Effects of the main secondary metabolites of *Physalis* plants on a specialist and a generalist species of Lepidoptera

Dissertation

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by M.Sc. Marie Pauline Sell

born on 30.10.1989 in Hamburg

Gutachter:

1. Prof. Dr. Ralf Oelmüller, Friedrich-Schiller-Universität, Jena

2. Prof. Dr. David G. Heckel, Max-Planck-Institut für Chemische Ökologie, Jena

3. Prof. Dr. Dietrich Ober, Christian-Albrechts-Universität, Kiel

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You will come to a place where the streets are not marked.
Some windows are lighted. But mostly they're darked.
A place you could sprain both your elbow and chin!
Do you dare to stay out? Do you dare to go in?
How much can you lose? How much can you win?
And IF you go in, should you turn left or right ...
or right-and-three-quarters? Or, maybe, not quite?
Or go around back and sneak in from behind?
Simple it's not, I'm afraid you will find,
for a mind-maker-upper to make up his mind.

[Dr. Seuss, *Oh the Places you'll go*]

Summary

The main secondary metabolites of *Physalis* plants - withanolides - are associated with a range of interesting biological properties; the compounds are antimicrobially active and seem to possess potent feeding deterrent, growth-inhibiting and immune-system-stimulating activity against herbivorous insects. However, the ecological role of these compounds in relation to their host plants and their mode of action remains poorly understood.

In this doctoral thesis, the effects of feeding on *Physalis peruviana* fruits and of the uptake of corresponding withanolide extracts and withaferin A (a withanolide from *Withania somnifera*) were analysed in two closely related Lepidoptera species. *Chloridea subflexa* is a dietary specialist feeding exclusively on plants of the genus *Physalis;* its sister species *C. virescens* represents a generalist pest with a broad host range. Although *C. virescens* is able to feed and thrive on *Physalis* plants under forced conditions, it has not been reported to do so in the field. This raises questions about the causes of the exclusion of this genus from *C. virescens*'s host-plant spectrum and about how *C. subflexa* may be adapted to circumvent potentially adverse effects of *Physalis* defences. Therefore, this doctoral thesis primarily sought to shed light on the general functions of withanolides and their putative involvement in the specialised adaptation of *C. subflexa* to its host plant.

In a series of comparative experiments, the performance and behaviour of larvae and adults from the specialist, *C. virescens*, and other non-adapted species were examined after the insects had fed on *P. peruviana* fruits and withanolide compounds. As *C. virescens* and *C. subflexa* can still produce viable offspring, *Chloridea* hybrids were also included in several experiments. While choice and oviposition assays confirmed differential behaviours of *C. subflexa* and *C. virescens* towards the *Physalis* fruits, interspecific differences were much less pronounced in feeding and survival studies. Interestingly, a correlation between the taxonomic relationship and larval performances was detected across several experiments. More distantly related heliothine larvae strongly rejected *Physalis* fruits as a food source and showed significantly lower survival and weight gains in comparison to *Chloridea* species and the hybrids.

Based on the observed moulting disorders in *C. virescens* and the structural similarity of withanolides to phytoecdysteroids and insect hormones, the potential hormonal activity of withanolides was also evaluated, but could not ultimately be confirmed in the present thesis.

In order to investigate specific transcriptional responses of the two *Chloridea* species to withanolides, transcriptomes from larvae feeding on *P. peruviana* fruits and on diet supplemented with withaferin

A or with *P. peruviana* withanolide extracts were compared. In addition, potential species-specific metabolic conversions were studied. Isotopic labelling of *P. peruviana* plants was performed to analyse the fate of withanolides in larvae upon ingestion. Frass samples were analysed via liquid chromatography mass spectrometry (LC-MS) and nuclear magnetic resonance spectroscopy (NMR). Neither transcriptomic screenings nor metabolic analyses point to an unequivocal species-specific adaptation strategy of the specialist. However, differences in the expression of the developmental Osiris gene family were observed between treatments, and may be worthy of further investigation.

Due to their evident antimicrobial activity, withanolides were further analysed in microbial inhibition assays. In contrast to previously published findings, inhibition zone assays revealed that withaferin A as well as *P. peruviana* extracts not only inhibit Gram-positive bacteria, but also possess potent antimicrobial properties against Gram-negative bacteria. In addition, the potential effects of withanolide uptake on the composition of the larval gut microbiome were analysed and compared between the two *Chloridea* species. Preliminary results indicate that the effect on the larval gut microbiota might not be biologically relevant.

In conclusion, the present findings support the theory that host specificity for *Physalis* plants may be guided primarily by adaptive traits that ensure low food competition and protection from predators and parasites. Specific behaviours as well as resistance to physiological host-plant defences seem to be important drivers in the dietary specialisation of *C. subflexa* rather than metabolic and transcriptional adaptations. Interestingly, withanolides do not seem to cause obvious repercussions on the fitness of *C. virescens*. However, in non-specialists, even small, difficult-to-quantify factors may add up to drive selection for avoidance. No direct involvement of the main *P. peruviana* withanolides in any one aspect of herbivore biology has yet been proven.

Zusammenfassung

Die ökologische Rolle der pflanzlichen Sekundärstoffklasse „Withanolide“ ist noch weitestgehend unerforscht. Studien weisen allerdings auf eine Funktion als effektive Abwehrstoffe gegen herbivoren Befall hin, da Withanolide in zahlreichen Fütterungsexperimenten mit Insekten sowohl effektive wachstumshemmende, fraßhemmende als auch Immunsystem-modulierende Eigenschaften zeigten. Zudem ist diese Stoffklasse sowohl antibakteriell als auch antimykotisch wirksam und könnte Pflanzen somit auch gegen Pathogene schützen.

Im Rahmen dieser Doktorarbeit wurde der Effekt von *Physalis peruviana* Früchten und Withanolidextrakten auf zwei verwandte Mottenarten sowie auf einige weitere Lepidoptera untersucht. *Chloridea subflexa* ist spezialisiert auf Physalispflanzen, wohingegen ihre nächstverwandte Schwesterart *C. virescens* eine Schädlingsspezies mit breitem Wirtspflanzenspektrum repräsentiert. Aufgrund ihrer starken genetischen Ähnlichkeit ist eine Verpaarung der Chloridea-Arten möglich, sodass auch zusätzlich die Hybride in Experimenten eingesetzt werden konnten. Mittels Choice- und Ovipositionassays sowie auch über Fütterungs- und Survivalstudien wurden die Auswirkungen der Withanolidaufnahme auf das Verhalten der Arten und auf ihre allgemeine physiologische Konstitution untersucht. Während *C. virescens* vermutlich wegen der ähnlichen genetischen Ausstattung nicht stark von *C. subflexas* biologischer Performance abwich, konnte u.a. eine Korrelation zwischen dem Verwandtschaftsgrad und dem Fitnessstatus anderer Larven beobachtet werden.

Aufgrund der in Experimenten beobachteten Häutungsprobleme in *C. virescens* und der strukturellen Ähnlichkeit der Withanolide zum Insektenhäutungshormon 20-Hydroxyecdyson und Phytoecdysteroiden, wurde außerdem untersucht, ob Withanolide hormonell wirksam sind. Eine direkte, ursächliche Wirkung konnte hierbei allerdings nicht abschließend nachgewiesen werden.

In weiteren Experimenten wurde untersucht, ob es Hinweise auf eine artspezifische, transkriptionelle und/oder metabolische Anpassung an Physalispflanzen und Withanolidaufnahme gibt. Hierfür wurde eine ^{13}C-Isotopenmarkierung an *P. peruviana* Pflanzen durchgeführt. Die so markierten Withanolide wurden anschließend extrahiert und verfüttert. Kotextrakte beider *Chloridea*-Arten wurden spektroskopisch analysiert. Zusätzlich wurden Transkriptomdaten von Larven, die sowohl auf Physalisfrüchten als auch auf künstlichem Futter mit zugesetzten Withanoliden gefressen hatten, auf differenzielle Genexpression untersucht. Eindeutige Hinweise auf eine Spezies-spezifische Anpassungsstrategie konnte jedoch in keinem der Ansätze gefunden werden. Die Transkriptomanalyse deutet allerdings auf eine differenzielle Regulierung der

Insektenspezifischen Osiris-Genfamilie hin. Diesen Genen wird eine Funktion in Detoxifizierungsmechanismen und der Insektenentwicklung zugeschrieben. Gerade in Bezug auf die beobachteten Häutungsschwierigkeiten in *C. virescens* Larven, sind Osirisgene daher interessante Kandidaten für zukünftige Experimente.

Durch die Untersuchung der antimikrobiellen Eigenschaften von Withanoliden konnte gezeigt werden, dass ihre Wirkungsweise weitaus effektiver und spezifischer ist als ursprünglich angenommen. Die Analyse der Darmflorazusammensetzung von *C. subflexa* und *C. virescens* Larven ergab jedoch keine signifikante Veränderung durch die Withanolidaufnahme im Vergleich zur Kontrollgruppe.

Zusammengefasst konnte in einem breiten experimentellen Ansatz gezeigt werden, dass die Spezialisierung von *C. subflexa* auf Physalispflanzen vielschichtig ist und womöglich nicht allein auf eine Anpassung an Withanolide zurückzuführen ist. Die vorliegenden Ergebnisse unterstützen die These, dass die Wirtspflanzenspezifizität von *C. subflexa* überwiegend durch Anpassungsstrategien bedingt sein könnte, welche geringe Nahrungskonkurrenz und Schutz vor Fraßfeinden und Parasiten ermöglichen. Spezifische Verhaltensweisen und Resistenzmechanismen gegen morphologische Abwehrbarrieren der Physalispflanzen scheinen hierbei entscheidendere Faktoren für die Spezialisierung zu sein als bspw. Anpassungstrategien auf metabolischer oder genregulatorischer Ebene. Interessanterweise scheint die Aufnahme von Physaliswithanoliden generell keinen gravierenden Einfluss auf den Generalisten *C. virescens* zu haben. Allerdings können auch viele geringfügigere Faktoren zusammenspielen und erst in ihrer Gesamtheit suboptimale Lebensbedingungen für die Raupen darstellen. Eine direkte, ursächliche Wirkung der *P. peruviana* Withanolide auf herbivore Insekten muss in zukünftigen Experimenten bestätigt werden.

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List of Abbreviations

Bt	*Bacillus thuringiensis*
Cs	*Chloridea subflexa*
CTAB	cetyltrimethylammonium bromide
Cv	*Chloridea virescens*
CYP	cytochrome P450
DTT	dithiothreitol
DRC	dose response curve
ED	effective dose
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
GC	gas chromatography
GPL	General Purpose Lepidoptera (diet)
HIPV	herbivore-induced plant volatile
HPLC	high-performance liquid chromatography
hy	hybrid(s)
IHZ	inhibition zone (assay)
indel	insertion/deletion
JH	juvenile hormone
LA	linolenic acid
LB	lysogeny broth
LC	liquid chromatography
L:D	light-dark (cycle)
MeOH	methanol
MS	mass spectrometry
MW	molecular weight
NMR	nuclear magnetic resonance
OD	optical density
OTU	operational taxonomic unit
PBS	phosphate-buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction
PDA	potato dextrose agar
PO	polyphenol oxidase
pv	pathovar
qPCR	quantitative real-time PCR
RNA-Seq	RNA sequencing
RT	room temperature
rt	retention time
SDS	sodium dodecyl sulfate
spp.	*species pluralis*
subsp.	subspecies
sv	serovar
TDH	Todd-Hewitt Bouillon
Tris-HCl	tris(hydroxymethyl)aminomethane
20E	20-hydroxyecdysone

Amino acids and nucleotides are abbreviated according the International Union of Pure and Applied Chemistry (IUPAC).

INTRODUCTION

General Introduction

The field of chemical ecology seeks to understand complex chemically-mediated interactions among and between plants, animals and microorganisms. It integrates research on the molecular basis of signalling, communication and defence mechanisms, and encompasses direct as well as indirect interactions. The identification and characterisation of specific molecules that play key roles in these mechanisms deeply interest chemical ecologists. Chemical signals are ubiquitous and basic research approaches in this field have paved the way for applied agricultural pest control, novel drug discovery and progress in crop production. Plant-herbivore interactions are a central subfield of chemical ecology and provide important insights into the dynamics of evolutionary co-adaptation and speciation mechanisms.

1. Plant-Herbivore Interactions

Research on plant-herbivore interactions addresses the dilemma that plants are sessile but surrounded by herbivorous enemies, and that the herbivores in turn are highly dependent on their well-defended host plants. The class of insects represents the most significant herbivorous group. Insects and plants have coexisted over millions of years (Labandeira, 2013; Bernays, 1998). Therefore, most plant defence strategies are directly aimed against insect herbivory, while defensive traits that are directed at vertebrates such as mammalian and bird species are often less pronounced. Herbivorous insects collectively impose a selective force on plants as they consume high proportions of the plant's biomass including reproductive organs such as inflorescences, fruits and seeds. To reduce or even circumvent this loss of biomass, plants have evolved a vast repertoire of highly dynamic defence strategies such as physical barriers and anti-herbivory chemicals. In turn, herbivorous insects respond to those defences by behavioural, morphological and physiological adaptions, including avoidance, detoxification and tolerance mechanisms. The wide range of insects's counteradaptations is diverse and just as effective as the plants' arsenal of defences. However, plant-insect interactions are not necessarily disadvantageous to either party. Plant-insect mutualistic relationships can also benefit both, as biotic pollination illustrates. Mutualistic interactions often also involve defence services provided by the insect to the plant in exchange for shelter or food (e.g. extrafloral nectars) rewarded to the insect by the plant (Nepi *et al.*, 2018).

In their classic paper, Ehrlich and Raven (1964) described a multistep model of "escape and radiation" coevolution over an extended period of time. According to their concept, the evolutionary arms race between plants and herbivores is continuous, and both experience reciprocal diversification.

Counteradaptations among species through coevolution have been seen as a driving force that promotes biodiversity. The underlying forces include the escape and radiation of genetic diversity. Plants that evolve novel defence traits may temporarily escape damage from herbivorous species. The resulting fitness benefit is strongly believed to catalyse rapid speciation processes. Insects, which in turn adapt to the novel plant defences over time, would be favoured in the newly established niche and confronted with fewer competitors; the recently evolved plant species would likely experience a burst in radiation. However, the escape-and-radiation hypothesis has been much debated. Sceptics often argue that the direct connection between coevolution and diversification of insects and plants lacks sufficient empirical support. Furthermore, it has been noted that speciation in plants is also influenced by factors such as plant-plant interactions and environmental conditions (Futuyma, 2000; Janz, 2011; Suchan and Alvarez, 2015). Thus, insect herbivory alone does not explain plant diversification. Although host-driven divergence appears to be a major cause of insect speciation, around 50% of insect-speciation events seem to take place without host shifts (Winkler and Mitter, 2008). It has been shown that insects also radiate within a host-plant species, becoming specialised on different plant tissues or organs and even on plant sexes (Zhang *et al.*, 2015, and references therein). Current consensus therefore posits that potential coevolutionary dynamics may occur in a diffuse manner and that the terms “coevolution” and “coevolutionary diversification” should be used cautiously (Suchan and Alvarez, 2015, and references therein). There is still much to learn about the dynamic nature of plant-insect interactions, and case studies of closely interacting plants and herbivores furnish excellent opportunities to broaden our understanding of speciation and diversification.

1.1 Plant defence strategies against insect herbivory

Despite the vulnerability of plants to biotic and abiotic stress, they have become the dominant organisms on the planet’s land surface (Whittaker and Feeny, 1971). All plant species have to defend themselves against numerous herbivorous and pathogenic attacks during their lifetimes. Generally, defence mechanisms can be classified according to three modes of action: (1) Escape mechanisms, to reduce the probability of being attacked by herbivores. For example, some plants avoid feeding damage by shifting their flowering season and seed production to a time of the year when common insect populations are relatively low in number (Parachnowitsch *et al.*, 2012; Mortensen, 2013). (2) Resistance strategies, to prevent severe feeding damage, including physical as well as chemical barriers. (3) Tolerance mechanisms, to mitigate fitness losses due to herbivory, such as rapid recovery from feeding damage (Strauss and Agrawal, 1999; Gong and Zhang, 2014).

Within the plant, the expression of defence traits and metabolites varies among tissues, and metabolites can also be transported to wounded spots and/or accumulate in plant parts most worthy of protection (Jacobo-Velázquez *et al.*, 2015; Cipollini *et al.*, 2017). Passive morphological resistance traits, such as thorns, trichomes, impenetrable barks, waxy cuticles and sticky entrapments, protect the plant from initial invasion by impeding larval locomotion and by reducing the accessibility of plant organs (Howe and Schaller, 2008; War *et al.*, 2012). Another, less well-understood plant characteristic that helps repel herbivores, is to become an unattractive food source by providing low-quality nutritional content (Augner, 1995).

In addition to this first line of defence, many plants also combat herbivory by producing defensive specialised (secondary) metabolites. Specialised metabolites are defined as organic compounds that are not directly required for essential (primary) metabolic pathways, reproduction or growth but that strongly enhance the survival and fitness of the plant in its natural environment (Böttger *et al.*, 2018). While highly conserved primary plant metabolites are universally distributed throughout the plant kingdom, certain taxonomic groups or individual plant species often possess distinct and complex set of specialised metabolites with unique functions (Mithöfer and Boland, 2012). Because many of these antiherbivory chemicals affect the survival, behaviour, growth and reproductive success of other organisms, they exert considerable selective pressure. They encompass three classes: (1) phenolics, (2) nitrogen- and sulphur-containing compounds (including common alkaloids and glycosides) and (3) terpenoids (Harborne, 1999). These chemicals confer protection as repellent factors, by discouraging herbivores from feeding or egg-laying; by reducing the palatability of plant tissues; by impairing larval growth and/or by causing developmental delays and moulting disorders. They also protect the plant by resulting in moderate to high levels of toxicity (Bennett and Wallsgrove, 1994; Mithöfer and Boland, 2012). More indirectly, plants can release herbivore-induced plant volatiles (HIPVs) that recruit the natural enemies of herbivores and warn neighbouring conspecifics upon insect attack (Kost and Heil, 2006; Allmann and Baldwin, 2010). Additionally, some nutritive and protective morphological features can attract other mutualistic species to defend the plant: for instance, *Acacia* trees and ants (Janzen, 1967).

