landscapes and the potential to detect a landscape effect of alfalfa crops cover on *Orius* spp. abundance in spite that alfalfa hosts a remarkable number of the predator in our area (Pons et al., 2005) with frequent spillover between the two crops along the season (Madeira et al., 2014). A third reason for the lack of detection of landscape effects on *Orius* populations could be the mismatch between the metrics used to measure landscape composition and the actual habitat that is being used by the organism (Veres et al., 2013).

The traditional terms semi-natural/natural/non-crop have been used to cover a wide range of distinct habitats, including hedgerows, shrublands, natural grasslands, forests and field margins, which are by no means homogenous in their structure or plant composition (Sarthou et al., 2014; Veres et al., 2013); by separating the effect of field edges and/or margins (configurational) to that of forest and schrubland (compositional) we aimed to detect the contrasting effects of these two variables on *Orius* spp.. Nevertheless great heterogeneity in the nature and/or structure of the habitat reflected by a landscape variable may occur between regions. In particular the nature of the semi-natural habitats differs between regions while forested land predominates in a Seu, it is scarce in Almacelles and practically non-existent in Bujaraloz where natural shrubland prevail (Supporting Information, Table S.3).

In the same direction the metrics Edge Density in our study may include several typologies of ecotones across the three regions: in La Seu, ecotones are greatly dominated by forest and urban habitats, while ecotones related to cultivated land would predominate in the other two regions. As a result when considering exclusively Almacelles and Bujaraloz, the most agricultural intensive regions sharing similar farming practices, we detected an increase of *Orius* spp. abundance with an increase in Edge Density in the landscape. This relation to agricultural field edges or margins is consistent with strategies described for *Orius* spp. diapause in arable dominated systems, in which fertilized females overwinter under fallen leaves, in litter, under tree bark or in plant stems, in field boundaries (Lattin, 1999; Lundgren, 2011; Saulich and Musolin, 2009) and in alfalfa field margins in the NE Iberian Peninsula (Nuñez, 2002). Our results suggest that the presence of field margins in the agricultural landscape is necessary for sustaining conservation biological control by *Orius* spp. in intensive agricultural regions.

Predator response to prey

The strong positive association between *Orius* spp. and the herbivore *Z. scutellaris* across the three regions reflects a consistent density-dependent process of predator aggregation towards prey (e.g. Tabic et al. 2010; Rutledge et al. 2004; Östman & Ives 2003). Even though the temporal resolution of this study does not allow to directly evaluate the effects of pest suppression derived from an increase of *Orius* spp. numbers (Chaplin-Kramer et al., 2013), our results suggest that *Z. scutellaris* colonization might trigger *Orius* spp. recruitment to maize fields. Then by providing a food source for *Orius* spp. and other predators, maize leafhoppers may increase the total population size of *Orius* spp. within a field, thereby causing an indirect negative effect on other pests arriving simultaneously or subsequently via predator population growth and avoid pest outbreaks of economic importance (Chailleux et al., 2014; Holt and Lawton, 1994; Rutledge et al., 2004; Yoo and O'Neil, 2009). As an example, the numerical aggregation of *Orius insidiosus* to thrips in soybean has been shown to be key for avoiding soybean aphid outbreaks: the later soybean aphid arrives in fields after *O. insidiosus*, the lower was the peak aphid density reaches during the season (Rutledge et al., 2004; Yoo and O'Neil, 2009). Future manipulative experiments should focus on the temporal interactions between *Orius* spp.-maize leafhopper- alternative pests and as a result in whether early colonization of a secondary pest can lead to a reduction of maize harvest loss.

Management implications

The design of the cropping systems mosaic in order to enhance the performance and abundance of the existing natural enemies in arable farm (Landis et al. 2000) or to control pests (Carrière et al., 2006; Kennedy and Storer, 2000) is one of the key strategies in conservation biological control, and is currently the only cost-effective pest control method in arable crop conditions of many Mediterranean regions. Long-term persistence of natural enemy populations might not necessarily require permanent semi-natural habitats, but field margins and complementary habitats that ensure resource continuity in space and/or time (Carrière et al., 2006; Costamagna et al., 2015; Schellhorn et al., 2015; Vasseur et al., 2013). To meet the challenge of adapting agricultural landscapes towards a better natural pest control further research is needed on the relative contribution of the agricultural mosaic - habitat complementation, crop rotation, management intensities- together with that of the semi-natural elements to natural enemy populations dynamics to ensure the stability of natural enemies over the seasons and years (Rand et al., 2006; Schellhorn et al., 2015).

ACKNOWLEDGEMENTS

We would like to thank Alejandro Juárez-Escario, Xavi Solé-Senan and Xoaquín Moreira for discussions on data analysis, Xavier Pons, Carmen López, Filipe Madeira and all farmers in Almacelles, Bujaraloz and La Seu d'Urgell (Cooperativa Pirinaica), who kindly allowed us to work in their fields. A.A. was funded with an FPU scholarship from the Ministerio de Educación. M.S.L. was funded with an FI scholarship from the Generalitat de Catalunya. The work was partially funded by the Spanish Government project AGL2011-23996.

REFERENCES

Albajes, R., Lumbierres, B., & Pons, X. (2009). Responsiveness of arthropod herbivores and their natural enemies to modified weed management in corn. Environmental Entomology, 38, 944–54.

Albajes, R., Lumbierres, B., & Pons, X. (2011). Two heteropteran predators in relation to weed management in herbicide-tolerant corn. Biological Control, 59, 30–36.

Bianchi, F. J. J. A, Booij, C. J. H., & Tscharntke, T. (2006). Sustainable pest regulation in agricultural landscapes: a review on landscape composition, biodiversity and natural pest control. Proceedings of the Royal Society of London B: Biological Sciences, 273, 1715–27.

Carrière, Y., Ellsworth, P. C., Dutilleul, P., Ellers-kirk, C., Barkley, V., et al. (2006). A GIS-based approach for areawide pest management : the scales of *Lygus hesperus* movements to cotton from alfalfa, weeds, and cotton. Entomologia Experimentalis et Applicata, 118, 203–210.

Castañera, P., Ortego, F., Hernández-crespo, P., Farinós, G. P., Biología, D. De, et al. (2010). El maíz Bt en España: experiencia tras 12 años de cultivo. Phytoma España, 219, 64-70.

Chaplin-Kramer, R., O'Rourke, M. E., Blitzer, E. J., & Kremen, C. (2011). A meta-analysis of crop pest and natural enemy response to landscape complexity. Ecology letters, 14, 922–932.

Chaplin-Kramer, R., de Valpine, P., Mills, N. J., & Kremen, C. (2013). Detecting pest control services across spatial and temporal scales. Agriculture, Ecosystems and Environment, 181, 206–212.

Comas, J., Lumbierres, B., Pons, X., & Albajes, R. (2013). Ex-ante determination of the capacity of field tests to detect effects of genetically modified corn on nontarget arthropods. Journal of Economic Entomology, 106, 1659–1668.

Costamagna, A. C., Venables, W. N., & Schellhorn, N. A. (2015). Landscape-scale pest suppression is mediated by timing of predator arrival. Ecological Applications, 25, 1114–1130.

Dormann, C. F., Elith, J., Bacher, S., Buchmann, C., Carl, G., et al. (2013). Collinearity: A review of methods to deal with it and a simulation study evaluating their performance. Ecography, 36, 027–046.

Fahrig, L., Baudry, J., Brotons, L., Burel, F. G., Crist, T. O., et al. (2011). Functional landscape heterogeneity and animal biodiversity in agricultural landscapes. Ecology Letters, 14, 101–112.

Fox, J., (2003). Effect Displays in R for Generalised Linear Models. Journal of Statistical Software, 8, 1-27.

Della Giustina, W. (2002). Migration in Auchenorrhyncha. Denisia, 4, 513–522.

Kennedy, G. G., & Storer, N. P. (2000). Life systems of polyphagous arthropod pests in temporally unstable cropping systems. Annual Review of Entomology, 45, 467–493.

de la Poza, M., Pons, X., Farinós, G. P., López, C., Ortego, F., et al. (2005). Impact of farm-scale Bt maize on abundance of predatory arthropods in Spain. Crop Protection, 24, 677–684.

Lattin, J. D. (1999). Bionomics of the Anthocoridae. Annual Review of Entomology, 44, 207-231.

Legendre, P. & Legendre, L. (1998). Numerical ecology. Amsterdam: Elsevier Science B.V.

Lundgren, J. G. (2011). Reproductive ecology of predaceous Heteroptera. Biological Control, 59, 37–52.

Madeira, F., di Lascio, A., Carlino, P., Costantini, M. L., Rossi, L., et al. (2014). Stable carbon and nitrogen isotope signatures to determine predator dispersal between alfalfa and maize. Biological Control, 77, 66–75.

Matson, P. A., Parton, W. J., Power, A. G., & Swift, M. J. (1997). Agricultural intensification and ecosystem properties. Science, 277, 504–509.

Nentwig, W., Frank, T., & Lethmaye, C. (1998). Sown weed strips: artificial ecological compensation areas as an important tool in conservation biological control. Conservation biological control, 8, 133–153.

Nickel, H. (2003). The leafhoppers amd planthoppers of Germany (Hemiptera, Auchenorrhyncha): Patterns and Strategies in a Highly Diverse Group of Phytophagous Insects. Moscow-Sofia: Pensoft Publishers.

Nuñez, E. (2002). La alfalfa como reservorio de enemigos naturales. PhD Thesis Universitat de Lleida.

Östman, Ö., & Ives, A. R. (2003). Scale-dependent indirect interactions between two prey species through a shared predator. Oikos, 102, 505–514.

Pons, X., & Albajes, R. (2002). Control of maize pests with imidacloprid seed dressing treatment in Catalonia (NE Iberian Peninsula) under traditional crop conditions. Crop Protection, 21, 943–950.

Pons, X., Núñez, E., Lumbierres, B., & Albajes, R. (2005). Epigeal aphidophagous predators and the role of alfalfa as a reservoir of aphid predators for arable crops. European Journal of Entomology, 102, 519–525.

Rand, T. A, Tylianakis, J. M., & Tscharntke, T. (2006). Spillover edge effects: the dispersal of agriculturally subsidized insect natural enemies into adjacent natural habitats. Ecology letters, 9, 603–14.

Rand, T. A., Waters, D. K., Blodgett, S. L., Knodel, J. J., & Harris, M. O. (2014). Increased area of a highly suitable host crop increases herbivore pressure in intensified agricultural landscapes. Agriculture, Ecosystems and Environment, 186, 135–143.

Rutledge, C. E., Neil, R. J. O., Fox, T. B., & Landis, D. A. (2004). Soybean Aphid Predators and Their Use in Integrated Pest Management Soybean Aphid Predators and Their Use in Integrated Pest Management. Annals of the Entomological Society of America, 97, 240–248.

Sarthou, J. P., Badoz, A., Vaissière, B., Chevallier, A., & Rusch, A. (2014). Local more than landscape parameters structure natural enemy communities during their overwintering in semi-natural habitats. Agriculture, Ecosystems and Environment, 194, 17–28.

Saulich, A. K., & Musolin, D. L. (2009). Seasonal development and ecology of anthocorids (Heteroptera, Anthocoridae). Entomological Review, 89, 501–528.

Schellhorn, N. A., Gagic, V., & Bommarco, R. (2015). Time will tell: resource continuity bolsters ecosystem services. Trends in Ecology & Evolution.

Symondson, W. O. C., Sunderland, K. D., & Greenstone, M. H. (2002). Can generalist predators be effective biocontrol agents? Annual Review of Entomology, 47, 561–594.

Tabic, A., Yonah, R., & Coll, M. (2010). Association between omnivorous *Orius* bugs and their thrips prey at different spatial scales of *Verbesina encelioides* flowers. Israel Journal of Plant Sciences, 58, 131-141.

Tscharntke, T., Klein, A. M., Kruess, A., Steffan-Dewenter, I., & Thies, C. (2005). Landscape perspectives on agricultural intensification and biodiversity - ecosystem service management. Ecology Letters, 8, 857–874.

Vasseur, C., Joannon, A., Aviron, S., Burel, F., Meynard, J. M., et al. (2013). The cropping systems mosaic: How does the hidden heterogeneity of agricultural landscapes drive arthropod populations? Agriculture, Ecosystems and Environment, 166, 3–14.

Veres, A., Petit, S., Conord, C., & Lavigne, C. (2013). Does landscape composition affect pest abundance and their control by natural enemies? A review. Agriculture, Ecosystems and Environment, 166, 110–117.

Wissinger, S. A. (1997). Cyclic colonization in predictably ephemeral habitats: A template for biological control in annual crop systems. Biological Control, 10, 4–15.

Yoo, H. J. S., & O'Neil, R. J. (2009). Temporal relationships between the generalist predator, *Orius insidiosus*, and its two major prey in soybean. Biological Control, 48, 168–180.

SUPPLEMENTARY INFORMATION

Table S.1. Spearman rank correlations among landscape variables in 0.5 km buffers surrounding maize fields in Bujaraloz, Almacelles and La Seu. In bold, correlations with a probability < 0.05. A, alfalfa; C, cereal; M, maize; SN, semi-natural; F, fallow; Ps, pastures; O, orchards; OA, other annuals; U, urban; Crop, total crop area; Non Crop, non-crop area; *Poaceae*, cover types with high abundance of *Poaceae* following Annex 4 (sum of cereals, orchards, fallow); ED, edge density; SDI, Shannon diversity index.

Landscape variables		ρ de Spearman	P > |ρ|
A	ED	-0.2387	0.1959
C	ED	-0.2567	0.1633
C	A	0.3302	0.0697
O	ED	0.16	0.3900
O	A	0.329	0.0707
O	C	-0.0046	0.9806
F	ED	0.4436	**0.0124**
F	A	0.1728	0.3527
F	C	-0.274	0.1358
F	O	0.0607	0.7457
M	ED	-0.4851	**0.0057**
M	A	-0.2794	0.1279
M	C	0.0234	0.9005
M	O	-0.1152	0.5370
M	F	-0.3629	**0.0448**
Ps	ED	0.5905	**0.0005**
Ps	A	-0.6497	**<.0001**
Ps	C	-0.3621	0.0453
Ps	O	-0.0439	0.8146
Ps	F	0.0361	0.8470
Ps	M	-0.2214	0.2312
OA	ED	-0.0111	0.9529
OA	A	0.3887	**0.0307**
OA	C	0.2639	0.1514
OA	O	-0.0521	0.7807
OA	F	0.3184	0.0809
OA	M	-0.0382	0.8383
OA	Ps	-0.3161	0.0832
SN	ED	0.2299	0.2134
SN	A	-0.292	0.1110
SN	C	-0.3152	0.0841
SN	O	-0.502	**0.0040**
SN	F	-0.0155	0.9341
SN	M	-0.2798	0.1274
SN	Ps	0.4293	**0.016**
SN	OA	-0.2612	0.1558
U	ED	0.656	**<.0001**
U	A	-0.2397	0.1939
U	C	-0.1806	0.3308
U	O	0.1377	0.4601
U	F	0.1282	0.4918
U	M	-0.4252	**0.0171**
U	Ps	0.5123	**0.0032**
U	OA	-0.1615	0.3853
U	SN	0.2321	0.2090
SDI	ED	0.7788	**<.0001**
SDI	A	0.0978	0.6007

SDI	C	-0.0379	0.8394
SDI	O	0.1876	0.3123
SDI	F	0.6204	**0.0002**
SDI	M	-0.5535	**0.0012**
SDI	Ps	0.373	**0.0388**
SDI	OA	0.0998	0.5931
SDI	SN	0.2136	0.2487
SDI	U	0.6002	**0.0004**
Poaceae	ED	-0.575	**0.0007**
Poaceae	A	-0.0927	0.6197
Poaceae	C	0.486	**0.0056**
Poaceae	O	-0.1723	0.3539
Poaceae	F	-0.4461	**0.0119**
Poaceae	M	0.8399	**<.0001**
Poaceae	Ps	-0.4302	**0.0157**
Poaceae	OA	0.1501	0.4202
Poaceae	SN	-0.4513	**0.0108**
Poaceae	U	-0.4437	**0.0124**
Poaceae	SDI	-0.5608	**0.0010**
Ncrop	ED	0.4077	**0.0228**
Ncrop	A	-0.2101	0.2567
Ncrop	C	-0.4383	**0.0136**
Ncrop	O	-0.4026	**0.0247**
Ncrop	F	0.3897	**0.0302**
Ncrop	M	-0.4105	**0.0218**
Ncrop	Ps	0.4259	**0.0169**
Ncrop	OA	-0.1152	0.5373
Ncrop	SN	0.8792	<.0001
Ncrop	U	0.2765	0.1321
Ncrop	SDI	0.4257	**0.0170**
Ncrop	Poaceae	-0.6335	**0.0001**
Crop	ED	-0.6125	**0.0002**
Crop	A	0.2323	0.2086
Crop	C	0.4494	**0.0112**
Crop	O	0.285	0.1202
Crop	F	-0.4177	**0.0194**
Crop	M	0.5194	**0.0028**
Crop	Ps	-0.4827	**0.0060**
Crop	OA	0.1539	0.4085
Crop	SN	-0.7887	**<.0001**
Crop	U	-0.4919	**0.0049**
Crop	SDI	-0.5939	**0.0004**
Crop	Poaceae	0.7185	**<.0001**
Crop	Ncrop	-0.9185	**<.0001**

Table S.2. Spearman rank correlations among landscape variables in 1 km buffers surrounding maize fields in Bujaraloz, Almacelles and La Seu. In bold, correlations with a probability < 0.05. A, alfalfa; C, cereal; M, maize; SN, semi-natural; F, fallow; Ps, pastures; O, orchards; OA, other annuals; U, urban; Crop, total crop area; Non Crop, non-crop area; *Poaceae*, cover types with high abundance of *Poaceae* following Annex 4 (sum of cereals, orchards, fallow); ED, edge density; SDI, Shannon diversity index.

Landscape variables		ρ de Spearman	P > |ρ|
Rich	ED	0.5211	**0.003**
A	ED	0.0544	0.771
A	Rich	0.337	0.064
C	ED	-0.1044	0.576
C	Rich	0.0632	0.735
C	A	0.496	**0.005**
O	ED	0.3213	0.078
O	Rich	0.5336	**0.002**
O	A	0.6413	**0.000**
O	C	0.2241	0.226
F	ED	0.2133	0.249
F	Rich	0.3405	0.061
F	A	0.2562	0.164
F	C	-0.0554	0.767
F	O	-0.0042	0.982
M	ED	-0.4754	**0.007**
M	Rich	-0.412	**0.021**
M	A	-0.1113	0.551
M	C	0.3077	0.092
M	O	-0.1303	0.485
M	F	-0.3943	**0.028**
Ps	ED	0.3893	**0.030**
Ps	Rich	0.2187	0.237
Ps	A	-0.4493	**0.011**
Ps	C	-0.5394	**0.002**
Ps	O	-0.1882	0.311
Ps	F	-0.0869	0.642
Ps	M	-0.3976	**0.027**
OA	ED	-0.0714	0.703
OA	Rich	0.1024	0.584
OA	A	0.3549	0.050
OA	C	0.3747	**0.038**
OA	O	0.07	0.708
OA	F	0.1792	0.335
OA	M	0.1468	0.431
OA	Ps	-0.3053	0.095
SN	ED	0.2803	0.127
SN	Rich	0.0764	0.683
SN	A	-0.6079	**<0.001**
SN	C	-0.6256	**<0.001**
SN	O	-0.5017	**0.004**
SN	F	0.0538	0.774
SN	M	-0.488	**0.005**
SN	Ps	0.5448	**0.002**
SN	OA	-0.4025	**0.025**
U	ED	0.6442	**<.0001**
U	Rich	0.1591	0.393
U	A	-0.1735	0.351
U	C	-0.2699	0.142

U	O	0.0201	0.915
U	F	0	1.000
U	M	-0.4744	**0.007**
U	Ps	0.3449	0.057
U	OA	-0.1945	0.294
U	SN	0.3414	0.060
SDI	ED	0.7577	**<.0001**
SDI	Rich	0.6121	**<0.001**
SDI	A	0.4556	**0.010**
SDI	C	0.0315	0.867
SDI	O	0.385	**0.033**
SDI	F	0.4311	**0.016**
SDI	M	-0.531	0.002
SDI	Ps	0.2097	0.258
SDI	OA	0.1192	0.523
SDI	SN	0.1203	0.519
SDI	U	0.4478	**0.012**
Poaceae	ED	-0.4903	**0.005**
Poaceae	Rich	-0.3521	0.052
Poaceae	A	0.0673	0.719
Poaceae	C	0.6109	**<0.001**
Poaceae	O	-0.0235	0.900
Poaceae	F	-0.3648	**0.044**
Poaceae	M	0.902	**<.0001**
Poaceae	Ps	-0.5756	**0.001**
Poaceae	OA	0.2383	0.197
Poaceae	SN	-0.6394	**<0.001**
Poaceae	U	-0.48	**0.006**
Poaceae	SDI	-0.4948	**0.005**
Crop	ED	-0.4585	0.010
Crop	Rich	-0.2007	0.279
Crop	A	0.4444	**0.012**
Crop	C	0.625	**<0.001**
Crop	O	0.3682	**0.042**
Crop	F	-0.3624	**0.045**
Crop	M	0.6754	**<.0001**
Crop	Ps	-0.5484	**0.001**
Crop	OA	0.3568	**0.049**
Crop	SN	-0.9025	**<.0001**
Crop	U	-0.4716	**0.007**
Crop	SDI	-0.3476	**0.055**
Crop	Poaceae	0.8056	**<.0001**
Non crop	ED	0.3153	0.084
Non crop	Rich	0.1045	0.576
Non crop	A	-0.5032	**0.004**
Non crop	C	-0.6629	**<.0001**
Non crop	O	-0.4876	**0.005**
Non crop	F	0.3976	**0.027**
Non crop	M	-0.5677	**0.001**
Non crop	Ps	0.514	**0.003**
Non crop	OA	-0.3186	**0.081**
Non crop	SN	0.9023	**<.0001**
Non crop	U	0.3356	0.065
Non crop	SDI	0.2302	0.213
Non crop	Poaceae	-0.7319	**<.0001**
Non crop	Crop	-0.9633	**<.0001**

Table S.3. Poaceae identity, presence and abundance in the different landscape variables considered in the study. This table was used for landscape variable selection to use in *Z. scutellaris* models (see M&M). *(w)*, winter grasses; *(s)*, summer grasses; and *(p)*, perennial grasses.

Landscape variable	**Presence in study areas**	**Disturbance**	**Abundance *Poaceae***	**Species**	**References**
Edge Density	3/3	0/1	+	See Annex 5.	M. Lee, sampling of study sites edges (summer 2012)
Alfalfa	3/3	1	-	*Lolium sp. (w/p)* (La Seu) *Poa annua* L. *(w)* and others *Bromus catharticus (w)* *Cynodon dactylon (p)* *Digitaria sanguinalis (s)* *Elymus repens (s)* *Lolium sp. (w)* *Oryzopsis miliacea (s)* *Setaria glauca (s)* *Setaria pumila (s)* *Setaria verticilata (s)*	Personal observation Chocarro, Lloveras, & Fanlo, 2005 M. Lee, sampling study sites (summer 2012)
Cereal	3/3	2	+	*Triticum spp*. (w) *Hordeum vulgare* L. (w) *Cynodon dactylon* (p) (stubble)	Correal et al. 2006
Orchards	1/3	1/2	+	28 species of Poaceae (commonly found on field margins)	Juárez-Escario, Solé-Senan, & Conesa, 2010
Fallow	3/3	1		Depends on rotation and management *Lolium sp. (w/p), Bromus sp. (w), Avena sp (w), Cynodon dactylon (p)*	Correal et al. 2006
Maize	3/3	2	+	*Zea mays (s)* Co-occurring weeds *Echinochloa sp.(s)* *Setaria sp.(s)* *Sorghum halepense (p)*	Albajes, Lumbierres, & Pons, 2011
Pastures	1/3	1	+	*Hordeum murinum (w)* *Lolium sp.(p)* La Seu (associacion) *Trapogono-Lolietum multiflori* *Ophioglosso-Arrhenatheretum*	Consell Comarcal de l'Alt Urgell, 2005
Other annuals	2/3	2	-	Mainly sunflower	-
Semi-natural*	3/3	0	+/-	Depends on typology and region	Almacelles Solé-Senan et al., 2015

					Bujaraloz Conesa Mor., Castañeda del Álamo & Pedrol Solanes, 2011 La Seu d'Urgell Consell Comarcal de l'Alt Urgell, 2005
Urban	3/3	1	?	Data not available	

*Semi-natural area. Almacelles: Steppe and forested area. Bujaraloz: Steppe. La Seu: Forested and riparian area.

REFERENCES

Albajes, R., Lumbierres, B. & Pons, X., 2011. Two heteropteran predators in relation to weed management in herbicide-tolerant corn. Biological Control, 59(1), pp.30–36.

Chocarro, C., Lloveras, J. & Fanlo, R., 2005. Effects of winter grazing on spring production of lucerne under Mediterranean conditions. Grass and Forage Science, 60, pp.146–150.

Juárez-Escario, A., Solé-Senan, X.O. & Conesa, J.A., 2010. Diversity and richness of exotic weeds in fruit tree orchards in relation to irrigation management. Aspects of Applied Biology, 104. pp. 79–87.

Solé-Senan, X.O. et al., 2015. Contribució del patrimoni biològic municipal al Segrià: flora d'afinitat estèpica dels secans. Shikar, Revista del Centre d'Estudis Comarcals del Segrià, 1, pp.117–122.

Table S.4. Poaceae cover (%) in field edges determined by 1 to 3 field edges per site for the three study regions. Main species recorded in each of the regions (M. Lee).

	Almacelles (n=15)	**Bujaraloz (n=9)**	**La Seu (n=5)**
	mean ±SE	mean ±SE	mean ±SE
Poaceae cover (%)	32.27 ±5.97	41.61 ±7.46	40.24 ±10.95
Total vegetation cover (%)	60.22 ±6.84	79.91 ±4.04	75.08 ±4.97
Main species	*Arundo donax (p)* *Avena sp.(w)* *Avena sterilis (w)* *Brachypodium phoenicoides (p)* *Bromus catharticus (p)* *Bromus diandrus (w)* *Bromus sp.(w)* *Cynodon dactylon (p)* *Desmazeria rigida(p)* *Digitaria sanguinalis(s)* *Echinochloa crus-galli (s)* *Elymus repens (p)* *Hordeum murinum (w)* *Lollium multiflorum (w)* *Lollium rigidum (p)* *Lygeum spartum (p)* *Panicum sp.(s)* *Phragmites australis (p)* *Poa pretense (p)* *Setaria adhaerens(s)* *Setaria sp.(s)* *Setaria verticilata (s)* *Sorghum halepense (p)*	*Bromus catharticus (p)* *Bromus sp.(w)* *Cynodon dactylon (p)* *Digitaria sanguinalis (s)* *Echinochloa crus-galli (s)* *Elymus repens(p)* *Hordeum murinum(w)* *Oryzopsis milliacea (s)* *Poa sp. (p?)* *Setaria spp.(s)* *Setaria adhaerens (s)* *Setaria glauca (s)* *Setaria pumila (s)* *Setaria verticilata (s)* *Setaria viridis(s)* *Sorghum halepense (p)*	*Bromus catharthicus(p)* *Cynodon dactylon (p)* *Dactylis glomerata (p)* *Digitaria sanguinalis (s)* *Echinochloa crus-galli (s)* *Elymus repens (p)* *Lolium sp. (p?)* *Panicum sp.(s)* *Setaria adhaerens (s)* *Setaria sp.(s)*

Plant species cover in 1x10m^2 rectangle was estimated using a modified Braun–Blanquet scale, the ordinal scale was later transformed into percentage values following van der Maarel (2007).

REFERENCE

van der Maarel , E., 2007. Transformation of cover-abundance values for appropriate numerical treatment: Alternatives to the proposals by Podani. *Journal of Vegetation Science* 18, pp.767 –770.

Table S.5. Correlations between *Z. scutellaris* and *Orius* spp. catches in maize, alfalfa and semi-natural habitat. In bold, correlations with substantial support (α=0.05).

	Habitat 1	Habitat 2	ρ de Spearman	Prob > |ρ|
Zyginidia scutellaris	Maize	Alfalfa	**0.5878**	**0.0005**
	Maize	Semi-natural	**0.6804**	**<.0001**
	Semi-natural	Alfalfa	**0.7109**	**<.0001**
Orius spp.	Maize	Alfalfa	**0.5771**	**0.0008**
	Maize	Semi-natural	0.2513	0.1803
	Semi-natural	Alfalfa	0.1588	0.4018

CHAPTER 2

Innate and learned prey-searching behavior in a generalist predator

Agnès Ardanuy, Ramon Albajes and Ted C. J.Turlings

ABSTRACT

Early colonization by *Zyginidia scutellaris* leafhoppers might be a key factor in the attraction and settling of generalist predators such as *Orius* spp. in maize fields. In this paper we aimed to determine whether our observations of early season increases in field populations of *Orius* spp. reflect a specific attraction to *Z. scutellaris*-induced maize volatiles, and how the responses of *Orius* predators to herbivore-induced volatiles (HIPVs) might be affected by previous experiences on plants infested by herbivorous prey. We therefore examined the innate and learned preferences of *Orius majusculus* towards volatiles from maize plants attacked by three potential herbivores with different feeding strategies, leafhopper *Z. scutellaris* (mesophyll feeder), lepidopteran *Spodoptera littoralis* (chewer) and leafhopper *Dalbulus maidis* (phloem feeder). In addition, we examined the volatile profiles emitted by maize plants infested by the three herbivores. Our results show that predators exhibit a strong innate attraction to volatiles from maize plants infested with *Z. scutellaris* or *S. littoralis*. Previous predation experiences in the presence of HIPVs influenced the predator's odor preferences. The innate preference for plants with cell or tissue damage can be explained by the fact that these plants released far more volatiles than plants infested by the phloem-sucking *D. maidis*. Yet, a predation experience on *D. maidis*-infested plants significantly increased the choices for *D. maidis*-induced maize volatiles. After *O. majusculus* experienced L3-L4 larvae (too large to serve as prey) on *S. littoralis*-infested plants they showed reduced attraction towards these plants and an increased attraction towards *D. maidis*-infested plants. When offered young larvae *S. littoralis*, which are more suitable prey, preference towards HIPVs was similar to that of naive individuals. The HIPVs from plants infested by herbivores with distinctly different feeding strategies showed clearly distinguishable quantitative differences for (Z)-3-hexenal and (E)-2-hexenal and methyl salicylate. These compounds might serve as reliable indicators of prey presence and identity for the predator. Our results support the notion that feeding by *Z. scutellaris* results in the emission of maize's HIPVs that initially recruit of *Orius* spp. into maize fields.