The synthesis, maintenance, transport and storage of defensive compounds is costly and each plant has to balance its needs for defence and growth (Herms and Mattson, 1992). Therefore, plants rely on the ability to detect when and where defences are most needed. Defensive metabolites are either constitutively expressed or induced in response to herbivory or pathogenic infections. Inducible defences allow the plant to invest in responses only when required, preventing costs if herbivores are absent. Insects might also induce the upregulation of constitutive defences: some plants synthesise toxins in constant but low concentrations (e.g. cardenolides in *Asclepias*) and rapidly

increase their production to deploy them if and when plant parts are injured (Malcolm and Zalucki, 1996; Agrawal *et al.*, 2012). Because all morphological defences also have a chemical basis, inducible defence traits also include morphological features. For example, the increased number of spines in the horse nettle *Solanum carolinense* is induced through feeding damage by *Manduca sexta* larvae (Kariyat *et al.*, 2013).

In addition to perceiving that they are under attack, some plants are able to identify their invader and to adjust their defence strategies accordingly (Agrawal, 2000; Züst and Agrawal, 2016). Insect salivary secretions, frass and ovipositional fluids can serve as distinctive indicators, stimulating specific defence responses in the plant (Ray *et al.*, 2015; Yoshinaga, 2016). Recent transcriptomic analyses have provided insight into gene clusters that are regulated differentially in the presence of herbivory (Howe and Schaller, 2008, and references therein). In a transcriptome screening, Ralph *et al.* (2006) showed that around 10% of a hybrid poplar (*Populus trichocarpa x deltoids*) transcriptome is differentially expressed when the plant is fed upon by *Malacosoma disstria* larvae.

1.2 Adaptation strategies of herbivorous insects

Herbivory implies insects are able to cope with the plant defences described above. Potent and directed species-specific defences may strongly limit the food sources herbivorous insects can exploit. During the course of coevolution, insects have evolved countermeasures to plant defensive traits by modifying their behavioural, physiological and morphological traits (Bernays, 1998; Opitz and Müller, 2009). These adaptations may include metabolic modifications, mechanisms to avoid contact with xenobiotic agents and physical defences. Morphologically, insects are equipped with highly sensitive chemosensory organs that help them recognise chemical plant compounds via olfactory and gustatory cues (Hansson and Stensmyr, 2011). Chemosensation not only alerts insects to the presence of noxious metabolites, it also helps to identify and select suitable plants for feeding and ovipositioning (Després *et al.*, 2007). Whereas some studies show that bitter-tasting cucurbitacins deter non-adapted herbivores (Tallamy *et al.*, 1997), some show that other species find them to be phagostimulants (Yousaf *et al.*, 2018, and references therein). Thus, plant metabolites can often be both: signals and weapons, feeding stimulants and deterrents.

Like those of plants, the behavioural adapations of insects include avoidance, e.g. by shifting their highest population peaks to a time in the year when leaves are fresh and easily digestible, or shifting to later, when the concentrations of some toxins have decreased with leaf age (Després *et al.*, 2007). Some milkweed bugs minimise their exposure to phytochemicals by cutting leaf veins in order to prevent canals from secreting latex near their feeding sites (Dussourd and Eisner, 1987). HIPVs are also a target of insect countertactics. Plants emit airborne compounds to warn fellow species and to

recruit parasitoids and predators in a tritrophic interaction, and those allelochemicals can in turn attract more herbivores to already damaged tissues. Insects have been shown to react behaviourally to plant volatiles by laying fewer eggs in order to suppress volatile emission. *Helicoverpa zea* larvae seem to prevent the release of HIPVs in several host plants by emitting salivary enzymes (glucose oxidases), which cause stomatal closure (Musser *et al.*, 2006; Lin *et al.*, 2021).

Toxin tolerance constitutes another adaptive strategy used by herbivorous insects. The most studied examples are metabolic detoxification mechanisms mediated by enzymes and transporter systems (Scanlan *et al.*, 2020). Phenotypic plasticity allows insects to adjust the production of defence compounds to varying concentrations of toxins. As it is unlikely that plants rely on only one defence strategy, insects need the ability to respond to more than one toxic compound or other combined plant defences (Mason and Singer, 2015). Often, resistance to toxins is achieved by a common set of detoxifying enzymes such as glutathione S-transferases and cytochrome P450 enzymes (Heidel-Fischer and Vogel, 2015). Many insects can prevent the activation of toxins post ingestion and metabolise them into non-toxic forms or compounds that can be excreted rapidly or even sequestered. Additionally, some insects possess target-site mutations that render them less sensitive to plant chemicals (Heckel, 2014). Detoxification enzymes are either constitutively expressed or induced by the presence of xenobiotics. Insects respond dynamically by up- or downregulating enzyme-encoding genes, which in turn catalyse reactions that disarm plant defence compounds.

Besides detoxification mechanisms, many herbivorous insects of various taxonomic groups accumulate plant-synthesised compounds in their tissues and utilise them for their own defence and reproduction (Opitz and Müller, 2009). A sophisticated form of sequestration has been shown in the arctiid moth *Tyria jacobaeae*, which stores pyrrolizidine alkaloids from *Senecio* species as an acquired anti-predator defence; for help in pheromone production and even for enhanced protection of eggs (Boppré, 1990; Hartmann and Ober, 2008).

2. Generalist and Specialist Herbivory in Insects

Herbivores are usually categorised as generalists or specialists depending on the breadth of their diet; generalists feed on a broad range of food plants, while specialists have a limited diet range. An estimated 80% of all insect species have a high degree of host specificity (Schoonhoven, 1999). Within the Lepidoptera clade, a proportion (25%) limits its host spectrum to a single plant family while around 50% feed on only one plant genus (Bernays and Chapman, 1994). Often, the causes, which have led to host shifts and specialisation, are not yet clear and even closely related sister species sometimes differ strongly in their diet breadth (Sheck and Gould, 1996; Liu *et al.*, 2012). Although both feeding types coexist, the numerical dominance of food specialists implies they have

a fitness advantage over generalists on selected host plants. Mounting and maintaining different defence strategies against a broad spectrum of anti-herbivory measures can be costly to herbivores. Consequently, most insect species are mono- or oligophagous specialists, feeding on a narrow range of related host-plant taxa with the same or similar defence profiles. Though specialists are not unaffected by host-plant defences, on average and in comparison to generalists, they suffer less from adverse defensive traits (Cornell and Hawkins, 2003; Ali and Agrawal, 2012, and references therein). To exploit their host plant(s) efficiently, dietary specialists require a high degree of specificity for the individual set of defensive traits, and they have evolved specialised resistance mechanisms that enable sequestration and metabolic modification of plant toxins (Petschenka and Agrawal, 2016). Insects often adapt to several traits of their specific host plant and show combined behavioural as well as physiological adaptations towards them (Pentzold *et al.*, 2014).

Generalists, or polyphagous species, need a wider range of detoxification and tolerance mechanisms to cope with the varying defence strategies of many different plant genera. For example, polyphagous species are thought to possess a broad spectrum of detoxification enzymes (Bansal and Michel, 2018). It has been further hypothesised that the chemosensation of generalists is less accurate than that of specialists and that specialists appear to choose their host plants more efficiently (Wang *et al.*, 2017). Although strategies for coping with defences of a few host plants have been extensively studied, our understanding of how generalists deal with the defences of a broad host-plant range remains fragmentary.

Ehrlich and Raven (1964) investigated the ecological implications of dietary specialisation on butterflies and their host plants. The authors postulated that plant defence compounds ultimately determine the host range of butterflies. Over the course of specialisation, insects consequently lose the ability to exploit many other non-host plants by evolving detoxification mechanisms towards only a specific set of similar chemical defence compounds from a rather narrow host plant array. Presumably, specialists lose mechanisms that counteract other potential host defences. Therefore, herbivores with a broad resource availability and high plasticity in feeding may have an advantage over species with a narrow host-plant array (Bernays and Minkenberg, 1997).

Specialisation may lower a species' adaptability to non-hosts and increases its sensitivity to the limited availability of host plants; such limitations may be due to climate change, novel invasive competitors and/or phytopathogenic infestations. Whereas specialists are often predicted to have a high probability of becoming extinct, other studies argue against the assumption that specialisation is an evolutionary dead end (Colles *et al.*, 2009). In comparative studies, particularly specialised species often represent the consequences of drastic environmental changes such as global warming (Condamine *et al.*, 2020).

3. Withanolides

Plant-based withanolides (or withasteroids) are defined as a class of bioactive steroidal lactones assembled on a polyoxygenated C-28 ergostane skeleton. More than 300 withanolide compounds and derivative forms are known to date and new compounds continue to be discovered (Veleiro *et al.*, 2005). All compounds share oxygenated carbon atoms at positions 1, 22 and 26 (Glotter, 1991). The characteristic feature of ergostane-type steroids is the side chain with a lactone/lactol ring bound to the backbone (**Figure 1A**) (Misico *et al.*, 2011). Their functional and structural complexity, ongoing discoveries of novel withanolide compounds and inconsistent nomenclature currently make a general classification difficult. Withanolides seem to occur almost exclusively in the nightshade family (Solanaceae), but knowledge of the diversity and overall distribution of these compounds within the plant kingdom is still fragmentary. Within the solanaceous clade, members of the genera *Acnistus*, *Datura, Deprea, Dunalia, Exodeconus, Iochroma, Jaborosa, Lycium, Mandragora, Nicandra, Physalis*, *Salpichroa*, *Trechonaetes*, *Vassobia, Withania* and *Witheringia* have been found to produce withanolides (Christen, 1989; Glotter, 1991; Veleiro *et al.*, 2005). Interestingly, a marine coral species (*Paraminabea acronocephala*) has also been reported to synthesise different withanolides (Chao *et al.*, 2011). The amount and number of withanolide variants seem to vary seasonally as well as with geographical factors (Veleiro *et al.*, 2005; Dhar *et al.*, 2013). The first isolated withanolide was withaferin A (**Figure 1A**), which represents the core structure of this class. Withaferin A has been used in traditional Indian folk medicine for centuries (Mishra *et al.*, 2000).

Many interesting biochemical activities have been ascribed to withanolide compounds *in vivo* and *in vitro*, including those with cytotoxic, anti-inflammatory, immunosuppressant and antimicrobial properties (Castro *et al.*, 2008; Misico *et al.*, 2011). Though early work on withanolide-producing plants strongly points to a defensive function against herbivorous attack (Misico *et al.*, 2011, and references therein), the basic ecological role for the plants and the mode of action of these biochemicals is still not fully understood. The regulatory and biosynthetic pathways involved in withanolide production are also poorly characterised and warrant further examination to understand their origin and the evolution of diversified structures.

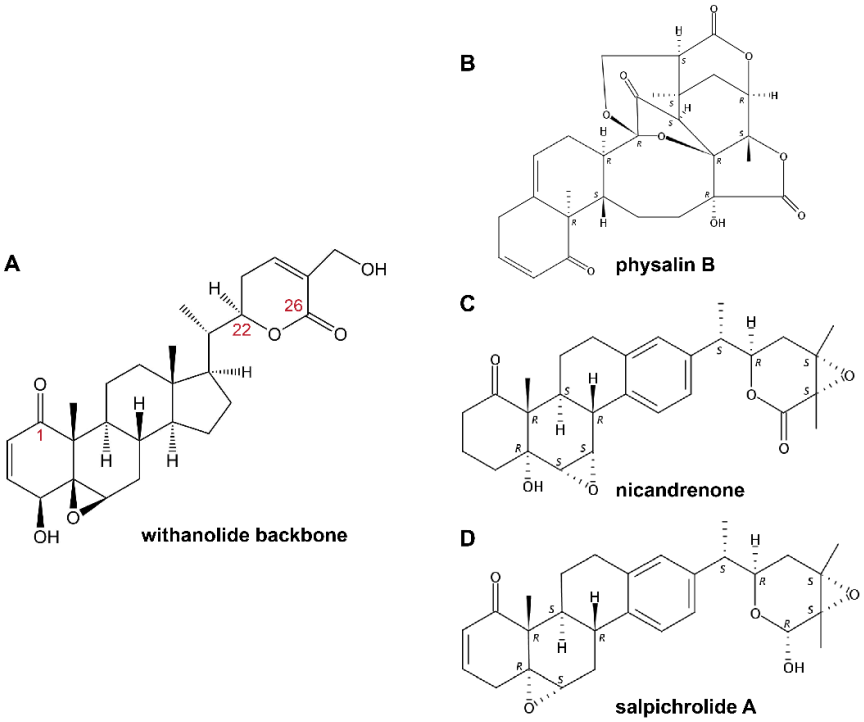

Figure 1: Chemical structures of a selection of prominent withanolides. A) The withanolide backbone structure (represented by withaferin A). Oxygenated carbons are labelled with red numbers. Withanolides known for anti-insect activity: **B)** *Physalis*-derived physalin B, **C)** nicandrenone and **D)** salpichrolide A.

It is known that withanolides are derived from 24-methylenecholesterol (Knoch *et al.*, 2018). The authors have discovered a key enzyme in withanolide biosynthesis - a sterol Δ^{24}-isomerase (ISO24) - that mediates the conversion of 24-methylenecholesterol to 24-methyldesmosterol in *W. somnifera*. Knoch *et al.* (2018), hypothesise that this conversion is a crucial step in the biosynthetic pathways and suggest that the enzyme might provide a target for genetic manipulation. Interestingly, this enzyme has been detected in medically relevant Solanoideae plants, but not in typical crop species like tomatoes or potatoes. The authors claim that this can be explained by a negative selective pressure during domestication of withanolide-containing food plants.

3.1 Biological function of withanolides and their effects on herbivorous insects

As mentioned above, withanolides are presumed to act as defence mechanisms against insect larvae and other herbivores by affecting development, feeding attractiveness and growth. Herbivores may be repelled by certain substances after initial testing; they may suffer decreased fitness due to post-digestive damage; and interruptions in feeding may lead to starvation, semi-starvation and growth inhibition. Detrimental downstream effects on development, reproduction and the immune system may result. Observations along those lines as well as different hypotheses on the mode of action are described in the literature, which is briefly summarised in this section.

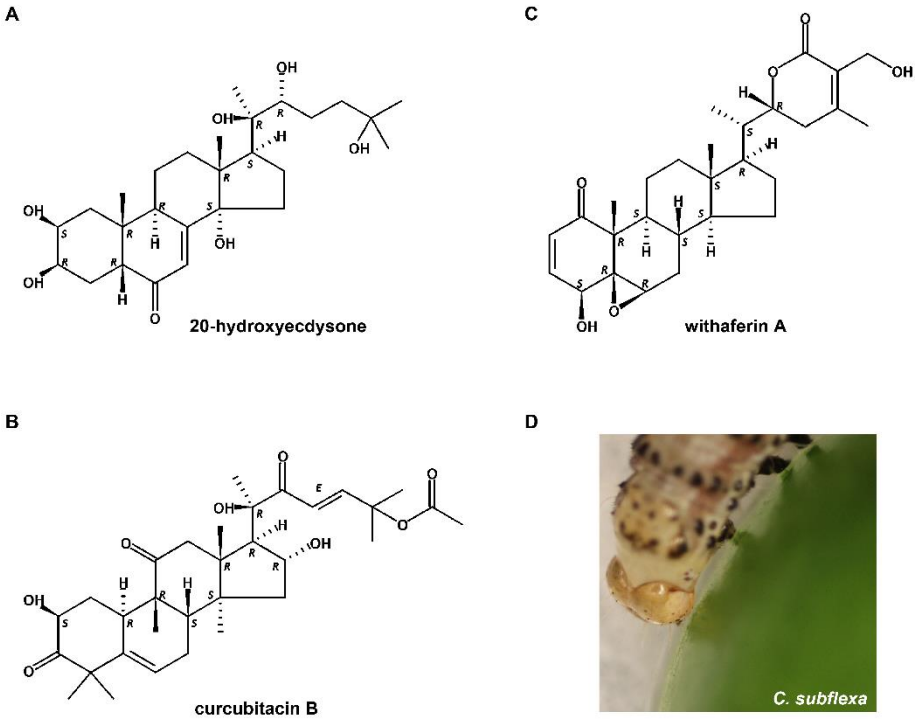

Figure 2: A) Chemical backbone of 20-hydroxyecdysone - the main moulting hormone in insects related in structure to **B)** cucurbitacin B, which is known to possess antagonistic functions as a phytoecdysteroid (Dinan *et al.*, 1997) and **C)** withaferin A. **D)** Head capsule of a *C. subflexa* larva close to be shed during moulting.

Interestingly, the chemical backbone structure of withanolides shares high similarity with the most commonly occurring hormone in insects: 20-hydroxyecdysone (**Figure 2A).** Together with juvenile hormones (JH), the steroid hormone 20-hydroxyecdysone (20E) is a major regulator of developmental processes in insects and coordinates larval moulting as well as the initiation of metamorphosis and diapause (Riddiford, 1993). Many plants are known to synthesise phytoecdysteroids, hormonal analogues that exhibit antagonistic or agonistic activity against matching hormone receptors in herbivores (Schmelz *et al.*, 1998). The function of this class of molecules might be to mimic the structures of hormone receptor ligands and disturb reproduction and development of phytophagous species. The binding of those hormone analogues can induce anomalous ecdysis and death (Jurenka *et al.*, 2017, and references therein). Some withanolide compounds show significant antagonistic activity to the action of 20E in *Drosophila melanogaster* BII cell lines expressing the ecdysteroid receptor (Dinan *et al.*, 1996). Radioligand-binding assays show that structurally similar cucurbitacin B (**Figure 2B**) and D also display ecdysone activity (Dinan *et al.*, 1997). These results indicate that observed delays and disorders in the development of insects might be caused by interference with the ecdysone receptor (EcR). Disruption of ecdysone metabolism is likely to influence cellular and humoral immune responses in addition to insect development. In recent experiments in which crude leaf, seed and root extracts of *W. somnifera*

plants were topically applied to lepidopteran *Pericallia ricini* or *Spodoptera litura* larvae and pupae, the authors found moulting disorders between larval-pupal stages as well as during pupal-adult ecdysis (Gaur and Kumar, 2010; Gaur and Kumar, 2018).

Early work identified and tested several insect repellent factors such as nicandrenone, salpichrolides and the two main withanolides in *Physalis peruviana* shrubs, withanolide E and its derivative form 4β-hydroxywithanolide E. Nicandrenone (Figure 1D) was discovered after larvae of the tobacco hawk moth *M. sexta* were observed to avoid plant parts of *Nicandra physalodes* (Nalbandov *et al.*, 1964). When later tested in feeding assays, nicandrenone was found to be lethal for the housefly *Musca domestica*. Larvae of *M. domestica* were also exposed to salpichrolides (Figure 1C) from *Salpichroa origanifolia*, which caused delays in development (Mareggiani *et al.*, 2000). Interestingly, similar developmental delays were observed when the insects were fed on low-nutrient diets, leading to the conclusion that these compounds might act as growth inhibitors. Furthermore, exposure to salpichrolides resulted in increased mortality and adverse developmental effects on the red flour beetle *Tribolium castaneum* (Mareggiani *et al.*, 2002) and the larvae of the Mediterranean fruit fly *Ceratitis capitata* (Bado *et al.*, 2004). Methanolic leaf extracts containing withanolide E and 4β-hydroxywithanolide E (Figure *3*) of *P. peruviana* leaves deterred feeding by African cotton leafworm *Spodoptera littoralis* larvae (Ascher *et al.*, 1980). In addition to these findings, it was shown that those leaf extracts had a strong inhibitory impact on the growth rate of *H. zea* larvae (Waiss, 1993; Elliger *et al.*, 1994). Larvae that were treated with the extracts formed smaller pupae and reached the adult stage significantly later than the control groups.

Baumann and Meier (1993) and Mareggiani *et al.* (2000) have monitored withanolide concentrations in *P. peruviana* plants throughout the year and during fruit development. The fact that the concentration of withanolides increases during summer months when insect populations are highest (Mareggiani *et al.*, 2000), as well as during the beginning of fruit development (Baumann and Meier, 1993), strongly indicates that these compounds have a protective function in plant defence against herbivores. Studies have demonstrated that the concentration of some plant chemicals can change based on variation in the abundance of generalist and specialist herbivores (Lankau, 2007).
So far, it is not known if withanolide synthesis or their accumulation in infested plant parts might be induced in response to herbivorous attack. However, a study testing if simulated herbivory impacts withanolide biosynthesis did not reveal any changes in the concentration of 4β-hydroxywithanolide E in *P. peruviana* (Calderón *et al.*, 2012).

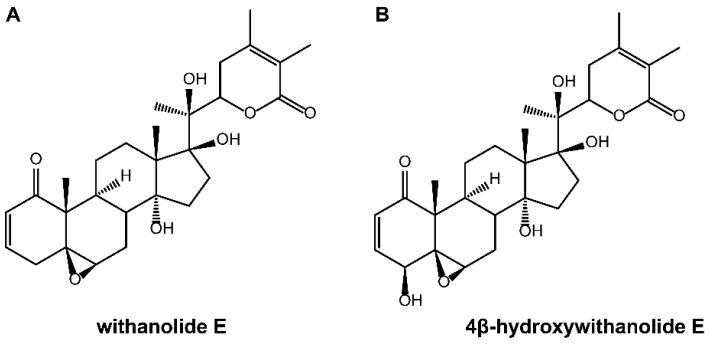

Figure 3: The main secondary metabolites of *P. peruviana* plants: A) withanolide E and B) its derivative 4β-hydroxywithanolide E.

Toxic compounds such as cardenolides and isothiocyanates are often characterised by an immediate adverse or acutely repellent effect against voracious insects (Karowe and Golston, 2006). However, many plants depend on secondary metabolites that seem to operate in a mild and often long-term mode of action, deterring feeding and oviposition instead of killing insects outright (Pino *et al.*, 2013). Accordingly, non-adapted herbivores would likely be affected only by ingesting withanolides constantly. Interestingly, according to the literature, the effect of these compounds is very selective. Since the efficacy of withanolides is variable and species-specific, the chemical structure alone is not sufficient to predict which insects the compounds will affect. Furthermore, the question of whether harmful effects occur as a result of feeding deterrence or whether a plant's overall chemical defences are toxic can confound cause and effect.

4. Study Organisms

The study system of the present thesis includes the two heliothine species *Chloridea subflexa* (Figure 4C & D) and *C. virescens* (Figure 4A & B). The Heliothinae (Lepidoptera) is a subfamily of the noctuid moths (Noctuidae) and consists of about 365 described species with a global distribution (Cho *et al.*, 2008). The majority of species are found in arid, subtropical regions and are severe agricultural pests of common herbaceous crops (Mitter *et al.*, 1993). Most Heliothinae are dietary specialists and only a few are considered generalists (Bateman, 2006). The larval stages usually feed on flower buds and fruits of shrubs and grasses such as tobacco, cotton, maize, soybean and tomatillo. A recent phylogenetic re-analysis (Figure 5) placed the two species of the former *Heliothis virescens*-group in the monophyletic clade of the genus *Chloridea* (Pogue, 2013). *C. tergemina* is a relatively unknown oligophagous species that feeds on tobacco plants and also belongs to the *Chloridea* clade but was not considered in the phylogenetic analysis (Mitter *et al.*, 1993, and references therein). The changes to the nomenclature are adopted in this thesis.

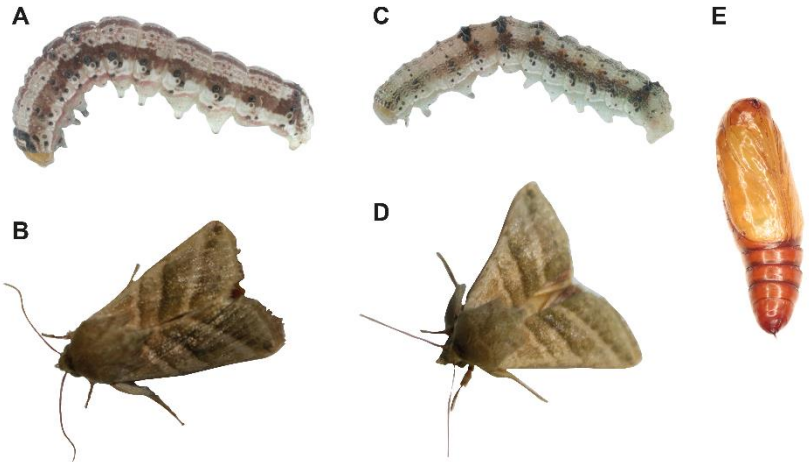

Figure 4: Study organisms: A) *C. virescens* last instar larvae and **B)** adult moth. **C)** *C. subflexa* last instar larva and **D)** adult moth with **E)** corresponding pupa. Due to their high morphological similarity, the moths were first identified as two separate species in 1941 (McElvare, 1941).

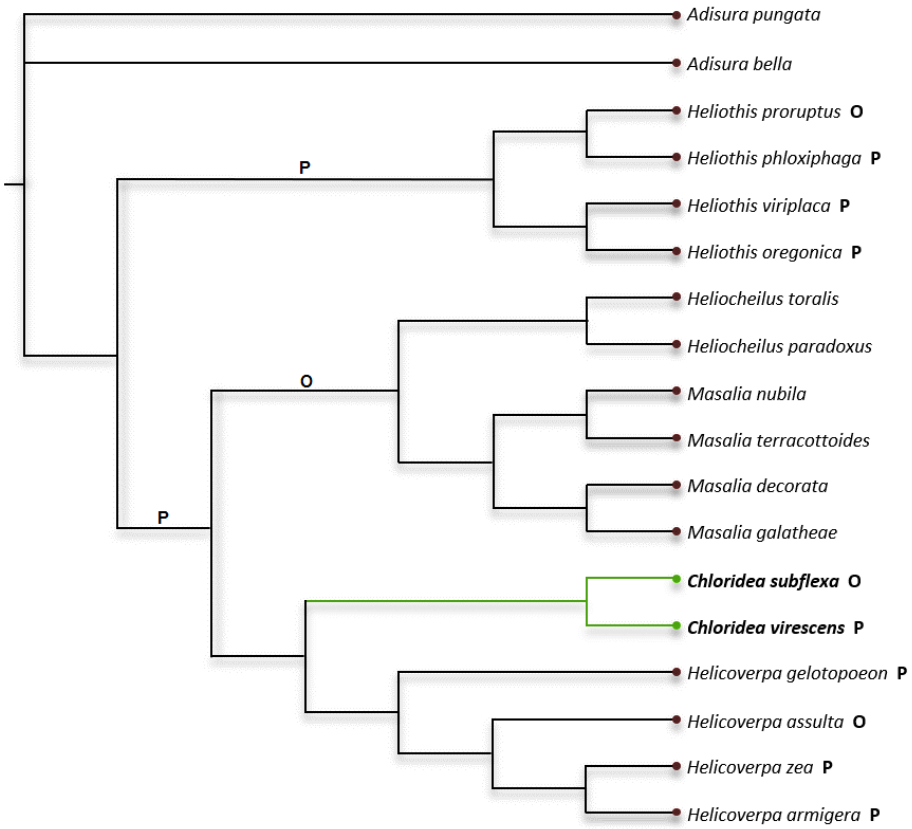

Figure 5: Simplified maximum likelihood tree of Heliothinae modified from Pogue (2013). Species in the monophyletic *Chloridea* clade are shown in bold. The letters O and P refer to oligophagous and polyphagous dietary behaviours.

Whereas *C. virescens* is a broad generalist on at least 37 species of 14 different host-plant families (Sheck and Gould, 1993), *C. subflexa* is narrowly specialised on plants of the *Physalis* genus. The latter species completes its entire life cycle associated with this single host genus (Mitter *et al.*, 1993; Bateman, 2006).

Though *C. virescens* and *C. subflexa* share an overlapping habitat in North and South America, *C. virescens* has not been observed feeding on *Physalis* plants in the field (Sheck and Gould, 1993). In the present thesis, *C. virescens* was included for a direct comparison of behavioural, physiological and genetic differences with specialised *C. subflexa* in response to *P. peruviana* defences. This species pair provides a model system that is under study with respect to the evolution of diet breadth (Sheck and Gould, 1996) and to the possible chemosensory mechanisms underlying the different feeding habits. It was speculated that the two species evolved from a generalist last common ancestor (Mitter *et al.*, 1993; Oppenheim *et al.*, 2012). The high degree of genetic similarity between *C. virescens* and *C. subflexa*, together with their ability to hybridise, further indicates that divergence of the two moth species occurred recently (Laster, 1972; Sheck and Gould, 1996; Benda *et al.*, 2011). That generalists and specialists can be hybridised provides an interesting genetic tool; with it, adaptations, speciation and host shifts can be studied, and the genetic mechanisms underpinning these processes can be dissected (Sheck and Gould, 1996). Studies of plant-herbivore interaction systems in lesser-known taxa add to our knowledge on the evolution of insect and plant adaptations and harbour great potential for novel discoveries in fundamental research. Often, research focusses on insect species with a long evolutionary association with their host. To understand how host specialisation develops and which factors might play a role in reciprocal interactions, it is relevant to study specialised insects that have diverged recently from a generalist group. The specialisation of *C. subflexa* is thought to have occurred rather recently; thus, *Physalis* plants and this moth species share a relatively short history of association.

5. The Specialised Adaptation of *Chloridea subflexa*

Frugivorous *C. subflexa* larvae, the only insects known to feed exclusively on the *Physalis* genus, spend their life cycle almost completely within the calyx of the fruits (Figure 6). Newly hatched neonates make their way to the calyx and cut out a hole to reach the fruit. During larval development, the caterpillars feed on several fruits before they finally leave the plant to pupate in the surrounding soils (Bateman, 2006). Nectarivorous adult *C. subflexa* moths were observed to visit *Physalis* flowers frequently, but they do not seem to be closely intertwined with *Physalis* plants as a feeding site or general mating location (Bateman, 2006). Most females, however, oviposit on the foliage and stems as well as on inflorescences of *Physalis* plants, but also lay their eggs on co-occurring non-host plants

(Benda *et al.*, 2011). Although mammals and other insects such as the three-lined potato beetle *Lema trilinea* (Kogan and Goeden, 1970) occasionally feed on *Physalis* fruits, *C. subflexa* represents the strongest permanent biotic pressure on those plants. However, *Physalis* plants are not defenceless against this major herbivore and in the course of reciprocal adaptation, *Physalis* plants have evolved protection mechanisms against larval attack: (1) Since *C. subflexa* larvae often consume the whole fruit including seeds, they can cause severe damage to the plant by significantly reducing its reproductive success. As a response to mechanical damage, herbivory-elicited fruit abscission was observed in at least three *Physalis* species (Petzold *et al.*, 2009). When occupied *Physalis* fruits are abscised, the larvae consequently have to crawl up the stem again; during this time, they are susceptible to predators and parasitoids. (2) Mechanical damage, not oral secretions were required to induce fruit abscission (Petzold *et al.*, 2009). However, oral secretions also induce a response in *Physalis* species. The plants release volatiles that in turn attract the parasitoid wasp *Cardiochiles nigriceps* (Oppenheim and Gould, 2002; De Moraes and Mescher, 2004). In laboratory experiments, *P. angulata* plants were shown to increase polyphenol oxidase (PPO) production after the application of jasmonic acid (Doan *et al.*, 2004). The expression of these enzymes was found to be involved in herbivore resistance (Constabel and Barbehenn, 2008). (3) Field studies showed that the oviposition of *C. subflexa* eggs induced a direct defence reaction in the leaves of *Physalis* plants, while egg-laying on neighbouring non-host plants did not (Petzold-Maxwell *et al.*, 2011). The leaves of *P. angulata* and *P. pubescens* responded to the eggs by morphologically altering the surface tissues: neoplastic growth (neoplasms) and necrosis were detected under the egg deposition sites. Overall, these morphological changes significantly decreased the ability of the eggs to stay attached to the leaves, which in turn decreased the probability of successful hatching. (4) *Physalis* plants are characterised by a lantern-like calyx that encloses and protects the fruit during maturation. This lantern-like calyx grows continuously throughout fruit development. Interestingly, calyx formation appears to have evolved several times independently within the Solanaceae, indicating the calyx benefits the plant's fitness (Hu and Saedler, 2007). This structure seems to contribute to shielding the berries from desiccation and herbivory (Li *et al.*, 2019). Smaller larvae that bore through the calyx to reach the fruit inside are confronted with empty space and distance between the calyx's inner surface and their food source. (5) If larvae do not take advantage of their ability to spin silk to reach the fruit, they are forced to move through a sticky coating that accumulates at the pedicels where fruit and calyx are connected. This viscous coating - seemingly a mixture of acylsugars (polyesters) - may serve as a trap for early instar larvae (Maldonado *et al.*, 2006, and references therein; Franco *et al.*, 2014). In the course of the ripening process, the coating eventually covers the entire fruit, aggravating larval locomotion and possibly also preventing larvae from penetrating the fruit's cuticle.

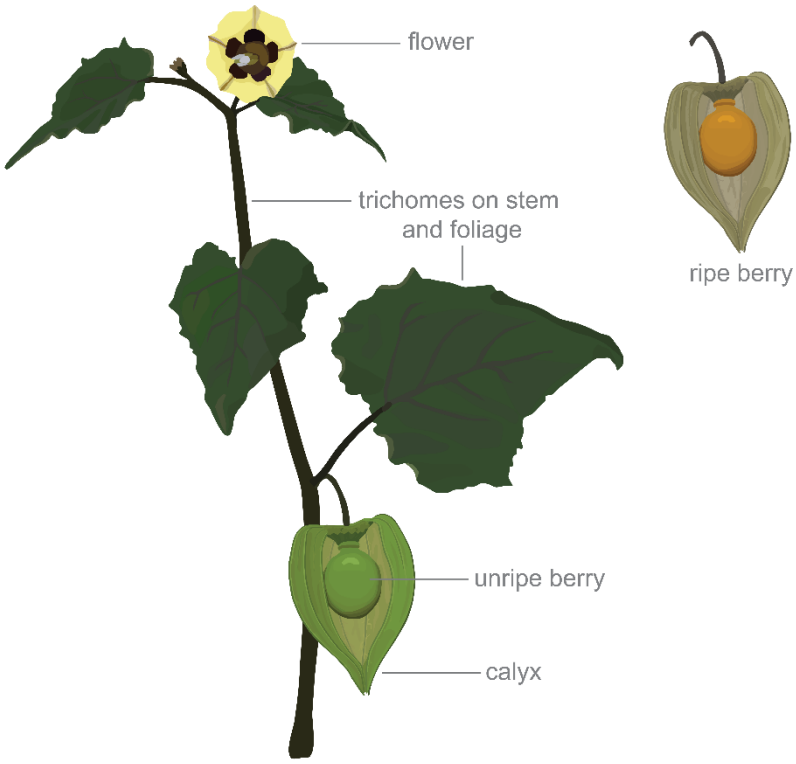

Figure 6: Schematic illustration of a *P. peruviana* plant showing calyx cross sections of an immature fruit and a ripe fruit. The inflated sepals forming the papery calyx turn brown during the ripening process.

Many other Solanaceous plants are known to secret acylsugars via glandular trichomes on their surface (Fan *et al*., 2019). Acylsugars in the wild tomato *Lycopersicon pennellii* were shown to affect the feeding and ovipositing ability of several insects and also appeared to be toxic to them (Liedl *et al.*, 1995; Chortyk *et al.*, 1996; McKenzie and Puterka, 2004). (6) Additionally, trichomes physically defend *Physalis* plants against herbivory. They delay or prevent smaller insects and larvae from using the surface for feeding and egg-laying as well as from reaching reproductive organs (Kariyat *et al.*, 2018). (7) Withanolides are the main secondary compounds in *Physalis* plants. As described above, these chemicals seem to play an important role in defence against herbivorous attack. However, just as no clear evidence of a direct interaction with herbivores has yet been found, little is known about potential other functions of withanolides, e.g. as signalling molecules in the plants themselves.

C. subflexa does not infest all species of the *Physalis* genus. It is thought that differences in plant chemistry and architecture within this genus influence host-plant use in the specialist (Benda, 2007). A screening study of potential hosts revealed that *C. subflexa* seems to be restricted to a subset of *Physalis* species, and non-preferred species were even shown to be suboptimal for larval survival and growth. Interestingly, the survival rate of larvae feeding on those *Physalis* plants did not differ noticeably from larvae feeding on non-*Physalis* species (Bateman, 2006).