Key words: *Orius* spp., *Zyginidia scutellaris*, *Spodoptera littoralis*, *Dalbulus maidis*, associative learning, innate, HIPVs, maize.

The content of this chapter has been published in Journal of Chemical Ecology (2016), 42(6), 497-507.

INTRODUCTION

Feeding on plants by herbivores triggers the emission of complex blends of volatile compounds (herbivore-induced plant volatiles, HIPVs). These volatiles can serve as signals for natural enemies to locate their prey (Dicke and Sabelis, 1987; Turlings et al., 1990), as they can provide them with valuable information on the identity and quality of potential prey on the plants (Dicke 1999; Clavijo McCormick et al. 2012). In the past two decades it has been reported that volatile blends released by plants vary widely between different combinations of plant and herbivore (De Moraes et al., 1998; Van Den Boom et al., 2004), between different herbivores on the same plant species (e.g Turlings et al. 1998, Leitner et al. 2005, Delphia et al. 2007, Gosset et al. 2009, Hare and Sun 2011) and between the same herbivore on different genotypes of the same plant species (Degen et al., 2004; Glinwood et al., 2011). It remains largely unclear whether the composition of the volatile blends induced by different herbivore species differs consistently enough to indicate not only that the plants are damaged by herbivores, but also the identity of the herbivore species causing the damage (Allison and Hare, 2009; Clavijo McCormick et al., 2012). Yet, several studies have shown that parasitoids are more attracted to volatiles emitted by plants under attack by their specific host than the volatiles from plants with non-hosts (Chabaane et al., 2014; De Moraes et al., 1998). These distinctive volatile profiles induced by various herbivores could be caused by different feeding modes and/or specific elicitors in the insects' oral secretions when they come in contact with the damaged plant tissue during the feeding process (Leitner et al., 2005; T. C. . Turlings et al., 1998).

Under Mediterranean conditions maize stands are colonized early in the season by the leafhopper *Zyginidia scutellaris* (Herrich-Schäffer), which is the first herbivore to establish in significant numbers on the aerial part of the maize plant (Pons and Albajes, 2002). Leafhopper populations may build up and reach high densities during summer in the maize fields, though direct damages are rarely of economic importance. *Z. scutellaris* is a mesophyll feeder, preferentially on the older leaves, where it causes pale stripes. In a previous study we observed a correlation between cumulative numbers per plot and season of *Orius* spp. and *Z. scutellaris* (Albajes et al., 2011). We therefore hypothesize that the early colonization of maize plants by maize leafhopper is a key factor for attraction and establishment in maize fields of generalist predators as *Orius spp*. which is the prevalent on-plant predator. *Orius* spp. preys on small insects (e.g. thrips, scales, aphids, psyllids, small caterpillars and the eggs of various insects) and mites (Lattin 1999) and are regularly found on cereals, maize and alfalfa (Madeira et al., 2014; Pons et al., 2005b), as well as on weeds, depending on plant phenology and crop management.

Generalist predators like *Orius* spp. may feed on multiple prey that are heterogeneously distributed in space and time and consequently face a challenging optimal foraging task. When prey declines to low levels, predatory arthropods switch from local searching to dispersal behavior (Symondson et al. 2002), which is also the case for *O. majusculus* (Reuter) (Montserrat et al. 2004). To locate their prey in an environment with numerous potential host plants and prey, the predators rely on both their innate olfactory and/or visual preferences and memory (Drukker et al., 2000; Dukas, 2008; Takabayashi et al., 2005). The behavioral responses of natural enemies to HIPVs are known to have a genetic basis (at least for mites Margolies et al. 1997, and parasitoids Gu and Dorn 2000), but are also plastic and can be modified through associative learning (Dukas, 2008). The ability to associate odors with rewards is well established for parasitoids (Papaj and Lewis, 1993; Turlings et al., 1993; Vet et al., 1995). This has been much less studied for predatory arthropods (see Drukker et al. 2000, Deboer et al. 2005, Glinwood et al. 2011, Lins et al. 2014).

In this paper we aim to determine whether our field observations of *Orius* spp. recruitment into maize fields reflect a specific attraction of the predators to HIPVs from *Z. scutellaris*-infested plants, and whether such attraction is affected by experience during previous prey encounters. To test this, we examined: (1) the innate preferences of *Orius majusculus* towards maize plants attacked by three herbivores with different feeding strategies, the leafhopper *Z. scutellaris* (mesophyll feeder), the leafhopper *Dalbulus maidis* (Delong y Wolcott) (phloem feeder), and the lepidopteran *Spodoptera littoralis* (Boisduval) (chewer); (2) the potential preference change in the case of previous prey experience on infested maize plants and the nature of this experience (rewarding/no-rewarding); and (3) the volatile profiles emitted by maize plants infested by the three herbivores.

MATERIAL AND METHODS

Plants and Insects

Maize seeds of the variety Delprim were sown in commercial soil (Ricoter Aussaaterde ®) in individual bottom-pierced plastic pots (ø 4 cm, 11 cm high). Plants were grown under natural light conditions (16:8 h L: D) in a greenhouse (24±5 ℃) and were watered as needed.

All insects were reared under controlled conditions (16:8 h L:D, 24±5 ºC) at the Université de Neuchâtel (Switzerland). A colony of the leafhopper *Z. scutellaris* was obtained from cereal fields at the Universitat de Lleida (Spain) and *D. maidis* was established from individuals provided by Dr. J. Bernal from a greenhouse colony maintained at Texas A&M (USA). Both colonies were reared on maize (varieties Delprim and B73). Eggs of *S. littoralis* were provided by Syngenta (Stein, Switzerland), and larvae were reared on wheat germ based artificial diet (Turlings et al., 2004).

The predator *O. majusculus* came from an established colony at the Universitat de Lleida, which is renewed every year with new individuals collected in maize fields. The colony was fed with frozen eggs of *Ephestia kuehniella* (Biotop S.A., France) as prey and green beans

(*Phaseolus vulgaris L.*) as egg-laying substrate. We considered that the predators that did not experience a maize plant were "naive" in the sense that they never encountered prey in association with maize and herbivore-induced maize volatiles. Females of *O. majusculus* were used in all experiments when they were more than a week old. In the innate preference bioassay “naïve” females coming directly from the rearing were used (12 per replicate), for the experience bioassay both “naïve” and “experienced” predators were used (12 per experience group). The day before the experiment each predator was placed individually in a 1,5mL eppendorf tube and provided with water by means of a wet cotton ball.

Odor Sources

Maize plants that were used for the experiments had 3 fully developed leaves. A day before the experiments plants were enclosed in glass bottles and infested with adults of *Z. scutellaris*, adults of *D. maidis*, or larvae of *S. littoralis*. Ten adult leafhoppers were freely released in the glass bottle with the help of an aspirator for both *Z. scutellaris* and *D. maidis* treatments. These densities were used in order to account for differences in the field abundances of leafhopper pests (Pons and Albajes, 2002) and to produce reliable response of maize plants without impairing their physiology. To infest plants with *S. littoralis*, five second instar were transferred with a brush to maize leaves. This density of *S. littoralis* was chosen because it resulted in a similar response in the maize plants as infestation by ten *Z. scutellaris* (see results), allowing for a better qualitative comparison of the plant’s HIPVs. After infestation, the bottles were maintained at laboratory temperature with a L16:D8 light cycle. The glass bottles were attached to the olfactometer setup (see Turlings et al., 2004).

Innate Prey Preference Bioassay

To test *O. majusculus* preference for *Z. scutellaris* as prey on maize we tested its attractiveness in a 4-arm olfactometer (for details see D’Alessandro and Turlings 2005) in a choice situation with a plant infested by each of the two other potential prey. In a first experiment, we tested *Z. scutellaris*-infested plants against *S. littoralis*-infested plants (n=7). In the second experiment we tested *Z. scutellaris*-infested plants against *D. maidis* (n=7). In both experiments we included an unharmed plant and an empty bottle as controls. The position of the odor sources was randomly assigned each experimental day to avoid any position-bias.

Purified and humidified air entered each odor source bottle at 1.2 l/min (adjusted by a manifold with four flowmeters, Analytical Research System, Gainesville, FL, USA) via Teflon tubing and carried the volatiles to the olfactometer compartment. Half of the air (0.6 l/min/olfactometer arm) was pulled out via volatile collection filters that were attached to the top of each odor source bottle (see “Collection and analyses of HIPVs”). These traps were connected to a vacuum pump via Tygon tubing and flow meters, and airflows were balanced with a pressure gauge.

Half an hour before an experiment started, Eppendorf tubes containing *O. majusculus* females were placed in a polystyrene box containing a plastic cooling block. In preliminary tests (not shown) we saw that this cooling pre-treatment suppressed the activity of the insects and as a consequence they were more receptive to odor sources and less likely to choose randomly. We adapted the olfactometer to the behavior of the predator by turning the central release arena upside-down (see design in D'Alessandro and Turlings 2005) so insects would orient downwards, escaping the light, towards the arms of the olfactometer. We released insects one by one and gave them 20 minutes to make a choice. When an insect entered an arm and reached the screw cap fitting we considered it to have made a choice. Twelve females were tested per replicate. The experiment was performed 7 times on different days. This resulted in 7 independent replicates. All olfactometer tests were conducted between 10 am and 4pm.

Prey Experience Bioassay

Two series of assays were conducted to test the influence of experience on *O. majusculus* preference. In the first series we evaluated the preference of *O. majusculus* when experienced to three herbivores with distinct feeding modes. In the second series we evaluated the response of the predator when experienced on prey, *S. littoralis* larvae*,* which we hypothesize that can provide both positive and negative experiences depending on their developmental stage (size). Small larvae (first instar) can be readily preyed upon by the minute predator, signifying a positive experience, whereas encounters with aggressive larger larvae could constitute a negative experience.

To provide the predators with odor experiences the following procedure was used. For the three herbivore bioassay on day one, 80 predators were individually placed in eppendorf tubes of 1mL, and plants were enclosed one-liter plastic (PET) bottles and exposed to one of the three herbivores *Z. scutellaris, D. maidis* or *S. littoralis* in the same density as in the olfactometer odor sources (2 plants per treatment). The following day (day two), additional prey of each of the herbivore treatments were added to each bottle to ensure sufficient prey to *O. majusculus* females. The extra prey consisted of either 25 nymphs for the leafhopper treatments or 20 second to third instar *S. littoralis* larvae. The predators were split in four groups of experience. The first three groups were transferred into the bottles of each of the herbivore treatments. A first group was transferred to the plants infested by *Z. scutellaris* (Zs experience), the second to *D. maidis*-infested plants (Dm experience), and the third group to S. *littoralis*-infested plants (Sl experience). The fourth group of predators served as the control (control experience) with insects that were placed in two plastic cages containing *E. kuehniella* eggs and a bean pod.

The same procedure was used to examine the importance of *S. littoralis* size (developmental stage) in affecting *O. majusculus* responses after the associative experiences. Based on the *Predation bioassay*, we hypothesized that preying on young larvae (L1-L2) would constitute a rewarding experience to *O. majusculus* and that older larvae (L3-L4) would constitute an unrewarding experience. Consequently, we experienced *O. majusculus* females with L1-L2 larvae (Sl-s) and L3-L4 larvae (Sl-B) following the procedure described earlier for the three herbivores with different feeding modes. In this case the additional prey added to the odor sources consisted of thirty L1-L2 larvae for the small larvae experience and eight L3-L4 larvae for the large larvae experience. A control group was also included (control experience).

The day before the experiment (day three), each predator was again placed in a 1,5 mL Eppendorf tube and provided with water by means of a wet cotton ball. Half an hour before an experiment started, predators were placed in groups of 6 according to experience group (2 Eppendorf tubes per experience group, 12 insects in total) and placed in a polystyrene box containing a plastic cooling block.

We tested Zs, Sl, Dm and empty odor sources for both experience bioassays. As in the innate bioassay the position of the odor sources was randomly assigned for each experimental run to avoid position-bias and we used the release arena of the olfactometer upside-down. We released insects in groups of six and gave them up to thirty minutes to make a choice. On each experimental day there were two releases per experience group, testing a total of twelve females experienced with the same herbivore/treatment for each olfactometer set-up. Once we had tested the first release of all the experience groups we rotated the olfactometer 90° and then tested the second release for all treatments. The order in which we tested the different experience groups was random. Again, when an insect entered an arm and reached the screw cap fitting we considered it to have made a choice. The experiment was performed 7 times on different days for the three herbivore experience bioassay and 8 times for the *S. littoralis* experience bioassay. Each of these days was considered as an independent replicate.

Predation Bioassay

A predation bioassay was conducted in order to evaluate the performance of O. *majusculus* on each of the offered prey. Arenas made of petri dishes (5cm in diameter) were used in the experiment. Each petri dish contained a filter paper moistened with water on which we placed a piece of maize leaf of approximately 4 cm of length. Prey corresponding to experience groups (see above) were added to the arena in groups of five. We tested four treatments: (1) *Z. scutellaris* and (2) *D. maidis* nymphs of 2nd to 4th stage (3) *S. littoralis* L2 instar larvae fed on maize leaves and (4) *S. littoralis* L3-L4 instar larvae fed on maize leaves. Thirty minutes later we introduced an *O. majusculus* female in each dish that had been starved for 24h, and left them for 24h. The next day we counted the number of killed prey in each of the arenas. We differentiated killed prey by *O. majusculus* females from missing prey. We compared the number of dead prey with those in control dishes without a predator. We performed the experiment two times with 8 replicates for each treatment.

Collection and Analysis of Volatiles

We collected volatiles of each odor source during the experience bioassays in the olfactometer, using adsorbent traps consisting of a glass tube (4 mm ID) packed with 25 mg Super-Q polymer (80–100 mesh) (Alltech Associates, Deerfield, Illinois, USA) for 5 hours. Each trap was attached horizontally to the top of an odor source bottle via a screw-cap outlet and connected via Tygon tubing to a flowmeter (Analytical Research System) and a vacuum pump. Air was pulled through each trap at a rate of 0.6 l/min for 5h, during each behavioral bioassay. Afterwards, the traps were extracted with 150 µl dichloromethane (Suprasolv, Merck, Dietikon, Switzerland), and 200 ng of n-octane and n-nonyl acetate (Sigma, Buchs, Switzerland) in 10 µl dichloromethane were added to the samples as internal standards. All extracts were stored at -80°C until analyses. Traps were washed with 3 ml dichloromethane before they were re-used for a next collection. Volatiles were identified with a gas chromatograph (Agilent 6890 Series GC system G1530A) coupled to a mass spectometer (Agilent 5975C VL MSD). A 2-µl aliquot of each sample was injected in the pulsed splitless mode onto an apolar capillary column (HP-1, 30 m, 0.25 mm ID, 0.25 µm film thickness; Agilent J&W Scientific, USA). Helium was used as carrier gas at constant pressure (15 psi). After injection, the column temperature was maintained at 40°C for 3 min and then increased to 100°C at 8°C/min and subsequently to 200°C at 5°C per min followed by a post-run of 5 min at 250°C. Chemstation software was used to estimate the quantities of all major components by comparison of the peak areas of each volatiles to the peak areas the internal standards. The detected volatiles were identified by comparison of their mass spectra with those of the NIST 05 library and by comparison of retention times with those from previous analyses.

Statistical Analysis

We analyzed data from innate preference bioassays with a generalized linear model (GLM) with a Poisson distribution, where the number of choices by *O. majusculus* females per replicate was the response variable, and the plant odor sources and the replicate and their interaction the explicative variables. A GLM with a Poisson distribution was also used for the experience bioassays' analyses. A global analysis was performed where the response variable was the number of *O. majusculus* females per arm, experience group and replicate; the explicative variables were treatment (odor sources), experience group, replicate, and their interactions. Next, we performed an individual analysis for each of the odor sources (Dm, Sl, Zs, empty) in order to test differences between the frequencies of choice by the four/three experience groups. We considered a response to be learned when we detected a change in the choice of odor sources in experienced insects respective of the control (naïve) insects. Multiple comparisons were calculated with Tukey's HSD test.

The proportion of *O. majusculus* females that fed on the offered prey or not in the predation bioassay were analyzed by a GLM with a Binomial distribution, with treatment, experiment and their interactions as explicative variables. As experimental day and the interaction were non-significant, they were removed from the final model. The number of prey eaten by *O. majusculus* females per treatment was analyzed with a GLM with a Poisson distribution; in this case the interaction was non-significant and was removed from the final model. Multiple comparisons were calculated with Tukey's HSD test.

The amounts of plant volatiles were analyzed in two different ways. Firstly, we compared the amounts for each compound among treatments using a nonparametric Kruskal-Wallis test followed by Dunn's test and adjusting p-values for multiple pairwise comparisons with the Bonferroni correction. When compounds were not detected in a treatment, analyses were performed excluding that treatment. Secondly, PLS-DA was used to determine whether samples belonging to specific herbivore treatments could be separated based on qualitative and quantitative differences in volatile emissions. The array of HIPVs may be composed of a large number of compounds and should be properly considered as an inter-correlated, multivariate suite of traits (Hare, 2011). Many of these compounds share common precursors and in some cases, particular ratios of several compounds can be the product of a single enzyme. One example is terpene synthase TPS10 in maize that forms (E)-β-farnesene, (E)- α-bergamotene, and other herbivory-induced sesquiterpene hydrocarbons from the substrate farnesyl diphosphate (Schnee et al., 2006). As a consequence compounds do no vary independently, and multivariate statistics that take into account the patterns of correlations of variables are required to determine statistically significant variation (van Dam and Poppy 2008, Hare 2011). The number of the model components was assessed graphically by checking plots of the error rate and the proportion of intergroup variance explained relative of the number of PLS components. Statistical significance of the obtained PLS-DA model was determined by m-fold cross-validation (m=7) and 999 permutations. An error rate value (%) was calculated to measure the accuracy of the classification by averaging the number of misclassifications (NMC) from each round of the cross-validation. The results of the PLS-DA analysis were represented in score plots, which reveal the sample structure according to the model components, and loading plots, displaying the contribution of the volatile emission to these components. Volatile compounds were subsequently ranked according to their respective variable importance of projection (VIP) score. The highest VIP scores reflect the relatively important contribution of compounds to the discrimination between groups. Data were log-transformed, mean-centered, and scaled to unit variance before they were subjected to the analysis. PLS-DA analysis and validation was performed using mixOmics (González et al., 2011) and RVAideMemoire (Hervé, 2014) packages. All statistical analyses were performed using R (R Development Core Team 2005).

RESULTS

Innate Behavior

We first tested the innate attraction of *O. majusculus* to the volatile blend emitted by plants infested with *Z. scutellaris*, relative to the attraction to volatiles from plants with any of the alternative prey or clean maize plants. *O. majusculus* females were attracted to *Z. scutellaris* infested plants, but when offered simultaneously, they did not distinguish between *Z. scutellaris*-infested plants and *S. littoralis*-infested plants (Fig. 1a, choice χ^2_3= 17.84, P< 0.001; replicate χ^2_1= 16.57, P=0.26; interaction χ^2_3= 15.98, P=0.90). However, *O. majusculus* markedly preferred Z. *scutellaris*-damaged plants when paired with *D. maidis*-damaged plants (Fig. 1b, choice χ^2_3= 17.12, P< 0.001; replicate χ^2_1= 16.94, P=0.68; interaction χ^2_3= 11.68, P=0.15). *D. maidis*-infested plants were as unattractive as uninfested plants or clean air (empty arm).

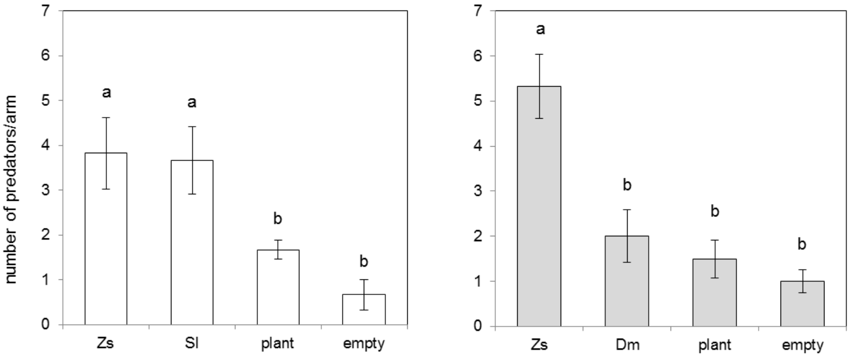

Fig. 1 Choices for herbivore-induced plant odors by *O. majusculus* shown as the average numbers (+SE) per trial. Four odor sources were tested in each of the two experiments Dm= *D. maidis*-damaged plant; Sl= *S. littoralis*-damaged plant; Zs= *Z. scutellaris*-damaged plant; Plant= maize plant; empty = empty arm. Different letters indicate significant differences between treatments ($p < 0.05$).

Learned Behavior

We also tested the effect of a previous prey experience on predator odor preferences when offered the "experienced" prey infested-plant and two alternative prey-infested plants as odor sources. *O. majusculus* females were given an experience by placing them on maize plants with *Z. scutellaris* (Zs), *S. littoralis* (Sl), or *D. maidis* (Dm), or providing them with a diet of only insect eggs without a plant (control, C).

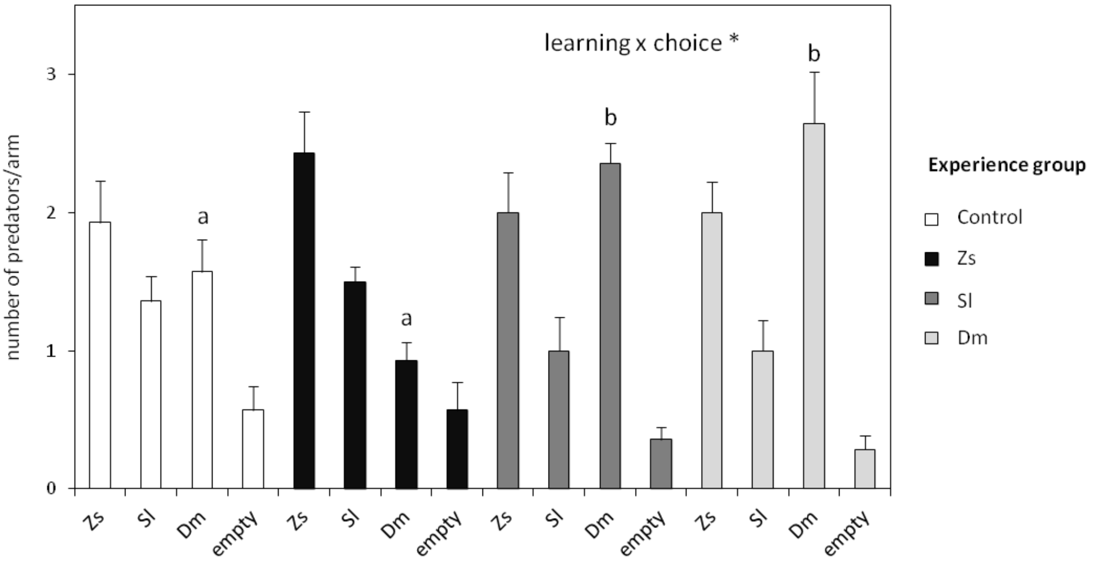

Fig 2 Choices for herbivore-induced plant odors by *O. majusculus* females with different previous prey-experiences, shown as the average numbers (+SE) of predators per release group of six. Four odor sources were tested Dm = *D. maidis*-damaged plant; Sl= *S. littoralis*-damaged plant; Zs= *Z. scutellaris*-damaged plant; empty = empty arm. Prey experience was provided on infested plants with extra prey of three herbivores Dm= *D. maidis*; Sl= *S. littoralis*; Zs= *Z. scutellaris*; and a Control with only *E. kuheniella* eggs (in the absence of a plant). Different letters indicate significant differences between treatments (p < 0.05). * indicates a significant interaction (p<0.05).

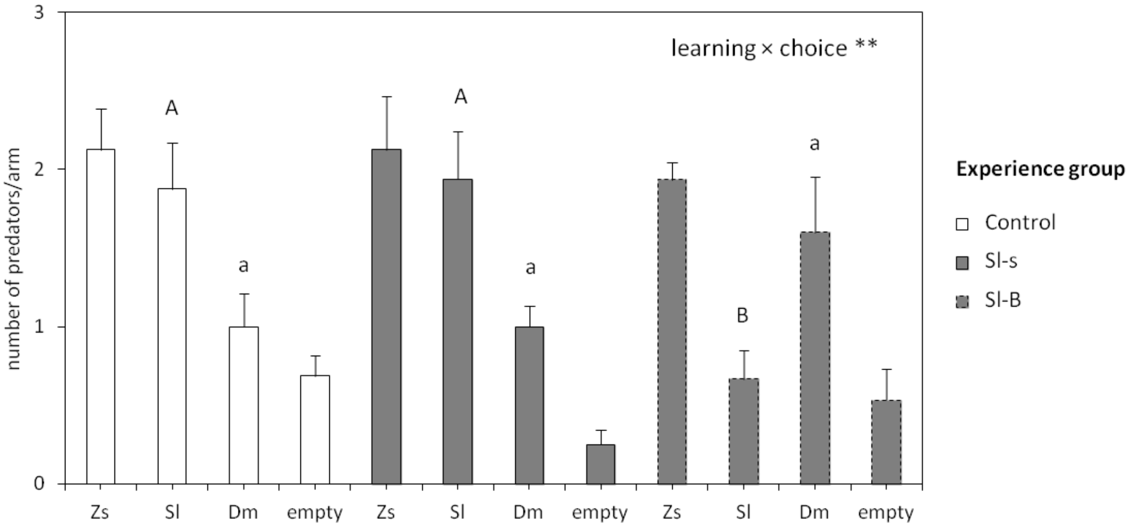

Fig. 3 Choices for herbivore-induced plant odors by *O. majusculus* females with different previous prey-experiences, shown as the average numbers (+SE) of predators per release group of six. Four odor sources were tested Dm = *D. maidis*-damaged plant; Sl= *S. littoralis*-damaged plant; Zs= *Z. scutellaris*-damaged plant; empty = empty arm. Prey experience was provided on *S. littoralis* infested plants of two sizes Sl-s= small and Sl-B= big, and a Control with only *E. kuheniella* eggs (in the absence of a plant). Different letters indicate significant differences between treatments (p < 0.05). ** indicates a significant interaction (p<0.01).

The prey-host plant experiences affected the predator's choices for the *D. maidis* odor source, but not the choices for the other two infested plant types (Fig. 2). This was reflected in a significant effect of the type of experience and the interaction term (choice × experience) in the model (interaction χ^2_9= 130.4, P=0.02). Compared with control predators, the number of choices for Dm was increased by two-thirds in Dm-experienced individuals (experience χ^2_3= 25.17, P=0.023; replicate χ^2_6= 20.61, P=0.60; interaction χ^2_{18}= 14.85, P=1; Fig. 2). Interestingly, this increase in preference for Dm was also observed for Sl-experienced predators, whereas Zs-experienced predators showed an increased tendency to avoid Dm in favor of the Zs treatment (Fig. 2).

Females of *O. majusculus* can experience *S. littoralis* prey positively or negatively depending on the larval instar encountered (see Results: *Predation on offered prey*), and their subsequent responses are affected accordingly (significant choice × experience term in the model χ^2_8= 178.15, P<0.01, Fig. 3). After 24h Sl-B experience predator females were mostly found hidden in the plant or bottle, and all big *S. littoralis* larvae were recovered alive; while for Sl-s treatment, predators were active in the bottle and few larvae from the Sl-s treatment were recovered. When experiencing a rewarding predation on *S. littoralis* (Sl-s), predator preference for odor treatments was similar to that of the predators from the control. In contrast, after facing an unrewarding experience on large larvae (Sl-B) *O. majusculus* were less attracted to the odor *S. littoralis*-infested plants (experience χ^2_2= 32.71, P<0.01; replicate χ^2_7= 23.50, P=0.24; interaction χ^2_{14}= 14.14, P=0.81) and tended to be more attracted to *D. maidis*-infested plants (Fig. 3), similar to what was found during the first experience bioassay. The proportion of females that did not choose was also similar for both bioassays.

Predation on Offered Prey

We performed a predation acceptance experiment to estimate the preference of *O. majusculus* females for the different prey offered in the experience bioassays. Predators fed on all prey offered (pie chart in Fig. 4), but the proportion of females that fed differed considerably between treatments (χ^2_3= 67.5, P< 0.001). Almost all predators that were offered small *S. littoralis* or *Z. scutellaris* fed on these prey, but only a small fraction of the predators managed to consume one of the large *S. littoralis* larvae. Overall, there were clear differences in the number of prey killed by females after 24h (experiment χ^2_1= 4.60, P=0.03; treatment χ^2_3= 102.9, P< 0.001; Fig 4). Predators were most successful feeding on small larvae and *Z. scutellaris* nymphs, followed by *D. maidis* nymphs and large *S. littoralis* larvae (Fig. 4). The large differences in consumption of small and large *S. littoralis* by *O. majusculus* females, are likely to reflect rewarding and non-rewarding experiences respectively, as is evident from their subsequent responses to the odor sources.

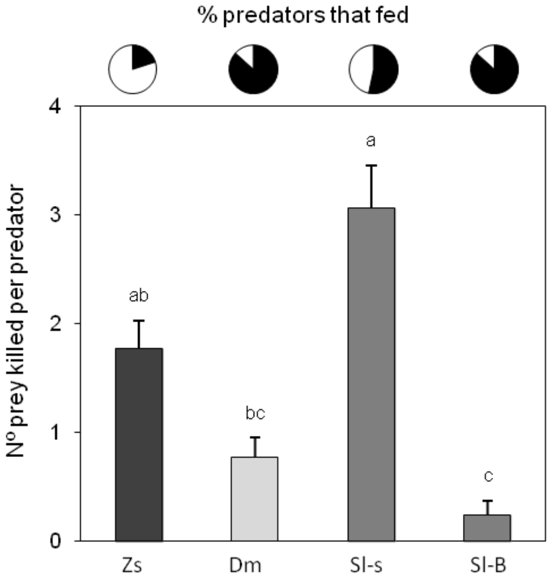

Fig. 4 Proportion of *O. majusculus* females that fed on offered prey during 24h (black proportion in pie charts), and average number of herbivorous prey eaten by the predators (bar graph) shown as the average (+SE). Six treatments were offered: Zs= 2nd to 4th instar *Z. scutellaris*; Dm= 2nd to 4th instar *D. maidis*; Sl-s= 1st to 2nd instar *S. littoralis*; Sl L3-B= 3rd to 4th instar *S. littoralis*. Different letters indicate significant differences between treatments ($p < 0.05$).

Volatile Profiles

Volatile blends from plants attacked by the herbivores *Z. scutellaris*, *D. maidis* and *S. littoralis* from the first experience experiment were analyzed. Twenty compounds were identified from previous studies (Table 1) (Degen et al., 2004; Erb et al., 2010; T. C. . Turlings et al., 1998), plus an unknown compound also detected by Turlings et al. (1998), probably a nitrogen containing compound present also in healthy plants. Twenty of those volatile compounds were quantified (Table 1). A PLS-DA analysis of volatiles emitted by plants infested with *Z. scutellaris*, *S. littoralis* and *D. maidis* showed two significant principal components (PLS), explaining 72.12 and 7.5 % of the total variance, respectively (Fig.5). The error rate value (%) calculated by permutation was < 3% (p=0.001). The first component (PLS1) separated the volatile blends based on the amount of emitted volatiles caused by the feeding of each of the three herbivores, exposing the quantitative differences in emission rates. The second component (PLS2) separated blends qualitatively according to the presence or absence of certain compounds or a difference in their proportions in the total blend. These discriminating compounds were the three that had a VIP value higher than 1 (Table 1). In decreasing order of importance, the compounds were the green leaf volatiles (GLVs) (Z)-3-hexenal and (E)-2 hexenal, and methyl salicylate (Table 1, Fig. 5). Globally, *Z. scutellaris* treated plants emitted the largest amounts of volatiles, followed by *S. littoralis* plants, whereas *D. maidis* plants emitted the smallest amounts and number of volatile compounds (Table 1, Fig. 5). Unlike *S. littoralis,* neither *Z. scutellaris* nor *D. maidis* feeding resulted in detectable release of (Z)-3-hexenal and (E)-2 hexenal. On the other hand methyl salicylate was detected in both Sl and Zs treatments, but its proportion was highest for Zs infested plants (Table 1, Fig. 5).

Table.1 Volatile emissions (ng/h) of plants infested by *Z. scutellaris*, *S. littoralis* or *D. maidis* and variable influence on projection (VIP) values for each compound for the PLS-DA model. Amounts of each compound were compared among treatments using a non-parametric Kruskal-Wallis test followed by Dunn's test and adjusting p-values for multiple pairwise comparisons with the Bonferroni correction (*p <0.05, **p < 0.01, ***p < 0.001). Compounds denoted with "N" were only tentatively identified by comparison of their MS to that reported in libraries. In bold compounds with VIP>1. n.d. not detected, d detected in a small fraction of samples.

		Z. scutellaris		***S. littoralis***		***D. maidis***			
	VIP	**ng/h**	**±SE**	**ng/h**	**±SE**	**ng/h**	**±SE**	**χ^2**	**P**
1. Unknown	0.16	3.02	0.11	3.10	0.13	3.39	0.14	1.21	n.s
2. (Z)-3-hexenal	**2.27**	**n.d**		**3.37**	**0.35**	**n.d**			
3. (E)-2-hexenal	**1.97**	**n.d**		**3.02**	**0.37**	**n.d**			
4. β-myrcene	0.52	3.78a	0.39	2.86a	0.18	0.83b	0.11	11.96	*
5. Z-3-hexenyl acetate	0.82	13.39	1.97	4.75	0.50	n.d		3.92	*
6. (Z)-β-ocimene	0.74	1.06	0.16	0.80	0.08	n.d		0.15	n.s
7. Linalool	0.77	100.97a	8.06	72.38a	3.78	19.51b	1.59	13.30	*
8. DMNT	0.68	45.22a	6.08	15.88a	1.27	0.92b	0.12	16.20	***
9. Phenyl-methyl acetate	0.82	6.98	1.68	1.79	0.23	n.d		3.43	·
10. Methyl salicylate	**1.07**	**2.01**	**0.29**	**0.49**	**0.08**	**n.d**		**4.48**	*****
11. 2-phenethyl acetate	0.83	4.19	0.58	1.40	0.15	n.d		3.92	*
12. Indole	0.89	102.57	14.40	43.30	2.54	n.d		1.47	n.s
13. Methyl anthranilate		d(3/7)		d(1/7)		n.d			
14. (E)-geranyl acetate	0.88	29.26	3.68	11.99	0.90	n.d		3.43	·
15. E-β-caryophyllene	0.80	22.98	5.02	6.72	0.54	d(2/7)		0.33	n.s
16. (E)-β-bergamotene	0.83	230.83a	29.98	81.39a	3.27	6.88b	1.22	15.15	***
17. E-β-farnesene	0.83	481.11a	59.90	165.19a	7.39	12.02b	2.51	15.38	***
18. α-zingiberene^{N}	0.87	8.70	1.50	1.95	0.12	n.d		4.44	*
19. β -bisabolene	0.87	14.80	2.39	4.05	0.23	n.d		3.43	·
20. β -sesquiphellandrene^{N}	0.87	41.19	6.74	10.72	0.55	n.d		4.44	*
21. TMNT	0.87	10.71	1.77	2.26	0.13	n.d		6.21	*

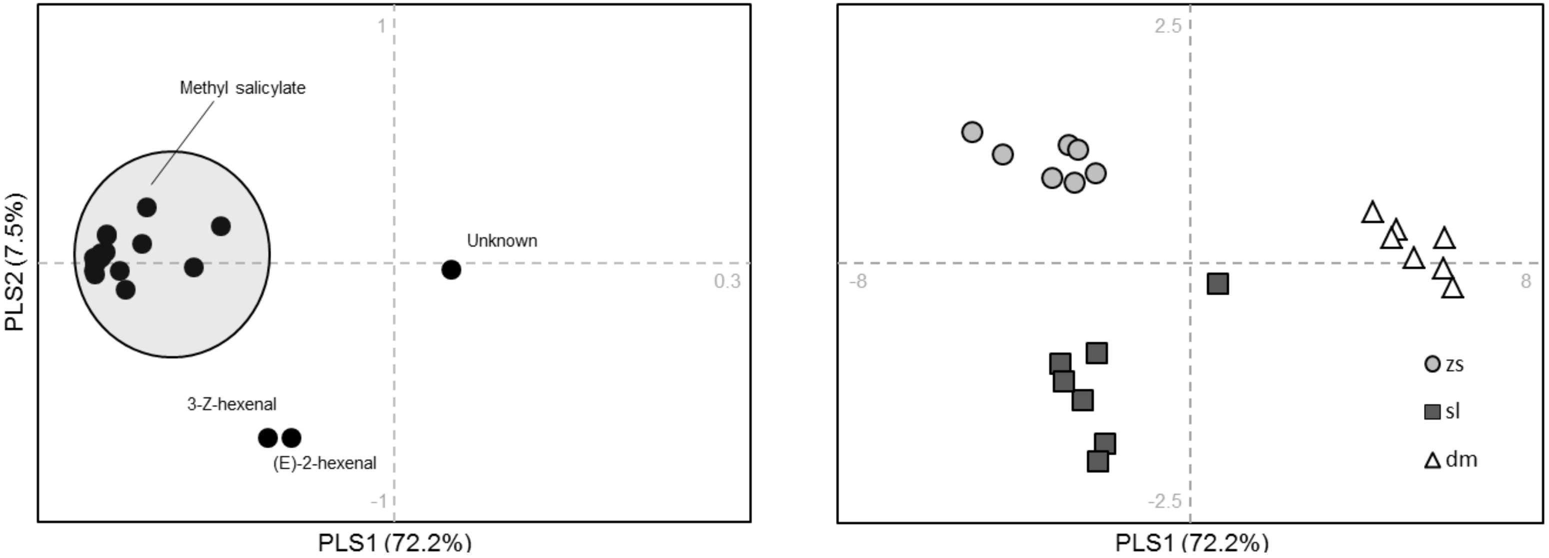

Fig. 5 Loading (a) and score (b) plots for the two components of the PLS-DA used to discriminate between volatile blends emitted by plants infested by *Z. scutellaris* (Zs), *S. littoralis* (Sl) and *D. maidis* (Dm).

DISCUSSION

Innate and learned preferences

We found that the anthocorid predator *O. majusculus* has an innate preference for *Z. scutellaris*- and *S. littoralis*-induced volatiles, and that this preference can be modified through experience. The innate preference suggests that the anthocorid predator is initially mainly attracted to volatiles that result from tissue and/or cell damage, as opposed to volatiles that are emitted in response to phloem feeding. This changed when they successfully fed on nymphs of the phloem feeder *D. maidis*. After preying on *D. maidis* nymphs on *D. maidis*-infested plants, the predator's preference shifted towards *D. maidis*-induced volatiles.

By contrast, the predator's odor preferences after a feeding experience on *S. littoralis* larvae depended on the developmental stage of the prey larvae. A reduced attractiveness towards *S. littoralis*-infested plants was observed when predators were experienced on large larvae. This can be explained by a possible negative association of the feeding experience (the larvae were too large for consumption) with the plant odor . After the predators were placed with small larvae, which can be considered a positive experience, their odor preferences did not differ from those of naïve predators. It is worth mentioning that during experience phase predators were exposed to plant volatiles and prey, but also to herbivore feces, associated products of herbivores and contact chemicals of infested plants that could modify the nature of their experience.

Learning of HIPVs by *O. majusculus* was expected, as it has been frequently observed in generalist carnivores (e.g. Drukker et al. 2000). Intriguingly, just as the discriminant analysis could separate volatile blends emitted by maize plants attacked by the three herbivores, the predators appear to be able to do the same. They appear to use this ability to discriminate between the odor blends in order to focus their foraging efforts on the most profitable odor source. Overall, a positive feeding experience resulted in or maintained a preference for the odor that was associated with this positive experience, whereas a negative experience (large *S. littoralis* larvae) reduced the response to the experienced odor. In agreement to our results Drukker et al. (2000), reported that the anthocorid predator *Anthocoris nemoralis* learnt to associate an odor source with the presence or absence of prey, and changed its attraction to aversion towards the odor source when experiencing prey deprivation. The predator feeding experiment revealed clear differences in the suitability of small and large *Spodoptera* larvae as prey (Fig. 4). This might be explained by prey quality (but see Venzon et al. 2002) and by differences in handling time and/or prey's aggressive and escape behavior (Heady and Nault 1985, Eubanks and Denno 2000). The flexibility in the predator's foraging behavior might facilitate its dispersal to plants where it will find prey and be more effective in controlling pests. In accordance De Boer et al. (2005) reported that predatory mites with multiple experiences (i.e. a non-rewarding experience followed by a rewarding experience) had the strongest preference for *T. urticae* (prey) versus *S. exigua* (non-prey) induced volatiles. The effects of negative associations are likely to quickly diminish upon dispersal, as it has been proposed for parasitoids (Takabayashi et al. 2006).

Under natural field conditions Janssen et al. (2014) reported the first case of associative learning by a predatory community in eucalyptus, where predators rapidly associated novel volatiles (*Mentha piperita* oil) with food and reduced pest populations. In this context, *O. majusculus* ability to learn by association is good news in the context of colonization by non-native pests, as potentially the predatory community could adapt to new prey species and their respective HIPVs, also if they have entirely different feeding habits.

Feeding strategies and HIPVs profiles

Plant responses to herbivore attack can strongly depend on the herbivore's feeding strategy and the amount of tissue damage occurring at the feeding site (Walling, 2000). For chewing herbivores like *S. littoralis*, it is well established that plant damage together with salivary enzymes, such as glucose oxidase, and non-enzymatic elicitors present in the oral secretions can trigger the release of plant volatiles (Alborn et al. 1997, Musser et al. 2002). Considerably less is known about the molecular mechanisms implicated in the differential plant defense responses to mesophyll and phloem-feeding insects. Most typhlocybine leafhoppers like *Z. scutellaris* feed using a sawing laceration strategy, leaving round, silvery-white marks called stipples (Marion-Poll et al. 1987, Backus et al. 2005). Phloem feeding insects, like *D. maidis*, form stylet-sheaths following intercellular (Sternorryncha, e.g. aphids) or intracellular (Auchenorryncha e.g. *D. maidis*) sucking pathways (Backus et al., 2005). Salivary enzymes and elicitors for Auchenorrhyncha are not well studied and it can only be inferred that cell degrading enzymes similar to those found in Thysanoptera or Heteroptera (reviewed by Sharma et al. 2014) play a critical role in their feeding behavior.

The discriminant analysis on herbivore-induced volatile blends shows that the plant's response to insects with distinctly different feeding strategies can be distinguished quantitatively (PLS1) and by discriminating compounds on the other (e.g. GLVs). Notably, mesophyll feeding *Z. scutellaris* induced volatile profiles that resembled the ones induced by the chewer *S. littoralis*, suggesting that the induction of plant volatile by *Z. scutellaris* adults can be as strong as caterpillars on a per capita basis. On the other hand, phloem feeding *D. maidis* induced only few volatiles (seven out of twenty-one detected), which were released in considerably smaller amounts. Hence, *Orius spp.* preference for maize plants damaged by a chewer and a mesophyll feeder can be explained by the fact that these plants released far more volatiles than *D. maidis* infested plants.

(Z)-3-hexenal and (E)-2 hexenal together with methyl salicylate were the discriminating compounds to distinguish the volatile profiles of the three herbivores (Fig. 5). These GLVs are cell wall breakdown products and commonly found to be released by plants under attack by chewing insects, but here they were not detected for either of the two leafhopper treatments. The lack in the emission of these GLVs has also been reported in maize for phloem-feeding aphids (T. C. . Turlings et al., 1998) and the leafhoppers *Euscelidius variegatus* (Erb et al., 2010) and *Cicadulina storeyi* (Oluwafemi et al., 2011). Interestingly, the reported overall volatile emission and number of detected compounds for *E. variegatus* and *C. storeyi* infested plants was much larger than the one observed for *D. maidis*. This could be explained by either the density of insects used for plant induction, thirty for *E. variegatus* and fifty *C. storeyi*, versus ten for *D. maidis,* but more likely by the type of damage inflicted by the phloem feeding insects or the elicitors that are implicated in the induction (Sharma et al., 2014). *D. maidis* has been reported to produce 10 mm thick single feeding tracks on maize leaves without depositing oral secretions within the sieve tubes, while *E. variegatus* produces 35mm thick branched feeding tracks and deposits large quantities of oral secretions in the phloem (Alivizatos 1982). On the other hand, *D. maidis* is a specialist on maize and its wild ancestor teosinte, and it may have evolved ways to avoid or suppress defense responses in these plants (Dávila et al., 2013; Nault and DeLong, 1980).

Methyl salicylate is one of the compounds that seems to be of particular importance in mediating attraction of several natural enemies, and a recent meta-analyses concluded that it acts as a broad spectrum attractant (Rodriguez-Saona et al. 2011 and references therein). Predatory taxa like *Orius* spp., Chrysopidae, Syrphidae, and Coccinellidae among others are attracted to synthetic methyl salicylate when deployed in the field (e.g. James and Price 2004, Mallinger et al. 2011). We detected methyl salicylate at high levels in *Z. scutellaris*-induced plants, reinforcing our hypotheses that *Z. scutellaris* mediated recruitment of generalist natural enemies into maize fields. *Z. scutellaris* colonizes maize fields early in the season and they may reach up to 100 individuals per plant before pollen shed (Pons and Albajes, 2002). At this early stage, colonization by key Lepidopteran pests like *Sesamia nonagrioides* (Lefebvre) and *O. nubilalis*, and occasional pests like *Helicoverpa armigera* (Hübner), *Mythimna unipuncta* (Haworth) and *Spodoptera* spp. is low (Pons and Albajes, 2002). These pests will arrive later and the presence of *Orius* spp.*,* thanks to early infestation by *Z. scutellaris,* may greatly reduce the negative impact of the Lepidopteran pests.

CONCLUSIONS

In summary, the results show that generalist insect predator *O. majusculus* is attracted to herbivore-induced plant volatiles and that its responses to these volatiles are flexible and affected by positive and negative experiences during prey encounters. The innate preference for volatiles released upon infestation by *Z. scutellaris* and *S. littoralis*, can be explained by the fact that these insects damage cause cell tissue damage, resulting in far larger amounts of volatiles than released from infested plants by the phloem feeder. Yet, the innate preference can be modified in favor of normally less preferred HIPVs after a rewarding experience with prey. Three compounds, (Z)-3-hexenal and (E)-2-hexenal and methyl salicylate were found to be most predictive in indicating whom was feeding on a plant and might be used by the predators to discriminate between plants with potential prey. Taken together, the results support the notion that feeding by *Z. scutellaris* results in the emission of maize's HIPVs that initially recruit of *Orius* spp. into maize fields.

AKNOWLEDGMENTS

We would like to thank Julio Bernal for kindly sharing the *D. maidis* colony, Hao Xu for help with olfactometer assays, Gregory Röder for help with the GC-MS analyses and Angela Köhler for maintaining the *Dalbulus* colony. A.A. was funded with an FPU scholarship and a visiting grant to FARCE laboratory from the Ministerio de Educación. The work was partially funded by the Spanish Government project AGL2011-23996.

REFERENCES

Albajes, R., Lumbierres, B., & Pons, X. (2011). Two heteropteran predators in relation to weed management in herbicide-tolerant corn. Biological Control, 59, 30–36.

Alborn, H. T., Turlings, T. C. J., Jones, T. H., Stenhagen, G., Loughrin, J. H., et al. (1997). An elicitor of plant volatiles from beet armyworm oral secretion. Science, 276, 945–949.

Allison, J. D., & Hare, J. D. (2009). Learned and naïve natural enemy responses and the interpretation of volatile organic compounds as cues or signals. New Phytologist, 184, 768–782.

Backus, E. A, Serrano, M. S., & Ranger, C. M. (2005). Mechanisms of hopperburn: an overview of insect taxonomy, behavior, and physiology. Annual Review of Entomology, 50, 125–151.

Van Den Boom, C. E. M., Van Beek, T. A., Posthumus, M. A., De Groot, A., & Dicke, M. (2004). Qualitative and quantitative variation among volatile profiles induced by *Tetranychus urticae* feeding on plants from various families. Journal of Chemical Ecology, 30, 69–89.

Cai, X. M., Sun, X. L., Dong, W. X., Wang, G. C., & Chen, Z. M. (2014). Herbivore species, infestation time, and herbivore density affect induced volatiles in tea plants. Chemoecology, 24, 1–14.

Chabaane, Y., Laplanche, D., Turlings, T. C. J., & Desurmont, G. A. (2014). Impact of exotic insect herbivores on native tritrophic interactions: a case study of the African cotton leafworm, *Spodoptera littoralis* and insects associated with the field mustard *Brassica rapa*. Journal of Ecology, 103, 109-117.

Clavijo McCormick, A., Unsicker, S. B., & Gershenzon, J. (2012). The specificity of herbivore-induced plant volatiles in attracting herbivore enemies. Trends in Plant Science, 17, 303–310.

D'Alessandro, M., & Turlings, T. C. J. (2005). In situ modification of herbivore-induced plant odors: a novel approach to study the attractiveness of volatile organic compounds to parasitic wasps. Chemical Senses, 30, 739–53.

Van Dam, N. M., & Poppy, G. M. (2008). Why plant volatile analysis needs bioinformatics - Detecting signal from noise in increasingly complex profiles. Plant Biology, 10, 29–37.

Dávila, A. M., Thomas, F., & Bernal, J. S. (2013). Facilitated by nature and agriculture : performance of a specialist herbivore improves with host-plant life history evolution , domestication, and breeding. Oecologia, 173, 1425-1437.

Deboer, J., Snoeren, T., & Dicke, M. (2005). Predatory mites learn to discriminate between plant volatiles induced by prey and nonprey herbivores. Animal Behaviour, 69, 869–879.

Degen, T., Dillmann, C., Marion-Poll, F., & Turlings, T. C. J. (2004). High genetic variability of herbivore-induced volatile emission within a broad range of maize inbred lines. Plant Physiology, 135, 1928–1938.

Delphia, C. M., Mescher, M. C., & De Moraes, C. M. (2007). Induction of plant volatiles by herbivores with different feeding habits and the effects of induced defenses on host-plant selection by thrips. Journal of Chemical Ecology, 33, 997–1012.

Dicke, M., & Sabelis, M. W. (1987). How plants obtain predatory mites as bodyguards. Netherlands Journal of Zoology, 38, 148–165.

Drukker, B. A. S., Bruin, J. A. N., & Jacobs, G. (2001). How predatory mites learn to cope with variability in volatile plant signals in the environment of their herbivorous prey. Experimental & applied acarology, 24, 881-895.

Drukker, B., Bruin, J., & Sabelis, M. W. (2000). Anthocorid predators learn to associate herbivore-induced plant volatiles with presence or absence of prey. Physiological Entomology, 25, 260–265.

Dukas, R. (2008). Evolutionary biology of insect learning. Annual Review of Entomology, 53, 145–160.

Erb, M., Foresti, N., & Turlings, T. C. J. (2010). A tritrophic signal that attracts parasitoids to host-damaged plants withstands disruption by non-host herbivores. BMC Plant Biology, 10, 247.

Eubanks, M. D., & Denno, R. F. (2000). Health food versus fast food: the effects of prey quality and mobility on prey selection by a generalist predator and indirect interactions among prey species. Ecological Entomology, 25, 140–146.

Glinwood, R., Ahmed, E., Qvarfordt, E., & Ninkovic, V. (2011). Olfactory learning of plant genotypes by a polyphagous insect predator. Oecologia, 166, 637–47.

Gosset, V., Harmel, N., Göbel, C., Francis, F., Haubruge, E., et al. (2009). Attacks by a piercing-sucking insect (*Myzus persicae* Sultzer) or a chewing insect (*Leptinotarsa decemlineata* Say) on potato plants (*Solanum tuberosum* L.) induce differential changes in volatile compound release and oxylipin synthesis. Journal of Experimental Botany, 60, 1231–1240.

Gu, H., & Dorn, S. (2000). Genetic variation in behavioral response to herbivore-infested plants in the parasitic wasp, *Cotesia glomerata* (L.) (Hymenoptera: Braconidae). Journal of Insect Behavior, 13, 141–156.

Hare, J. D. (2011). Ecological role of volatiles produced by plants in response to damage by herbivorous insects. Annual Review of Entomology, 56, 161–180.

Hare, J. D., & Sun, J. J. (2011). Production of induced volatiles by *Datura wrightii* in response to damage by insects: effect of herbivore species and time. Journal of Chemical Ecology, 37, 751–764.

Heady, S. E., & Nault, L. R. (1985). Escape behavior of *Dalbulus* and *Baldulus* Leafhoppers (Homoptera: Cicadelidae). Environmental Entomology, 14, 154–158.

James, D. G., & Price, T. S. (2004). Field-testing of methyl salicylate for recruitment and retention of beneficial insects in grapes and hops. Journal of Chemical Ecology, 30, 1613–1628.

Lattin, J. D. (1999). Bionomics of the Anthocoridae. Annual Review of Entomology, 44, 207-231

Leitner, M., Boland, W., & Mithöfer, A. (2005). Direct and indirect defences induced by piercing-sucking and chewing herbivores in Medicago truncatula. New Phytologist, 167, 597–606.

Lins, J. C., van Loon, J. J. A., Bueno, V. H. P., Lucas-Barbosa, D., Dicke, M., et al. (2014). Response of the zoophytophagous predators *Macrolophus pygmaeus* and *Nesidiocoris tenuis* to volatiles of uninfested plants and to plants infested by prey or conspecifics. BioControl, 59, 707-718.

Madeira, F., di Lascio, A., Carlino, P., Costantini, M. L., Rossi, L., et al. (2014). Stable carbon and nitrogen isotope signatures to determine predator dispersal between alfalfa and maize. Biological Control, 77, 66–75.

Mallinger, R. E., Hogg, D. B., & Gratton, C. (2011). Methyl salicylate attracts natural enemies and reduces populations of soybean aphids (Hemiptera: Aphididae) in soybean agroecosystems. Journal of Economic Entomology, 104, 115–124.

Margolies, D. C., Sabelis, M. W., & Boyer, J. E. (1997). Response of a phytoseiid predator to herbivore-Induced plant volatiles: Selection on attraction and effect on prey exploitation. Journal of Insect Behavior, 10, 695–709.

Montserrat, M., Albajes, R., & Castañé, C. (2004). Behavioral responses of three plant-inhabiting predators to different prey densities. Biological Control, 30, 256–264.

Montserrat, M., Albajes, R., & Castañé, C. (2000). Functional response of four heteropteran predators preying on Greenhouse Whitefly (Homoptera: Aleyrodidae) and Western Flower Thrips (Thysanoptera: Thripidae). Environmental Entomology, 29, 1075–1082.

De Moraes, C. M., Lewis, W. J., Paré, P. W., Alborn, H. T., Tumlonson, J. H., et al. (1998). Herbivore-infested plants selectively attract parasitoids. Nature, 393, 570–573.

Musser, R. O., Hum-Musser, S. M., Eichenseer, H., Peiffer, M., Ervin, G., et al. (2002). Herbivory: caterpillar saliva beats plant defences. Nature, 416, 599–600.

Nault, L. R., & DeLong, D. M. (1980). Evidence for co-evolution of leafhoppers in the genus *Dalbulus* (Cicadellidae: Homoptera) with maize and its ancestors. Annals of the Entomological Society of America, 73, 349–353.

Oluwafemi, S., Bruce, T. J. A., Pickett, J. A., Ton, J., & Birkett, M. A. (2011). Behavioral responses of the leafhopper, *Cicadulina storeyi* China, a major vector of maize streak virus, to volatile cues from intact and leafhopper-damaged maize. Journal of chemical ecology, 37, 40–8.

Papaj, D. R. (1994). Optimizing learning and its effect on evolutionary change in behavior. Behavioral mechanisms in evolutionary ecology, 133–153.

Papaj, D. R., & Lewis, A. C. (1993). Insect Learning: Ecological and Evolutionary Perspectives. Springer Science & Business Media.

Pons, X., & Albajes, R. (2002). Control of maize pests with imidacloprid seed dressing treatment in Catalonia (NE Iberian Peninsula) under traditional crop conditions. Crop Protection, 21, 943–950.

Pons, X., Núñez, E., Lumbierres, B., & Albajes, R. (2005). Epigeal aphidophagous predators and the role of alfalfa as a reservoir of aphid predators for arable crops. European Journal of Entomology, 102, 519–525.

Riudavets, J., Castañé, C., & Gabarra, R. (1995). Native predators of western flower thrips in horticultural crops. In Thrips Biology and Management (pp. 255–258). Springer.

Rodriguez-Saona, C., Kaplan, I., Braasch, J., Chinnasamy, D., & Williams, L. (2011). Field responses of predaceous arthropods to methyl salicylate: A meta-analysis and case study in cranberries. Biological Control, 59, 294–303.

Schnee, C., Köllner, T. G., Held, M., Turlings, T. C. J., Gershenzon, J., et al. (2006). The products of a single maize sesquiterpene synthase form a volatile defense signal that attracts natural enemies of maize herbivores. Proceedings of the National Academy of Sciences of the United States of America, 103, 1129–1134.

Sharma, A., Khan, A., Subrahmanyam, S., Raman, A., Taylor, G. S., et al. (2014). Salivary proteins of plant-feeding hemipteroids- implication in phytophagy. Bulletin of Entomological Research, 104, 117–36.

Symondson, W. O. C., Sunderland, K. D., & Greenstone, M. H. (2002). Can generalist predators be effective biocontrol agents? Annual Review of Entomology, 47, 561–594.

Takabayashi, J., Sabelis, M. W., Janssen, A., Shiojiri, K., & Van Wijk, M. (2005). Can plants betray the presence of multiple herbivore species to predators and parasitoids? The role of learning in phytochemical information networks. Ecological Research, 21, 3–8.

Turlings, T. C. J., Bernasconi, M., Bertossa, R., Bigler, F., Caloz, G., et al. (1998). the induction of volatile emissions in maize by three herbivore species with different feeding habits: possible consequences for their natural enemies. Biological Control, 11, 122–129.

Turlings, T. C. J., Davison, A. C., & Tamò, C. (2004). A six-arm olfactometer permitting simultaneous observation of insect attraction and odour trapping. Physiological Entomology, 29, 45–55.

Turlings, T. C. J., Tumlinson, J. H., & Lewis, W. J. (1990). Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. Science, 250, 1251–1253.

Turlings, T. C. L., Wäckers, F. L., Vet, L. E. M., Lewis, W. J., & Tumlinson, J. H. (1993). Learning of host-finding cues by hymenopterous parasitoids. In Insect learning (pp. 51–78). Springer.

Venzon, M., Janssen, A., & Sabelis, M. W. (2002). Prey preference and reproductive success of the generalist predator *Orius laevigatus*. Oikos, 97, 116–124.

Vet, L. E. M., Lewis, W. J., & Carde, R. T. (1995). Parasitoid foraging and learning. In Chemical ecology of insects 2 (pp. 65–101). Springer.

Walling, L. (2000). The myriad plant responses to herbivores. Journal of Plant Growth Regulation, 19, 195–216.

Zhu, J., & Park, K. C. (2005). Methyl salicylate, a soybean aphid-induced plant volatile attractive to the predator *Coccinella septempunctata*. Journal of Chemical Ecology, 31, 1733–1746.

CHAPTER 3

Leafhoppers as indicators for risk assessment of GM biofortified crops

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ABSTRACT

As striking advances have been made in the last years to produce a range of biofortified GM crops with an increased level of nutrients, new approaches for environmental risk assessment on non-target arthropods have to be developed. In particular we focus on a new multivitamin maize developed at our university (Naqvi et al., 2009), producing an increased level of beta carotene, ascorbate and folate. We argue that problem formulation becomes extremely complex for this GM maize both at the plant and arthropod level. First, although the functions of carotenes and other vitamins are relatively well studied in plants, little is known about how biofortified plants modulate the metabolic pathways to increase the production of these compounds and which are their associated trade-offs. Second, studies on vitamins in insect systems are scarce, especially on their movement among trophic levels.