Laster *et al.* (1982) proposed that host specificity of *C. subflexa* combines larval preference and oviposition. Several noticeable behavioural traits that may help larvae overcome *Physalis* defences have been described in the literature (Oppenheim and Gould, 2002; Benda, 2007). Those adaptive traits might have initiated a host shift and specialisation: (I) *C. subflexa* larvae gain protection from the endoparasitoid *C. nigriceps* by using the *Physalis* calyx as an "enemy-free space" (Sisterson and Gould, 1999; Oppenheim and Gould, 2002). *C. nigriceps* are known to be specialised on *C. subflexa* and *C. virescens* as their main hosts (Lewis and Vinson, 1971). As studies clearly demonstrate, larvae that use the calyx suffer less parasitism and have a significantly reduced probability of being attacked by wasps. The advantage of using the calyx raises the question of why no other insect utilises it for protection. Studies indicate that *C. virescens* would immediately benefit from the protective calyx (Oppenheim and Gould, 2002). (II) Besides using the calyx as shelter, larvae rely on behavioural adaptation such as feeding strategies and fast-decision making. Usually, *C. subflexa* larvae move from plant to plant feeding on more than one fruit (Benda, 2007). In a behavioural study, Oppenheim and Gould (2002) observed that *C. subflexa* larvae were faster to cut an entrance hole into the calyx and initiate feeding than *C. virescens*. Furthermore, *C. virescens*, but not *C. subflexa* adopt feeding strategies more suited to cotton fruiting structures when feeding on *Physalis* fruits. Consequently, the larvae do not fully crawl into the calyx. This behaviour, together with slower decision-making, makes them more likely to be spotted by predators and parasitoids than *C. subflexa*. (III) Interestingly, feeding damage by *C. subflexa* differs from that of other species feeding occasionally on the fruit (Bateman, 2006). Whereas *C. subflexa* bores holes into the fruit, larvae of *Lineodes fontella*, for example, scrape the inner calyx tissue and the fruit surface. One might thus speculate that the feeding behaviour of *C. subflexa* is strongly manifested in the impulse to hide inside *Physalis* by boring holes and entering the calyx with the whole body. (IV) Another study examined the oviposition behaviour of *C. subflexa* adult moths and found that around 20% of the females laid their eggs in close proximity to *Physalis* plants, but on non-host species (Benda *et al.*, 2011). The oviposition site generally has a high influence on the fitness of the offspring (Janz, 2011). Egg-laying on plants that are not suitable for optimal development entails costs as vulnerable neonates have to travel to reach their desired host. Benda *et al.* (2011) hypothesised that as monophagy has evolved rather recently, females have yet to fully convert their host perception and oviposition behaviour to *Physalis* plants. The same authors alternatively proposed that ovipositing on non-hosts a short distance from *Physalis* shrubs could be a trade-off strategy; that is, the costs might be outweighed by other fitness benefits such as the avoidance of above-described plant defences responses (neoplasms and necrosis). (V) To use its host plant, a larva must be able to digest the plant material and to tolerate potentially defensive metabolites. Little is known about how efficiently insects are able to digest these metabolites. To date, few papers have detailed post-

ingestive mechanisms for detoxification or improved nutrient accessibility. *C. subflexa* may be more efficient than *C. virescens* at overcoming low-nutritive food sources and exploiting *Physalis* plants as a food source. A study in this direction has been conducted by De Moraes and Mescher (2004). The authors propose that *Physalis* plants lack an essential fatty acid, linolenic acid (α-18:3 LA), and that in contrast to *C. virescens, C. subflexa* does not require α-18:3 LA to survive. In their experiments, *C. virescens* larvae were unable to develop normally on the fruits unless they were treated with α-18:3. However, results from testing larvae on artificial diet supplemented with different concentrations of α-18:3, have shown that *C. subflexa* larvae do in fact have a dietary α-18:3 requirement for normal growth (Sörensen, 2012). In addition, Bateman (2006) has detected concentrations of α-18:3 in different *Physalis* species equal to or greater than the amounts added to the experimental diets on which both *C. virescens* and *C. subflexa* developed normally. It can therefore be postulated that specialisation in *C. subflexa* took place for reasons other than adaptation to this apparent dietary deficiency.

A previous study proposed that *C. subflexa* larvae benefit from the uptake of withanolide extracts by experiencing increased growth and immune system stimulation (Barthel *et al.*, 2016). While *C. subflexa* larvae showed increased phenoloxidase (PO) activity when fed on artificial diet supplemented with purified withanolide extracts from *P. peruviana,* the PO levels in its sister species *C. virescens* were unaffected. RNA-Seq analyses showed that in response to the extracts, immune-related genes were upregulated in *C. subflexa* larvae, whereas an opposite or non-significant response was observed in its close relative. The faster individual larvae can grow after hatching, the lower their mortality risk. Thus, the ability to use *Physalis* fruits as a food source might have been a trait under strong selection. It is, however, questionable if a stimulated immune system is a generally advantageous effect, especially in the absence of an infection. The adaptive mechanism by which *C. subflexa* seems to circumvent the adverse effects of withanolides, together with behavioural changes, might have originally led to a host shift in the last common ancestor of the two Heliothine species (Oppenheim and Gould, 2002).

6. Aim of the Study

Though the exact ecological role and the mode of action are not fully understood, withanolides are presumed to act as a defence to inhibit herbivorous attack. The specialised adaptation and tight association of *C. subflexa* with its withanolide-producing host plants represents an interesting study system. However, the mechanistic underpinnings by which *C. subflexa* overcomes the inhibitory effects of withanolides are unknown. This dissertation primarily aims to widen our understanding of the function and mode of action of *Physalis*-derived withanolides. What role do these compounds

play in the adaptation of *C. subflexa* to its host-plant genus? And why are *Physalis* plants excluded from the host range of the broad generalist *C. virescens*? By comparing the effects of withanolides and a diet based on *Physalis* fruits in a specialist and a closely related species, I hoped to 1) identify traits and adaptive key strategies that contribute to the specialisation process of *C. subflexa* and 2) to understand the factors that may lead to divergent development between both species.

Barthel *et al.* (2016) showed that *P. peruviana* withanolide extracts exert inhibitory activity against the spores of the insect pathogen *Bacillus thuringiensis*. The chronic uptake of antimicrobial withanolides may significantly shape the microbial composition in insect guts by reducing the load of entomo- and/or phytopathogens or by promoting the growth of beneficial microbial strains. These consequences would particularly apply to insects like the specialist *C. subflexa* whose larvae are exposed to withanolide-producing plants over a long time as they use them as their only food source. It is as of yet unknown if withanolides can significantly influence the intestinal microflora of insects. In short, this thesis is based on three major objectives: (1) further investigations of the consequences of a *P. peruviana* fruit and withanolide-based diet on a specialised and on non-adapted moth species, (2) the analysis of a putative withanolide metabolism in *Chloridea* larvae and (3) the study of the antimicrobial activity of withanolides and a potential impact of withanolide-feeding on the larval gut microbiome.

Chapter I explores the effects of a *Physalis* fruit-based diet and artificial diet supplemented with withanolide extracts on the development and growth of *C. subflexa*, *C. virescens* and their hybrids. In order to assess their overall larval performance together with other lepidopterans, they were additionally tested in choice and survival assays.

The observed moulting disorders in non-adapted insects feeding on withanolide-producing plants, as well as the structural similarity of withanolides to 20E, suggest a putative interaction with the ecdysteroid receptor, which is involved in the regulation of development as well as in immune responses. Therefore, the ecdysteroid receptor sequences of the two sister species were further analysed in chapter II. This chapter also presents the results of the transcriptome analysis of hybrid, *C. subflexa* and *C. virescens* gut RNA samples after feeding on *P. peruviana* fruits, on a withanolide-spiked diet or on control diet. Digital gene expression analysis was performed in order to detect interesting candidate genes that respond specifically to withanolide ingestion.

The unsaturated carbonyl-system in withanolides might be a target for enzymatic reactions. A metabolic conversion could potentially initiate the activation of these compounds or may lead to their rapid elimination. Thus, chapter III focusses on the analysis of a potential species-specific withanolide metabolism in *C. subflexa*. Experimental approaches included the isotopic labelling of *P. peruviana* plants for *in vivo* feeding assays with ^{13}C-labelled withanolides. In order to detect

putative differences in the withanolide metabolism of the specialist and *C. virescens*, the frass content was analysed with LC-MS/MS and NMR.

Since withanolides are known to have antimicrobial properties against certain microbes, metagenomic analyses were conducted to detect possible changes and differences in the microbial composition in the test organisms feeding on withanolide-containing diet compared to control groups. Chapter IV summarises the results of antimicrobial inhibition assays and analyses metabiome data.

MATERIALS AND METHODS

Materials and Methods

1. Insect Rearing and Plant Growth Conditions

1.1 Insect rearing

The two moth species were originally collected in Florida (*C. subflexa; Cs*) and North Carolina (*C. virescens; Cv*) and were reared at the North Carolina State University until their transfer to Jena in 2005 (*Cv*) and 2006 (*Cs*). All *Cs* larvae were grown on a wheat germ and soy flour-based General Purpose Lepidoptera (GPL) artificial diet containing 19 g agar added to 144 g Dry Mix F9772 (Frontier Scientific Services) per 875 ml of distilled water. The performance of *Cv* and *Cs* was assessed on three different diets in preliminary tests (Suppl. Figure 45 & Figure 46). Originally, *Cv* were reared on pinto bean-based diet (see page 143). Since the pinto bean mix contained the antibiotic tetracycline (Sigma-Aldrich), GPL diet became the diet of choice in all experimental settings. From 2018 onwards, also *Cv* sub-colonies were reared on GPL diet. Upon pupation, individual larvae were kept in single 20 ml plastic cups (Ultra Clear™ PET Soufflé Portion; Dart Container Corporation) until they eclosed. Pupae and mating couples were kept in a Snijder climate chamber at 26°C, 55 ± 10% relative humidity and 16:8 h light/dark cycle (L:D). Emerged adults were kept at 12°C 55 ± 10% relative humidity and 16:8 h (L:D) and fed with a 10% honey water solution (v/v). For a balanced breeding, single pair mating was performed. It was avoided to mate individuals of the same family. All mating couples were set-up in plastic coffee cups covered with egg cloths made of gauze. Egg cloths with fertilised eggs were collected and transferred to Petri dishes containing GPL diet. F1 *Chloridea* hybrids (hereafter referred to as hybrids or (hy)) were bred by combining both sexes from *Cs* and *Cv*. To avoid a high degree of inbreeding, stocks of both insect strains were frequently refreshed by adding larvae from a collaborating group at the University of Amsterdam.

1.2 Plant growth conditions

P. peruviana (in the Materials and Methods section abbreviated to *Physalis*) and *Gossypium hirsutum* (cotton) plants were reared in the greenhouse of the Max Planck Institute for Chemical Ecology, Jena. *Physalis* seedlings were first cultivated in York climate chambers. Cuttings were reared in cabins with a 14:10 h (L:D) and a temperature range of 18 - 21°C (night) and 21 - 23°C (day). The average humidity range was set to 50 - 60%. Older plants were kept in a chamber with 16:8 h (L:D) at 19 - 23°C (night) and 23 - 25°C (day) with a humidity range of 45 - 55%. Cotton plants were grown in chambers with 16:8 h (L:D) and temperatures between 16 - 18°C (night) and 22 - 24°C during the day

(humidity range 60 - 70%). All plant species were grown on a (70/200 v/v) mix of Futterpflanzensubstrat/Tonsubstrat TS1 substrate (Klasmann-Deilmann).

2. Feeding Assays

A series of laboratory assays was conducted to compare the relative impact of withanolide extracts, *Physalis* fruits and immature cottonseeds on the larval performance. If not otherwise mentioned, third instar larvae were used in all feeding assays. Feeding assays were performed in 24-well Falcon polysterene plates (Thermo Fisher Scientific™), which were placed in plastic clip boxes and kept in a Snijder climate chamber at 21°C, 55 ± 10% relative humidity and 16:8 (L:D). Each larva was weighed at the beginning and again on the last day of the experimental period. Potential family effects were controlled for by tracking back larvae individually and by distributing moth families randomly throughout all treatments. Detailed descriptions of the statistical analyses of feeding assays are provided in section 11.1.

2.1 Extraction and purification of withanolide compounds

In most experiments, *Physalis* withanolide leaf extracts were used that were previously prepared in the Entomology Department at the Max Planck Institute for Chemical Ecology. Those extracts were obtained as described in Barthel *et al.* (2016). Crude leaf homogenates were produced by crushing leaf material and adding an equal volume of 40% methanol (MeOH). The resulting liquid was used in inhibition zone assays. Fresh and/or labelled withanolide compounds were obtained in a slightly modified procedure: see section 8.2. All withanolide extracts were stored at - 20°C.

2.2 Withaferin A

Withaferin A [CAS 5119-48-2] with a purity of ≥ 96.0% was purchased from BOC Sciences (#B0084 - 120734) and stored at - 20°C according to the manufacturer's recommendations.

2.3 Feeding assays on artificial diet

The methods for making withanolide-supplemented diets were based on the formula described in (section 1.1). For feeding assays, 1 ml GPL diet was pipetted into each well of 24-well plates and allowed to solidify. Then, the diet was surface-sterilised under UV light for 15 min. Withaferin A and *Physalis* withanolides extracts (100 µg/ml) were diluted in 40% MeOH and pipetted onto solidified diet. Control group larvae were provided with normal diet, but with addition of the same volume of 40% plain MeOH. Plates were allowed to dry under a fume hood for at least four hours to ensure complete evaporation of the solvent. For minimal diet acetone instead of MeOH was used, but was

otherwise processed the same way (see page 143). Every second day, larvae were provided with freshly prepared diet.

2.4 Feeding assays with *Physalis* fruits and cottonseeds

Quarters of equally sized *Physalis* fruits and whole immature seeds picked from cotton bolls were provided to larvae and distributed randomly across the 24-well plates. In preliminary test series, ripe fruits revealed rapid decay and a strong fermented odour, which was considered adverse test conditions for the larvae. Therefore, unripe (still green) *Physalis* fruits were used instead. For both treatments, fruits and seeds were changed daily to avoid mould and rotting of the plant material under room temperature (RT) conditions. The cottonseed diet treatment was chosen as cotton is part of the natural food range of *Cv* larvae but not of that of *Cs* larvae. As no ideal control was available for cottonseeds and *Physalis* fruits, normal larval growth and development was tested in parallel on artificial diet.

2.5 Calculation of the relative larval weight gain

Relative larval weight gain during the experimental period was calculated as follows:

$$\frac{\text{end weight - starting weight}}{\text{starting weight}}$$

2.6 Comparative analysis of larval development

Differences in developmental processes of *Chloridea* and hybrid larvae were assessed. Early third instar larvae (n = 24 per species) were reared on a *Physalis* fruit diet, and also on artificial (GPL) diet as a control for normal larval development in the lab populations. The diet was replaced daily. Larvae were reared as described in section 2.3 & 2.4. After pupation, individuals were transferred to plastic cups in which they later eclosed. The developmental stage of each larva and the number of moulting steps were checked daily. Details on statistical analyses are provided in section 11.2.

2.7 Survival assays

The probability of survival of six different Noctuidea third instar larvae and *Chloridea* hybrid larvae was determined over a 10-day experimental period. Insects (n = 48) were fed on *Physalis* fruits or on artificial (GPL) diet. The diet was changed every day. After seven days, the weight gain of a subset (n = 24 per species) from each diet treatment group was assessed. Statistical analysis was performed using the Cox proportional hazard model from the survival package in R (Therneau, 2021). Larval survivorship was displayed as Kaplan-Meier survival curves.

3. Behaviour assays

3.1 Hole-boring efficiency of neonate larvae

Whole *Physalis* fruits were impaled on pins to fix them in an upright position (**Figure 7**) in PET plastic cups (Dart Container Corporation). Per cup (n = 24), five neonates were used. Fruits were inspected for holes after 12 h. In order to compare the number of feeding sites in *Physalis* fruits across different Noctuidea species (*Cs*, *Cv*, hybrids, *H. zea* and *H. armigera*), larvae were first placed directly on unripe fruits. In another assay, the number of holes were compared between *Cv* and *Cs* larvae fed on ripe or unripe berries (n = 24). In a third experiment, *Cv* and *Cs* neoneates (n = 24) were transferred on top of unripe fruits of which the calyx was not completely removed; a calyx "platform" was left from which the larvae had to crawl down to reach the fruit. The ability to establish feeding sites was additionally compared between *Cs* and *Cv* larvae fed on fruits with and without a sticky fruit coating. The coating was washed-off by using moist cotton buds. Detailed information on the statistical analyses is provided in **section 11.4**.

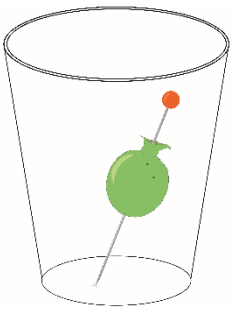

Figure 7: Schematic drawing of the experimental setup.

3.2 Larval choice assays

Neonates and second instar larvae of *Cs*, *Cv* and their hybrids were used in choice assays. In a ternary choice assay, larvae were offered three different food options: a) *Physalis* fruits, b) immature cottonseeds and c) artificial diet. Binary choice assays tested larval food choice on a) *Physalis* fruits or c) artificial diet. The food chunks were placed in 20 ml plastic cups with a distance of 10 mm to each other (**Figure 8**). Diet chunks were cut into cubes of 5 x 5 mm. *Physalis* fruits and cottonseeds were cut equally sized to the artificial diet chunk.

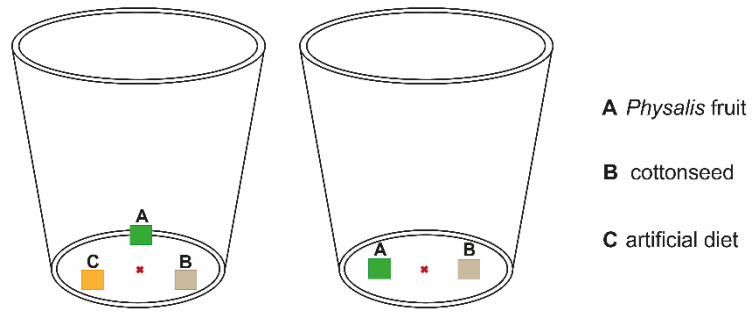

Figure 8: Experimental design of ternary and binary choice assays. Single second instar larvae or neonates were placed in the centre of the cup (red cross). Larvae were provided with cottonseeds and *Physalis* fruit chunks and optionally with artificial diet (ternary choice assays).

By using a brush, single larvae were placed in the middle of the food sources offered. Per cup, only single larvae were used in order to avoid cannibalism and reciprocal interactions between them. Naïve neonates were picked right after hatching to avoid any imprinting on a diet that might potentially influence their diet choice. As neonates are susceptible to handling, slightly bigger early second instar larvae were additionally tested. All second instar larvae and their parental generations were reared on artificial diet. Prior to the experiment, second instars were starved for two hours. Per species, 24 neonates and 72 second instar larvae were used in the assays. The position of each larva was recorded after 1 h and again after 12 and 24 h. Details on the statistical analyses are described in the **section 11.3**.

3.3 Analysis of oviposition behaviour

Standard matings were set-up in coffee cups as described above. Female *Chloridea* moths usually oviposit at the gauze (egg cloth) of mating cups from bottom-up. In a collaborating research group (Astrid Groot, University of Amsterdam; personal communication), ripe *Physalis* berries were frequently added on top of the gauze to stimulate egg-laying in *Cs*. In the course of standard *Cv* and *Cs* rearings, a ripe, unripe or no *Physalis* fruit was placed on the gauze (centre or periphery) with the cut-side down (**Figure 9**). By visual inspection, the position of eggs on the gauze was determined in relation to the fruits. Oviposition was first checked after four days and then on a daily base. Coloured, paper-based fruit dummies (green or orange), which correspond to unripe/ripe fruits were additionally tested as oviposition stimuli to investigate if moths respond to the colour only. For each experiment, a total number of 24 mating couples were used per treatment group.