We propose Zyginidia scutellaris (Auchenorryncha: Cicadellidae) as an indicator species to assess risks of GM maize to non-target herbivores guided by the use of the best predicted power versus replication relationships from previous field trials. Additionally, we hypothesize that this species is the base to build an indicator maize trophic chain given that it is the most abundant herbivore in maize fields. To explore the suitability of leafhoppers as indicators we present a literature review on the effects of insect resistant and herbicide tolerant GM crops and non-GM varieties on different leafhopper species. Finally we suggest an ecological risk assessment as the only way to detect the potential cascading effects of multivitamin crops.

Key words: cicadellidae, biofortified maize, multivitamin maize, risk assessment, problem formulation

The content of this chapter has been published in IOBC-WPRS Bulletin (2013) vol. 97.

INTRODUCTION

New generations of GM crops are being developed globally. Many of these new generation crops imply generally a modified metabolism of the plant, as the recent advances in genomics have allowed to target new genes of tolerance to biotic stresses (e.g. involving lectins, RNAi, etc) abiotic stress (e.g. tolerant to drought, salt, heat, and future “climate ready” crops), and to engineer other crops with modified metabolism that confer desired attributes to the plant, like biofortified crops. The scientific principles underlying the environmental risk assessment for non-target arthropods (NTAs), completed for herbicide-tolerant and insect-protected GM crops commercialized to date, need now to be applied to these new biofortified crops.

In this paper we deal in the first place with vitamin biofortified crops, and we explore the basis for the current regulatory frameworks in the potential countries of adoption, mainly the African continent. Secondly we study the case of GM Multivitamin Maize (MVM), present the potential changes in the MVM that may have occurred due to the genetic modification. In the third place we introduce the leafhopper *Zyginidia scutellaris* (Hemiptera: Cicadellidae as the candidate indicator species of GM maize impacts on the maize food web; and propose an NTA Environmental Risk Assessment for MVM that follows a tiered approach through trophic relationships.

REGULATORY SYSTEMS: EUROPE, US AND AFRICA

Regulatory frameworks governing GM crops vary widely throughout the world, but essentially they are either developed specifically for GM crops, or they are adapted from existing legal instruments that apply to conventional agriculture (Ramessar et al., 2008). In the EU there is a process-based approach for the regulation of GMOs as the breeding techniques used for their production are considered new and raise specific safety concerns and thus a specific legislation was developed. The actual directive 2001/18/EC on the release of GMOs into the environment stresses the need for a common methodology for Environmental Risk Assessment (ERA), and broadens the risk assessment criteria from the older directive to include direct, indirect, immediate, delayed and cumulative long-term adverse effects and establishes an obligatory Post Market Environmental Monitoring.

In contrast, in the US there is a product-based approach to regulate GMOs, where the legislation focuses on the risks of the products and not the breeding techniques. Thus, GM plants and products are regulated by the existing regulatory system. Most developed countries have introduced regulations that share features of both the EU and US systems, the regulation of GM crops worldwide has been reviewed by Ramessar et al. (2008) and Paoletti et al. (2008).

The adoption of GMOs in developing countries and particularly in Africa has been strongly influenced by developed countries, and particularly the EU and USA. In fact, there is the opinion that the polarized debate about GM crops and their regulation has been an obstacle for the adoption of this new technology in Africa (Adenle, 2011; Paarlberg, 2010). In contrast, GM crop biofortification has been developed to reach malnourished rural populations in the African continent and deliver micronutrients, like minerals and vitamins, that may alleviate chronic diseases. At the present time there are still some African countries with no biotechnology regulatory systems (e.g. Angola, Chad and Somalia) while others have established legal instruments that enable them to regulate GMOs to varying extents (e.g. Burkina Faso, Egypt, Ghana, Kenya, Mali, Namibia, Nigeria, South Africa, Uganda, and Zimbabwe). So far, only three countries, South Africa, Burkina Faso, and Egypt, have commercialized GM crops, while a few others have or are conducting confined field trials (AU-NEPAD African Biosafety Network of Expertise, 2011 www.nepadbiosafety.net).

In all regulatory systems comparative risk assessment is a fundamental principle of GM plant ERA, and it is mostly based on the concept of substantial equivalence. The principle of substantial equivalence stipulates that new GM varieties should be assessed for their safety by comparing them with an equivalent, conventionally bred varieties that have an established history of safe use (Codex, 2003, EFSA, 2011). GM crop lines have to be screened for phenotypic and compositional equivalence in order to confirm or falsify the risk hypothesis that the GM crop is not different from the non-GM crop other than the presence of the introduced gene(s), the expression of the gene(s), and the intended phenotype (Nickson, 2008). Thus, the biologically meaningful differences observed between the GM plants and its comparators are an outcome of the genetic modification (Wolt et al., 2010) and are the ones to evaluate when developing an ERA for NTAs.

EMERGING BIOFORTIFIED CROPS

Biofortification aims to reach malnourished rural populations who may have limited access to a diverse diet, dietary supplements and commercially fortified foods. The most popular traits used for plant biofortification are high mineral and vitamin density (Beyer, 2010). As the Table 1 reflects there is a particular interest in breeding crops containing provitamin A or carotenes and iron, both through transgenesis and conventional breeding.

Some of these biofortified crops obtained through programs of conventional breeding are already cultivated and others obtained by transgenesis are on the pipeline, including the famous Golden Rice II. Conventionally bred provitamin A maize varieties were released in Zambia (three varieties) and Nigeria (two varieties) in 2012 (Saltzman et al., 2013). As for Golden Rice II, two seasons of multi-location field trials have been completed in The Philippines (for details http://www.philrice.gov.ph/?page=golden) and data from these trials must next be submitted to Philippine government regulators for their evaluation as part of the biosafety approval process.

Curiously though Golden Rice has been a flagship biotech crop for the last 10 years, to our knowledge, no scientific (public) literature on potential impacts on NTA is available. So, either an ERA has not been developed yet or it is for developers and regulators eyes only. It has been argued that when the introduced gene has no reasonable mechanism for conferring toxicity to organisms, like in the case of biofortified crops, it is unlikely that detailed knowledge of the mechanism by which a gene confers the desired properties will be necessary for the risk assessment (Nickson, 2008). Still, experience shows that unintended effects might still take place, and with the existing GMO regulation in Europe a sound ERA for biofortified crops has to be developed. Both scientists and regulators appeal to establish which is the basis for comparability and the parameters to identify "meaningful changes" in the transformed plant as to date no limits of concern have been set (Wolt et al., 2010).

Table 1. Summary of the provitamin A and iron biofortified crops that are developed or under development, and the country and year of their past or expected deployment (adapted from Saltzman et al., 2013)

	Conventional breeding			Genetically modified		
Nutrient	Crop	Country	Release	Crop	Country	Release
Provitamin A/ Carotenoids	Banana	Nigeria Ivory Coast Cameroon Burundi DR Congo	?	Rice*	Philippines Bangladesh Indonesia India	2014?
	Cassava	DR Congo Nigeria Brasil	2008 2011 2009	Sorghum	Kenya Burkina Faso Nigeria	2018 all countries
	Maize	Zambia Nigeria Brazil China India	2012 2012 2013 2015 ?			
	Pumpkin	Brazil	2015			
	Sweet potato	Uganda Mozambique Brazil China	2007 2002 2009 2010			
Provitamin A/ Carotenoids + Iron				Banana	Uganda	2019
				Cassava	Nigeria Kenia	2017 both

*Golden Rice II

THE PLANT: BIOFORTIFIED MULTIVITAMIN MAIZE

The Applied Plant Biotechnology Laboratory at UdL created an elite inbred South African transgenic maize plant in which the levels of 3 vitamins were increased specifically in the endosperm through the simultaneous modification of 3 separate metabolic pathways (Naqvi et al., 2009). The kernels of this multivitamin maize contain 169-fold the normal amount of beta-carotene (provitamin A), 6-fold the normal amount of ascorbate (vitamin C), and double the normal amount of folate (vitamin B9).

The selectable marker bar and 4 genes/cDNAs encoding enzymes of the metabolic pathways for the vitamins were introduced: 1) the maize (*Zea mays*) phytoene synthase (*psy1*) cDNA under the control of the wheat LMW glutenin promoter and the *Pantoea ananatis* (formerly *Erwinia uredovora*) *crtI* gene (encoding carotene desaturase) under the control of the barley D-hordein promoter were introduced to increase beta-carotene levels; 2) the rice dehydroascorbate reductase (*dhar*) cDNA to increase ascorbate levels; 3) the *E. coli folE* gene encoding GTP cyclohydrolase (GCH1) under the control of the barley D-hordein promoter to increase folate levels.

European ERAs requires a thorough evaluation of environmental effects of crops obtained through transgenesis by exploring the possible scenarios of harm. As the traits introduced to maize do not have toxic properties, the potential impacts on the arthropod maize community will mainly derive either from a diet enrichment for herbivores or from unintended changes in the plant. To explore these impacts we summarize the potential plant changes that MVM may have experienced and its potential implications for NTA.

POTENTIAL IN CAROLIGHT TISSUES AND IMPLICATIONS FOR INSECTS

In order to develop a sound ERA for NTAs for the case of MVM we have to define and identify potential differences in the plant that may plausibly lead to an impact to the herbivore community and the subsequent trophic levels. To our understanding the potential changes between the MVM and its isogenic counterpart may be due either to the intended effects, i.e., a vitamin overexpression in the endosperm, or unintended effects that may take place throughout the plant, consequence of the changes of metabolic pathways in the endosperm or other cascading effects derived from the gene insertion, regulation or interaction of products.

1) Vitamin overexpression in the endosperm

The overexpression of vitamins in MVM variety is not constitutive as the 3 metabolic pathways have been engineered with endosperm specific promoters and thus we expect the vitamin overexpression to be tissue specific. In fact, Diretto et al. (2007) obtained a GM carotenoid rich potato achieved both under constitutive and tuber-specific overexpression of a bacterial pathway. In this work the authors found that the constitutive expression of the *crtY* and/or *crtI* (the same as MVM) genes interferes with the accumulation of leaf carotenoids, but that the expression of the genes under tuber specific promoter control results in tubers with a "golden" phenotype without any adverse leaf phenotypes. Consequently we expect that the accumulation of vitamins in MVM takes place in maize kernels and that affects predominantly insects that feed directly on the maize cob. A good surrogate to test the effect of a vitamin rich food on insects in our conditions might be the secondary pest *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae).

Although the function of carotenes and ascorbate is relatively well studied in plants (Asensi-Fabado and Munné-Bosch, 2010; DellaPenna and Pogson, 2006), studies on plant-derived vitamins in insect systems are scarce. Carotenes and ascorbate act as antioxidants in living organisms but in addition they fulfil other physiological and ecological roles. Two recent papers review carotene and ascorbate function in plants and insects and give insight to the complex plant-insect and insect-insect interactions mediated by these vitamins (Goggin et al., 2010; Heath et al., 2012); a summary of the functions of carotenes and ascorbate in plants and insects is summarized below (Tables 2 and 3).

Multiple hypothesis can be derived from the literature. The most straightforward would be that if an insect species had a shortage of any of these vitamins in its diet, these multivitamin cobs may produce fitter insects (e.g. reinforce their immune response) than the ones feeding on conventional maize. This fitter insects may be able to live longer or/and produce more offspring, and might result in a population explosion of the pest. In southern Africa *Chilo partellus* (Pyralidae: Lepidoptera) and *H. armigera* may be the species provided with an extra vitamin content if MVM was to be cultivated, and thus become focal pest species for risk assessment.

Table 2. Carotenoid synthesis and function in plants and insects (based on Heath et al., 2013).

Plants	Insects
Synthesized in plastids	*Most insects cannot synthesize them (exceptions related to fungus gene transfer: Acyrthosiphon pisum, Bemisia tabaci, Tetranychus urticae)**
Harvests light energy during photosynthesis	Is involved in coloration, vision, diapause and photoperiodism
Quenches Reactive Oxygen Species produced during photosynthesis and plant stress	Serves as antioxidants (UV radiation/oxidative stress)– Immune response
Is a precursor of signaling molecules that influence development and biotic/abiotic stress responses	
Precursor of semiochemicals	Is a precursor of pheromones and mediates interspecific interactions

*(Moran & Jarvik, 2010; Altincicek et al., 2011; Sloan & Moran, 2012

Table 3. Ascorbate (vitamin C) synthesis and functions in plants and insects (based on Goggin et al., 2010)

Plants	Insects
Synthesized in mitochondria	*Not clear whether insects can synthesize it.*
Controls gene expression and cell growth	Controls molting process
Quenches Reactive Oxygen Species produced during photosynthesis and plant stress	Modulates humoral and cellular immune responses.
	Regulates accumulation of energy reserves in the haemolymph.
Is a signaling molecule involved in plant response to plant stress	Detoxifies plant allelochemicals
Participates in the regeneration of VitE and in the synthesis of organic acids	
Is involved in phythormone and flavonoid biosynthesis and in the xanthophyll cycle	

2) Unintended effects

Unintended effects are difficult to hypothesize and may be sometimes rather speculative. In the case of MVM, we have identified two possible unintended effects: (1) Physiological trade-offs as a consequence of vitamin overexpression; and (2) Other unintended effects derived from the transgenesis.

a) Physiological trade-offs as a consequence of vitamin overexpression

The precursors of the vitamin synthesis are the sugar pools in the cell. These assimilates are produced in the green tissues of the plantduring the process of photosynthesis and translocated through the phloem to the sink organs. Our hypothesis is that, in the case of MVM, the endosperm and its vitamin production pathways might act as a stronger sink as more assimilates are needed to produce more vitamins. The alterations of the metabolic fluxes toward these vitamin production pathways might affect the availability of intermediates for correlated pathways, or limit the amount of assimilates in other tissues of the plant, with relevant consequences for plant development and fitness. Nevertheless, the relationships between carbohydrate availability and secondary compounds synthesis cam be extremely complex and difficult to decode (Fanciullino et al., 2013).

On the other hand as vitamins are part of the plant secondary metabolism probably the impact of the "stronger sink" will not be as relevant as for other modified metabolism crops involved in the primary metabolism of the plant or that express genes of the secondary metabolism constitutively. It remains to be seen whether this overproduction of vitamins in MVM is really free of endogenous regulation.

b) Other unintended differences

Unintended differences in transgenic and non-GM plants can be predictable or unpredictable as a function of whether they are expected and explicable in terms of the present knowledge of plant metabolism and physiology or whether they fall outside our present level of understanding (Cellini et al., 2004). Unintended effects may occur as a consequence of (1) pleiotropic effects of the integrated DNA on the host plant genome as a result of transgene products interacting with the regulation of other genes or the activity of other proteins (transgene specific) (2) host gene disruption or DNA sequence rearrangements at the insertion site (event specific) (3) host plant genome modification by the process to obtain GM plants.

Both targeted and untargeted approaches can be used to explore unintended effects. Current risk assessment of GM maize includes a targeted analysis of nutrients, anti-nutrients, allergens and secondary metabolites identified by an OECD consensus document (OECD, 2002) as the key compounds for maize, using validated analytical methods.

Targeted approaches have been able to detect unintended differences in GM maize. For example (Saxena and Totzky, 2001) detected higher lignin levels in insect resistant transgenic maize stems than in conventional isogenic lines, and (Poerschmann, et al., 2005) observed differences in lignin composition. It has been suggested that untargeted profiling techniques at different biological levels (transcripts, proteins and metabolites) may be the future to screen any of the potential unexpected differences among GM and conventional lines (Cellini et al., 2004; Ricroch et al., 2011). Using transcriptome, proteome and metabolome profiling, Barros et al. (2010) found that the environment (plants were grown over three seasons in one location) affected more strongly gene expression, protein distribution, and metabolite content of kernels of two GE maize lines (MON810 and glyphosate tolerant) than the genetic modification. The main drawback is that both approaches and most studies usually target GM food and feed safety issues, and consequently the unintended changes in the GM plant as a whole are not explored further than at the phenotype level.

One of the most well-known unintended effect of GM crops on the arthropod food web is the case of higher abundance of homopterans in Bt maize. Lumbierres et al. (2004) and Pons et al. (2005) found a significantly higher rate of offspring production by colonizing alate mothers of *Rhopalosiphum padi* (Hemiptera: Aphidae) and consequently higher densities of this species on Bt maize. These unexplained differences between GM crops and its comparators may scale up to the following trophic levels, as it was reported by Faria et al. (2007). The authors observed a positive effect of Bt maize on the performance of the aphid *Rhopalosiphum maidis* that led to an enhanced the performance of parasitic wasps that feed on aphid honeydew. They also showed that two of the three transgenic/isogenic plant pairs studied differed significantly in the amino acid concentrations of the phloem sap.

We believe that the MVM and its isogenic counterpart provide a case study in which, apart from the endosperm, we can compare effects of plants with few genetic differences (3 genes + promoters-metabolic pathways) on insects, and as a consequence the potential unintended effects of the breeding technique in non-targeted tissues may be inferred.

LEAFHOPPERS AS INDICATORS OF IMPACTS OF GM CROPS

Zyginidia scutellaris is a widely distributed species in Europe and is considered a secondary pest of maize in Spain, France and Germany, though it is rarely of economic importance. It is an oliphagous feeder on Poaceae and it may build up high density populations during summer in the maize. As a mesophyll feeder, the species causes damages in by producing pale stripes on the leaves, with a preference for the older ones. This leafhopper species has been recorded for years in maize field trials in Spain (Eizaguirre et al., 2006; Pons et al., 2005) and in Germany (Rauchen et al., 2008, 2010).

Why do we choose the maize leafhopper as an indicator species? First of all for its relevance; population densities of *Z. scutellaris* in maize are often high and can exceed those of other herbivores (Pons et al., 2005, Albajes et al., 2009, 2011). They perform an important functional role as herbivores in maize arthropod communities and their populations have been reported to be the base of an indicator food web (Albajes et al., 2011).

Secondly, this leafhopper shows high statistical power in field trials, in fact it is the taxon with the best detectability both in meta-analysis and single field trials (Comas et al., 2013). Statistical power, which represents the probability that an incorrect null hypothesis will be correctly rejected by a particular test, has been suggested to be an important criterion for selecting indicator species and it can indicate the quality of sampling in a way that addresses the adequacy of experimental designs (Prasifka et al., 2008).

In the third place, homopterans are insects with a high potential sensitivity to plant quality and environmental changes. It has been seen that selected aphid species prefer and perform better in some genotypes or in plants that differ in quality (Mooney et al., 2012; Powell et al., 2006; Zytynska and Preziosi, 2011). Less information is available for leafhoppers but we think they might behave similarly. For example a recent article reported that *Empoasca* leafhoppers are able to identify jasmonate mutants in natural populations of *Nicotiana attenuata* (Kallenbach et al., 2012).

To explore the topic of leafhopper performance on transgenic crops we did a literature compilation of laboratory and field studies that tested leafhoppers on transgenic crops and their isogenic counterparts. We performed this search in the Scopus database using the keywords: cicadellidae, GM crop, Bt, Ht. From the output of the search we selected those published studies that were dealing with taxonomically determined leafhopper species and withdrew those studies that evaluated the "Cicadellidae" all together. Also we selected those studies that were "clear" in their choice of GM varieties (mentioned the variety name and the trait/s introduced) and in their methods and results. We selected 4 laboratory studies (Table 4) and 10 field studies (Table 5).

From the laboratory studies we can conclude that the parameters mortality, development and fecundity and plant choice have been able to detect differences between the GM crop and its isogenic counterpart. Though the number of laboratory studies is limited, and two of the studies focus on GM rice varieties designed to control homopteran pests, we believe that the above mentioned life history traits should be assessed when considering the potential effects of MVM on Z. scutellaris.

In contrast, field studies focus mostly on arthropod abundance. Results show that differences in the abundance of leafhoppers between the GM varieties and their isogenic counterparts (and treatments in some case, e.g. Ht maize) can be detected, but multi-year studies show that these differences are depending on the year and probably also on the method used.

Table 4. Published laboratory studies testing the effects of a GM crop on leafhoppers.

Crop	Varieties	Stressor	Species	Parameters	Effect*	Country	Reference
Maize	Bt(Event 176)	Cry1Ab	*Zyginidia scutellaris*	Bt content on predator	+	Spain	(Obrist et al., 2006)
	Bt germplasm/ isogenic	Cry1F	*Dalbulus maidis*	Oviposition Egg haching rate	+ -	Argentina	(Virla et al., 2010)
Rice	Lectin transgenic/isogenic	GNA	*Nephotettix virescens*	Mortality Feeding preference GNA on honeydew	+ + n.d.	UK	(Foissac et al., 2000)
	Lectin transgenic/isogenic	ASAL + GNA	*Nephotettix virescens*	Mortality Development Fecundity Feeding activity	+ - - -	India	(Bharathi et al., 2011)

*(+) The effect detected for the GM plant is higher/faster than in the isogenic counterpart; (-) The effect detected for the GM plant is lower/slower than in the isogenic counterpart; n.d No differences detected

Table 5. Published field studies testing the effects of a GM crop on leafhoppers.

Crop	Varieties	Stressor	Species	Parameters	Effect*	Country	Reference
Maize	Bt (MON810)/ isogenic	Cry1Ab	*Zyginidia scutellaris*	Abundance (visual) Damage (SPAD)	+ 0	Spain	(Pons et al., 2005a)
	Ht/ isogenic plus herbicide regime	Ht + management	*Zyginidia scutellaris*	Abundance (visual)	+/0 (year dependent)	Spain	(Albajes et al., 2011, 2009)
	Bt (Event 176)	Cry1Ab	*Zyginidia scutellaris*	Bt content (ELISA)	+	Spain	(Obrist et al., 2006)
	Bt (MON810)/ isogenic	Cry1Ab	*Zyginidia scutellaris*	Abundance (visual, sweep netting, yellow traps, custom made sticky traps)	+/0 (year/method dependent)	Germany	(Rauschen et al., 2008)
	Bt (event MON88017)/ isogenic	Cry3Bb1	*Zyginidia scutellaris*	Abundance (sweep netting, custom made sticky traps)	+/0 (year dependent)	Germany	(Rauschen et al., 2011)
	Bt (Herculex Elite)/ isogenic	Cry1F	*Dalbulus maidis*	Abundance (visual)	+	Argentina	(Virla et al., 2010)
Potato	2 Newleaf/ isogenic	Cry3a	*Empoasca fabae*	Abundance (sweep netting, visual) Damage (visual %)	0 0	USA	(Kaplan and Dively, 2008)
	Bt (Newleaf)	Cry3a	*Empoasca fabae*	Abundance (meta-analysis)	+/0	Canada	(Cloutier et al., 2008)
Rice	Bt (TT9-3)/ isogenic	Cry1Ab+Cry1Ac	*Nephotettix cincticeps* *Thaia subrufa* *Recilia dorsalis*	Composition Abundance (yellow sticky traps, Malaise traps, vacuum-suction)	0 0	China	(Chen et al., 2006)
Cotton	3 Bt/3 isogenic	Cry1Ac	*Amrasca biguttula*	Abundance (visual)	+/0 (year dependent)	India	(Sharma and Pampapathy, 2006)

*(+) The effect detected for the GM plant is higher/faster than in the isogenic counterpart; (-) The effect detected for the GM plant is lower/slower than in the isogenic counterpart; 0 No differences detected

THE LEAFHOPPER Z. SCUTELLARIS AS AN INDICATOR OF IMPACTS OF MULTIVITAMIN MAIZE ON NON-TARGET ORGANISMS

In summary, we can say that leafhoppers, and in particular the maize leafhopper, might be a good species to evaluate the impact of transgenic plants on the arthropod food web. Following a recent case study concerning the development of risk hypotheses for invertebrates exposed to a GM ryegrass with elevated triacylglyceride levels (Barratt et al., 2011) we developed the change hypotheses for *Z. scutellaris* feeding on MVM and the maize food-web. The process employs a stepwise analysis of the trophic relationships within the community following the tiered-approach recommended for ERA of NTAs (Romeis et al., 2008). Again, for MVM hypotheses are not as explicit as in the above mentioned study on GM rygrass, where the higher density of lipids in the plant is a constitutive trait, and consequently it is the main factor to cause changes in the insect community. For this motive we think that an ecological approach for the risk assessment of NTAs is the only way to detect the potential cascading effects of multivitamin crops, especially in the scope of the current GM regulation in the EU.

Table 6. Hypothesized changes in *Z. scutellaris* life history traits, and on the maize food web, when feeding on MVM (following Barratt et al., 2011).

Level 1. *Zyginidia scutellaris* feeding on MVM exhibit the following changes:	
Physiological	Improved survival Nymphs grow more rapidly Nymphs and adults have higher biomass Adult females have higher fecundity
Phenological	More generations per year
Behavioral	Larger individuals consume more vegetation
Level 2. Some Level 1 effects are demonstrated, so consider:	
Population effect	Species has increased fitness, density and competitive ability, stronger immune system/reserves
Tritrophic effect	Natural enemies benefit by changes in host fitness and phenology
Effect on vegetation	MVM under increased pressure from herbivores
Level 3. Some Level 2 effects are demonstrated, so consider:	
Trophic cascade effect	Other prey/hosts at increased risk from fitter natural enemies Reduced impact on plants from other herbivores which are under an increased NE pressure

The nature of the trait introduced into the GM crop greatly influences the kind of risk assessment studies that need to be conducted to effectively evaluate these novel crops. An example of this is a recent paper that compares the regulation of GM crops containing dsRNA between three countries and suggests improvements to be made in risk assessments

(Heinemann et al., 2013). In all GM crops unintended effects may occur. One big challenge for regulatory systems will be to establish which differences are considered "acceptable" differences between a GM crop and its isogenic counterpart (Nickson, 2008; Wolt et al., 2010), and to consider at the same time the nature of all new generation GM crops.

IMPLICATIONS FOR ENVIRONMENTAL RISK ASSESSMENT

Formulation of risk hypotheses is extremely challenging for insects feeding on multivitamin maize plants and other crops with modified metabolism as little is known about how these plants modulate the metabolic pathways to increase the production of these compounds and which are their associated trade-offs. Problem formulation is complex also in the scope of current regulatory frameworks as we have to define what to protect from harm when we no longer deal with a transgene that produces toxic compounds, or that may affect directly other organisms by its associated practices (e.g. herbicide tolerant crops, and herbicide applications that may cause flora changes).

Due to the above mentioned reasons we propose that the ERA of NTAs of biofortified crops should focus on species that (1) feed on tissues that accumulate the biofortified elements, e.g. *H. armigera* in the case of MVM as it feeds on the cob that overexpresses the 3 metabolic pathways; (2) are key players in the crop's food web (representive of trophic levels), and if possible that have proved statistical power. The leafhopper *Z. scutellaris* meets these last requirements and is thus a suitable indicator herbivore to detect unintended effects of MVM on arthropods. For this, ERA for MVM on NTA should focus in traits related to the physiology, phenology and behavior of the leafhopper to hypothesize which effects might be expected on the maize food web. With our selected system MVM-*Z. scutellaris* we will be able to test whether our indicator species is sensitive enough to detect small nutritional changes in plant tissues.

In the near future, regulatory frameworks will have to adapt to GM crops with enhanced nutritional traits (and other "new generation" traits) and probably we will see how the established ERA, inherited from toxicological analysis, is revisited.

ACKNOWLEDGMENTS

This work is possible thanks the project AGL 2011-23996, Ministerio de Ciencia y Tecnología, Spain. A. Ardanuy is supported by a FPU fellowship from the Ministry of Education, Spain.

REFERENCES

Adenle, A. A. (2011). Response to issues on GM agriculture in Africa: Are transgenic crops safe? BMC Research, 4, 388.

Albajes, R., Lumbierres, B. & Pons, X. (2009). Responsiveness of arthropod herbivores and their natural enemies to modified weed management in corn. Environtal Entomology, 38, 944–954.

Albajes, R., Lumbierres, B. & Pons, X. (2011). Two heteropteran predators in relation to weed management in herbicide-tolerant corn. Biological Control, 59, 30–36.

Altincicek, B., Kovacs, J. L. & Gerardo, N. M. (2011). Horizontally transferred fungal carotenoid genes in the two-spotted spider mite *Tetranychus urticae*. Biology Letters, 8, 253-257.

Asensi-Fabado, M. A. & Munné-Bosch, S. (2010). Vitamins in plants: occurrence, biosynthesis and antioxidant function. Trends in Plant Science 15, 582–592.

Barratt, B. I. P., Todd, J. H., Burgess, E. P. J. & Malone, L. A. (2011). Developing biosafety risk hypotheses for invertebrates exposed to GM plants using conceptual food webs: a case study with elevated triacylglyceride levels in ryegrass. Environmental Biosafety Research 9, 163–179.

Barros, E., Lezar, S., Anttonen, M. J., Dijk, J. P. Van, Ro, R. M., Kok, E. J. & Engel, K. (2010). Comparison of two GM maize varieties with a near-isogenic non-GM variety using transcriptomics, proteomics and metabolomics. Plant Biotechnology Journal, 8: 436–451.

Beyer, P. (2010). Golden Rice and "Golden" crops for human nutrition. New Biotechnology, 27, 478–81.

Bharathi, Y., Vijaya Kumar, S., Pasalu, I. C., Balachandran, S. M., Reddy, V. D. & Rao, K. V. (2011). Pyramided rice lines harbouring *Allium sativum* (*asal*) and *Galanthus nivalis* (*gna*) lectin genes impart enhanced resistance against major sap-sucking pests. Journal of Biotechnology, 152, 63–71.

Cellini, F., Chesson, A., Colquhoun, I., Constable, A., Davies, H. V, & Engel, K. H. (2004). Unintended effects and their detection in genetically modified crops. Food and Chemical Toxicology, 42, 1089–1125.

Chen, M., Ye, G. Y., Liu, Z. C., Yao, H. W., Chen, X. X., Shen, Z. C., Hu, C., et al. (2006). Field assessment of the effects of transgenic rice expressing a fused gene of Cry1Ab and Cry1Ac from *Bacillus thuringiensis* Berliner on Nontarget Planthopper and Leafhopper Populations. Environmental Entomology, 35, 127–134.

Cloutier, C., Boudreault, S. & Michaud, D. (2008). Étude originale Impact de pommes de terre résistantes au doryphore sur les arthropodes non visés: une méta-analyse des facteurs possiblement en cause dans l'échec d'une plante transgénique Bt. Cahiers Agricultures, 17, 388–394.

Comas, C., Lumbierres, B., Pons, X. & Albajes, R. (2013). No effects of Bacillus thuringiensis maize on nontarget organisms in the field in southern Europe: a meta-analysis of 26 arthropod taxa. Transgenic Research, 23, 135-143.

DellaPenna, D. & Pogson, B. J. (2006). Vitamin synthesis in plants: tocopherols and carotenoids. Annual Review of Plant Physiology, 57, 711–38.

Diretto, G., Al-Babili, S., Tavazza, R., Papacchioli, V., Beyer, P. & Giuliano, G. (2007). Metabolic engineering of potato carotenoid content through tuber-specific overexpression of a bacterial mini-pathway. PloS one 2(4): e350.

Eizaguirre, M., Albajes, R., López, C., Eras, J., Lumbierres, B. & Pons, X. (2006) Six years after the commercial introduction of Bt maize in Spain: field evaluation, impact and future prospects. Transgenic Research, 15, 1–12.

European Food Safety Authority Panel on Genetically Modified Organisms (GMO) (2011). Guidance document on selection of comparators for the risk assessment of GM plants. EFSA Journal, 9, 2149-2169.

Fanciullino, A. L., Bidel, L. P. R. & Urban, L. (2013). Carotenoid responses to environmental stimuli: integrating redox and carbon controls into a fruit model. Plant Cell & Environment, 37, 273-289.

Faria, C. A, Wäckers, F. L., Pritchard, J., Barrett, D. A, & Turlings, T. C. J. (2007). High susceptibility of Bt maize to aphids enhances the performance of parasitoids of lepidopteran pests. PloS one 2(7): e600.

Foissac, X., Thi Loc, N., Christou, P., Gatehouse, A. M. R. & Gatehouse, J. A. (2000). Resistance to green leafhopper (*Nephotettix virescens*) and brown planthopper (*Nilaparvata lugens*) in transgenic rice expressing snowdrop lectin (*Galanthus nivalis* agglutinin; GNA). Journal of Insect Physiology 46, 573–583.

Goggin, F. L., Avila, C. A, & Lorence, A. (2010). Vitamin C content in plants is modified by insects and influences susceptibility to herbivory. BioEssays, 32, 777–790.

Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants (2003). Rome, Italy: Codex Alimentarius.

Heath, J. J., Cipollini, D. F. & Stireman III, J. O. (2013). The role of carotenoids and their derivatives in mediating interactions between insects and their environment. Arthropod-Plant Interactions, 7, 1–20.

Heinemann, J. A, Agapito-Tenfen, S. Z. & Carman, J. A. (2013). A comparative evaluation of the regulation of GM crops or products containing dsRNA and suggested improvements to risk assessments. Environtment International 55, 43–55.

Kallenbach, M., Bonaventure, G., Gilardoni, P. A, Wissgott, A. & Baldwin, I. T. (2012). *Empoasca* leafhoppers attack wild tobacco plants in a jasmonate-dependent manner and identify jasmonate mutants in natural populations. PNAS 109(24): E1548–1557.

Kaplan, I. & Dively, G. P. (2008). Variation in tolerance and resistance to the leafhopper *Empoasca fabae* (Hemiptera: Cicadellidae) among potato cultivars : implications for action thresholds. Journal of Economic Entomology 101, 959-968.

Lumbierres, B., Albajes, R. & Pons, X. (2004). Transgenic Bt maize and *Rhopalosiphum padi* (Hom. Aphididae) performance. Ecological Entomology, 29, 309-317.

Mooney, K. A., Pratt, R. T. & Singer, M. S. (2012). The tri-trophic interactions hypothesis: interactive effects of host plant quality, diet breadth and natural enemies on herbivores. PloS one 7(4): e34403.

Moran, N. A. & Jarvik, T. (2010). Lateral transfer of genes from fungi underlies carotenoid production in aphids. Science, 328, 624–627.

Naqvi, S., Zhu, C., Farre, G., Ramessar, K., Bassie, L., Perez, D., Ros, G., et al. (2009). Transgenic multivitamin corn through biofortification of endosperm with three vitamins representing three distinct metabolic pathways. PNAS, 106, 7762–7767.

Nickson, T. E. (2008). Planning environmental risk assessment for genetically modified crops: problem formulation for stress-tolerant crops. Plant Physiology, 147, 494–502.

Obrist, L. B., Dutton, A., Albajes, R. & Bigler, F. (2006). Exposure of arthropod predators to Cry1Ab toxin in Bt maize fields. Ecological Entomology, 31, 143–154.

Paarlberg, R. (2010). GMO foods and crops: Africa's choice. New Biotechnology, 27, 609–613.

Paoletti, C., Flamm, E., Yan, W., Meek, S., Renckens, S., Fellous, M. & Kuiper, H. (2008). GMO risk assessment around the world: Some examples. Trends in Food Science & Technology, 19, S70–S78.

Poerschmann, J., Gathmann, A., Juergen Augustin, Langer, U. & Górecki, T. (2005). Molecular composition of leaves and stems of genetically modified Bt and near-isogenic non-Bt maize: characterization of lignin patterns. Journal of Environmental Quality, 34, 1508–1518.

Pons, X. A., Lumbierres, B. E., López, C. A. & Albajes, R. A. (2005). Abundance of non-target pests in transgenic Bt-maize: A farm scale study. European Journal of Entomology, 102, 73–79.

Powell, G., Tosh, C. R. & Hardie, J. (2006) Host plant selection by aphids: behavioral, evolutionary, and applied perspectives. Annual Review of Entomology, 51, 309–30.

Prasifka, J. R., Hellmich, R. L., Dively, G. P., Higgins, L. S., Dixon, P. M. & Duan, J. J. (2008). Selection of nontarget arthropod taxa for field research on transgenic insecticidal crops: using empirical data and statistical power. Environmental Entomology, 37, 1–10.

Ramessar, K., Capell, T., Twyman, R. M., Quemada, H. & Christou, P. (2008). Calling the tunes on transgenic crops: the case for regulatory harmony. Molecular Breeding, 23, 99–112.

Rauschen, S., Eckert, J., Schaarschmidt, F., Schuphan, I. & Gathmann, A. (2008). An evaluation of methods for assessing the impacts of Bt-maize MON810 cultivation and pyrethroid insecticide use on Auchenorrhyncha (planthoppers and leafhoppers). Agricultural and Forest Entomology, 10, 331–339.

Rauschen, S., Schultheis, E., Hunfeld, H., Schaarschmidt, F., Schuphan, I. & Eber, S. (2011). *Diabrotica*-resistant Bt-maize DKc5143 event MON88017 has no impact on the field densities of the leafhopper *Zyginidia scutellaris*. Environtal Biosafety Research, 9, 87–99.

Ricroch, E., Berge, J. B. & Kuntz, M. (2011). Evaluation of genetically engineered crops using transcriptomic, proteomic, and metabolomic profiling techniques. Plant Physiology, 155, 1752–1761.

Romeis, J., Bartsch, D., Bigler, F., Candolfi, M. P., Gielkens, M. M. C., Hartley, S. E., Hellmich, R. L., et al. (2008). Assessment of risk of insect-resistant transgenic crops to nontarget arthropods. Nature Biotechnology, 26, 203–8.

Saltzman, A., Birol, E., Bouis, H. E., Boy, E., De Moura, F. F., Islam, Y. & Pfeiffer, W. H. (2013). Biofortification: Progress toward a more nourishing future. Global Food Security, 2, 9–17.

Saxena, D. & Totzky, G. S. (2001). Corn has a higher lignin content than non-Bt corn. American Journal of Botany, 88, 1704–1706.

Sharma, H. C. & Pampapathy, G. (2006). Influence of transgenic cotton on the relative abundance and damage by target and non-target insect pests under different protection regimes in India. Crop Protection, 25, 800–813.

Sloan, D. B. & Moran, N. A. (2012). Endosymbiotic bacteria as a source of carotenoids in whiteflies. Biology Letters, 8, 986–989.

Virla, E. G., Casuso, M. & Frias, E. A. (2010). A preliminary study on the effects of a transgenic corn event on the non-target pest *Dalbulus maidis* (Hemiptera: Cicadellidae). Crop Protection 29, 635–638.

Wolt, J. D., Keese, P., Raybould, A., Fitzpatrick, J. W., Burachik, M., Gray, A., Olin, S. S., et al. (2010). Problem formulation in the environmental risk assessment for genetically modified plants. Transgenic Research, 19, 425–436.

Zytynska, S. E. & Preziosi, R. F. (2011). Genetic interactions influence host preference and performance in a plant-insect system. Evolutionary Ecology, 25, 1321–1333.

CHAPTER 4

A vitamin enriched maize does not impact on plant-insect interactions

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ABSTRACT

A number of plant traits influence the arthropod community composition through bottom-up mechanisms, and therefore ecosystem services delivered by arthropods may be impacted by the cultivation of novel crops. Here we tested the hypothesis that a vitamin enriched maize (Carolight^R) is similar in terms of plant-arthropod interactions to its wild type counterpart when compared in a controlled environment and under field conditions. We used the maize arthropod field abundance, the behaviour and fitness of a maize herbivore keystone species and above ground chemistry of maize plants (volatile, hormone and metabolite profiling) as indicators of potential changes in plant-insect interactions. In order to broaden the range of growth environments in the study we tested two nitrogen availability regimes. We show that nitrogen availability was the key driver of herbivore abundance and behavior. Nitrogen availability also determined direct and also indirect chemical defense in Carolight^R and its isoline M37W. Both genotypes performed similarly in constitutive expression of phytohormones, and in hormone response to herbivory. Interestingly, feeding by the herbivore *Zyginidia scutellaris* suppressed the levels of JA-Ile and SA in both genotypes without impairing the release of HIPVs independently of the nitrogen level or maize variety. Carolight^R and M37W differed to some degree in the concentrations of phenolics (e.g. caffeic acid, chlorogenic acid and lignans) and in the abundance of the volatile compound (β -sesquiphellandrene). Nevertheless, the impact of these changes on the herbivore were smaller than the effect of nitrogen fertilization. Overall we conclude that Carolight^R and its near isogenic line behave similarly in terms of plant-insect interactions.

Key words: plant-insect interactions, maize, novel traits, modified metabolism, Zyginidia scutellaris, hormone suppression, nitrogen fertilization

A manuscript with the contents of this chapter is being prepared for submission.

INTRODUCTION

One of the issues regarding the cultivation of novel crops (GM or otherwise) is their possible impact on insect biodiversity and associated ecosystem services in agriculture. The mandatory environmental risk assessment (ERA) for cultivation of novel crops addresses the hypothesis that the traits introduced into the novel crops do not adversely affect the non-target arthropods (NTAs). Risk assessments are comparative in the sense that novel crops are screened for phenotypic and compositional equivalence to their near isogenic counterpart, and the biologically meaningful differences observed between them are a consequence of the novel trait (Wolt et al., 2010) and are subsequently evaluated when developing an ERA for NTAs.

An elite South African maize inbred (Carolight^{R}) was engineered as a vehicle to deliver pro-vitamin A (and other nutritionally important carotenoids such as lutein, zeaxanthin and lycopene) in the diet and thus address vitamin A and other nutritional deficiencies in at-risk populations in developing countries. The kernels of Carolight^{R} accumulate higher levels of 3 vitamins in the endosperm through the simultaneous engineering of 3 separate metabolic pathways: 169-fold the normal amount of beta-carotene (provitamin A), 6-fold the normal amount of ascorbate (vitamin C), and double the normal amount of folate (vitamin B9) (Naqvi et al., 2009).

The compositional characterization of Carolight^{R} seeds (transcript, proteome, and metabolite profiles) indicated changes in sugar and lipid metabolism in the endosperm due to the higher up-stream metabolite demand by the extended biosynthesis capacities for terpenoids and fatty acids (Decourcelle et al., 2015). Nevertheless under field conditions the metabolic phenotype of vitamin enriched maize kernels under contrasting soil nitrogen conditions were indistinguishable from the wild type in terms of carotenoid accumulation in leaves, photosynthetic activity, sensitivity to source limitation and yield (Zanga et al., 2016). Authors concluded that the additional metabolic requirements of Carolight^{R} endosperm did not affect agronomic performance. Interestingly gravid females of the key Mediterranean maize pest *Sesamia nonagrioides* preferred the volatiles of the near isogenic line to Carolight^{R} in an olfactometer setting (Cruz and Eizaguirre, 2015), which led to the idea that vitamin enriched maize could modify the outcome of plant-insect interactions.

The strong influence of plant chemical traits on food webs has been demonstrated experimentally both above and below ground (van der Putten et al. 2001, Ode 2006). As it is not possible to measure all ecological interactions between a plant and its associated insect species, we used the arthropod field abundance, the behaviour and fitness of a herbivore keystone species and above ground chemistry of maize plants as indicators of possible changes in plant–insect interactions. We therefore tested the hypothesis that Carolight^{R} is similar in terms of plant-arthropod interactions to its near-isogenic line (M37W) when compared in a controlled test that hypothesis were: (i) to determine if Carolight^{R} and M37W affect the abundance and dynamics of herbivores and natural enemies in the field; (ii) to determine potential effects of both genotypes on herbivore choice and performance under controlled conditions; and (iii) to characterize the chemical profiles of leaves that are usually consumed by most herbivores (and thus involved in plant–insect interactions) in both genotypes. Characterization was done by means of volatile, hormone and metabolite profiling. In order to broaden the range of environments in the study and to test the consistency of performance between Carolight^{R} and M37W, we compared both genotypes under different substrate nitrogen availability regimes. The data and conclusions from our studies not only validate the use of plant-insect interactions in the ERA of biofortified crops but importantly also sheds light into the biochemical and metabolic components that underpin the mechanism of maize-insect interactions.

MATERIAL AND METHODS

Plants and nitrogen treatments

Seeds of the elite South African maize (Zea mays L.) inbred cv. M37W and its biofortified derived line CarolightR were obtained from the Applied Plant Biotechnology Group at Universitat de Lleida- Agrotecnio Center.

A field experiment was carried out in order to evaluate the performance of CarolightR and M37W in terms of arthropod community composition and dynamics. The experimental design encompassed a factorial combination of the two maize genotypes and two nitrogen treatments. Plots were randomized with four replicates per genotype-nitrogen combination, each consisting of six rows, 70 cm apart and 6.47 m in length (approximately 4 plants per meter). Maize was planted on the 5th May 2013. Two different fertilization regimes were applied on the 9th July: Control = 0 kg ha^{-1} and +N = 200 kg ha^{-1} as urea at the V6 stage (six fully-expanded leaves). Each plot was fully irrigated.

For laboratory experiments, seeds from each variety were sown in plastic pots (10 cm high, 5 cm diameter) in vermiculite, and germinated in the greenhouse. Forty maize plants (seven to ten days old) were placed in plastic containers and provided 2.5 l of hydroponic solution for 10-12 days. Two hydroponic solutions were tested: a control solution and a solution with an increased content in nitrogen (+N). The control solution consisted of a half-strength modified Hoagland solution with micro-nutrients provided at full strength. A modified Hoagland solution contains all of the known mineral elements needed for rapid plant growth, the concentrations of these elements are set at the highest possible levels without producing toxicity symptoms or salinity stress and thus may be several orders of magnitude higher than those found in the soil around plant roots (Taiz and Zeiger, 2010); for this reason macronutrients were supplied at half strength. Nitrogen was supplied as both ammonium (NH_4^+) and nitrate (NO_3^-), this balanced mixture of cations and anions tends to reduce the rapid rise in the pH of the medium that is commonly observed when the nitrogen is supplied solely as nitrate anion (Asher and Edwards 1983, in Taiz and Zeiger, 2010). The solution with nitrogen (+N) consisted of a control solution in which 8 mM of NH_4NH_3 was added. The hydroponic solutions were adjusted to pH 5.9, and were buffered with MES tampon. The solution was replaced every 3-4 days.

Insects and herbivory treatments

A colony of the leafhopper *Z. scutellaris* was established from cereal fields and maize fields at the Universitat de Lleida (Spain). The colony was reared in controlled conditions (16:8 h L:D, 24±5 ºC) on maize var. Delprim and B73.

Plants were transferred to an experimental chamber equipped with full spectrum light benches (24±2 ºC, 40±10% r.h., 16:8 h L/D, and 8000 lm m^{-2}) the day prior the experiments started. Plants used for Volatile Collection were enclosed in custom made Nalophan bags (Omya AG, Oftringen, Switzerland, 150 mm diameter) closed with a parafilm seal at the top of the plastic pot Plants used for hormone and untargeted metabolome profiling were enclosed in bottom cut PET plastic bottles covered with muslin cloth. On the following day, herbivore treatment was initiated by exposing plants to ten *Z. scutellaris* adults for 24 h in the case of volatile analysis and untargeted metabolome profiling, and 24, 48 and 96h for hormone profiling. The timing was chosen based on a previous study showing a strong induction of plant volatiles at 24h after the start of leafhopper feeding (Ardanuy et al., 2016).

Effects of variety and nitrogen on herbivore and natural enemy abundance in field conditions

Visual sampling of arthropod fauna was conducted on whole plants from the 8th of July (V6-7 stage) to the 16th of September, 2013 every other week (6 samplings in total, 1 before nitrogen fertilization and 5 subsequently) according to Albajes et al. (2011). We sampled four plants from each plot randomly, and we recorded the number of herbivores and their natural enemies per plant. Herbivore counts were grouped in five taxonomic units: Thysanoptera (thrips), Hemiptera\Aphididae (aphids), Hemiptera\Cicadellidae (leafhoppers, mainly *Zyginidia scutellaris*), and Hemiptera\ Delphacidae (planthoppers, *Laodelphax striatellus*) and Lepidoptera (*Spodoptera spp.*, *Helicoverpa armigera*, corn borers). Later we transformed aphid counts into an abundance scale (0, no aphids; 1, isolated aphids; 2, small colony; 3, medium colony; 4, large colony). Natural enemy counts were grouped in Hemiptera\Anthocoridae, Hemiptera\Miridae, Neuroptera, Coccinellidae, Thysanoptera (thrips) and Arachnida.

We obtained the sum of abundances per plot and sampling date for all taxonomic units. We tested the effects of genotype, nitrogen, and sampling date on herbivore and natural enemy community with a permutational MANOVA using the Adonis function in the package vegan in R (Oksanen et al., 2013). We then performed univariate analysis at the species level for herbivore abundance data with a generalized linear model following a Negative Binomial distribution in which sampling date, nitrogen treatment and genotype and their interactions were used as fixed factors. Aphid abundance was analyzed with an ordinal logistic regression. All statistical analyses were performed using R (R Development Core Team) unless otherwise indicated.

Effects of variety and nitrogen on herbivore performance and plant choice

Leafhopper performance was tested by transferring 1-day old leafhopper nymphs from the colony to maize plants and letting them develop until adult stage. Plant treatments consisted of a factorial combination of the two maize genotypes and two N treatments (control and +N) (n=13-15 plants per treatment). Plants were enclosed in plastic bottles with their bottom open, covered by cloth to avoid leafhoppers from escaping; each plant contained 3 leafhoppers. Plants were monitored daily until leafhoppers reached adult stage. Leafhoppers were then removed and placed in 0.5 mm eppendorfs and frozen at -20ºC until sexed and weighed. When there was more than one leafhopper per sex in a plant we averaged final weight and developmental time. Final weight of leafhopper individuals and developmental time was analyzed with a GLM following a Gaussian distribution using the variables insect sex, nitrogen regime and genotype and their interactions as factors.

The effects of plant volatiles emitted by the different combination of varieties and nitrogen treatments on the behavior of the leafhopper were investigated in a six-arm-olfactometer (for details see Turlings, Davison, & Tamò, 2004). A plant of each genotype-nitrogen treatment was enclosed into glass vessels one hour before the assay started. Two empty vessels were used as blanks. Purified and humidified air entered each odor source bottle at 0.8 l/min via Teflon tubing (adjusted by a manifold with four flow-meters; Analytical Research System, Gainesville, FL, USA) and carried the volatiles through to the olfactometer compartment. The position of the odor sources in the olfactometer was randomly assigned each experimental day to avoid position-bias.

At least half an hour before the experiment started groups of six *Z. scutellaris* females were isolated in pipette tips by means of a manual aspirator, and covered in parafilm. Twelve leafhoppers were freed at the base of the olfactometer and left for 45 minutes. Only when an insect entered an arm and passed the screw cap fitting or was recovered in the bulb we considered it made a choice. Three times twelve females were tested per experimental day. All olfactometer tests were conducted between 10 am and 4 pm under light benches (24±2 ºC). Each experiment was performed 7 times on different days. This resulted in 7 independent replicates for each olfactometer setup.

Olfactometer choice counts were analyzed with a GLM following a Poisson distribution, with nitrogen regime and genotype and their interactions as factors. Pair-wise comparisons were performed with using Tukey's HSD.

Effects of variety, nitrogen and herbivory on volatile profiles

VOCs were collected simultaneously from herbivore damaged plants and from control non-damaged plants for all the treatments consisting of the factorial combination of genotype and nitrogen treatments. Two tubular glass outlets (23x17x12 mm) with a screw cap were attached to the bottom and top of the bag respectively (as described by Turlings et al. 1998). Clean air was supplied to the system through the top outlet via Tygon tubing connected to a flowmeter (Analytical Research Systems) and though the bottom device air was pulled through a volatile adsorbent trap at a rate of 1 l/min using a vacuum pump. We collected volatiles of each odor source for 5h using adsorbent traps consisting of a glass tube (4 mm ID) packed with 25 mg Super-Q polymer (80–100 mesh) (Alltech Associates, Deerfield, Illinois, USA). We performed 7 experimental replications of all treatments on different days.

The traps were then extracted with 150 µl dichloromethane (Suprasolv, Merck, Dietikon, Switzerland), and 200 ng of n-octane and n-nonyl acetate (Sigma, Buchs, Switzerland) in 10 µl dichloromethane were added to the samples as internal standards. All extracts were stored at -80°C until analyses. Traps were washed with 3 ml dichloromethane before they were re-used for the next collection. Volatiles were identified with a gas chromatograph (Agilent 6890 Series GC system G1530A) coupled to a mass spectrometer (Agilent 5975C VL MSD). A 2-µl aliquot of each sample was injected in the pulsed splitless mode into an apolar capillary column (HP-1, 30 m, 0.25 mm ID, 0.25 µm film thickness; Agilent J&W Scientific, USA). Helium was used as carrier gas at constant pressure (15 psi). After injection, the column temperature was maintained at 40°C for 3 min and then increased to 100°C at 8°C/min and subsequently to 200°C at 5°C per min followed by a post-run of 5 min at 250°C. Chemstation software was used to estimate the quantities of all major components by comparison of the peak areas of each volatile to the peak areas the internal standards. The detected volatiles were identified by comparison of their mass spectra with those of the NIST 05 library and by comparison of retention times with those from a library from earlier assays.

We used permutational MANOVA to evaluate whether the VOC blend varied between herbivore treatments, nitrogen availability regimes and among genotypes. The abundance of the components of the volatile blend was used as the response variable, while herbivore treatment, nitrogen regime, plant genotype, and their double interactions were used as independent variables. A principal components analysis (PCA) was used to determine whether control or herbivore induced samples belonging to specific genotype-nitrogen treatments could be separated based on qualitative and quantitative differences in volatile emissions. Data were log-transformed, mean-centered, and scaled to unit variance before they were subjected to the PCA.

Effects of plant variety, nitrogen level and herbivory on endogenous plant hormones and phenolic compounds

We performed metabolite profiling of herbivore damaged plants (n=3) and control plants (n=3) for each combination of genotype-nitrogen levels at three time points (24, 48 and 96h) after the experiment started. The aboveground part of the plants was flash frozen with liquid nitrogen and stored at -80ºC until freeze dried. The experiment was repeated three times. The hormones 12-oxo-phytodienoic acid (OPDA), jasmonic acid (JA), JA-isoleucine (JA–Ile), abscisic acid (ABA) and salicylic acid (SA), and the hydroxycinnamic acids caffeic acid, chlorogenic acid and ferulic acid were analyzed by ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS), as described by Camañes et al. (2012)

Effects of plant variety, nitrogen levels and herbivory on metabolite fingerprint

We performed metabolite profiling of herbivore damaged (n=5) and control plants (n=5) for each combination genotype-nitrogen levels. The aboveground part of the plants was flash frozen with liquid nitrogen 24h after the experiment started and stored at -80ºC. Each sample was ground to powder using a mortar previously frozen in liquid nitrogen. The frozen plant powder was weighed (100 mg ±1mg) in an Eppendorf tube, and 500 ul of extraction solvent (MeOH:H_2O:formic acid 80:20:0.5) and a few glass beads were added. Samples were briefly vortexed and then extracted in a bead mill for three minutes at 30Hz. After centrifugation at 10,000 rpm for 10 min (Hettich mikrolitter D 7200, Buford, GA, USA) the supernatant was transferred to a new Eppendorf tube, to which 350ul of dichloromethane was added. Samples were vortexed and centrifuged again to separate the two phases. The upper phase was recovered (150ul) and transferred to HPLC vials.

Metabolite analysis was performed using an Acquity UPLC^{TM} system (Waters) coupled to Synapt G2 QTOF mass spectrometer (Waters) through an electrospray interface (ESI). The separation was performed on an Acquity BEH C18 column (50 × 2.1 mm i.d., 1.7 μm particle size) at a flow rate of 0.6 mL min-1. The injection volume was 3 μl and the autosampler and column temperatures were kept at 15 and 40 °C, respectively. The mobile phase consisted of 0.05% formic acid (FA) in water (phase A) and 0.05% FA in acetonitrile (phase B). The segmented gradient program was as follows: 2% B to 35% B in 3.0 min, 35% B to 100% B in 3.0 min, hold at 100% B for 1.5 min, re-equilibration to initial conditions (2% B) for 1.5 min. Acquisitions were performed in both ESI-negative and ESI-positive modes over a mass range of 100–1000 Da. The MSe mode, in which the instrument alternatively acquires data at low (4 eV; 0.15 s scan time) and high (10-30 eV ramp; 0.15 s scan time) collision energies, was employed. The mass spectrometer was internally calibrated by infusing a 500 ng/mL solution of leucine-enkephalin at a flow rate of 15 ul/min through the LockSpray^{TM} probe. All aspects of the system were controlled by Masslynx v4.1.

Metabolite raw data was transformed to CDF using Databridge provided by the Masslynx package. The CDF data was processed with R for statistical computing using XCMS package for relative quantification (Smith et al., 2006). ESI-negative and ESI-positive data were combined, log-transformed and Pareto scaled prior analysis. Pareto scaling gives each variable a variance equal to the square root of its standard deviation. The advantage of using this technique in comparison to scaling to unit variance lies in the fact that it reduces the relative importance of large values but keeps data structure partially intact (van den Berg et al., 2006). First a permutational MANOVA was used to evaluate whether the metabolite fingerprint consistently varied among genotypes, nitrogen availability regimes and herbivore treatments and the influences of the interactions of the factors (permutations=999). Next, a principal component analysis (PCA) was used as an unsupervised method to visualize variability and clustering in the data set. Later partial least squares–discriminant analyses (PLS–DA) were conducted to detect differently detected ions between plant experimental factors - wild type vs. Carolight^{R}, control nitrogen vs. nitrogen treatment, and controls vs. leafhopper-induced plants - given that interactions between factors were non-significant in the perMANOVA. PLS–DA is a supervised multivariate analysis technique, which maximizes the covariance between the X–matrix (spectral intensities) and the Y–matrix (group information). New components were only added to the model when significant according to the cross–validation function of the software. R^2X and R^2Y represent the fraction of the variance of X matrix and Y matrix, respectively, while Q^2Y suggests the predictive accuracy of the model. We assessed model reliability using CV-ANOVA. Then, variable influence on projection (VIP) was used to select the most influential metabolites to group separation in the validated PLS-DA models. The VIP values summarize the overall contribution of each X-variable to the model, summed over all components and weighted according to the Y variation accounted for by each component. The Sum of squares of all VIP's is equal to the number of terms in the model - the average VIP is equal to 1- and thus terms with large VIP are the most important for explaining Y. We considered that metabolites with a VIP> 2 were extremely influential for treatment separation. The ions with VIP>2 for each experimental factor (plant variety, nitrogen and herbivory, as no interactions were significant in the MANOVA model) were screened for putative identification using the pathway tool from MarVis (Kaever et al., 2014). The MS/MS fragmentation of the metabolites was compared with candidate compounds identified in databases or earlier publications, especially when the metabolites were already reported in maize. Metabolite multivariate analysis (PCA, PLS-DA) was performed with SIMCA–P software (v. 11.0, Umetrics, Umeå, Sweden).

RESULTS

Variation in non-target arthropod communities in the field

The most prominent source of variation in insect abundances in the field was plant developmental stage, which reflects seasonal insect dynamics in the plot (Table 1). Abundance of maize herbivores was mainly influenced by the developmental stage of the plant (perMANOVA $R^2 = 0.62$, p=<0.001) and to a minor extend by nitrogen regime ($R^2 = 0.13$, p=0.085) while no effects were attributable to genotype ($R^2 = 0.07$, p=0.213) or genotype x nitrogen interaction ($R^2 = 0.02$, p=0.684). Similarly, maize developmental stage was the main factor explaining the variation in the abundance of the natural enemies recorded in the study ($R^2 = 0.28$, p=<0.001) whilst genotype and nitrogen were not significant for determining community composition.

Leafhoppers and thrips were the most abundant herbivore taxa in the field, and Anthocoridae and spiders the most abundant natural enemy taxa (Supplementary material, Fig. S1). Univariate analysis revealed that Hemipteran herbivores (leafhoppers, planthoppers and aphids) were more abundant in treatments with a nitrogen surplus independently of population dynamics (Table 1). Only leafhopper populations were influenced by plant genotype: Carolight^R plots supported lower populations of leafhopper nymphs than the wild type (Table 1). Levels of other herbivores such as thrips and Lepidoptera were not invfluenced by nitrogen treatment or genotype (Table 1). Overall the variation of natural enemy taxa was attributable to population dynamics, and no differences were detected between any of the treatments (Table 1).