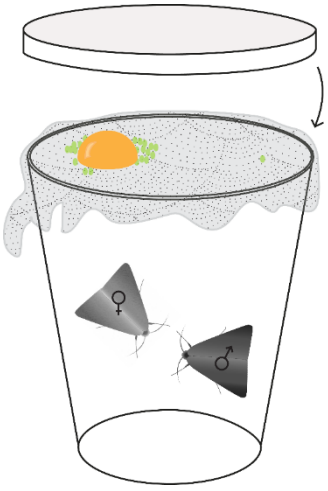

Figure 9: Scheme of a typical mating cup setup with a ripe *Physalis* fruit half (orange). Eggs are shown in green.

4. Molecular Biology Methods

4.1 Genomic DNA/RNA extraction

Genomic DNA from larval guts was extracted according to the standard CTAB-chloroform (cetyltrimethylammonium bromide) method (see **section 10.2**). In all other experiments, the Quick-DNA Tissue/Insect kit (Zymo Research) was used to isolate genomic DNA. RNA extraction was performed with TRIzol™ reagent (Thermo Fisher Scientific™) according to the manufacturer's instructions. DNA samples were stored at - 20°C; RNA extracts at - 80°C.

4.2 Purification of DNA/RNA

DNA was resuspended in 100 µl of sterile water and cleaned using the DNeasy® PowerClean® Pro Cleanup Kit (Qiagen) following the company's instructions. To remove DNA from RNA samples, DNase digestion was performed with the TURBO™ DNase (Thermo Fisher Scientific™). RNA samples were purified by using the RNeasy® MinElute® Cleanup Kit (Qiagen) according to the recommended protocol. RNA quantity was measured spectrophotometrically with a NanoDrop ND-1000. All RNA samples used for sequencing were additionally tested in an Agilent 2100 Bioanalyzer with the RNA 6000 Nano Kit (Agilent Technologies) to verify RNA integrity.

4.3 Polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify ecdysteroid receptor sequences (RACE PCR) and for DNA barcoding. The AccuPrime™ *Taq* DNA-Polymerase (Thermo Fischer Scientific™) was used for amplification. The *Taq* PCR Master Mix kit by QIAGEN was used for colony PCR only. PCR reactions were carried out according to the company's instructions. PCR products were cleaned with the GeneMATRIX PCR/DNA Clean-up Purification Kit (Roboklon) for further use. Amplification products were extracted from agarose gel using the Zymoclean™ Gel DNA Recovery kit (Zymo Research). All PCR reactions were performed in a BIO-Rad thermocycler. For the RACE PCR method, RNA was extracted from a single third instar *Cs* and from a *Cv* larvae. The cDNA synthesis and RACE PCR was performed using the SMARTer® RACE 5'/3' kit (Takara Bio) according to the user manual. A list of all primers used in experiments is shown in (Table 1).

Oligoname	Sequence (5' - 3')
DNA barcoding primers (COI gene)	
LCO1490 (for)	GGTCAACAAATCATAAAGATATTGG
HCO2198 (rev)	TAAACTTCAGGGTGACCAAAAAATCA
RACE primers *Csub* EcR	
3' RACE (for)	GCCAAGATCTCGCAGTCGGATCA
5' RACE (rev)	GAACGGCGGCAGCTTCCTGT
RACE primers *Cvir* EcR	
3' RACE (for)	TTGAACGTTGCGTAGACGAG
5' RACE (rev)	AACAAAAGGCTTGCTCGTGT
16S rRNA primers	
515f (for)	GTGYCAGCMGCCGCGGTAA
806r (rev)	GGACTACNVGGGTWTCTAAT

Table 1: List of all primers used in this doctoral thesis. Barcoding primers were frequently used to verify the identity of *Chloridea* species. RACE primers were designed to complete the *Chloridea* EcR sequences. 16S rRNA primer pairs were used to study the taxonomic composition of the *Chloridea* gut microbiome.

4.4 Molecular cloning

PCR fragments of interest were processed with the TOPO® Cloning Kit (Thermo Fisher Scientific™) according to the manufacturer's instructions. Plasmid DNA was recovered and purified with the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific™). Sequencing was performed using the pCR™4-TOPO® sequencing vector (Thermo Fisher Scientific™) and corresponding M13 primers.

5. Transcriptome Analysis

Three gene expression datasets were analysed, that span gut samples of early third instar larvae (*Cs*, *Cv*, and hy) fed for 72 h either on *Physalis* fruits, on untreated artificial diet (control), or on artificial diet supplemented with 100 µg/ml *Physalis* withanolide extracts or with 100 µg/ml withaferin A. After 72 h, larval guts were removed and immediately frozen in liquid N_2. The gut was cleaned by pulling out the food bolus. Total RNA was extracted from gut tissues and purified as described in section 4.1 & 4.2 From each diet treatment, 5 pools were sequenced, each containing RNA from 3 larval intestines.

5.1 Sequencing and transcriptome assembly

Sequencing was carried out by the Max Planck Genome Centre, Cologne using the Illumina HiSeq-3000 Genome Analyser platform (http://mpgc.mpipz.mpg.de/home/). Poly-A mRNA was isolated from 1 µg of total RNA using oligo(dT) magnetic beads and fragmented to an average of 250 bp before sequencing libraries were generated using the TruSeq RNA Library Preparation Kit v2 (Illumina). Paired-end (2 × 150 bp) read technology was used for sequencing, resulting in ~ 20 million reads per sample. All reads generated by the sequencing provider were processed using an in-house assembly and annotation pipeline. In brief, quality control measures, including the filtering of high-quality reads, the removal of reads containing primer/adaptor sequences, and the trimming of read lengths, were applied using CLC Genomics Workbench v17.1. For transcriptome assembly, RNA-Seq data from all replicate samples and treatments were combined per species (reads from hybrid samples were not used in the assemblies) and *de novo* transcriptome assemblies were prepared using CLC Genomics Workbench v17.1 with standard settings and two additional CLC-based assemblies with different parameters. The presumed optimal consensus transcriptome for each species was then selected, as previously described (Vogel *et al.*, 2014).

5.2 Transcriptome annotation

The transcriptomes were annotated using BLAST, Gene Ontology, InterProScan and EggNOG in OmicsBox (https://www.biobam.com/omicsbox) as described by (Götz *et al.*, 2008). For BLASTx searches against the non-redundant NCBI protein database (NR database), up to 20 best NR hits per transcript were retained, with an e-value cutoff of $\leq 10^{-3}$ and a minimum match length of 15 amino acids. To assess transcriptome completeness, a Benchmarking Universal Single-Copy Orthologs (BUSCO) analysis (http://busco.ezlab.org) was performed by comparing the assembled transcriptomes against a set of highly-conserved single-copy orthologs. This was accomplished using the BUSCO v3 pipeline (Waterhouse *et al.*, 2017), comparing the predicted proteins of the *Cv* and *Cs* transcriptomes to the predefined set of 1658 Insecta single-copy orthologs from the OrthoDB v9.1 database.

5.3 Read mapping and differential gene expression analysis

For sequence read mappings, the *Cv* and *Cs* transcriptome assemblies were concatenated into a single reference file and digital gene expression analysis was carried out using CLC Genomics Workbench v17.1 to generate BAM files, and then counting the sequences to estimate expression levels, using previously described parameters for read mapping and normalisation (Pöppel *et al.*, 2015). To account for highly similar sequences (orthologous genes) between the two *Chloridea* species and to optimise differential read mappings in the F1 hybrids, the settings in the mapping parameters were modified. Briefly, the following stringent parameters were used: mismatch, insertion and deletion costs were all set to three, and read assignment quality options required at least 90% of the total read bases (how much of the sequence should be able to map in order to include it) and at least 97% of bases matching (minimum similarity fraction; defines how exact the matching part of the read should be) within each read to be assigned to a specific contig; maximum number of hits for a read (reads that match to more distinct places than this number will not be mapped) = 4; mer repeat settings were automatically determined while other settings were not changed. Gene expression levels were estimated by normalising mapped read values as implemented in CLC Genomics Workbench v17.1 and ArrayStar, calculating the reads per kilobase per million mapped reads (RPKM) and transcripts per million (TPM) values to obtain correct estimates for relative expression levels. To identify differentially expressed genes, Student´s t-test (as implemented in Qseq) was used, correcting for multiple testing using the Benjamini-Hochberg procedure to control the false discovery rate (FDR). As an alternative approach for comparative gene expression analysis, mapped reads were log2-transformed and normalised using the quantile method and statistical analysis of the normalised data was carried out using the “empirical analysis

of digital gene expression" (EDGE) tool, implemented in CLC Genomics Workbench v17.1. For both methods, the thresholds for differentially expressed genes were a minimum two-fold change in expression and FDR-corrected *p*-value of < 0.05.

5.4 Principal component analysis

In order to identify potential outliers and to assess the global differences between the *Cv*, *Cs* and hybrid samples, a principal component analysis (PCA) was performed using CLC Genomics Workbench v17.1, clustering samples in 2D. The large set of variables (here counts (mapped reads) for each individual contig of each sample) were transformed to a smaller set of orthogonal principal components. Log CPM (counts per million) were calculated for each contig, using the effective library sizes as calculated by the TMM normalisation. Afterwards, z-score normalisation was performed across samples for each gene where the counts for each gene are mean centred and scaled to unit variance. Genes or transcripts (contigs) with zero expression across all samples were removed before clustering. The clustering visualisation plot shows the projection of the samples onto the two-dimensional space spanned by the first and second principal components of the covariance matrix. The expression levels used as input are normalised log CPM values.

5.5 Gene ontology term enrichment analysis

Fisher's exact test as part of the FatiGO package implemented in OmicsBox (v2.1) was used to identify the over- and underrepresentation of gene ontology (GO) terms among lists of differentially expressed genes between treatment groups. The GO-enriched word clouds were simplified to display only the most specific GO terms by removing parent terms representing existing child terms using the function "reduce to most specific terms" in OmicsBox. A GO term was considered significantly enriched if the *p*-value corrected by FDR control was less than 0.01.

5.6 Identification of true orthologous genes

The Osiris gene sequences of *Cv* and *Cs* were annotated manually from draft genomes of the corresponding species. Osiris genes from the model Lepidoptera *Bombyx mori* were used as a query (Shah *et al.*, 2012). The obtained coding sequences were translated into amino acid sequences before being aligned with an online version of MAFFT (https://mafft.cbrc.jp/alignment/server/) using default parameters. A maximum likelihood analysis was then performed on the resulting amino acid alignment using the web server version of IQ-TREE (Trifinopoulos *et al.*, 2016). Best-fit substitution model was determined automatically in IQ-TREE using ModelFinder (Kalyaanamoorthy *et al.*, 2017) and FreeRate heterogeneity (+R) were included in the model selection process. Branch support was

assessed using the ultrafast bootstrap approximation (UFBoot) with a maximum of 1000 iterations (Hoang *et al.*, 2018).

6. Protein Extraction and Analysis of Proteomic Data

6.1 Extraction of larval microsomes

Larval guts from *Cs* and *Cv* were cleared from gut content and collected in 1 ml hypotonic buffer [20 mM Tris-HCl pH 7.5 (Sigma-Aldrich), 5 mM EDTA (Invitrogen), 1 mM dithiothreitol (DTT; Thermo Fisher Scientific™) and cOmplete™ protease inhibitor (Roche)]. Each 1 g of gut tissue was resuspended in 9 ml hypotonic buffer and homogenised on ice using a Dounce homogeniser. The same volume of sucrose buffer pH 7.5 [20 mM Tris-HCl, 5 mM EDTA, 1 mM DTT, 500 mM sucrose (Roth®) and protease inhibitor] was added to reach a final sucrose concentration of 250 mM. The mix was centrifuged for 10 min at 1200 x g. The supernatant was collected and the homogenisation procedure was repeated with the pellet. The resulting supernatant was pooled with the first one. The mix was then centrifuged for 15 min at 10000 x g. Afterwards, the supernatant was centrifuged again for 1 h at 100000 x g. The resulting pellet was resuspended in 1 ml resuspension buffer [1 x phosphate-buffered saline (PBS; Bio-Rad) and protease inhibitor] using a Dounce homogeniser. Centrifugation was performed again for 1 h at 100000 x g. The final pellet was resuspended again in resuspension buffer and stored at - 80°C. All centrifugation steps were carried out at 4°C. The integrity and concentration of microsomal proteins was further analysed via Bradford assays (section 6.2) and SDS-PAGE (section 6.3).

6.2 Determination of protein concentration

The concentration of protein samples was determined using the Protein Assay (Bio-Rad) which is based on the Bradford method (Bradford, 1976). For every sample of interest, duplicates were measured containing either 2 µl or 4 µl of the sample. Dye reagent was prepared by mixing the Dye Reagent Concentrate with distilled water (1:5). Then, 20 µl protein samples were mixed with 200 µl of Bradford reagent and incubated in the dark for 10 - 15 min. The absorption of the samples was determined at a wavelength of 595 nm and standardised based on a calibration curve with 0 - 11 mg/ml BSA standard.

6.3 SDS-polyacrylamide gel electrophoresis

Denatured proteins (20 µg) were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% Criterion XT gradient gels (Bio-Rad) with XT MES running buffer at 125 V for 1 h. The molecular weight (kDa) was determined with pre-stained (Novex™ Sharp,

Thermo Fisher Scientific™) and unstained (peqGOLD protein marker II, Peqlab) protein standards. All gels were stained overnight using a 1:1 mix of Coomassie Brilliant Blue R-250 and colloidal Coomassie Brilliant Blue G-250 (Thermo Fisher Scientific™).

7. Enzyme Assays

7.1 *In vitro* cytochrome P450 enzyme activity (microsome assays)

In vitro microsome assays using withaferin A as substrate were performed by mixing the following reagents in amber glass vials: microsomal protein (100 µg), 0.3 M potassium phosphate buffer (33.3 µl) for an overall 0.1 M in 100 µl total volume, withaferin A substrate (1 µg in 2 µl), milli-Q water (add. 100 µl) and NADPH regeneration system (Promega) (7.5 µl). This mix was incubated for 4 h at 30°C. A volume of 200 µl MeOH was added to stop the reaction. The mix was centrifuged for 5 min at 300 x g. To remove precipitated proteins, the supernatant was taken off and dried under an N_2 stream. The pellet was afterwards redissolved in 200 µl MeOH and centrifuged for 5 min at 300 x g. As negative controls, components of the NADPH system, withaferin A and microsomes were omitted in the assays. Measurements were performed in triplicate for each treatment.

For non-target analysis, ultra-high-performance LC-electrospray ionisation-high resolution mass spectrometry (UHPLC–ESI–HRMS) was performed with a Dionex Ultimate 3000 series UHPLC (Thermo Fisher Scientific™) and a Bruker timsToF mass spectrometer (Bruker Daltonik, Bremen, Germany). UHPLC was used applying a reversed-phase Zorbax Eclipse XDB-C18 column (100 mm × 2.1 mm, 1.8 µm, Agilent Technologies, Waldbronn, Germany) with a solvent system of 0.1% formic acid (A) and acetonitrile (B) at a flow rate of 0.3 ml/min. The elution profile was the following: 0 to 0.5 min, 5% B; 0.5 to 11.0 min, 5% to 60% B in A; 11.0 to 11.1 min, 60% to 100% B, 11.1 to 12.0 min, 100% B and 12.1 to 15.0 min 5% B. Electrospray ionisation (ESI) in negative/positive ionisation mode was used for the coupling of LC to MS. The mass spectrometer parameters were set as follows: capillary voltage 4.5 KV/3.5 KV, end plate offset of 500 V, nebuliser pressure 2.8 bar, nitrogen at 280°C at a flow rate of 8 l/min as drying gas. Acquisition was achieved at 12 Hz with a mass range from *m/z* 50 to 1500. At the beginning of each chromatographic analysis 10 µl of a sodium formate-isopropanol solution (10 mM solution of NaOH in 50/50 (v/v %) isopropanol/water containing 0.2% formic acid) was injected into the dead volume of the sample injection for re-calibration of the mass spectrometer using the expected cluster ion *m/z* values. Peak areas of extracted ion chromatograms (EIC) were extracted with QuantAnalysis (Bruker Daltonik).

7.2 Phenoloxidase activity assay in larval haemolymph

After seven days of feeding on artificial diet with and without withaferin A or *Physalis* withanolide extracts or on immature cottonseeds and *Physalis* fruits, 10 µl of haemolymph was collected from individual third instar larva. To ensure comparability, for each individual the third true leg on the right side was cut off with sterile scissors. The haemolymph was pipetted separately into 500 µl of ice-cold sodium cacodylate buffer (0.01 M Na-cacodylate, 0.005 M $CaCl_2$; Sigma-Aldrich), snap frozen in liquid N_2 and later stored at - 80°C. Haemolymph samples were thawed on ice and subsequently centrifuged for 15 min at 4°C and 2800 x g. The supernatant (100 µl) was transferred to a pre-chilled 96-well polystyrene plate (VW) with 200 µl of 3 mM L-Dopa (Sigma-Aldrich) per well. The absorbance was determined every minute at a wavelength of 490 nm for a total run time of 45 min at 30°C in a Tecan Life Sciences multiplate reader. The fastest change in absorbance (from 15 - 26 min [Vmax]) of the reaction was used, which is within the linear range of 5 - 45 min after adding the substrate. Details on the statistical analyses are described in the section 11.1.

8. Withanolide Metabolite Analysis

8.1 Isotopic labelling of *Physalis* plants

Stable ^{13}C-isotope labelling was performed in a growth chamber with a $^{13}CO_2$ atmosphere as previously described in Feistel *et al.* (2018). Three *P. peruviana* greenhouse plants were pruned before they were transferred to the chamber. After 75 days, plants were removed and newly grown leaves were harvested and snap frozen in liquid N_2. Normal plant growth was tested under the growth chamber conditions prior to the actual labelling procedure.

8.2 Extraction and isolation of (labelled) withanolides

A total of 5.83 g of untreated plant material was additionally processed to obtain unlabelled withanolide extracts for antimicrobial activity studies and feeding assays. Unlabeled leaf material was exhaustively extracted in MeOH, and afterwards subjected to PS/DVB SPE cartridges (Chromabond HR-X, Macherey & Nagel) according to company's instructions. The cartridges were eluted with MeOH and the collected flow-through was evaporated again using a rotory evaporator. Afterwards, the residue was diluted in Milli-Q water. The aqueous extract was then filtered again in through the SPE columns and eluted with Milli-Q water (fraction 1), 50% MeOH (fraction 2; withanolides & flavonoids) and 100% MeOH (fraction 3; main withanolide fraction). All three fractions were tested in inhibition zone (IHZ) assays.