Effects of plant variety and nitrogen levels on herbivore choice and performance

Plants from both genotypes obtained from the nitrogen surplus hydroponic treatments (+N) were taller and shoots were more robust than plants grown under control nitrogen conditions (Supplementary material Fig. S.1). Genotype and nitrogen factors did not impact on herbivore performance as sex was the only significant predictor of final weight ($F_{1,62}$=121.40, p<0.001) and developmental time ($F_{1,62}$=8.71, p=0.032). Overall, plants from both genotypes grown under high nitrogen attracted more female leafhoppers than plants grown with no additional nitrogen (χ^2_1= 25.22, p<0.001); although interestingly, when only considering the nitrogen surplus treatment Carolight maize was preferred to its isogenic counterpart in (χ^2_1= 4.19, p=0.04) (Fig. 2). Leafhopper chose maize plants over empty bottle control treatments (χ^2_5= 30.70, p<0.001) a result that validates the experimental setup.

Table 1. Effects of maize genotype, nitrogen treatment, their interaction and maize's developmental stage on field abundances of herbivores and natural enemies. Arthropod abundance was determined by visual sampling on five maize developmental stages. Significant effects (α=0.05) appear in bold.

Herbivores		**Leafhoppers**		**Planthoppers**		**Thrips**		**Lepidoptera**		**Aphids**	
Factors	df	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p
genotype	1	4.09	**0.046**	0.09	0.779	1.41	0.233	2.19	0.076	0.64	0.423
nitrogen	1	5.28	**0.023**	11.9	**<0.002**	0.15	0.693	0.18	0.613	10.91	**0.001**
genotype × nitrogen	1	2.1	0.150	0.43	0.547	0.45	0.501	1.38	0.161	2.57	0.109
develop. stage	4	223.14	**<0.001**	32.98	**<0.001**	157.96	**<0.001**	181.53	**<0.001**	45.65	**<0.001**

Natural enemies		**Anthocoridae**		**Chrysopidae**		**Thrips**		**Coccinellidae**		**Miridae**		**Arachnida**	
Factors	df	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p
genotype	1	0.012	0.914	0.29	0.564	0.161	0.686	0.05	0.83	1.17	0.286	1.14	0.306
nitrogen	1	0.49	0.49	0.29	0.564	0.875	0.346	0.21	0.67	0.16	0.69	1.14	0.306
genotype × nitrogen	1	1.95	0.168	0.02	0.881	0.24	0.619	0.15	0.72	1.90	0.175	0.00	0.992
develop. stage	4	52.41	**<0.001**	16.45	**<0.001**	45.08	**<0.001**	22.68	**<0.001**	57.08	**<0.001**	16.93	**0.004**

Effects of plant variety, nitrogen levels and herbivory on volatile compounds

Seven volatile compounds were quantified in our study (Table 2) and all seven had been previously reported for maize (Degen et al., 2004). We expected a small number and amount of volatile compounds in both control and herbivore induced plants given that (i) the wild type line W37 produces low amounts of volatile inducible terpenes (Richter et al., 2016) and that (ii) *Z. scutellaris* induced plants do not emit the green leaf volatiles (Z)-3-hexenal and (E)-2-hexenal (Ardanuy et al., 2016). Herbivory explained the most variability in volatile blends (perMANOVA R^2=0.647, p=0.001), and a clear separation between control and herbivore induced plants was observed in PC1 (Fig. 2). Herbivore damaged plants emitted DMNT, indole, E-β-farnesene and (E)-β-bergamotene in addition to α-copaene, E-β-caryophyllene and β-sesquiphellandrene. However a significant genotype per nitrogen interaction was detected (perMANOVA R^2=0.036, p=0.007). In particular individual differences in volatile emission between nitrogen regimes could be attributed for α-copaene and E-β-caryophyllene (Table 1), while differences between genotypes were only detected for β-sesquiphellandrene in the treatment with nitrogen surplus which is consistent with the preference of *Z. scutellaris* females for Carolight+N in the olfactometer assay. An effect of the experimental day of volatile collection was detected on the volatile blend (R^2=0.028, p=0.011).

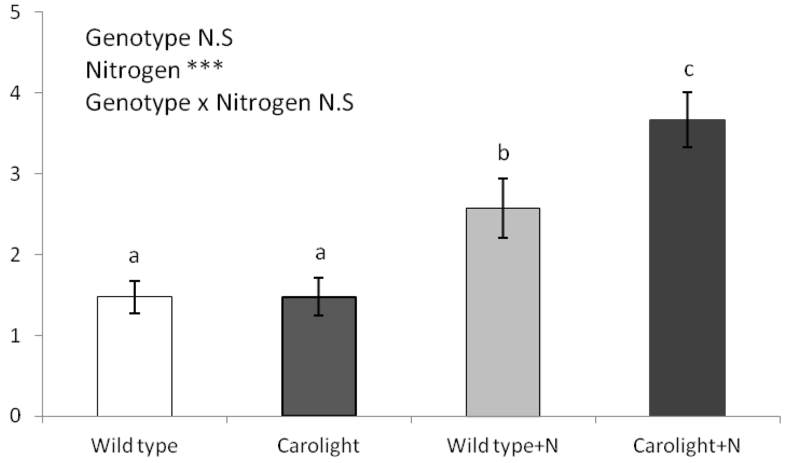


Fig 1. Choice of maize volatiles by leafhopper *Z. scutellaris* on the olfactometer. Tested plants consisted of Wild type and Carolight plants grown under control or surplus nitrogen (+N) conditions. Different letters indicate differences between treatments (α=0.05).

Effects of plant variety, nitrogen availability and herbivory on phytohormone and hydroxycinnamic acid accumulation

To further investigate the effect of genotype, nitrogen and herbivore attack on plant defenses, the concentrations of the phytohormones JA, OPDA, JA-Ile, SA, ABA and IAA were measured together with the hydroxycinnamic acids caffeic, ferulic and chlorogenic acid. The concentration of SA, ABA, and JA-Ile was significantly influenced by herbivory, time point and experimental repetition (Fig. 3). Interestingly, feeding by the herbivore *Z. scutellaris* significantly repressed basal levels for JA-Ile, as mean levels of JA-Ile in herbivore damaged plants was lower than in their respective undamaged controls. This trend was also significant but not so clear for SA and ABA accumulation after herbivory by maize leafhopper. However, all hormone concentrations were similar among genotype per nitrogen treatments for all time points with the exception of SA levels that were lower in Carolight^{R} relatively to M37W (Fig 3).

Overall, caffeic and chlorogenic acid concentrations were up to 2-fold lower in Carolight^{R} than in the wild type genotype, and were significantly affected by the experimental repetition (Fig 4). Caffeic acid concentration depended on herbivory, time point and time point per nitrogen interaction (Fig 4), whereas chlorogenic accumulation varied greatly between nitrogen regimes, its concentration practically doubled under control nitrogen regime with respect to the nitrogen surplus treatment (Fig 4).

Effects of plant variety, nitrogen availability and herbivory on the metabolite fingerprint

In total 4271 and 2002 markers were detected in ESI-positive and ESI-negative mode, respectively. Overall, nitrogen availability was the main factor contributing to the observed chemotypes (perMANOVA $R^2=0.124$, $p=0.001$), followed by genotype (perMANOVA $R^2=0.038$, $p=0.030$) and herbivory (perMANOVA $R^2=0.034$, $p=0.048$) while interactions of the experimental factors were non-significant. An unsupervised approach (PCA) showed that nitrogen metabolites from plants subjected to control and nitrogen surplus treatments clearly grouped in the first two PCs (Fig. 5), independently of the plant genotype and herbivore treatment. In contrast, genotype and herbivory related profiles could not be separated by PCA. However a supervised partial least squares discriminant analysis (PLS-DA) models separated (i) nitrogen regimes (ii) maize genotypes, and (iii) healthy and herbivore damaged plants (Table 3, validated through CV-ANOVA). These PLS-DA models were used to identify the metabolites showing the maximum difference between treatments with VIP values>2 (Table 3), and subsequently the selected metabolites for each experimental factor (variety, nitrogen and herbivory) were screened for putative identification using the pathway tool from MarVis (Table 3, Table 4).

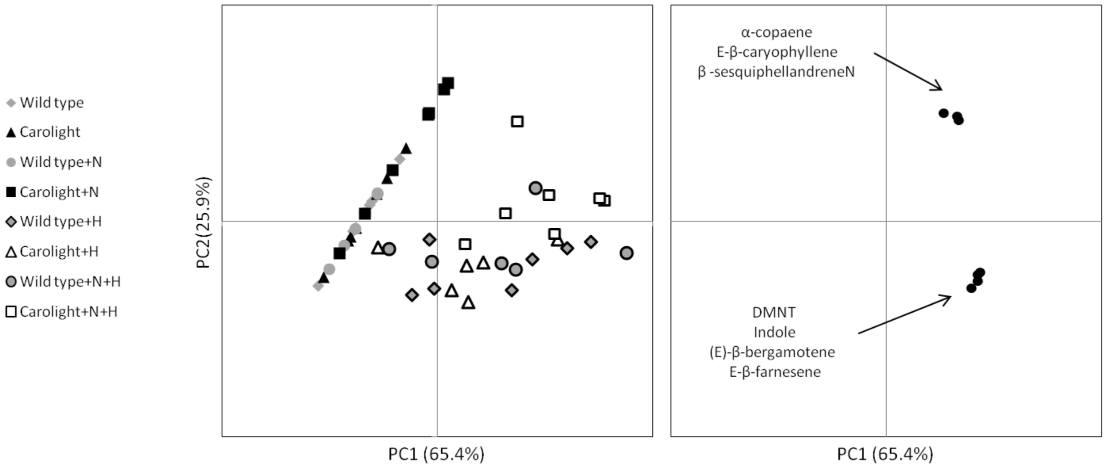


Fig 2. Score-plot (a) an loading-plot (b) of PCA analysis based on the emission rates of VOCs (Table 2) of control and herbivore-attacked (+H) maize plants from two genotypes (Carolight,Wild type) at two different N availability treatments (control, +N). Compounds that appear close to each other are co-varying.

Table 2. Volatile emissions (ng/h) of control and herbivore-induced maize plants from two genotypes (Carolight,Wild type) at two different N availability treatments (control, +N) (n=7). Amounts of each compound were compared among treatments using a non-parametric Kruskal-Wallis test followed by Dunn's test (*p<0.05, **p<0.01, ***p<0.001). Compounds denoted with "N" were only tentatively identified by comparison of their MS to that reported in libraries.

	Wild type		**Carolight**		**Wild type+N**		**Carolight+N**			
	mean	**±SE**	**mean**	**±SE**	**mean**	**±SE**	**mean**	**±SE**	**χ^2**	**P**
Control										
α-copaene	0.59	0.13	0.67	0.17	0.51	0.09	1.21	0.22	6.11	0.11
E-β-caryophyllene	0.44	0.06	0.35	0.06	0.32	0.05	0.78	0.15	6.31	0.10
β -sesquiphellandrene^N	1.36	0.26	1.16	0.34	1.01	0.19	2.96	0.69	5.53	0.14
Induced										
DMNT	3.74	0.79	3.44	0.80	3.51	1.46	4.27	0.94	0.98	0.80
Indole	7.79	2.55	5.29	1.40	7.64	4.00	6.85	1.68	1.13	0.77
α-copaene	0.75ab	0.16	0.60a	0.11	1.19bc	0.25	1.35c	0.12	9.10	**0.03**
E-β-caryophyllene	0.45a	0.08	0.40a	0.07	0.79b	0.18	0.92b	0.13	9.00	**0.03**
(E)-β-bergamotene	1.33	0.34	1.01	0.20	1.54	0.49	1.37	0.22	1.97	0.58
E-β-farnesene	4.97	1.45	2.81	0.85	5.37	1.90	4.32	1.03	1.48	0.69
β -sesquiphellandrene^N	2.08b	0.44	1.57ab	0.32	0.80a	0.14	3.68c	0.45	13.90	**<0.001**

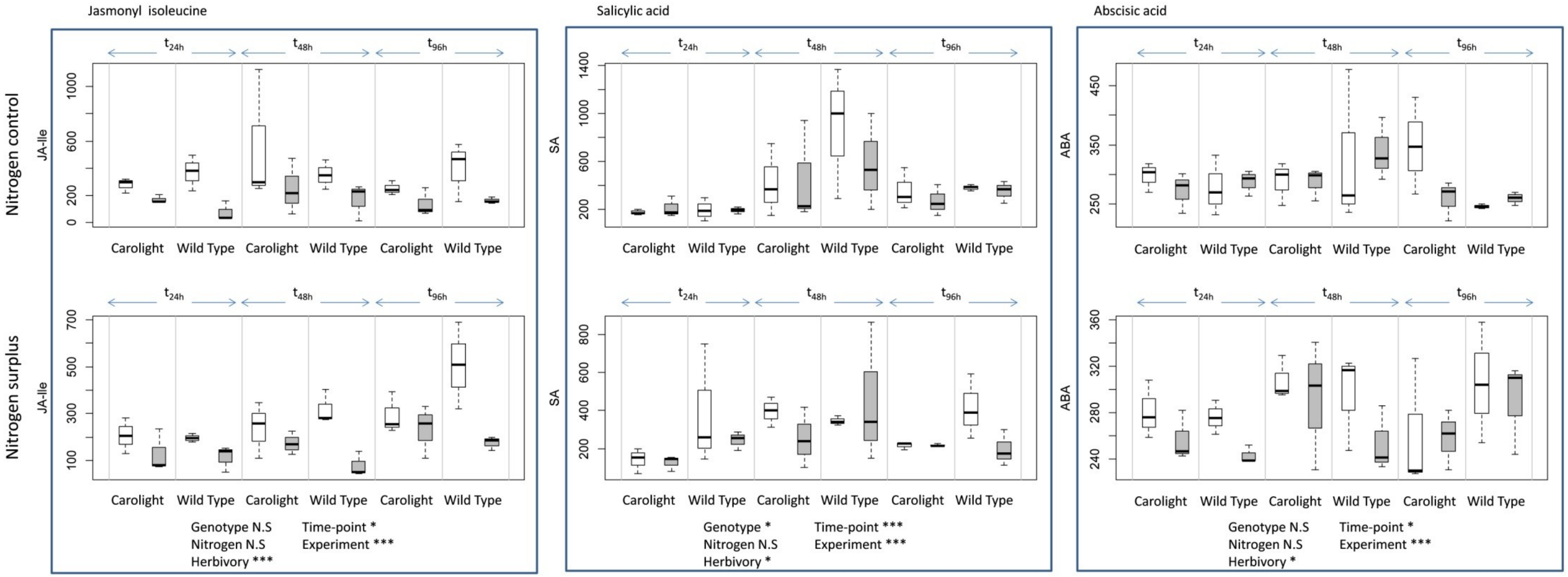


Fig. 3 Hormonal content (ng/g dry weight) in Carolight and Wild type plants grown in two different nitrogen regimes (control and N+) upon *Z. scutellaris* feeding. Control and herbivore-damaged plants were collected at different timepoints (24h, 48h and 96h after herbivore feeding), and JA-Ile, SA and ABA levels were determined in freeze-dried material by UPLC-MS. The experiment was repeated 3 times with similar results. The results shown are mean hormone levels of one experiment, the statistical analysis was performed on the three experiments with the Experiment as a blocking factor. White bars indicate control plants an grey bars indicate herbivore damaged plants. Asterisks indicate differences between control and herbivore damaged plants.

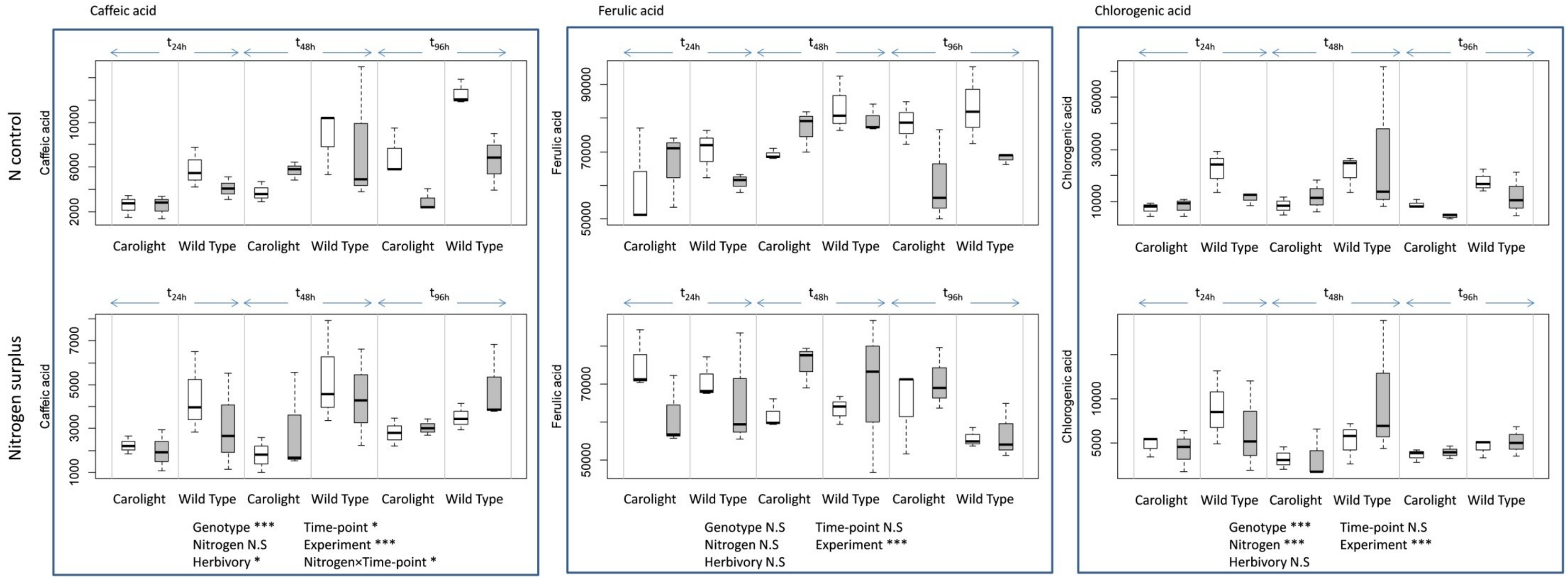


Fig. 4 Caffeic, ferulic and chlorogenic acid content (ng/g dry weight) in Carolight and Wild type plants grown in two different nitrogen regimes (control and N+) upon *Z. scutellaris* feeding. Control and herbivore-damaged plants were collected at different time-points (24h, 48h and 96h after herbivore feeding), and hydroxycinnamic levels were determined in freeze-dried material by UPLC-MS. The experiment was repeated 3 times with similar results. The results shown are mean levels of one experiment, the statistical analysis was performed on the three experiments with the Experiment as a blocking factor. White bars indicate control plants an grey bars indicate herbivore damaged plants.

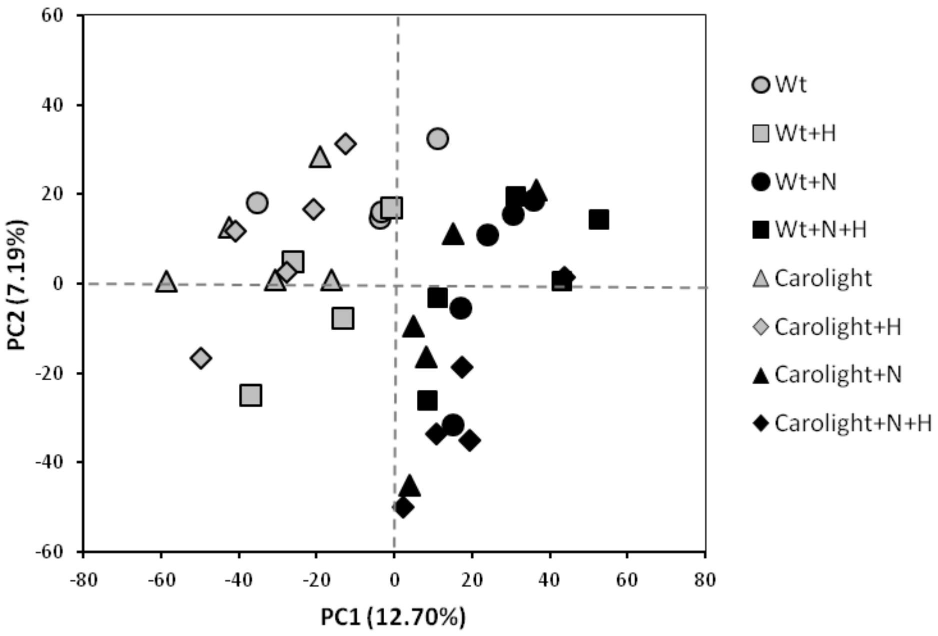


Fig 5.Principal component analysis (PCA) showing groups generated from signals obtained in ESI+ and ESI− by non-targeted analysis.

Table 3. PLS-DA models of the factors nitrogen, genotype and herbivory, number of components and valitation through CV-Anova. For each factor, the number of ions with a VIP>2 for the best model and the number of Marvis Pathway IDs are specified.

Factor	Components	R^2	Q^2	CV-ANOVA		VIP>2	MarVis ID
Nitrogen	**1**	**0.875**	**0.744**	**$F_{2,35}$=50.89**	**p=4.38x10^{-11}**	**405**	**48**
	1+1	0.968	0.999	$F_{4,33}$=34.80	p=2.08x10^{-11}		
Genotype	**1+1**	**0.956**	**0.581**	**$F_{4,33}$=7.87**	**p=0.00014**	**69**	**19**
	1+1+1	0.993	0.803	$F_{6,31}$=6.25	p=0.00022		
Herbivory	**1+1**	**0.967**	**0.486**	**$F_{4,33}$=3.97**	**p=0.0097**	**163**	**15**
	1+1+1	0.99	0.634	$F_{6,31}$=2.79	p=0.027		

Table 4. Metabolites with higher loadings on the best PLS-DA models (VIP>2) for the factors Nitrogen, Genotype and Herbivory that could be tentatively identified. .Metabolites in A were identified by comparison of the MS/MS to online databases, while metabolites in B were assigned after comparing the accurate mass to reference compound databases. Mean abundances of the matabolites can be found in Supplementary material Fig. S.4, S.5 and S.6.

A. Identified	**Mass neutral**	**ESI**	**RT (min)**	**Factor**	**Pathway**
Kynurenic acid	189.0431	+	0.99	Herbivory (H↑)	Tryptophan-kynurenine pathway
11-trans-LTD4	496.2592	-	3.43	Herbivory (H↑)	Leukotriene- Arachidonic acid metabolism
19-HETE/ 20-HETE	320.236	+	2.06	Genotype (Wt↑)	Arachidonic acid metabolism
Thiamine	265.1153	+	0.86	Genotype (Wt↑)	Vitamin and cofactor - Thiamine metabolism (primary metabolism)
Phytosphingosine	317.2932	+	4.41	Genotype (Wt↑)	Sphingolipid metabolism (primary metabolism)
L-Tryptophan	204.0897	-	0.93	Nitrogen (+N↑)	Tryptophan pathway (primary metabolism)

B. Assigned to	**Mass neutral**	**ESI**	**RT (min)**	**Factor**	**Pathway**
Sinapoyl malate	340.0791	-	2.73	Herbivory (H↓)	Biosynthesis of phenylpropanoids
Porphobilinogen	226.0965	-	0.76	Herbivory (H↑)	Porphyrin and chlorophyll metabolism
(-)-Jasmonoyl-L-isoleucine	323.2097	+	4.97	Herbivory (H↓)	Biosynthesis of plant hormones
A-tocopherol	430.3777	+	5.92	Herbivory (H↑)	Biosynthesis of plant secondary metabolites
2,3-Dihydroxybenzoate	154.0263	-	1.44	Herbivory (H↑)	Biosynthesis of phenylpropanoids
Coniferol	180.0788	+	0.84	Genotype (Wt↑)	Biosynthesis of phenylpropanoids
Cis-hinokiresinol	252.1195	-	0.84	Genotype (Wt↑)	Biosynthesis of phenylpropanoids
Unknown flavonoid	306.0775	-	1.36	Genotype (Wt↓)	Flavonoid biosynthesis
Unknown flavonoid	578.1634	+	1.78	Genotype (Wt↓) Nitrogen (+N↓)	Flavone and flavonol biosynthesis
Unknown flavonoid	610.1544	+	1.79	Genotype (Wt↓) Nitrogen (+N↓)	Flavone and flavonol biosynthesis
Unknown flavonoid	448.1016	+/-	1.59/2.05	Nitrogen (+N↓)	Flavone and flavonol biosynthesis
Unknown flavonoid	594.1579	-	1.61	Nitrogen (+N↓)	Flavone and flavonol biosynthesis
Unknown flavonoid	286.0483	+	1.99	Nitrogen (+N↓)	Flavone and flavonol biosynthesis
Unknown flavonoid	464.0949	-	1.86	Nitrogen (+N↓)	Flavone and flavonol biosynthesis
Unknown flavonoid	592.1802	+	2.73	Nitrogen (+N↓)	Flavone and flavonol biosynthesis
Zeatin/Pantothenate	219.1113	+	0.8	Nitrogen (+N↓)	Biosynthesis of plant hormones
DIMBOA-Glu	373.1009	-	1.46	Nitrogen (+N↑)	Benzoxazinoid biosynthesis
HBOA	149.0471	-	0.73	Nitrogen (+N↑)	Benzoxazinoid biosynthesis
DHBOA/ DIBOA-Glc	343.0891	-	1.27	Nitrogen (+N↑)	Benzoxazinoid biosynthesis
Dhurrin	311.1002	-	0.73	Nitrogen (+N↑)	Cyanoamino acid metabolism

DISCUSSION

Insect abundance and performance on Carolight^R in contrasting nitrogen availability conditions

We addressed the hypothesis that a nutritionally enhanced maize (Carolight^R), similar in terms of biomass and yield to its near isogenic line M37W (Zanga et al., 2016), will also be equivalent in terms of the associated arthropod community under field conditions. The performance of Carolight^R and M37W was tested under two different nitrogen regimes in order to broaden the range of environments in which plants were grown and to assess the robustness of equivalence.

Overall, the community of herbivores was similar for both Carolight^R and M37W genotypes. Nevertheless in the case of Hemiptera (leafhoppers, planthoppers and aphids) higher abundances were detected in plots with nitrogen surplus while only the leafhopper *Z. scutellaris* nymph abundances were significantly higher for M37W. Nitrogen is one of the most frequently used anthropogenic fertilizers in agricultural production and is known to exert a variety of bottom-up effects and potentially alter tritrophic interactions through various mechanisms (Chen et al., 2010), especially in herbivorous Hemiptera (Butler et al., 2012). Hemipterans are insects with a high potential sensitivity to plant quality as they have been reported to prefer and perform better on some genotypes or on plants that differ in quality in terms of nutritional requirements (e.g. nitrogen content), physical or chemical plant defense (e.g. Kallenbach et al., 2011; Zytynska and Preziosi, 2011). A number of reports suggest that herbivore Hemiptera (especially leafhoppers and aphids) are more abundant and/or perform better on Bt maize lines than on the near isogenic counterparts (Lumbierres et al., 2010, 2004; Obrist et al., 2006; Pons et al., 2005a; Rauschen et al., 2011; Virla et al., 2010) although mechanisms involved in such differences have not been clearly attributed to specific factors, rather to pleiotropic effects. Pleiotropic effects reported for Bt maize that might influence Hemipteran densities are higher lignin content in the stem of Bt plants (Saxena and Totzky, 2001), reduced amount of VOC emission in a Bt line (Turlings et al., 2005) and sap amino acid content (Faria et al., 2007).

The leafhopper *Z. scutellaris* was the most abundant herbivore in our maize fields (total of 5,951 nymphs in all plots though the season, Supplementary Material Fig S.1) followed by thrips (Albajes et al., 2011) and consequently a change in their abundance could have great impact on the abundance of arthropods in higher trophic levels. The differences in herbivore abundances in plots with increased nitrogen content were not detected on the next trophic level, as the composition and abundance of natural enemies did not vary between genotype x nitrogen treatments. The most abundant predator during the season was *Orius* spp. that is known to respond numerically to *Z. scutellaris* abundance at the field level (Albajes et al. 2011, Ardanuy et al. unpublished). Nevertheless we did not detect any differences in *Orius* spp. between treatments probably due to the high abundance of prey in the treatment plots (leafhoppers and thrips) or small differences in density between plots, small size of the experimental plots and the dispersal capacity of *Orius* spp. (Madeira and Pons, 2015). To the best of our knowledge no studies have reported differences in natural enemy composition in GM crops not related to the variation in available prey (Marvier et al. 2007; Naranjo, 2009; Comas et al. 2014) (e.g. numbers of hymenopteran parasitoids might be reduced due to the low availability of target lepidopteron hosts in Cry1Ab maize fields), and a recent analysis of food web structures in Bt and herbicide tolerant maize revealed a complex and stable food web in maize agroecosystems (Szénási et al., 2014).

Insect herbivores are limited by low nitrogen concentrations in food plants, and therefore herbivore performance is generally thought to be positively related to increases in nitrogen content in plants (Awmack and Leather, 2002; Behmer, 2009; Butler et al., 2012). The performance of *Z. scutellaris* nymphs was similar when fed on Carolight^R and M37W grown under control and surplus nitrogen conditions. This result was unexpected as we hypothesized that nitrogen availability would be the main factor contributing to adult final weight as a proxy for reproductive fitness. However, leafhopper females preferred maize plants grown under nitrogen surplus conditions in the olfactometer, and even preferred Carolight^R plants over M37W when plants were grown under high nitrogen availability conditions. This fact - together with field data on leafhopper abundance - supports the notion that host plant quality (resulting from enhanced nitrogen fertilization) might indeed offer other advantages to the species, such as reproductive success, that are not reflected by adult body weight or duration of nymphal development. Prestidge (1982) reported an increasing oviposition of *Z. scutellaris* as the nitrogen fertilization increased in the grass *Holcus lanatus*. Therefore the lack of differences in adult weight and developmental time for maize leafhoppers in our experiments could be a product of a mismatch between adult size and fecundity in *Z. scutellaris* as it has been previously described for grasshoppers (Joern and Behmer, 1998). Therefore, several features including field abundance, plant preference and fecundity could provide the best measures of performance for *Z. scutellaris* in general.