The ^{13}C-labelled plant material was lyophilised resulting in 5.47 g of dried material. Leaves were further processed in a laboratory sample mill (IKA® M20 universal mill). The powder was transferred to a 500 ml Erlenmeyer flask and was extracted five times in 300 ml MeOH gently shaking on a table shaker. The methanolic crude extract was filtered through a Whatman® filter by decanting to separate insoluble matter. The flow-through was evaporated in a rotary evaporator. Afterwards, the residue was resuspended in 100 ml Milli-Q H_2O and treated 3 x with 100 ml n-hexane. This step was carried out to remove unwanted plant substances such as plant fats and waxes. The addition of n-hexane, however, resulted in a stable suspension. Therefore, the extract was resuspended in 100 ml Milli-Q H_2O and treated with ultrasound. The extract was afterwards transferred to Falcon tubes and centrifuged for 2 min at 12000 x g. This step was repeated three times. The resulting supernatant (500 ml) was loaded onto a (20 x 5 cm) column filled with MCI GEL polymer resin (CP20P Supelco®) for chromatographic separation.

8.3 Feeding assays using ^{13}C-labelled 4β-hydroxywithanolide E

Per species, four individual *Chloridea* fourth instar larvae were fed with ^{13}C-labelled 4β-hydroxywithanolide E. For this purpose, a 24-well plate containing GPL artificial diet was prepared as described in section 2.3. Fourth instar larvae were used since smaller larvae did not feed sufficient amounts before dessication of the treated diet and produced less frass. Isolated ^{13}C-labelled 4β-hydroxywithanolide E (1.5 mg) was redissolved in 1 ml 60% MeOH and 125 µl were transferred onto the diet of each well and evaporated for 4 h. Each well contained ~ 190 µg of labelled compound. Larvae were allowed to feed for 48 h. The frass was pooled per species and afterwards lyophilised.

8.4 Extraction of ^{13}C-labelled 4β-hydroxywithanolide E from frass

Freeze-dried frass samples were extracted three times with MeOH (5 ml) and were afterwards homogenised in a Minilys® cell disruptor (Bertin Technologies SAS) using 1.4 mm ZrO_2 beads. For qualitative analysis, methanolic extracts were analysed LC-MS/MS and NMR.

8.5 Mass spectrometric analysis

The characterisation of ^{13}C-labelled 4β-hydroxywithanolide E after the ingestion by *Cs* and *Cv* was performed by means of HPLC-MS analysis. UHPLC-HR-ESI-MS analyses were performed on an Agilent 1260 UPLC system, consisting of a combined degasser/quaternary pump G1311B, an autosampler G1367E, a column oven G1316A and a photodiode array detector G1315D (Agilent Technologies GmbH, Waldbronn, Germany) connected to a Bruker Compact QToF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Mass spectra were obtained by ESI in both positive (ESI^+) and

negative (ESI^-) ionisation detection modes using a mass range of *m/z* 50 to *m/z* 1300. Standard parameters for small molecule analysis were used as implemented in Bruker Compass ver.1.9. HPLC separations were accomplished using an Agilent 1100 HPLC system, consisting of a degasser G1322A, a binary pump G1312A, an autosampler G1313A and a photodiode array detector G1315B (Agilent Technologies GmbH, Waldbronn, Germany). The column outlet was connected to an Advantec CHF122SB fraction collector (Advantec Toyo Kaisha Ltd., Tokyo, Japan) triggered by the HPLC via a relay contact board. All HPLC separations were carried out using Macherey-Nagel columns (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Medium pressure chromatographic (MPLC) separations were accomplished using a Biotage Isolera One chromatograph (Biotage Sweden AB, Uppsala, Sweden) using linear gradient elution on a 20 x 5 cm MCI GEL column (CP20P Supelco®; solvents: H_2O + 0.1% FA and MeCN + 0.1%FA).

8.6 NMR spectroscopy analysis

The presence of ^{13}C-labelled 4β-hydroxywithanolide E in *Physalis* leaf extracts was confirmed before feeding them to *Cs* and *Cv* larvae. In addition, ^{13}C-labelled breakdown products detected in pooled frass samples (*Cv* and *Cs* frass combined) were fractionated and subjected to NMR analysis for chemical structure elucidation. NMR spectra were recorded on a Bruker Avance III HD 700 MHz spectrometer, equipped with a cryoplatform and a 1.7 mm TCI microcryoprobe or on a Bruker Avance III HD 500 MHz NMR spectrometer equipped with a cryoplatform and a 5 mm TCI cryoprobe (Bruker Biospin GmbH, Rheinstetten, Germany). NMR spectra were recorded at 298 K with MeOH-d_3 as solvent. Chemical shifts were referenced to the residual solvent peaks at δ_H 3.31 and δ_C 49.15. Data analysis was performed using the standard pulse programs implemented in the Bruker TopSpin software ver.3.6.1.

9. Microbial Methods

9.1 Culturing of bacterial and fungal strains

Except for *Enterococcus mundtii*, which was cultivated in Todd Hewitt broth (THB; VWR), all bacterial strains were grown at their optimal temperature range in either liquid lysogeny broth (LB; Roth®) or on LB agar plates. *Escherichia coli* (DH5α), *Bacillus subtilis, Bacillus thuringiensis* sv. *kurstaki* strain (HD73), *E. mundtii* and *Agrobacterium tumefaciens* were obtained from stocks at the Max Planck Institute for Chemical Ecology, Jena. Plant pathogenic bacteria *Ralstonia solanacearum* (DSM9544), *Rhodococcus fascians* (ST107849) and *Clavibacter michiganensis* subsp. *michiganensis* (ST35867) were obtained from the Jena Microbial Resource Collection (JMRC). *Xanthomonas campestris* pv. *campestris*, *Xanthomonas campestris* pv. *horticola, Pantoea ananatis* (CV2093) and

Pseudomonas syringae (g59) were kindly provided by the research group "Microbial Communication" at the Friedrich Schiller University, Jena. For culturing *B. thuringiensis* (Bt), and for *Bt* spore production, a method published in Milutinović *et al.* (2013) was followed. The three fungal species *Fusarium brachygibbosum*, *Fusarium oxysporum* f. sp. *lycopersici* and *Alternaria* spp. were reared on potato dextrose agar (PDA; Merck) at 28°C. The "Molecular Plant Pathology" group at the University of Amsterdam kindly provided samples of *F. oxysporum* f. sp. *lycopersici*.

9.2 Inhibition zone assays

To ensure comparability with previous work, IHZ assays were performed as described in Barthel *et al.* (2016). In brief, bacterial strains were plated on agar plates and grown overnight, single colonies were collected and grown in liquid cultures to an optical density (OD) = 1 ± 0.1 at a wavelength of 600 nm (BioPhotometer, Eppendorf). Fungal spores were harvested from PDA plates and washed twice in sterile water. After centrifugation, the pellet was dissolved and filtered through a filter membrane to remove remaining agar chunks. Bacterial and fungal spores were counted in a Thoma counting chamber. For bacterial IHZ assays, 500 µl of overnight cultures or 25 µl of *Bt* spores (≙ 4 x 10^9 CFU) were added to 500 ml liquid LB agar and 10 ml of the mix was subsequently poured into Petri dishes. Holes were made with 5 ml pipette tips (⌀ = 2 mm) and 2 µl of test solution (20 µg/µl) was added. In addition to withanolide extracts, the antimicrobial activity of crude leaf homogenates was assessed. Crude leaf homogenates were obtained by grinding fresh *Physalis* leaf material in an Eppendorf tube with plastic mortar and pestle. Fungal spores (≙ 1.4 x 10^6 CFU) were mixed with liquid PDA [+ Rifampicin (GERBU)] and poured into Petri dishes (10 ml per plate). Holes were punctured with a sterile glass (⌀ = 7 mm). To determine the susceptibility, inhibition zones were correlated to known standard antibiotics [gentamicin (Fluka), erythromycin (Merck), amphotericin B (MP) and chloramphenicol (Serva)] as positive controls. A potential microbial susceptibility against 40% MeOH (solvent for withanolide test compounds) was eliminated beforehand by testing it against all microbes. Plates were incubated until CFUs were clearly visible (usually after 24 h). Normal microbial growth was monitored on control plates without any test solutions. All assays were repeated twice and were performed as triplicates to ensure reproducibility.

9.3 Effective dose estimation

In order to assess the median effective dose (ED_{50}) of *Physalis* withanolide extracts and withaferin A against *B. thuringiensis* sv. *kurstaki* strain HD73 96-well microplate assays were performed. Cells were plated on LB agar and grown at 30°C. Afterwards, single colonies were picked and grown overnight in 5 ml LB at 30°C and 250 rpm. The next day, 50 µl of overnight cultures were used to

inoculate 5 ml LB and were grown for another 1 - 3 h to an OD_{600} = 0.003 ± 0.002. A 1:2 dilution series of 80 µg (20 µl of a 4 mg/ml stock solution) of gentamicin (positive control), withaferin A and the *Physalis* withanolide extracts was pipetted into the wells and mixed with 20 µl LB. 100 µl of bacterial culture was added into each well except for the blanks. Normal bacterial growth was determined by measuring 100 µl bacterial culture in 40 µl LB. For each treatment, duplicate samples were run in a Tecan Life Sciences multiplate reader; the absorbance was determined at 490 nm every minute for a total run time of 24 h at 30°C. A fitted dose response curve (DRC) model was generated in R using the drc package (Ritz *et al.*, 2016).

10. Metagenomic Analysis of the Larval Gut Microbiome

10.1 Extraction of larval guts

A total of 15 third instar *Cs* and *Cv* larvae (n = 45 per species) were reared on artficial diet supplemented with either 100 µg/ml *Physalis* withanolide extracts or with 100 µg/ml withaferin A. The control group was allowed to feed on artificial diet treated with only the solvent (40% MeOH). Feeding assays were otherwise performed as described in section 2.3. After 48 h, individual larvae were placed in 15 ml Falcon tubes on ice in order to sedate them for dissection. Each larva was surface-sterilised to remove potential bacteria from the cuticle: larvae were washed twice (10 - 15 s) each in 1% (w/v) SDS solution, sterile water, 90% EtOH and finally again in sterile water. Dissection tools were sterilised with bleach. Larvae with disrupted gut tissues or with flattened guts, indicating that not much food was consumed, were replaced by back-up larvae from the same treatment group. Dissected guts were snap frozen in liquid N_2 and stored at - 80°C until DNA extraction. Diet chunks that were collected from each treatment served as background control.

10.2 Extraction and purification of genomic gut bacterial DNA

Genomic DNA was extracted via the CTAB/chloroform method. Gut tissues were processed for 5 min (30 Hz) in a TissueLyser LT (Qiagen) in 500 µl TES Buffer (VWR) and 4 µl (100 mg/ml) lysozyme (Sigma-Aldrich). Samples were incubated for 30 min at 37°C. To remove remaining proteins, 2.5 µl of proteinase K (20 mg/ml by Thermo Fisher Scientific™) was added to each sample and incubated for 4 h at 56°C. To adjust the salt concentration to 1.4 M, 170 µl of 5 M sodium chloride (NaCl) was added. After adding 80 µl of 10% CTAB, the mix was incubated at 65°C for 10 min. An additional 750 µl of (24:1) chloroform-isoamyl alcohol (Sigma-Aldrich) was added and samples were incubated on ice for 30 min by gently shaking. After centrifugation for 10 min at 16000 x g (4°C), the supernatant was transferred into a new tube. One volume of pre-cooled 100% isopropanol (Sigma-Aldrich) was added and samples were centrifuged again for 10 min at 16000 g at 4°C. The

supernatant was discarded by decanting. Pellets were washed with 500 µl of ice-cold 70% EtOH (Roth®) and centrifuged again as described above. The EtOH was discarded and pellets were allowed to dry at RT. Finally, pelletd were dissolved in 90 µl distilled H_2O. A blank was run to control for potential contaminations during the extraction procedure.

10.3 Sequencing of genomic gut bacterial DNA

From each sample, genomic DNA was pooled to a final concentration of 22.5 ng/µl. For sequencing and library preparation of the bacterial 16S rRNA (variable region V4) pools of 20 µl were shipped to MR DNA (Shallowater, Texas, USA). Sequencing primers (see **Table 1**) were used based on Caporaso *et al.* (2012). Sequencing was performed on an Illumina MiSeq platform using the 2 x 300 bp paired-end sequencing technology. Sequences were processed using the MR DNA pipeline (www.mrdnalab.com, MR DNA, Shallowater, Texas, USA) and afterwards deposited on BASESPACE (basespace.illumina.com).

10.4 Processing of DNA libraries via QIIME2

DNA sequences were recovered from BASESPACE and the libraries in QIIME2 (2018.6). Prior to analysis, sequences were trimmed at 249 and 250 bp to remove short sequences. Additionally, sequences were demultiplexed via the default cut-off (quality score 2). Non-bacterial sequences (mostly insect DNA and chloroplasts) as well as unassigned reads were removed from the data. During the genomic DNA extraction procedure, an additional blank extraction was conducted and the blank was sequenced together with the other samples. Sequences with a proportion higher in the blank than in any other gut sample was removed from analysis in order to eliminate potential contaminants. In this analysis, sequences were not clustered into OTUs, but instead compared to each other by using the DADA2 sample inference method (version 1.9.1) based on Callahan *et al.* (2017); Callahan *et al.* (2019). The sequence taxonomy was determined by using the Greengenes 16S rRNA gene 21 database (version 13.8) using a Naïve Bayes classifier (DeSantis *et al.*, 2006). Details on the statistical analyses of the datasets are described in the **section 11.4**.

11. Statistical Analyses

All statistical analyses were performed using the statistical software R version 4.1.1 (R Core Team, 2021). If not provided in the corresponding Materials and Methods sections, detailed information on the statistical methods is listed below.

11.1 Relative larval weight gain and PO activity

To test for the effect of withanolide supplementation on the RWG of the larvae as well as on the PO activity of the larval haemolymph, a generalised least squares method (GLS, implemented in the nlme library (Pinheiro and Bates, 2022)) was used to account for the heterogeneity of the data. The varIdent variance structure was applied to allow different spread for each treatment group. *P*-values were obtained by removing non-significant variables and comparison of models with a likelihood ratio test. To identify treatments that are different to each other, factor level reduction was applied or data were analysed by a *post hoc* multiple comparison of estimated means using Tukey contrasts.

11.2 Larval development

To test for the effect of the diet treatment on the proportion of successfull pupations and number of moulting events until pupation, generalised linear mixed effects (GLM) models were applied. In two separate models the status of the insect (pupation/dead) or the number of moulting steps (once/twice) were treated as response variable. The influence of the proposed explanatory variables (species and treatment) was obtained by model simplification using a likelihood ratio test (Zuur *et al.*, 2009). In addition, χ^2-tests were performed on individual species level to investigate if the diet treatment affected the number of moulting steps and the proportion of successful pupations. *P*-values were corrected for multiple comparisons using the Bonferroni correction.

11.3 Choice assays

Larvae that died and/or did not choose any diet during the course of the experiment were not included in the statistical analyses. In order to test whether the subset of larvae that made a choice depends on the time point and/or the species, GLMs (glmer with the binomial error structure in the lme4 package (Bates *et al.*, 2015)) were applied. The different time points and the species were treated as fixed factors whereas the larval identity was used as random intercept. The influence of fixed effects was obtained by model simplification using a likelihood ratio test (Zuur *et al.*, 2009). Factor level reduction was performed in order to reveal differences between time points, species or time point/species combinations (Crawley, 2013). In order to test whether larvae of the different species choose their food in the same way, and whether certain diet options were preferred to others, χ^2-tests were performed. In case of significant differences, additional single comparisons χ^2-tests were executed. In these cases, *p*-values were corrected for multiple comparisons using the Bonferroni correction.

11.4 Hole-boring efficiency and larval distribution of fruits

In order to test if the number of holes bored depended on species and treatment, GLMs with the poisson error structure were applied. Significant differences between species/treatments were determined by *post hoc* multiple comparison of estimated means using Tukey contrasts (emmeans from the emmeans library; Lenth (2022)). In order to analyse if the presence of the *Physalis* fruit coating affected the distribution of the larvae on the fruit, χ^2-tests were run. Dead larvae (including larvae that died through cannibalism or that were caught in the fruit coating) and larvae that were neither sitting on the fruit nor on the fruit calyx (off-fruit) were excluded from this statistical analysis.

11.5 Analysis of the gut microbial α-diversity/richness

Per gut sample pool (n = 5), the Shannon-Wiener index (diversity index) was used as a quantitative measure to estimate the taxonomic diversity at the family level for a given diet treatment. The α-diversity was calculated per treatment as the mean of the Shannon-Wiener index. Family richness of each pool was calculated as the number of detected bacterial families per diet treatment (n = 5). The mean of richness calculated for each gut pool was used as the observed richness. Afterwards, the differences in α-diversity and richness between *Cv* and *Cs* gut communities were statistically analysed using a Wilcoxon rank sum test. To test the normality assumption, a Shapiro-Wilk test was run on the residuals of a between-subjects full-factorial ANOVA model. Secondly, a Kruskal-Wallis test was performed to assess whether the species' α-diversity and richness were affected by the diet treatments.

CHAPTER I

Chapter I: Larval Performance and Behaviour on *Physalis* Fruits

Background

Many studies neglect the possibility that secondary defensive metabolites play a role in adaptation processes to *Physalis* plants and instead focus on the morphological defence of the plants. Physiological traits that have been reported to be negatively affected by withanolide extracts in non-adapted insects are growth rate, survival and development (reviewed in Sell *et al.*, 2021). Counter-adaptations to withanolide-producing plants may also incur costs, as there is a trade-off between decreased fitness and the expansion of ecological niches with e.g. reduced competition for resources or better protection from predators and parasites. By investing in efficient adaptation mechanisms, growth rate, developmental speed, reproductive success and longevity might be improved.

Whether C. *subflexa* larvae benefit from putative nutritive or regulatory effects of *Physalis* metabolites is as yet unknown. So far, Barthel *et al.* (2016) were the only ones to describe enhanced growth of the specialist when feeding on a withanolide-containing diet. The increased larval weight gain suggests that withanolides might have nutritional effects. The uptake of withanolides may influence metabolic processes, which in turn stimulate larval growth. On the other hand, withanolides may simply act as feeding stimulants, which cause larvae to consume more food and consequently gain more weight. However, *C. virescens* does not differ noticeably from *C. subflexa* in their willingness to initiate feeding on *P. angulata* and *P. peruviana* fruits (Laster *et al.*, 1982; Oppenheim *et al.*, 2012). Although *C. virescens* and *C. subflexa* were observed to feed equally on *P. angulata* fruits, only the larvae of *C. subflexa* were shown to increase body weight relative to the amount of food they consumed (Laster *et al*., 1982).