Maize defense responses to Z. scutellaris feeding

Plant damage together with salivary secretions of phytophagous arthropods can trigger plant inducible defense responses (Alborn et al. 1997, Musser et al. 2002). Inducible plant defenses can be major determinants of ecological interactions, in particular defenses depending on JA and SA pathways appear to play important roles in determining community composition (Kallenbach et al., 2011; Thaler, 2002; Thaler et al., 2001). Hence it was vital to determine whether Carolight^R and its near-isogenic line behave similarly in terms of constitutive expression of JA and SA and in hormonal response when facing herbivory. Phytohormone content in undamaged plants was similar for Carolight^R and M37W and was not influenced by nitrogen fertilization. Leafhopper *Z. scutellaris* feeding/oviposition did not trigger the accumulation of phytohormones JA-Ile, salicylic acid, ABA and IAA involved in induced plant defenses in maize at any of the time-points considered (1, 2 and 4 days). This was reflected in that phytohormone levels in leafhopper damaged plants were similar or even lower than the constitutive levels in healthy control plants, in particular those of JA-Ile.

Cell content feeders, such as the spider mite *Tetranychus urticae* Koch (Acari: Prostigmata) and the thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera:Thripidae) usually stimulate JA-inducible genes (known as the oxylipin pathway) upon attack (Vos et al., 2005), although there are reports that confirm the activation of both SA- and JA-inducible genes (Kant et al., 2004; Kawazu et al., 2012). Given that typhlocybine leafhoppers such as *Z. scutellaris* feed on the mesophyll using a sawing laceration strategy (Marion-Poll et al. 1987, Backus et al. 2005) we predicted that feeding by the leafhopper would activate either the JA or/and SA pathways; however feeding by this herbivore appears to decrease the constitutive levels of JA and SA on maize plants.

Suppression of plant defenses is a well-known phenomenon in plant pathogens such as pathogenic bacteria, rust fungi, oomycetes, viruses, and herbivores such as nematodes and spider mites (reviewed by Kant et al. 2015 and Zhang et al. 2017). Spider mite *Tetranychus evansi* suppresses both JA and SA dependent defenses in tomato, which enhances their performance (Alba et al., 2015; Sarmento et al., 2011). In the case of insects, to our knowledge the majority of cases of plant defense suppression has been attributed to JA-SA hormonal crosstalk (Walling, 2000; Zhang et al., 2017) and not to a direct blocking of JA or SA defenses. Some examples of hormonal crosstalk are glucose oxidases in lepidopteran larvae saliva that suppress wound-induced plant defense against herbivores via activation of the SA pathway, or the oviposition by Lepidoptera that also triggers SA accumulation and signaling and suppresses JA-regulated plant defense against herbivores. However aphids and mites have been reported to deliver effectors when feeding as a strategy to overcome host-plant defenses and improve their fitness (Hogenhout and Bos, 2011; Kant et al., 2015; Mugford et al., 2016; Villarroel et al., 2016).

In our experimental system, *Z. scutellaris* - by feeding and ovopositing - suppresses JA and does not induce SA in maize plants, and hence hormonal suppression appears to occur independently of SA-JA cross-talk (as was the case for *T. evansi*). The mechanism by which maize leafhopper might suppress plant defense is unknown but it impairs hormone accumulation without disturbing plant indirect defense by means of HIPVs emission. Previous work showed that maize plants damaged by ten *Z. scutellaris* adults emitted a similar amount of HIPVs than plants damaged by the five 2nd instar *Spodoptera littoralis*, although the blend emitted by plants damaged by maize leafhopper did not contain the GLVs (Z)-3-hexenal and (E)-2-hexenal (Ardanuy et al., 2016). We hypothesize that the suppression of JA defenses ultimately benefits leafhopper reproduction and nymphal performance, but still the plant would remain protected by means of top-down control by natural enemies. However, defense manipulation by maize leafhoppers might also have consequences for subsequent colonizing herbivores since maize plants with suppressed defenses might promote the performance of co-occurring herbivores (Kant et al., 2015; Stam et al., 2014).

Nitrogen determines the chemical defense attributes of Carolight and M37W

Evaluating Carolight^R and wild type genotypes in contrasting nitrogen conditions allowed for (i) a broader characterization of the resulting chemotypes and their impact on insect behavior/performance; and (ii) a comparative analysis of the impact of experimental factors (nitrogen, genotype, herbivory) on the final chemotypes. We demonstrated that nitrogen is the main factor determining the metabolite fingerprint in Carolight^R and M37W, followed by the introduced traits and herbivory. There were no significant effects of nitrogen x genotype interactions, suggesting that both genotypes behaved similarly when grown under the same nitrogen conditions. Our results corroborate the work of Coll et al. (2010) where transcript analysis in two maize MON810/near isogenic lines in the field indicated that differences between lines (genetic background) exerted the highest impact on gene expression patterns, followed by nitrogen availability, while the Cry1Ab trait had the lowest impact. Barros et al. (2010) compared two GM pairs (Bt and glyphosate tolerant) using transcriptome, proteome, and metabolome profiling and reported that the environment affected gene expression, protein distribution, and metabolite content more strongly than the genetic modification. Present results would therefore be in accordance with the overall research up to date that shows that environmental factors (e.g field location, sampling time during the season or at different seasons, mineral nutrition) consistently exert a greater influence on crop GM pairs than the genetic modification itself (reviewed by Ricroch et al. 2011).

In general, nitrogen fertilization increases plant growth and reproduction, decreases concentrations of carbon-based secondary compounds (e.g. phenolics and terpenoids), and increases nitrogenous compounds (Hermans et al., 2006; Koricheva et al., 1998; Kusano et al., 2011; Lou and Baldwin, 2004; Scheible et al., 2004). Nitrogen levels influenced Carolight^R and M37W phenotypes at the metabolite level substantially, including compounds involved in direct and indirect plant defenses. Of the potential 405 markers with a VIP>2 only few were putatively identified. Some of these are secondary metabolites and contribute to the plant's constitutive defense as flavonoids or hydroxamic acids (benzoxazinoids) (Table 4, Supplementary material Fig. S.6).

Targeted analysis of constitutive direct defense metabolites showed that chlorogenic acid greatly varied with the nitrogen regime - at higher concentrations in plant tissues when nitrogen was limiting - but also with the plant genotype - M37W had higher levels of both chlorogenic and caffeic acid. Higher concentration of constitutive phenolics in plants under low nitrogen conditions is consistent with results in *Nicotiana atenuata* (Lou and Baldwin, 2004) and tomato (Stout et al., 1998). Carolight accumulated up to 2-fold lower amounts of plant hydroxycinnamic acids (caffeic and chlorogenic acids) depending on the nitrogen treatment and time-point, and higher amounts of lignans (specially at low nitrogen conditions) than the wild-type counterpart. This revealed an effect of the genetic modification on the phenylpropanoid biosynthetic pathway. In addition phenolics in form of unidentified flavonoids were more abundant in underfertilized maize plants (Table 4, Supplementary material Fig. S.6).

Metabolite fingerprinting showed that nitrogen surplus increased the accumulation of tryptophan in plants, which we identified as a marker of nitrogen surplus treatment. Tryptophan serves as precursor of a wide variety of nitrogen-containing aromatic secondary metabolites, such as hydroxamic acids (Fig. S.6), which play crucial roles in plant defense against herbivore feeding (Balmer et al., 2013; Niemeyer, 2009). Higher levels of constitutive phenolics and hydroxamic acids would theoretically increase plant tolerance towards herbivores, as increased levels of these secondary compounds have been associated to reduced herbivory (Balmer et al., 2013; Mithöfer and Boland, 2012). Nevertheless, we have no data on how levels of these phenolics and hydroxamic acids might impact maize-*Z. scutellaris* interactions. Olfactometer plant choice might indicate a preference towards plants with lower concentration of phenolics and higher concentration of hydroxamic acids in the plant; however it fails to explain higher abundance of nymphs in wild type plots in field conditions.

In addition the VOC blend was also modified by nitrogen availability: a higher concentration of the sesquiterpenes α-copaene and E-β-caryophyllene was detected for Carolight^R and M37W plants subjected to higher nitrogen availability, and which could be responsible for leafhopper preference towards those plants. These results contrast with previous findings by Schmelz et al. (2003) that reported higher VOC emission in maize with limited nitrogen availability, though differences could be explained by maize varieties or by the source of nitrogen used in each study. While we applied nitrogen as both nitrate and ammonium, in the later study nitrogen was applied as nitrate. VOC blend was unaffected by nitrogen conditions in soybean (Winter and Rostás, 2010) and *Nicotiana attenuata* (Lou and Baldwin, 2004). Carolight damaged plants grown under nitrogen surplus conditions emitted a larger amount of β -sesquiphellandrene, however the change in the volatile blend did not influence the community of natural enemies in the field. A blend of VOCs that varies in the composition or quantity of its components may constitute a signal with altered information content and may potentially modify the host finding behavior of herbivores and natural enemies as it is the case for maize leafhopper *Z. scutellaris*, that prefers Carolight plants to the wild type when grown in surplus nitrogen conditions. It remains unclear whether leafhoppers respond in a dose-dependent manner to the total blend or if other compounds at doses too small to be detected (D'Alessandro et al. 2006) triggered leafhopper preference in the olfactometer and in field conditions.

To date several reports have shown that the VOC profile of plants might be altered as a result of the genetic modification although very few reports address how these modifications impact herbivore plant choice and indirect defense. In maize, Turlings et al. (2005) analyzed volatiles from the Bt line and its near isogenic counterpart and reported that the isogenic line released a larger amount of most volatiles, although this change did not impact on plant choice as both lines were equally attractive to two parasitoid species in olfactometer bioassays. The changes in the Bt maize volatile blend could be partly responsible for early settlement of aphids in the field (Lumbierres et al., 2004), although differences in total aphid abundance were probably better accounted for the year conditions and variety background (Degen et al., 2004; Lumbierres et al., 2010).

In summary we have shown the separate and interactive effects of nitrogen and genotype availability on (i) the arthropod community and on(ii) the performance and behavior of an herbivore, and related these changes to the constitutive and inducible defenses of maize. In summary nitrogen availability greatly shapes the maize metabolism and the resulting plant chemotypes and promotes *Z. scutellaris* preference through the emission of a more attractive blend of VOCs. Feeding by *Z. scutellaris* suppresses the accumulation of JA and SA phytohormones while triggering the emission of HIPVs. Minor differences were defected among plant genotypes related to the phenylpropanoid pathway and a volatile compound. Overall the high-carotenoid maize Carolight^{R} does not behave in a significantly different manner to its wild-type counterpart in terms of aboveground plant-arthropod interactions.

ACKNOWLEDGMENTS

We would like to thank Michael Gaillard and Daniel Maag for help in sample preparation, Gemma Camañes for advice on nitrogen treatments, Diana LaForgia for help in arthropod sampling, Joan Safont, Carmen López and Matilde Eizaguirre for providing Z. scutellaris, Daniela Zanga for discussions on Carolight agronomic performance, Victor Flors and the Metabolic Integration and Cell Signaling Group for discussion on results and data analysis, and all the members of the FARCE and E-Vol groups in Neuchâtel for their collaboration. A.A. was funded with an FPU scholarship and a visiting grant to FARCE laboratory from the Ministerio de Educación. The work was partially funded by the Spanish Government project AGL2011-23996, Agrotecnio (Fundació Centre de Recerca en Agrotecnologia, VitaMaize 01 project) and Fundació la Caixa (Grant P13005).

REFERENCES

Alba, J. M., Schimmel, B. C. J., Glas, J. J., Ataide, L. M. S., Pappas, M. L., et al. (2015). Spider mites suppress tomato defenses downstream of jasmonate and salicylate independently of hormonal crosstalk. New Phytologist, 205, 828–840.

Albajes, R., Lumbierres, B., & Pons, X. (2011). Two heteropteran predators in relation to weed management in herbicide-tolerant corn. Biological Control, 59, 30–36.

Alborn, H. T., Turlings, T. C. J., Jones, T. H., Stenhagen, G., Loughrin, J. H., et al. (1997). An elicitor of plant volatiles from beet armyworm oral secretion. Science, 276, 945–949.

Ardanuy, A., Albajes, R., & Turlings, T. C. J. (2016). Innate and Learned Prey-Searching Behavior in a Generalist Predator. Journal of Chemical Ecology, 42, 497–507.

Awmack, C. S., & Leather, S. R. (2002). Host plant quality and fecundity in herbivorous insects. Annual Review of Entomology, 47, 817–844.

Backus, E. a, Serrano, M. S., & Ranger, C. M. (2005). Mechanisms of hopperburn: an overview of insect taxonomy, behavior, and physiology. Annual review of entomology, 50, 125–151.

Balmer, D., Flors, V., Glauser, G., & Mauch-Mani, B. (2013). Metabolomics of cereals under biotic stress: current knowledge and techniques. Frontiers in Plant Science, 4, 82.

Barros, E., Lezar, S., Anttonen, M. J., Dijk, J. P. Van, Ro, R. M., et al. (2010). Comparison of two GM maize varieties with a near- isogenic non-GM variety using transcriptomics, proteomics and metabolomics. Plant Biotechnology Journal, 8, 436–451.

Behmer, S. T. (2009). Insect herbivore nutrient regulation. Annual Review of Entomology, 54, 165–87.

van den Berg, R. A., Hoefsloot, H. C. J., Westerhuis, J. A., Smilde, A. K., & van der Werf, M. J. (2006). Centering, scaling, and transformations: improving the biological information content of metabolomics data. BMC genomics, 7, 142.

Butler, J., Garratt, M. P. D., & Leather, S. R. (2012). Fertilisers and insect herbivores: A meta-analysis. Annals of Applied Biology, 161, 223–233.

Camañes, G., Pastor, V., Cerezo, M., Garcia-Andrade, J., Vicedo, B., et al. (2012). A deletion in NRT2.1 attenuates *Pseudomonas syringae*-induced hormonal perturbation, resulting in primed plant defenses. Plant Physiology, 158, 1054–1066.

Chen, Y., Olson, D. M., & Ruberson, J. R. (2010). Effects of nitrogen fertilization on tritrophic interactions. Arthropod-Plant Interactions, 4, 81–94.

Coll, A., Nadal, A., Collado, R., Capellades, G., Kubista, M., et al. (2010). Natural variation explains most transcriptomic changes among maize plants of MON810 and comparable non-

GM varieties subjected to two N-fertilization farming practices. Plant Molecular Biology, 73, 349–362.

Cruz, D., & Eizaguirre, M. (2015). Do Sesamia nonagrioides (Lepidoptera; Noctuidae) Gravid Females Discriminate Between Bt or Multivitamin Corn Varieties? Role of Olfactory and Visual Cues. Journal of Insect Science, 15, 33–33.

Decourcelle, M., Perez-Fons, L., Baulande, S., Steiger, S., Couvelard, L., et al. (2015). Combined transcript, proteome, and metabolite analysis of transgenic maize seeds engineered for enhanced carotenoid synthesis reveals pleotropic effects in core metabolism. Journal of Experimental Botany, 66, 3141–3150.

Degen, T., Dillmann, C., Marion-Poll, F., & Turlings, T. C. J. (2004). High Genetic Variability of Herbivore-Induced Volatile Emission within a Broad Range of Maize Inbred Lines. Plant Physiology, 135, 1928–1938.

Faria, C. A, Wäckers, F. L., Pritchard, J., Barrett, D. A, & Turlings, T. C. J. (2007). High susceptibility of Bt maize to aphids enhances the performance of parasitoids of lepidopteran pests. PloS One, 2, e600.

Hermans, C., Hammond, J. P., White, P. J., & Verbruggen, N. (2006). How do plants respond to nutrient shortage by biomass allocation? Trends in Plant Science, 11, 610–617.

Hogenhout, S. A., & Bos, J. I. B. (2011). Effector proteins that modulate plant-insect interactions. Current Opinion in Plant Biology, 14, 422–428.

Joern, A., & Behmer, S. T. (1998). Impact of diet quality on demographic attributes in adult grasshoppers and the nitrogen limitation hypothesis. Ecological Entomology, 23, 174–184.

Kaever, A., Landesfeind, M., Feussner, K., Mosblech, A., Heilmann, I., et al. (2014). MarVis-Pathway: integrative and exploratory pathway analysis of non-targeted metabolomics data. Metabolomics, 764–777.

Kallenbach, M., Bonaventure, G., Gilardoni, P. A, Wissgott, A., & Baldwin, I. T. (2011). Empoasca leafhoppers attack wild tobacco plants in a jasmonate-dependent manner and identify jasmonate mutants in natural populations. Proceedings of the National Academy of Sciences of the United States of America, 109, E1548-57.

Kant, M. R., Ament, K., Sabelis, M. W., Haring, M. A, & Schuurink, R. C. (2004). Differential timing of spider mite-induced direct and indirect defenses in tomato plants. Plant Physiology, 135, 483–495.

Kant, M. R., Jonckheere, W., Knegt, B., Lemos, F., Liu, J., et al. (2015). Mechanisms and ecological consequences of plant defence induction and suppression in herbivore communities. Annals of Botany, 115, 1015–1051.

Kawazu, K., Mochizuki, A., Sato, Y., Sugeno, W., Murata, M., et al. (2012). Different expression profiles of jasmonic acid and salicylic acid inducible genes in the tomato plant against herbivores with various feeding modes. Arthropod-Plant Interactions, 6, 221–230.

Koricheva, J., Larsson, S., Haukioja, E., & Keinänen, M. (1998). Regulation of woody plant secondary metabolism by resource availability: hypothesis testing by means of meta-analysis. Oikos, 83, 212–226.

Kusano, M., Fukushima, A., Redestig, H., & Saito, K. (2011). Metabolomic approaches toward understanding nitrogen metabolism in plants. Journal of Experimental Botany, 62, 1439–1453.

Lou, Y., & Baldwin, I. T. (2004). Nitrogen Supply Influences Herbivore-Induced Direct and Indirect Defenses and Transcriptional Responses in *Nicotiana attenuata*. Plant physiology, 135, 496–506.

Lumbierres, B., Albajes, R., & Pons, X. (2004). Transgenic Bt maize and *Rhopalosiphum padi* (Hom ., Aphididae) performance. Ecological Entomology, 29, 309–317.

Lumbierres, B., Starý, P., & Pons, X. (2010). Effect of Bt maize on the plant-aphid–parasitoid tritrophic relationships. BioControl, 56, 133–143.

Madeira, F., & Pons, X. (2015). Effects of weed density on the dispersal of *Orius majusculus* Reuter (Heteroptera Anthocoridae) within maize. Journal of Applied Entomology, 139, 712–720.

Marvier, M., McCreedy, C., Regetz, J., & Kareiva, P. (2007). A meta-analysis of effects of Bt cotton and maize on nontarget invertebrates. Science, 316, 1475–1477.

Mithöfer, A., & Boland, W. (2012). Plant defense against herbivores: Chemical aspects. Annual Review of Plant Biology, 63, 431–450.

Mooney, K. A, Pratt, R. T., & Singer, M. S. (2012). The tri-trophic interactions hypothesis: interactive effects of host plant quality, diet breadth and natural enemies on herbivores. PloS one, 7, e34403.

Mugford, S., Barclay, E., Drurey, C., Findley, K., & Hogenhout, S. (2016). An immuno-suppressive aphid saliva protein is delivered into the cytosol of plant mesophyll cells during feeding. Molecular Plant-Microbe Interactions, 29, 854-861.

Musser, R. O., Hum-Musser, S. M., Eichenseer, H., Peiffer, M., Ervin, G., et al. (2002). Herbivory: caterpillar saliva beats plant defences. Nature, 416, 599–600.

Naqvi, S., Zhu, C., Farre, G., Ramessar, K., Bassie, L., et al. (2009). Transgenic multivitamin corn through biofortification of endosperm with three vitamins representing three. Proceedings of the National Academy of Sciences of the United States of America, 106, 7762–7767.

Niemeyer, H. (2009). Hydroxamic Acids Derived from 2-Hydroxy-2 H-1, 4-Benzoxazin-3 (4 H)-one: Key Defense Chemicals of Cereals. Journal of Agricultural and Food Chemistry, 57, 1677–1696.

Obrist, L. B., Dutton, A., Albajes, R., & Bigler, F. (2006). Exposure of arthropod predators to Cry1Ab toxin in Bt maize fields. Ecological Entomology, 31, 143–154.

Ode, P. J. (2006). Plant chemistry and natural enemy fitness: Effects on Herbivore and Natural Enemy Interactions. Annual Review Entomology, 51, 163–185.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., et al. (2013). Package "vegan". Community Ecology Package. R package.

Pons, X., Lumbierres, B., López, C., & Albajes, R. (2005). Abundance of non-target pests in transgenic Bt-maize: A farm scale study. European Journal of Entomology, 102, 73–79.

Prestidge, R. A. (1982). Instar duration, adult consumption, oviposition and nitrogen utilization efficiencies of leafhoppers feeding on different quality food (Auchenorrhyncha: Homoptera). Ecological Entomology, 7, 91–101.

Van Der Putten, W. H., Vet, L. E. M., Harvey, J. A., & Wäckers, F. L. (2001). Linking above- and belowground multitrophic interactions of plants, herbivores, pathogens, and their antagonists. Trends in Ecology and Evolution, 16, 547–554.

Rauschen, S., Schultheis, E., Hunfeld, H., Schaarschmidt, F., Schuphan, I., et al. (2011). Diabrotica-resistant Bt-maize DKc5143 event MON88017 has no impact on the field densities of the leafhopper *Zyginidia scutellaris*. Environmental Biosafety Research, 9, 87–99.

Richter, A., Schaff, C., Zhang, Z., Lipka, A. E., Tian, F., et al. (2016). Characterization of the (herbivore-induced) biosynthetic pathways of the volatile homoterpenes 3,8-dimethyl-1,4,7-nonatriene (DMNT) and 4,8,12-trimethyltrideca-1,3,7,11-tetraene, in *Zea mays*. The Plant Cell, 28, 2651–2665.

Ricroch, E., Berge, J. B., Kuntz, M., Ricroch, A. E., Bergé, J. B., et al. (2011). Evaluation of genetically engineered crops using transcriptomic, proteomic, and metabolomic profiling techniques. Plant Physiology, 155, 1752–1761.

Sarmento, R. A., Lemos, F., Bleeker, P. M., Schuurink, R. C., Pallini, A., et al. (2011). A herbivore that manipulates plant defence. Ecology Letters, 14, 229–236.

Saxena, D., & Totzky, G. S. (2001). Corn has a higher lignin content than non-Bt corn. American Journal of Botany, 88, 1704–1706.

Scheible, W.-R., Morcuende, R., Czechowski, T., Fritz, C., Osuna, D., et al. (2004). genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of *Arabidopsis* in response to nitrogen. Plant Physiology, 136, 2483–2499.

Schmelz, E. A, Alborn, H. T., Engelberth, J., & Tumlinson, J. H. (2003). Nitrogen deficiency increases volicitin-induced volatile emission, jasmonic acid accumulation, and ethylene sensitivity in maize. Plant Physiology, 133, 295–306.

Smith, C. A., Want, E. J., O'Maille, G., Abagyan, R., Siuzdak, G., et al. (2006). XCMS: processing mass spectrometry data for metabolite profiling using Nonlinear Peak Alignment,Matching,and Identification. Analytical Chemistry, 78, 779–87.

Stam, J. M., Kroes, A., Li, Y., Gols, R., van Loon, J. J. A. A, et al. (2014). Plant interactions with multiple insect herbivores: from community to genes. Annual review of plant biology, 65, 689–713.

Stout, M. J., Brovont, R. A., & Duffey, S. S. (1998). Effect of nitrogen availability on expression of constitutive and inducible chemical defenses in tomato, Lycopersicon esculentum. Journal of Chemical Ecology, 24, 945–963.

Szénási, A., Pálinkás, Z., Zalai, M., Schmitz, O. J., & Balog, A. (2014). Short-term effects of different genetically modified maize varieties on arthropod food web properties: an experimental field assessment. Scientific Reports, 4, 5315.

Thaler, J. S. (2002). Effect of jasmonate-induced plant responses on the natural enemies of herbivores. Journal of Animal Ecology, 141–150.

Thaler, J. S., Stout, M. J., Karban, R., & Duffey, S. S. (2001). Jasmonate-mediated induced plant resistance affects a community of herbivores. Ecological Entomology, 26, 312–324.

Turlings, T. C. J., Davison, A. C., & Tamò, C. (2004). A six-arm olfactometer permitting simultaneous observation of insect attraction and odour trapping. Physiological Entomology, 29, 45–55.

Turlings, T. C. J., Jeanbourquin, P. M., Held, M., & Degen, T. (2005). Evaluating the induced-odour emission of a Bt maize and its attractiveness to parasitic wasps. Transgenic Research, 14, 807–16.

Turlings, T. C. J., Lengwiler, U. B., Bernasconi, M. L., & Wechsler, D. (1998). Timing of induced volatile emissionsin maize seedlings. Planta, 207, 146–152.

Villarroel, C. A., Jonckheere, W., Alba, J. M., Glas, J. J., Dermauw, W., et al. (2016). Salivary proteins of spider mites suppress defenses in *Nicotiana benthamiana* and promote mite reproduction. Plant Journal, 86, 119–131.

Virla, E. G., Casuso, M., & Frias, E. a. (2010). A preliminary study on the effects of a transgenic corn event on the non-target pest *Dalbulus maidis* (Hemiptera: Cicadellidae). Crop Protection, 29, 635–638.

Vos, M. De, Oosten, V. R. Van, Poecke, R. M. P. Van, van Pelt, J. A., Pozo, M. J., et al. (2005). Signal Signature and Transcriptome Changes of Arabidopsis During Pathogen and Insect Attack. Molecular Plant-Microbe Interactions, 18, 923–937.

Walling, L. (2000). The Myriad Plant Responses to Herbivores. Journal of Plant Growth Regulation, 19, 195–216.

Winter, T. R., & Rostás, M. (2010). Nitrogen deficiency affects bottom-up cascade without disrupting indirect plant defense. Journal of Chemical Ecology, 36, 642–651.

Wolt, J. D., Keese, P., Raybould, A., Fitzpatrick, J. W., Burachik, M., et al. (2010). Problem formulation in the environmental risk assessment for genetically modified plants. Transgenic Research, 19, 425–36.

Zanga, D., Capell, T., Slafer, G. A., Christou, P., & Savin, R. (2016). A carotenogenic mini-pathway introduced into white corn does not affect development or agronomic performance. Scientific Reports, 6, 38288.

Zhang, L., Zhang, F., Melotto, M., Yao, J., & He, S. Y. (2017). Jasmonate signaling and manipulation by pathogens and insects. Journal of Experimental Botany, erw478.

Zytynska, S. E., & Preziosi, R. F. (2011). Genetic interactions influence host preference and performance in a plant-insect system. Evolutionary Ecology, 25, 1321–1333.

SUPPLEMENTARY INFORMATION

Fig S.1 Control and surplus nitrogen plants grown hydroponically under greenhouse conditions.

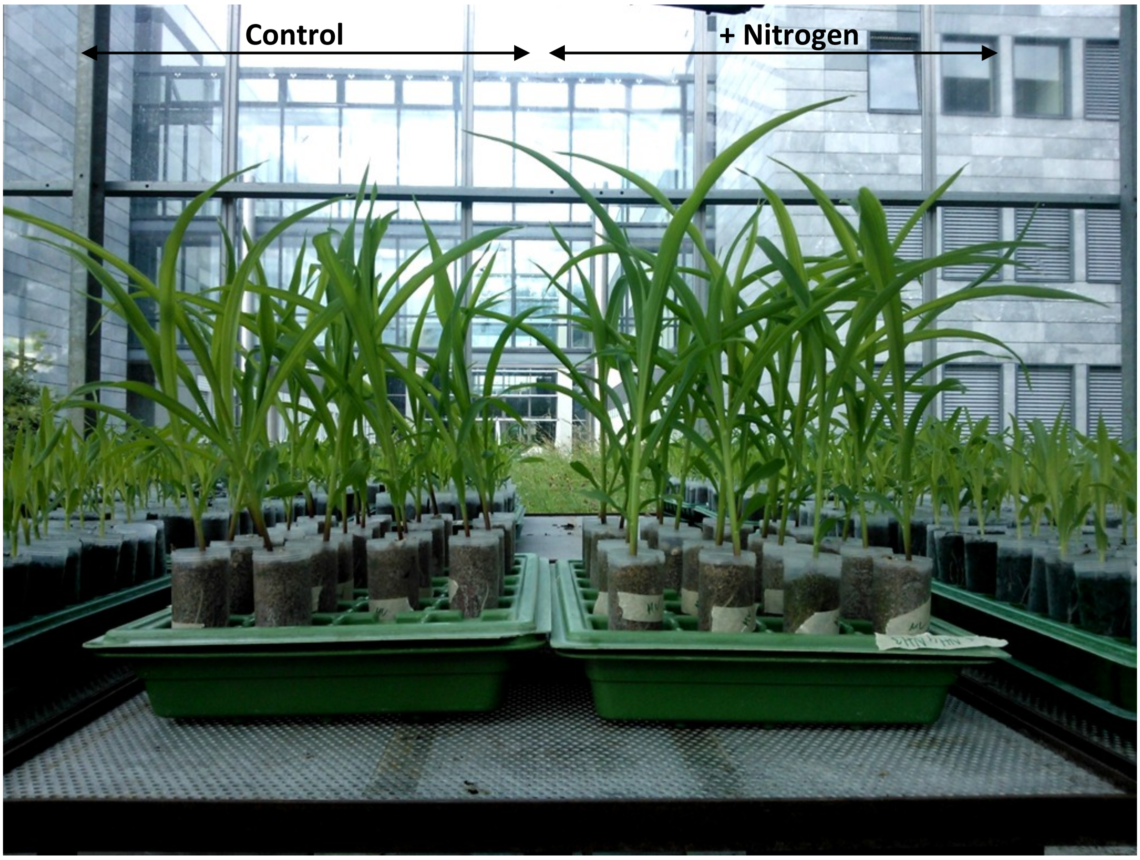


Fig S.2 Accumulated number of field herbivore abundances according to genotype x nitrogen treatment.

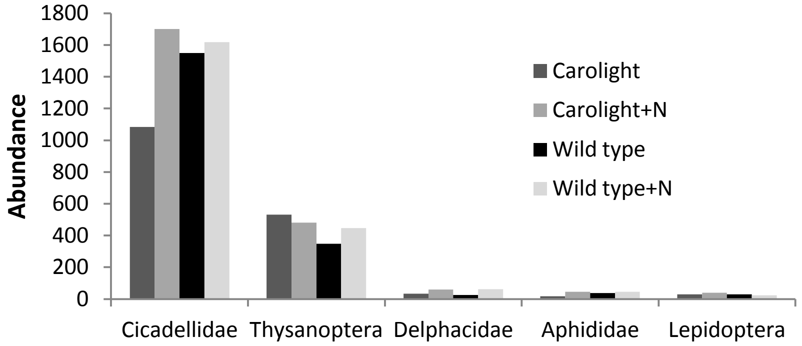


Fig S.3 Accumulated number of field natural enemy abundances according to genotype x nitrogen treatment.

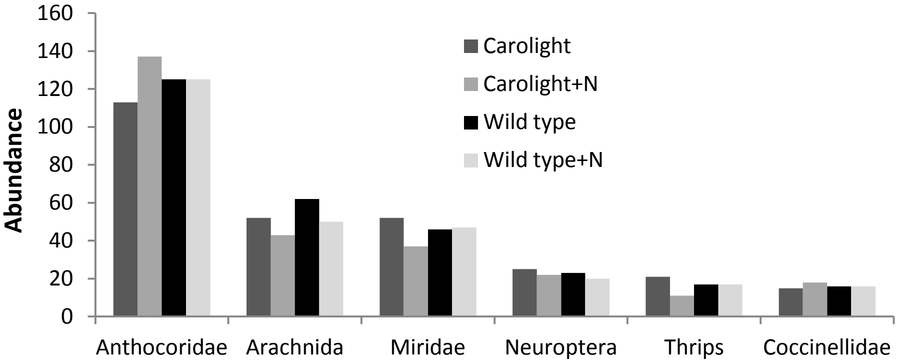


Fig S.4 Seasonal dynamics of *Z. scutellaris* nymph abundance per genotype x nitrogen treatment

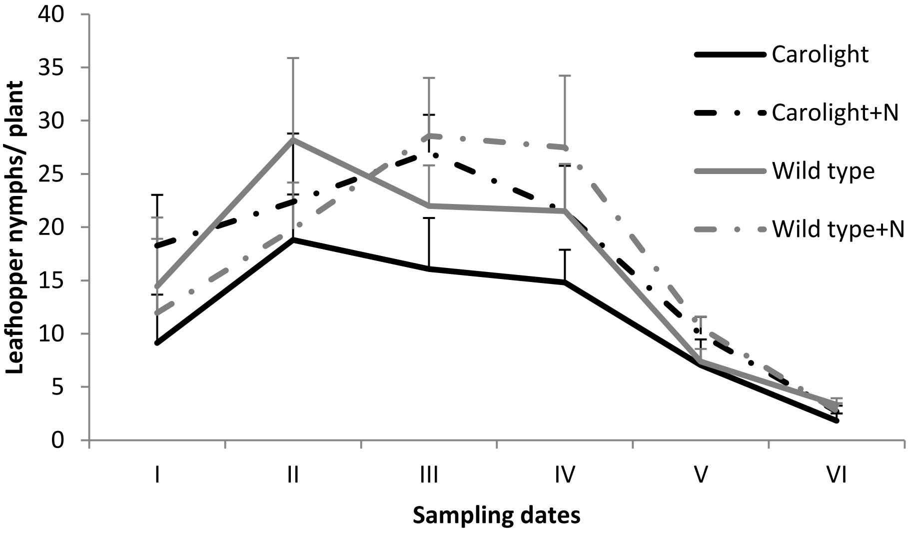


Fig. S.5. Mean intensity (±SE) of 6 biomarkers in herbivore-attacked (+H) and control plants from two genotypes (Carolight,Wild type) at two different N availability treatments (control, +N) that differ between the herbivory treatments. The white bars represent control plants and the grey bars herbivore-attacked plants (+H) after 24 h .

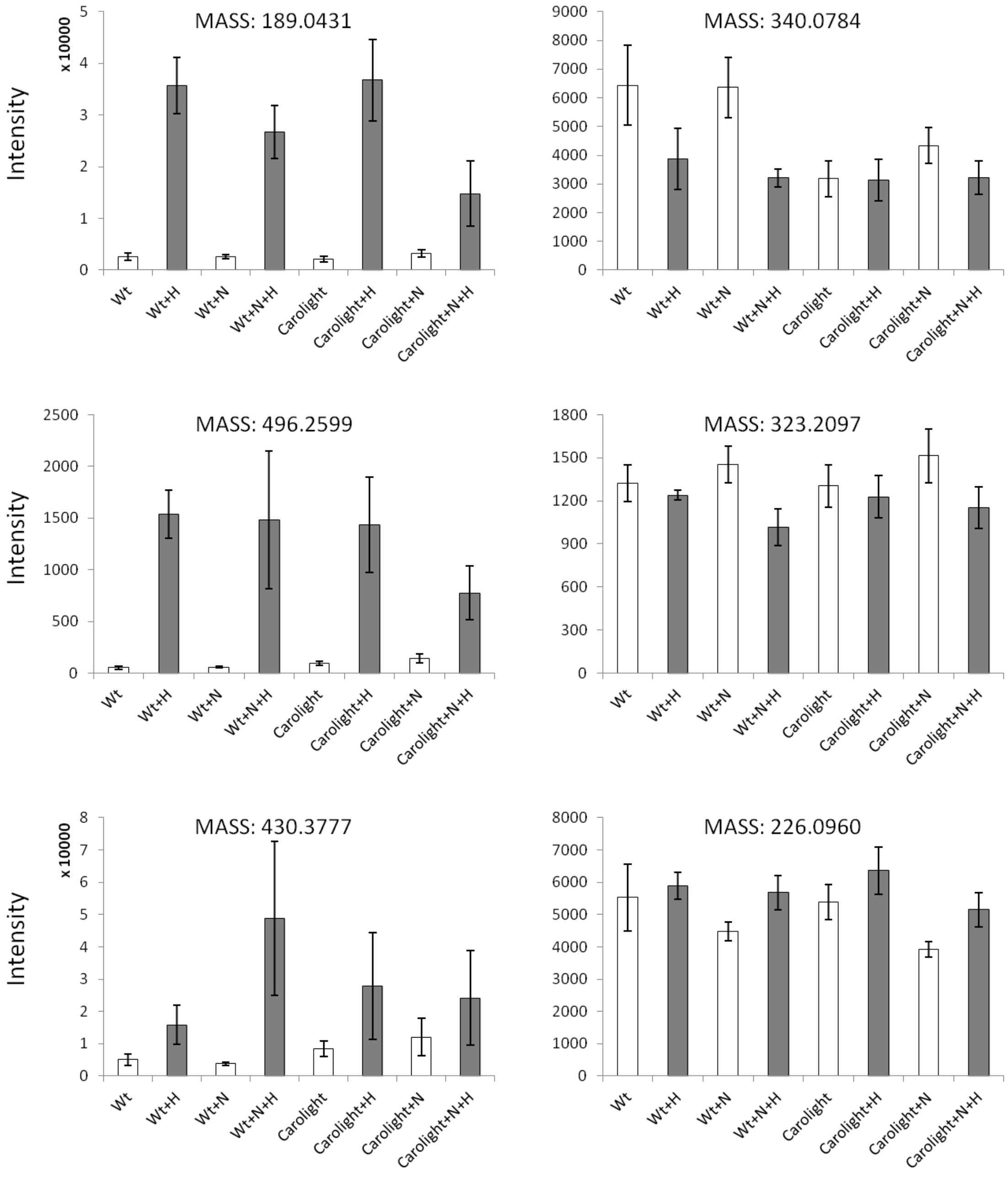


Fig. S.6. Mean intensity (±SE) of 6 biomarkers in herbivore-attacked (+H) and control plants from two genotypes (Carolight,Wild type) at two different N availability treatments (control, +N) that differ between genotypes.. The white bars represent Wild type plants and the grey bars Carolight plants (+H) after 24 h .

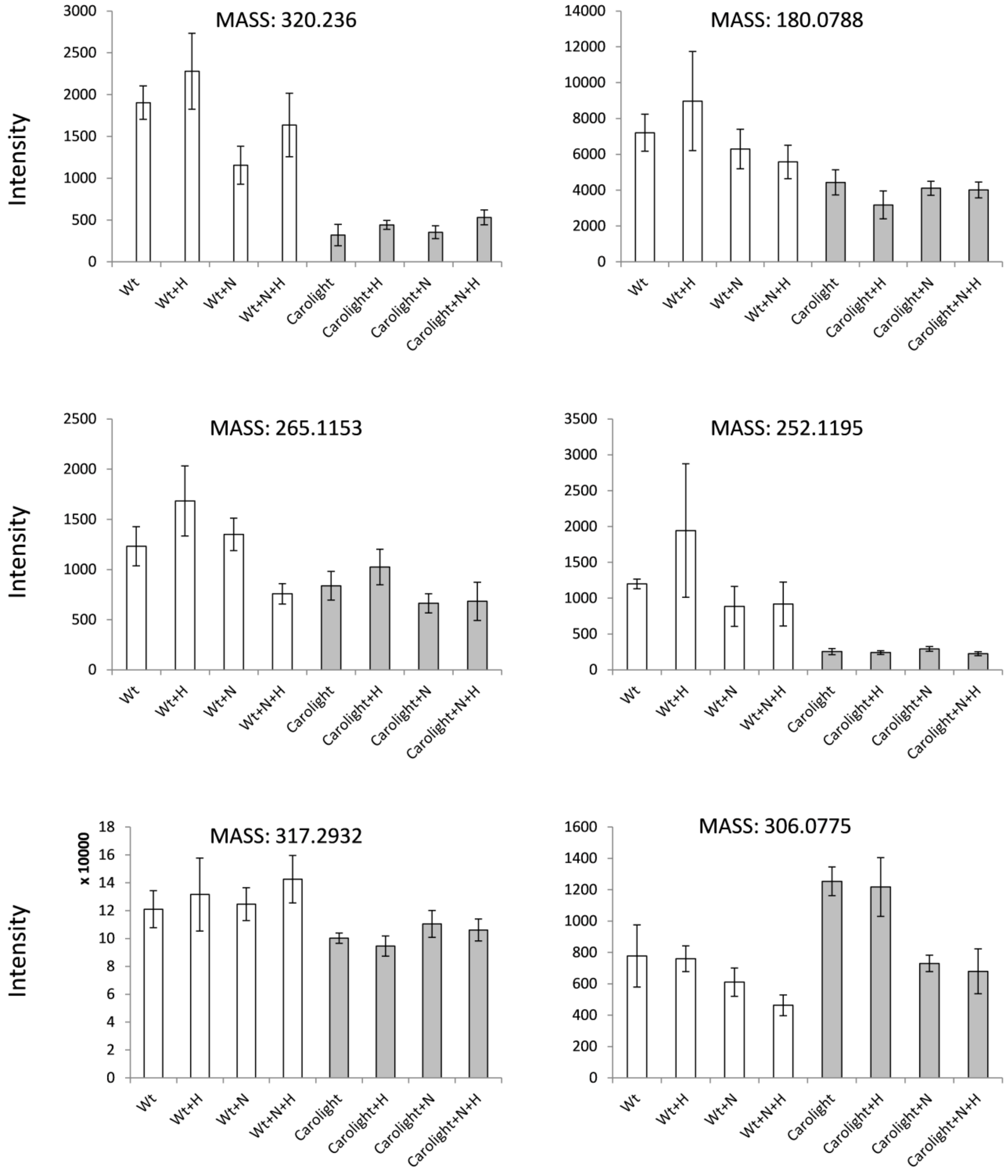
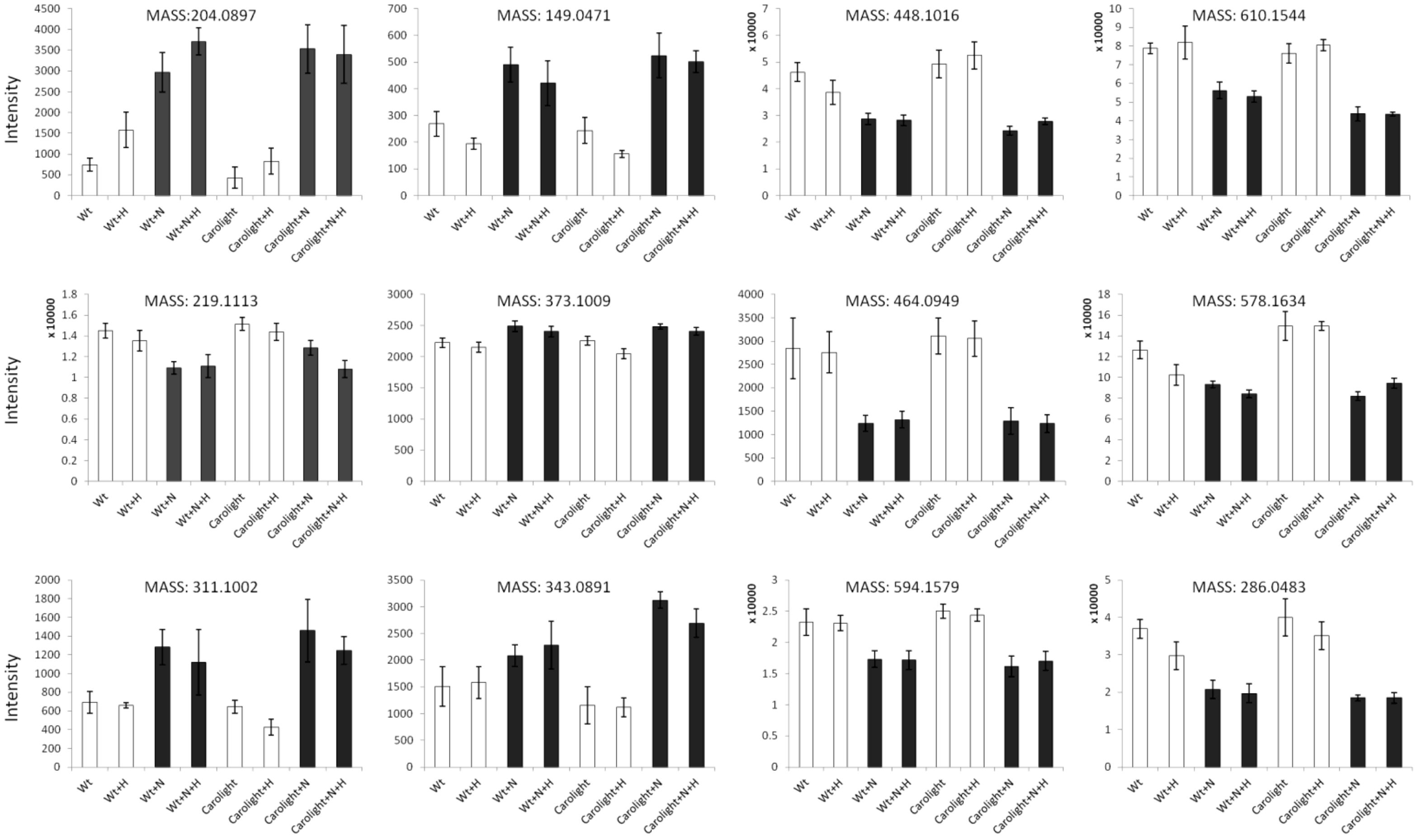


Fig. S.6. Mean intensity (±SE) of 12 biomarkers in herbivore-attacked (+H) and control plants from two genotypes (Carolight,Wild type) at two different N availability treatments (control, +N) that differ between nitrogen treatments. The white bars represent control nitrogen and the grey bars surplus nitrogen treatment (+N) after 24 h .



GENERAL DISCUSSION

The results of my thesis provide new insights into the ecology of the tri-trophic maize- *Z. scutellaris* -*Orius* spp. interactions. In addition to their scientific relevance for the study of tri-trophic relationships in maize ecosystems, this work sets a baseline framework for the assessment of potential effects of changing cultural practices on current Integrated Pest Management in maize. Here, we illustrate how generation of new ecological knowledge on maize keystone insect species *Z. scutellaris* and *Orius* spp. and their interactions with the host plant can validate their use as indicators of change in maize agroecosystems. Finally, we highlight open questions and future directions of research in this system.

Recent research in our group highlighted the potential of *Z. scutellaris* as an indicator of the herbivore group in maize agroecosystems (Albajes et al., 2011). Earlier work had already remarked on its early colonization of maize stands and high abundances through the season. Consequently, this leafhopper species could account most years for the largest proportion of above-ground herbivore biomass, and therefore their abundances could greatly impact on predators at higher trophic levels. On the other hand cumulative numbers of generalist predators - mainly *Orius* spp. and spiders - correlated with numbers of *Z. scutellaris* in the fields (Albajes et al., 2011), which led to the hypothesis that predator recruitment might be mediated by early season herbivory by *Z. scutellaris*. *Orius* spp. are known to be effective predators of small insects (Lattin, 1999) and are one of the most abundant predator taxa in arable crops in NE Spain. Both *Z. scutellaris* and *Orius* spp. are the taxa with the best detectability in GM field trials (Albajes et al., 2012; Comas et al., 2015). Any modification introduced into maize cultivation that affects the leafhopper can alter directly or indirectly the density and activity of predatory fauna and therefore natural control as it was shown for the modification of weed management (Albajes et al. 2009). Summing it all up, *Z. scutellaris* and *Orius* spp. could effectively be a keystone herbivore and predator indicators of agricultural change in maize systems. Nevertheless as *Z. scutellaris* has not been a key pest in terms of production loss, data on their biology was limited as well as their functional importance as prey for *Orius* spp.

In this thesis we show that the density or activity of leafhoppers might be affected by plant identity (maize genotype), plot characteristics like nitrogen fertilization (Chapter 4) and by region-wide characteristics and the associated agricultural landscape (Chapter 1). *Orius* spp. density in maize fields in turn is mainly determined by in-field leafhopper abundance (Chapter 1) and their attraction to maize fields may be largely mediated by HIPVs released by maize plants upon herbivore attack, mainly *Z. scutellaris* leafhoppers (Chapter 2).

Multi-trophic interactions and arthropod community structure can change substantially among genotypes within a single plant species and are influenced by biotic and abiotic factors, which ultimately might shape plant traits of importance for tri-trophic interactions. In Chapter 4, we tested the importance of bottom-up effects in form of nitrogen fertilization and plant genotype in structuring community composition in the field, and the mechanisms that might influence community structure by focusing of the keystone herbivore *Z. scutellaris* performance and preference in laboratory conditions. In the field, leafhoppers (*Z. scutellaris*) and other Hemipteran herbivores responded positively to enhanced nitrogen at the plot level, and overall the mean densities of leafhoppers were slightly higher in the wild type genotype than in Carolight. Higher abundances of leafhoppers in the nitrogen might indicate preference for plots with nitrogen fertilization as it was seen in olfactometer bioassays, and higher abundances in the wild type genotype could be a product of increased fecundity as no differences were found in the lab in nymph development.

Targeted and untargeted metabolite analysis revealed nitrogen to be the main factor defining plant chemical composition, followed by genotype and herbivory by *Z. scutellaris*. Chemotypes of maize plants were mainly influenced by nitrogen fertilization, and plants grown under nitrogen surplus conditions presented an altered composition of secondary defense compounds (lower hidroxamic acids and higher hydroxynamic acids) than plants grown under control nitrogen conditions and emitted higher amounts of three plant volatiles. Carolight plants with enhanced fertilization emitted a higher amount of the volatile β-sesquiphellandrene, that did the wild type and were preferred by leafhoppers in laboratory conditions. Changes in volatile blend agree with trends in plant selection by *Z. scutellaris*, nevertheless as stated above this preference for high nitrogen Carolight plants was not translated to higher abundances in the field. Yet, novel maize varieties that would present altered emission of constitutive or induced plant volatile profiles could disturb herbivore colonization and natural pest suppression in maize fields.

Several studies have shown that bottom-up effects of soil fertility can influence plant-herbivore interactions (Awmack and Leather, 2002; Chen et al., 2010; Garratt et al., 2011), however apparently nitrogen (and genotype) differences in leafhopper abundances, tissue quality and volatile production, did not impact on the third trophic level at the plot scale. Several reasons might be responsible for these lack of effects: (i) smaller differences in plant chemical chemotypes in the field in comparison with plants obtained under controlled conditions; (ii) similar quality and high abundance of prey in all treatments (and therefore relatively small differences between treatment abundances might not impact on predators abundances); and (iii) the small size of plots probably diluted recruitment effects to higher quality treatment plots.

Some herbivores have selected a variety of traits to overcome plant induced defenses (Kant et al., 2015). In Chapter 4, we show that leafhopper *Z. scutellaris* manipulates the host plant when feeding by suppressing the JA and SA defense pathways probably independently of JA-SA hormonal cross-talk. According to theory, maize leafhopper population should benefit from defense suppression by enhanced performance of their eggs, nymphs or adults; however feeding by the leafhopper also triggered indirect plant defense by the emission of HIPVs. Early season herbivory by *Z. scutellaris* might therefore be key in shaping the interactions with subsequently arriving herbivores and natural enemies (Stam et al., 2014; Poelman and Kessler, 2016).

In Chapter 2, we investigated whether the early colonization of *Z. scutellaris* might actually trigger *Orius* spp. colonization of maize fields. We show that *Z. scutellaris* damaged plants are as attractive to *Orius majusculus* females as *Spodoptera littoralis* damaged plants. The total amount of volatiles emitted by feeding of both herbivores is similar, and far superior tan feeding by a phloem feeding leafhopper. The HIPVs from plants infested by herbivores with distinctly different feeding strategies showed clearly distinguishable quantitative differences for (Z)-3-hexenal and (E)-2-hexenal and methyl salicylate. These compounds might serve as reliable indicators of prey presence and identity for the predator, given that experienced predators were able to distinguish differences in HIPVs profiles between *Z. scutellaris* and *S. littoralis* damaged plants when subjected to a prior unfavorable experience. Notably *Z. scutellaris* is a suitable prey for *Orius* spp. in all life stages, unlike Lepidoptera, which may strengthen the leafhopper-*Orius* spp. association in maize. On the other hand as *Z. scutellaris* is the first herbivore in sufficient numbers to colonize maize in our conditions can be largely responsible the early release of HIPVs and the subsequent colonization by generalist predatory arthropods and parasitoids.

A growing body of evidence suggests that simplified agricultural landscapes - based on bigger field sizes, smaller number of crop species, high agrochemical inputs, and very little natural or semi-natural areas - lead to disturbances in the community composition of herbivorous insects (Kennedy and Storer, 2000), their natural enemies (predators and parasitoids) and ultimately to an erosion of natural pest regulation (Matson et al., 1997; Tscharntke et al., 2005). The main goal of Chapter 1 was to estimate how landscape variables influenced predator *Orius* spp. and its prey *Z. scutellaris* abundances in maize fields; and how the importance of the landscape context compared to that of prey levels and habitat complementation for *Orius* spp.. Results show that the positive relationship detected between the maize leafhopper and *Orius* spp. is maintained regionally, although their abundances in maize fields depend on the surrounding landscape. We obtained the baseline variation for the potential indicator taxa in a two-year in three regions study sampling different co-existing habitats, and confirmed that while *Z. scutellaris* greatly predominates in maize fields *Orius* spp. are as abundant in maize than in alfalfa. On the other hand the landscape context might influence mean insect abundances at a particular site within a region. In particular, *Orius* spp. populations might vary in function of the landscape configuration in intensive agricultural regions, as the most complex sites supported more predators. Leafhoppers populations in maize were found to be negatively influenced by non-agricultural habitat and positively related to an increase of the winter cereal proportion in their surroundings. These results are particularly useful when developing a monitoring plans to assess the impacts on new agricultural management as it permits selecting the regions and landscapes supporting the most stable indicator populations in time. Additionally, results provide a baseline of biotic relationships that could transfer changes in maize fields to related habitats.

In summary we confirmed that (1) maize leafhopper is sensitive to detect differences in plant chemistry at the field scale and may be used as indicator of changes produced by modification of plant metabolism, (2) feeding by *Z. scutellaris* results in the emission of large amount of HIPVs comparable to feeding by a chewing herbivore, and it suppresses the accumulation of the defense hormones JA-Ile and SA, (3) the recruitment of *Orius* spp. by maize fields might be chemically mediated by the release of HIPVs resulting of leafhopper feeding, given that *Orius* spp. have a robust attraction towards *Z. scutellaris* infested plants, (4) the strong positive association between *Orius* spp. and the herbivore *Z. scutellaris* across the three regions reflects a consistent density-dependent process of predator aggregation towards prey (5) temporal, regional and landscape variation greatly influence maize leafhopper and *Orius* spp. populations in NE Spain. All these results provide insights when understanding the plant-herbivore-predator relationships and consequently to predict consequences of agricultural management modifications on the food web and natural control.

REFERENCES

Albajes, R., Farinos, G. P., Perez-Hedo, M., de la Poza, M., Lumbierres, B., et al. (2012). Post-market environmental monitoring of Bt maize in Spain: Non-target effects of varieties derived from the event MON810 on predatory fauna. Spanish Journal of Agricultural Research, 10, 977–985.

Albajes, R., Lumbierres, B., & Pons, X. (2011). Two heteropteran predators in relation to weed management in herbicide-tolerant corn. Biological Control, 59, 30–36.

Awmack, C. S., & Leather, S. R. (2002). Host plant quality and fecundity in herbivorous insects. Annual Review of Entomology, 47, 817–844.

Chen, Y., Olson, D. M., & Ruberson, J. R. (2010). Effects of nitrogen fertilization on tritrophic interactions. Arthropod-Plant Interactions, 4, 81–94.

Comas, J., Lumbierres, B., Comas, C., Pons, X., & Albajes, R. (2015). Optimising the capacity of field trials to detect the effect of genetically modified maize on non-target organisms through longitudinal sampling. Annals of Applied Biology, 166, 183–195.

Garratt, M. P. D., Wright, D. J., & Leather, S. R. (2011). The effects of farming system and fertilisers on pests and natural enemies: A synthesis of current research. Agriculture, Ecosystems and Environment, 141, 261–270.

Kant, M. R., Jonckheere, W., Knegt, B., Lemos, F., Liu, J., et al. (2015). Mechanisms and ecological consequences of plant defence induction and suppression in herbivore communities. Annals of Botany, 115, 1015–1051.

Lattin, J. D. (1999). Bionomics of the Anthocoridae. Annual Review of Entomology, 44, pp.207-231.

Meissle, M., Mouron, P., Musa, T., Bigler, F., Pons, X., et al. (2009). Pests, pesticide use and alternative options in European maize production: current status and future prospects. Journal of Applied Entomology, 134, 357–375.

Poelman, E. H., & Kessler, A. (2016). Keystone Herbivores and the Evolution of Plant Defenses. Trends in Plant Science, 21, 477–485.

Stam, J. M., Kroes, A., Li, Y., Gols, R., van Loon, J. J. A. a, et al. (2014). Plant interactions with multiple insect herbivores: from community to genes. Annual review of plant biology, 65, 689–713.

CONCLUSIONS

1. Maize leafhopper *Z. scutellaris* abundance is positively related to winter cereal cover in the landscape and negatively related to semi-natural habitat across the three regions. Therefore overlap of cereal and maize crops in the rotation might favor the colonization of maize fields by the leafhopper.
2. *Orius* spp. are unresponsive to shifts in habitat composition despite being present in maize and associated habitats. However the presence of permanent field margins and complementary crops -like alfalfa- are necessary for sustaining *Orius* spp. populations in intensive agricultural regions.
3. The strong positive association between *Orius* spp. and the herbivore *Z. scutellaris* across the three regions reflects a consistent density-dependent process of predator aggregation towards prey.
4. The predator *O. majusculus* presents an innate preference for plants with cell or tissue damage, like the mesophyll feeder *Z. scutellaris* and the chewer *S. littoralis*. This innate preference can be modified by positive and negative experiences during prey encounters, however preference for *Z. scutellaris* is maintained.
5. Plants with cell or tissue damage release a higher amount of volatiles than plants infested by the phloem-sucking *D. maidis*. The induction of plant volatiles by mesophyll-feeding *Z. scutellaris* adults is as strong as that of caterpillars on a per capita basis.
6. Three compounds (Z)-3-hexenal and (E)-2-hexenal and methyl salicylate were found to be most predictive in indicating whom was feeding on a plant and might be used by the predators to discriminate between plants with potential prey.
7. Maize leafhopper is sensitive to detect differences in plant chemistry at the field scale and may be used as indicator of changes produced by modification of plant metabolism. The best measures of performance for *Z. scutellaris* in general are field abundance, plant preference and probably fecundity.

8. Among the factor studied, nitrogen availability is the main factor that shapes the maize metabolism followed by genotype and herbivory by *Z. scutellaris*. Plants grown under nitrogen surplus conditions presented an altered composition of secondary defense compounds (lower hidroxamic acids and higher hydroxynamic acids) than plants grown under control nitrogen conditions, and promote *Z. scutellaris* preference through the emission of a more attractive blend of plant volatiles.

9. Carolight plants with enhanced fertilization emit a higher amount of the volatile β-sesquiphellandrene, that did the wild type and were preferred by leafhoppers in laboratory conditions. However this preference for high nitrogen Carolight plants is not reflected in field conditions.

10. Feeding by *Z. scutellaris* suppresses maize inducible defenses by decreasing the content of basal levels of JA and SA phytohormones. The mechanism by which maize leafhopper might suppress plant defense is unknown but it impairs hormone accumulation without disturbing plant indirect defense by means of the emission hervivore induced plant volatiles.

11. Untargeted metabolomics fingerprinting allows the identification of markers of the experimental conditions plant genotype, nitrogen and herbivory by *Z. scutellaris*. This opens a door to further research about the mechanisms involved in the definition of plant chemotypes by abiotic (nitrogen) and biotic factors (plant genotype, *Z. scutellaris* feeding).

12. Globally *Z. scutellaris* and *Orius spp*. populations can be keystone meaningful indicators of change in maize agroecosystems.