Based on the findings from Barthel *et al.* (2016), feeding assays were repeated to elucidate a reproducible benefit of withanolide extracts on *C. subflexa* larvae. Following up on these assays, long-term experiments were conducted on *C. subflexa* and *C. virescens* in order to assess the influence of *Physalis* fruit feeding as measured by the duration of larval development, the number of moulting steps and the percentage of successful pupation events. A separate experiment was performed to investigate the effect of consuming a diet of *Physalis* fruits on the survival and the weight gain of *C. virescens* and *C. subflexa* larvae (+ hybrids) and of larvae from four non-adapted moth species. Choice and oviposition assays were implemented in order to test for specific patterns of natural avoidance behaviour/attraction exhibited by the two *Chloridea* species.

Results

1. Feeding studies with early third instar *Chloridea* larvae

1.1 Artificial diet feeding studies

To verify the above-described positive effect of dietary withanolides on the growth of *C. subflexa*, early third instar larvae were reared on artificial diet supplemented with *Physalis* withanolide extracts or withaferin A as a novel test treatment. Larvae of the control group received artificial diet treated only with the solvent (40% MeOH). The extraction of *Physalis*-derived withanolides is time consuming and does not yield high amounts of the desired substances. It was therefore aimed to replace less pure *P. peruviana* extracts (hereafter referred to as "*Physalis* extracts" or "withanolides [P]") with withaferin A in later experiments. Prior to running experiments, preliminary tests were conducted to validate if withaferin A causes comparable effects on *C. virescens* and *C. subflexa* larvae. In contrast to previous findings in Barthel *et al.* (2016), no significant effects (ns) on relative weight gain were observed in larvae that feed on either withanolide treatment (**Figure 10**). In addition to common box plots, violin plots were generated to visualise the probability density and to identify potentially interesting variance structures in the datasets. The variable distribution was very similar in all three treatments. Feeding assays with *C. subflexa* and *C. virescens* larvae were run using higher concentrations of *Physalis* extracts (150 - 200 µg/ml), but did not reveal any statistically significant differences in the weight gain (Suppl. **Figure 47**).

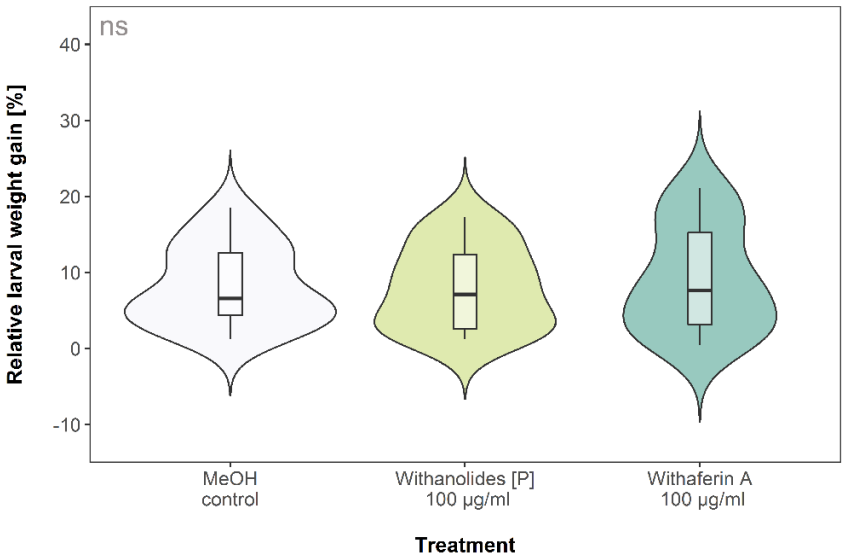

Figure 10: Relative larval weight gain of third instar *C. subflexa* larvae after feeding for seven days on control diet or on artificial diet supplemented with either 100 µg/ml withaferin A or 100 µg/ml *Physalis* withanolide extracts. NS = no statistically significant influence (GLS, $p > 0.05$; n = 21 - 24).

In addition, feeding assays were repeated including third and second instars of *C. virescens* larvae (Figure 11). Second instar *C. virescens* were included as it was discovered that the average starting weight of both species differed greatly (*Cv:* Ø = 9.3 mg/*Cs:* Ø = 27.5 mg) in the experiments of Barthel *et al.* (2016). This discrepancy suggests that different instars were used for each species in these experiments. Under the same experimental conditions, no significant effects were observed in *C. virescens* larvae across all three treatments. Likewise, the weight gain did not differ significantly between *C. subflexa* and *C. virescens* larvae. The violin plots displayed a generally higher variance in the growth rate of second instar *C. virescens* larvae compared to third instars. Though differences across treatment groups were found to be non-significant within species and within the same instar, the larval instar itself significantly affected the overall weight gain. However, expected intraspecific differences in weight gain between second and third instars are likely due to the continuous feeding of second instar larvae, while the third instar reduced feeding earlier (upon reaching the pre-pupal stage). In this experimental setting, early third instar larvae with an equal average weight around Ø = 20 - 25 mg were used. In contrast to Barthel *et al.* (2016), weight gain was not found to vary significantly between the species when they started with a similar average weight. This result underlines the importance of thoroughly synchronising developmental stages and starting weights when comparing different species. To rule out the possibility that withanolide extracts might have been inactive, new withanolide extracts were freshly isolated from *P. peruviana* plants and additionally tested in feeding assays (Suppl. Figure 48). *Physalis* extracts and withaferin A were frequently tested in inhibition zone assays against *Bt* spores to verify biological activity. Both fresh and older extracts demonstrated the expected biological activity even after up to six years of storage.

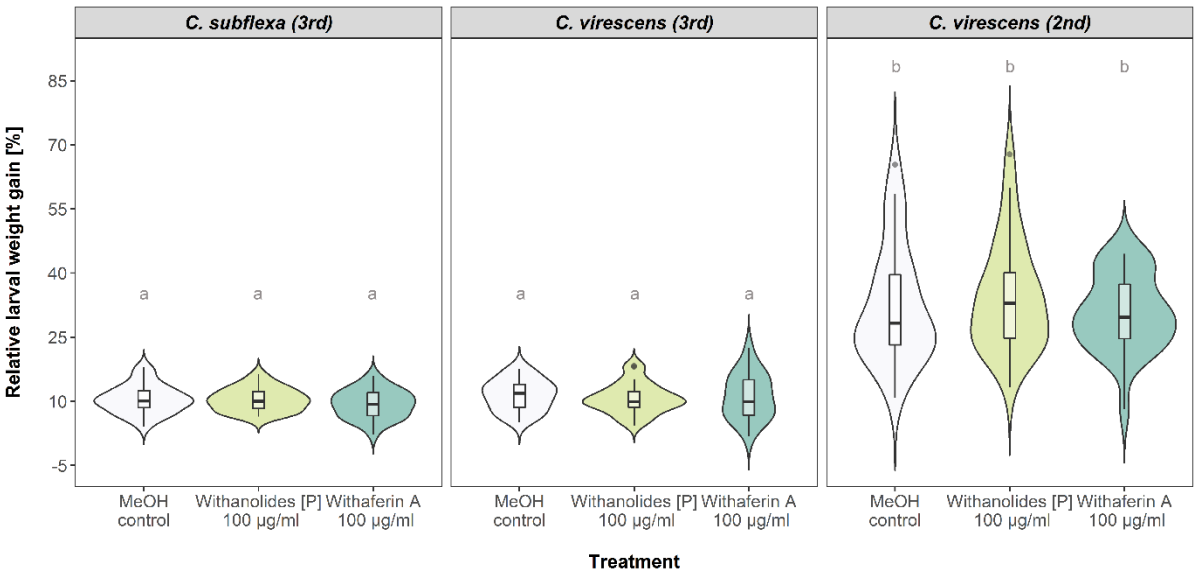

Figure 11: Relative larval weight gain in third instar *C. subflexa* larvae and two different instars of *C. virescens* fed on a control artificial diet or on diet containing either 100 µg/ml withaferin A or *Physalis* extracts. Larvae were exposed to the treatments for seven days. Statistically significant differences between larval instars are indicated by letters using a *post hoc* multiple comparison of estimated means using Tukey contrasts (GLS, $p < 0.001$; n = 21 - 30).

In order to test whether the effects of withanolides on the weight gain of the larvae might be covered by rich artificial diet, another feeding experiment was conducted on diet with reduced nutrient content (Suppl. **Figure 49**). Though growth rate decreased in larvae raised on a low-nutrient diet, the addition of withanolides did not significantly affect the two species or the hybrids. Further details on the feeding experiments are provided in the Supplementary Data (from **page 139** onwards).

1.2 Feeding assays using *P. peruviana* fruits and *G. hirsutum* seeds

The relative weight gain of C*. virescens*, *C. subflexa* and their hybrids was assessed in larvae fed on *P. peruviana* fruits (for simplicity, hereafter referred to as *Physalis* fruits) and on cottonseeds, a natural host plant of *C. virescens*. Artificial diet served as a control for normal growth and survival of the experimental populations. Although switching to fruits prevents us from linking withanolides with the experimental outcome, any difference in the performance between species points to potentially divergent strategies in host-plant utilisation. Furthermore, withanolides alone might not be the only explanation of effects (positive or negative) of *Physalis* plant consumption on *C. subflexa*. Plant defence compounds may work synergistically or together with other plant substances. The diet treatment and the species had a statistically significant influence on the relative weight gain of larvae (**Figure 12**). Although there was a slight trend in *C. subflexa* (lower weight gain on cotton) and *C. virescens* (lower weight gain on *Physalis* fruits) larvae, differences in the weight gain between the treatments were not significant. All test organisms gained less weight on natural diet than on rich artificial diet. Violin plots illustrate the notably high variance in all species of larvae that fed on the artificial diet treatment. In contrast, the shape of the violin plots was similar across fruit and seed treatments. Interestingly, the data show more weight gain in hybrid larvae compared to those of *C. virescens* when fed on natural diets, but weight gain of *C. subflexa* larvae that fed on the *Physalis* fruits was unchanged. Cottonseeds were offered as an additional food source to study further the general development of larvae on non-artificial food. Most remarkably, the strict specialist *C. subflexa* was able to feed and thrive on cottonseeds if no other food source was available. Although *C. virescens* larvae did not gain significantly less weight when fed on *Physalis* fruits than on cottonseeds, other occasional indications of physiological stress were observed. In the *Physalis* group, several *C. virescens* larvae had difficulty shedding their cuticles when moulting into the fourth instar. In addition, some *C. virescens* larvae seemed to perform an unusual additional moulting step. No such moulting disorders and supernumerary moulting were observed in any treatment group of hybrid and *C. subflexa* larvae.

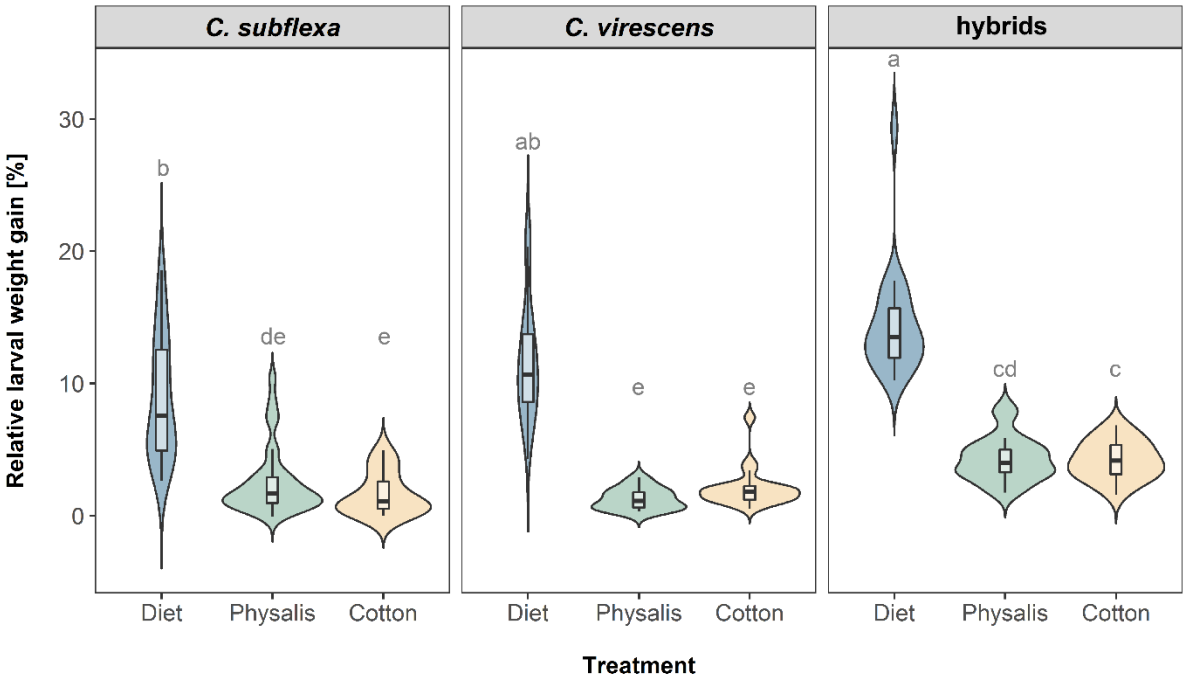

Figure 12: Relative larval weight gain of *C. subflexa*, *C. virescens* and hybrids. Third instar larvae were allowed to feed on *Physalis* fruits, cottonseeds and artificial diet for seven days. Letters indicate significant differences between treatment/species combinations by a *post hoc* multiple comparison of estimated means using Tukey contrasts (GLS, $p < 0.001$; n = 19 - 35).

1.3 Larval development on *Physalis* fruits

As feeding assays on *Physalis* fruits did not show a clear difference between the weight gain of the specialist and that of its non-adapted sibling species, the long-term larval development on *Physalis* fruits was studied. Early third instar larvae were reared individually in plastic cups until the last larva pupated. Though larvae fed on artificial diet do not represent an ideal control, they indicate generally normal development and may point to unusual mortality caused e.g. by infections.

Fed on a *Physalis* diet, 75% of the *C. subflexa* larvae pupated successfully, while 88% completed pupation when fed on artificial diet. Only 52% of *C. virescens* larvae survived the experiment on *Physalis* diet compared to 95% in the control group. Hybrids exhibited a pupation rate of 63% when fed on *Physalis* fruits and 95% when fed on artificial diet (**Figure 13**). Treatment and species both had a statistically significant influence on the pupation rate. Compared to conspecifics fed on artificial diet, *C. virescens* larvae fed on *Physalis* fruits had a significantly lower pupation rate; but the rate for *C. subflexa* larvae did not differ. In addition, how long individual larvae took to pupate on both diets was monitored (**Figure 14**). While most pupations occurred after 9 - 11 days of being fed on artificial diet, the pupal stage was reached later by *Physalis* fruit feeders: all *C. subflexa* larvae managed to pupate within less than 16 days (Ø = 12 days). In contrast, in *C. virescens* and hybrid larvae feeding on the fruits, pupation occurred after an average of 18 days (hy) and 21 days (*Cv*). The datasets were

split into larvae that pupated (**Figure 15**) and dead individuals (**Figure 16**) to assess the number of moulting steps required until pupation or until death. Ecdysis took place only once before pupation in all test species fed on artificial diet (moulting from third into fourth instars). In larvae fed on *Physalis* fruits, 31% of *C. virescens* larvae moulted twice before they pupated (**Figure 15**). Third instar larvae moulted from third to fourth stages, and some moulted to an additional fith instar stage. The proportion of moulting events was significantly different between the two treatment groups of *C. virescens* larvae. Generally, very few larvae died when fed on artificial diet, and the ones that died moulted only once (**Figure 16**). No obvious moulting disorders were detected in dead larvae fed on artificial diet, indicating that the larvae probably died for reasons other than developmental difficulties. Among larvae fed on on *Physalis* fruits that did not survive the entire experimental time, two or three moulting steps were likely. Before they died, a remarkably large proportion of *C. virescens* larvae (75%) had completed ecdysis more than once. Interestingly, no triple moulting steps occurred in *C. virescens* larvae that managed to pupate successfully. Due to the low sample sizes among dead larvae (n < 6), no statistical analysis could be performed to compare differences in the number of moulting steps between the diets. Overall, results show a significantly higher number of supernumerary moulting steps and delayed onset of pupation in *C. virescens* and hybrid larvae fed on *Physalis* fruits.

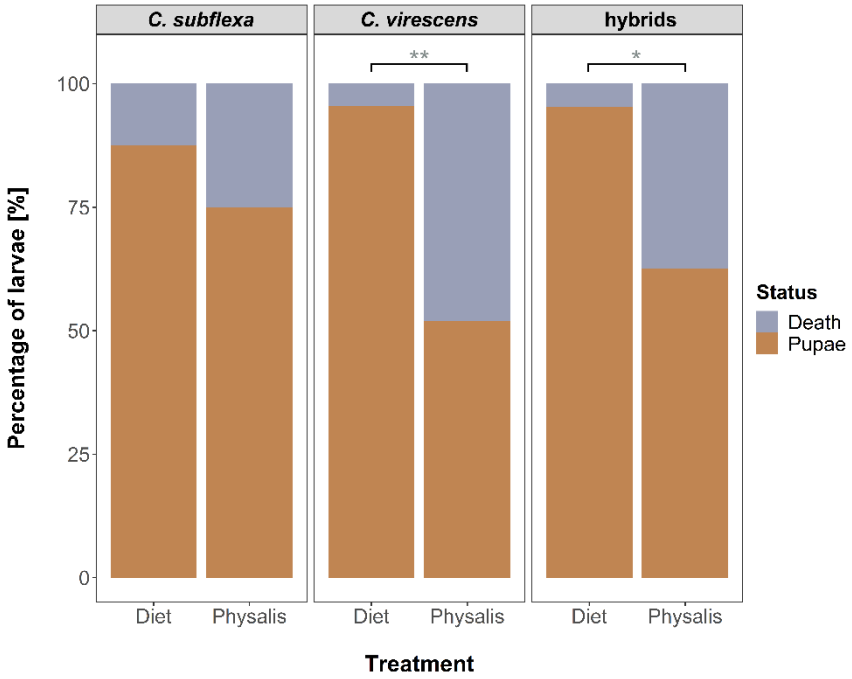

Figure 13: Analysis of the pupation rate in *C. subflexa*, *C. virescens* and hybrid third instar larvae fed on artificial diet or *P. peruviana* fruits. The colour code on the right shows the status of the larvae. Significant differences are indicated by asterisks ($p < 0.01$** and $p < 0.05$*; n = 21 - 24).

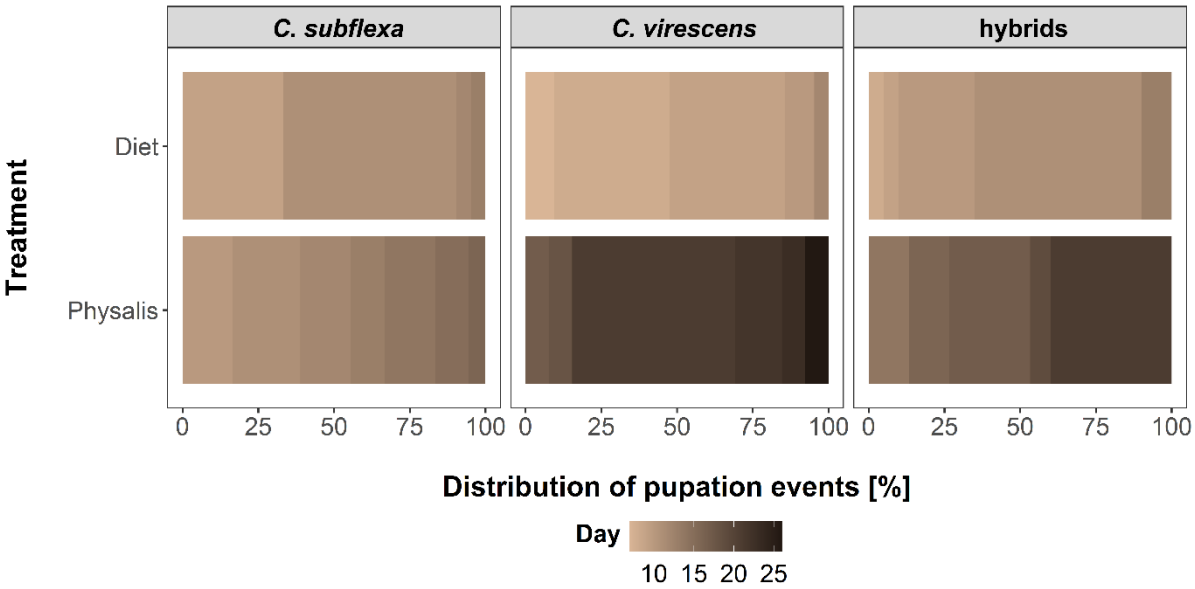

Figure 14: Distribution of pupation events (in days) over the experimental time. Larvae of *C. subflexa*, *C. virescens* and hybrids were fed on artificial diet or *P. peruviana* fruits. The colour code on the right represents the time frame within which pupation occurred (n = 20 - 21).

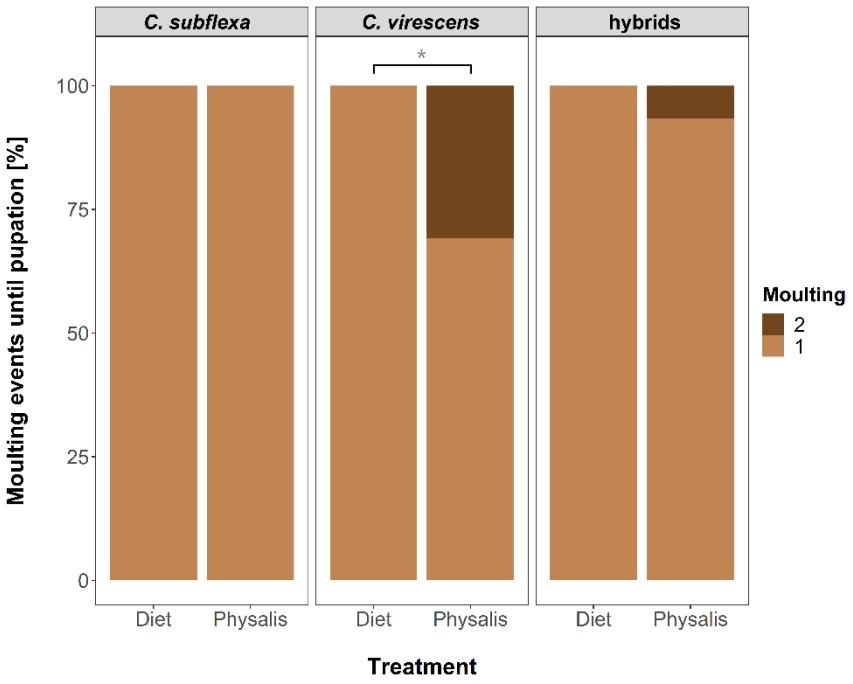

Figure 15: Analysis of the proportion of moulting steps until pupation in *C. subflexa*, *C. virescens* and hybrid larvae fed on artificial diet or *P. peruviana* fruits. Significant differences are indicated by an asterisk ($p < 0.05$*; n = 13 - 21). The number of moulting events corresponds to the colour code on the right.

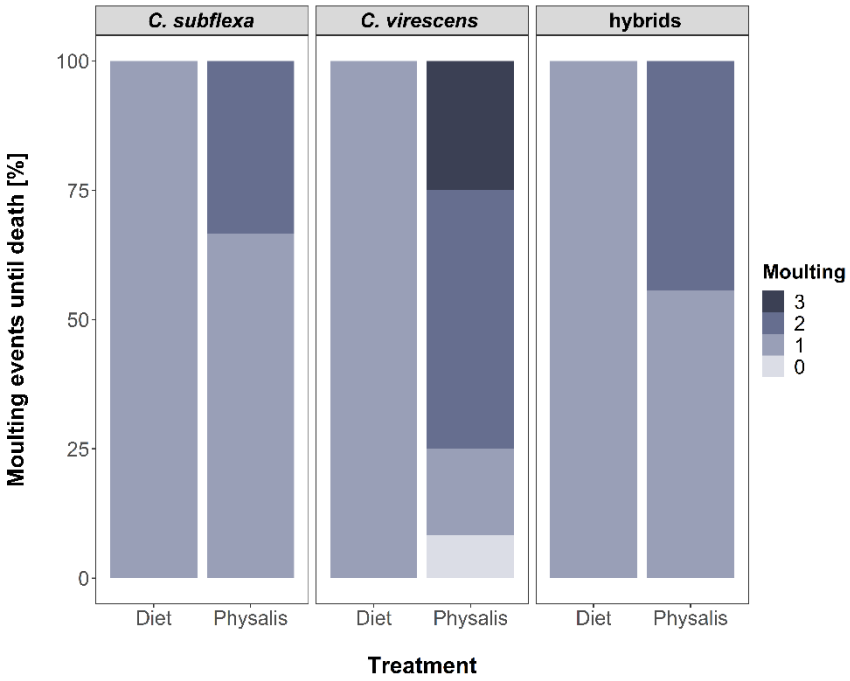

Figure 16: Analysis of the proportion of moulting steps in dead *C. subflexa*, *C. virescens* and hybrid larvae that were fed on artificial diet or *Physalis* fruits (n = 1 - 6). The number of moulting events corresponds to the colour code on the right.

2. Survival analysis on *Physalis* fruits and on artificial diet

The survival rates of both *Chloridea* species and the hybrids were analysed in this assay. To broaden understanding of the likelihood larvae can survive on *Physalis* fruits, *H. armigera* and *H. zea* (Heliothinae) and two other noctuid moth species (*S. frugiperda* and *S. littoralis*) were included in the experiment. Synchronised third instar larvae were fed for ten days on artificial diet (**Figure 17**) or *Physalis* fruits (**Figure 18**). Few or no numbers of dead individuals were counted in the control group across all test species and all minor differences were found to be non-significant. Therefore, it was assumed that all species were healthy and had developed normally; differences in survival rates, therefore, were most likely caused by the different diet treatments. However, the probability of survival differed significantly between species feeding on *Physalis* fruits. *C. subflexa* larvae displayed the highest likelihood of survival on a diet of *Physalis* fruits: their mortality rate did not surpass 4%. This rate did not differ significantly from that of larvae of *C. virescens* and the hybrids. In contrast, *H. armigera* and *S. frugiperda* larvae experienced high mortality rates (46% and 42%) when fed on *Physalis* fruits. The likelihood of survival was lowest for non-heliothine *S. littoralis* larvae (mortality rate of 81%). Interestingly, the monophyletic *Chloridea*-group (+ hybrids) showed a significantly higher survival rate compared to the two other heliothine species, *H. zea* and *H. armigera,* and the *Spodoptera* species. Interestingly, the more distantly related the species were from the *Chloridea* genus, the worse their survival rate when fed on the fruits.

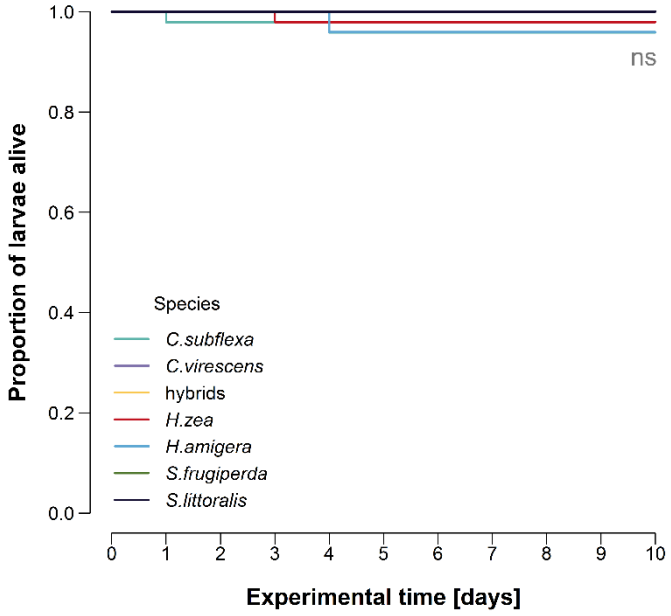

Figure 17: Kaplan-Meier survival plot of third instar Noctuidea larvae and the *Chloridea* hybrids fed on artificial diet. NS = no stastistically significant differences (Cox regression survival analysis).

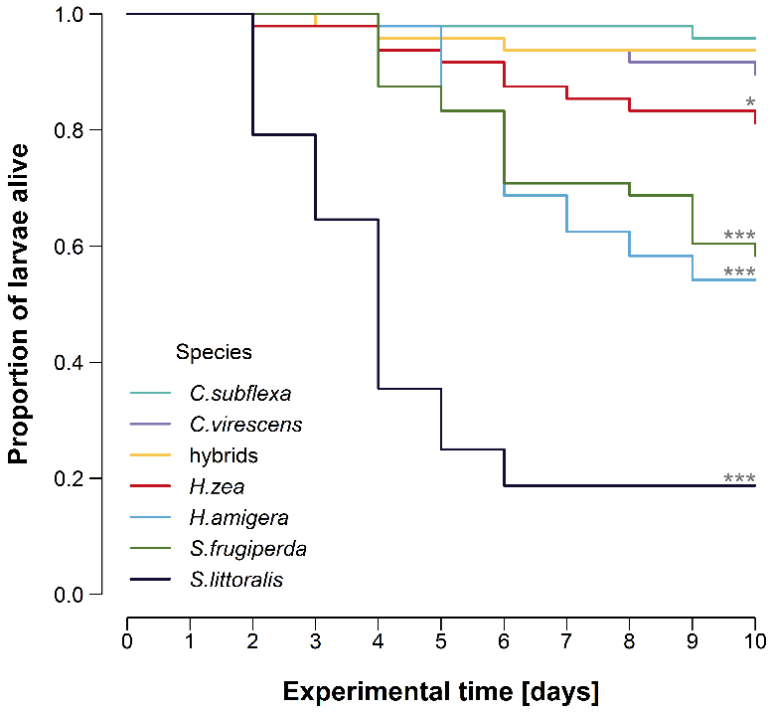

Figure 18: Kaplan-Meier survival plot of third instar Noctuidea larvae and the *Chloridea* hybrids fed on *Physalis* fruits. Significant differences in the survival probability determined by a Cox regression survival analysis are indicated by asterisks ($p < 0.001$***, $p < 0.05$*).

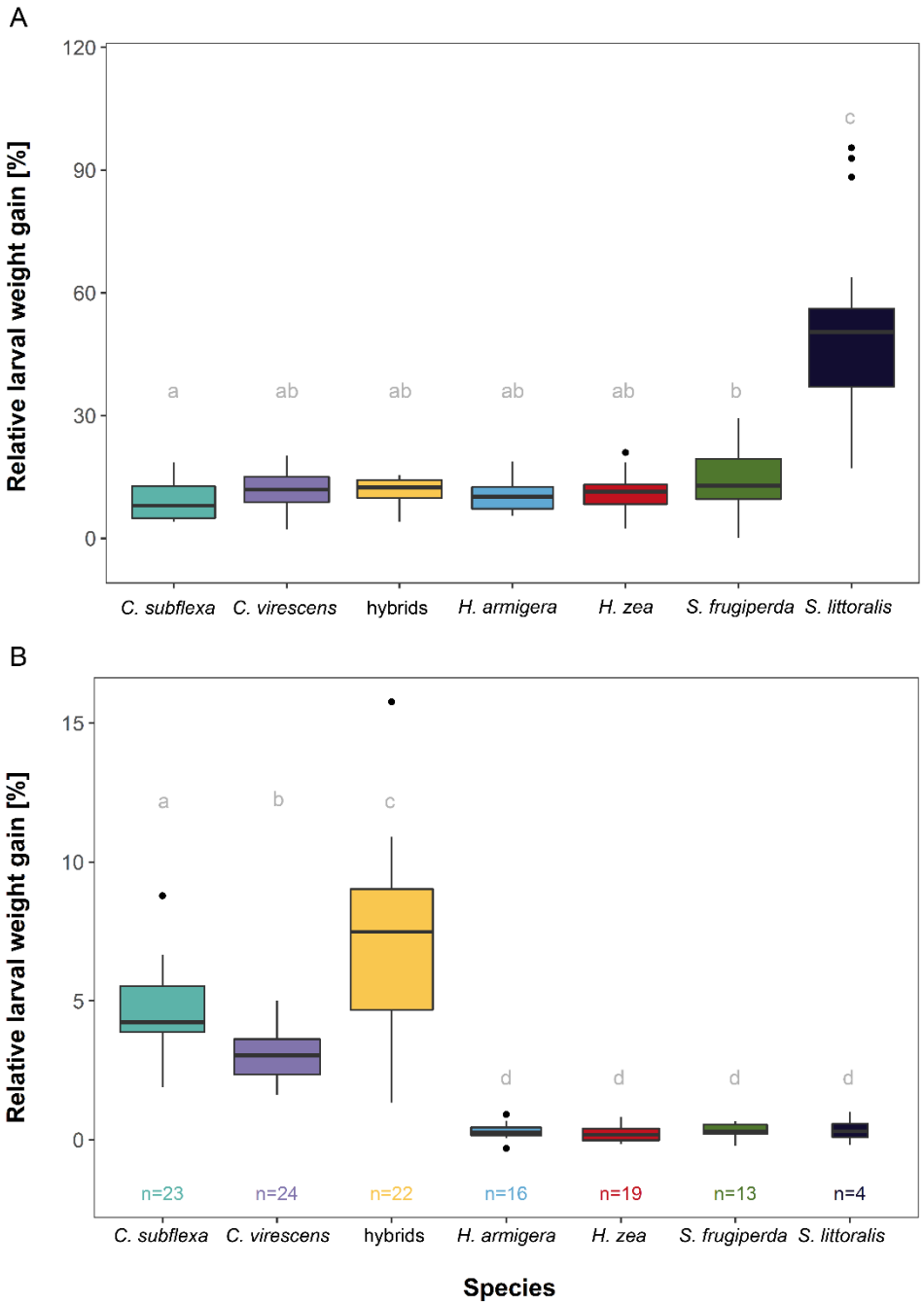

Figure 19: Relative larval weight gain of Noctuidea larvae after seven days of feeding on **A)** artificial diet (n = 24) and **B)** *Physalis* fruits. The box width corresponds to the sample size (n). Letters indicate significant differences between species by a *post hoc* multiple comparison of estimated means using Tukey contrasts (GLS, $p < 0.001$).

Both *Helicoverpa* generalists were observed to consume moderate amounts of the fruits, whereas the *Spodoptera* species rejected them outright. Therefore, in addition to the survival status, relative weight gain was examined in a subset of larvae from each diet. The factor "species" had a statistically significant influence on the weight gain in both diet treatments. No differences in weight gain were found between larvae in both the *Chloridea* species and in *H. zea* on artificial diet (**Figure 19A**). The weight gain of larvae that fed on *Physalis* fruits also differed significantly across the six species and

the hybrids (Figure 19B). As not all larvae survived equally well, the number of weighted individuals (n) differed widely across the species. In line with the survival data and the observation that larvae seemed to feed normally, the weight gain of the *Chloridea* group (+ hybrids) that fed on *Physalis* fruits was significantly higher compared to that of all other species. There was no significant difference between the weight gain of *Spodoptera* and *Helicoverpa* species fed on *Physalis* fruits. Except for *H. armigera* larvae, the weight gain correlates well with the probability of survival. However, this experimental setup does not reveal the ultimate cause of larval death. Though consumption observations and weight gain data strongly suggest a correlation between survival and low food uptake, death could have also been caused by post-digestive effects.

3. Choice behaviour assays

Food selection by specialist herbivores may be governed by a high degree of sensitivity to (non-) host metabolites. As previous data show that a) *C. virescens* larvae survive equally well on a *Physalis* fruit diet and b) weight gain does not differ strongly between the species, it was aimed to assess if they differ in their food choice. In a set of choice assays, *Chloridea* larvae (+ hybrids) were confronted with either two or three diet options. Only larvae that made a diet choice (*Physalis* fruits, cottonseeds or artificial diet) were analysed. Non-choosing larvae were excluded from the statistical analyses but incorporated into the figures. In the first two experiments, naïve neonates were used right after hatching to avoid any imprinting of diets. As often less than 50% of the neonates had chosen after one hour, this time point was omitted in statistical analysis. Neonates were first tested in a ternary choice assay (Figure 20). The ratio of neonates that had chosen a diet within the timeframe of the experiment (24 h) was not significantly different between species. It was then examined if species chose their food differently. *C. subflexa* selected its food differently compared to *C. virescens* and hybrids. Further statistical analysis showed that *C. virescens* larvae and hybrids preferred cottonseeds and artificial diet to *Physalis* fruits at time point two, and cottonseeds over all other diets at the last time point. Interestingly, *C. subflexa* neonates did not prefer a particular food source at either time point. However, a large proportion of the larvae did not choose cottonseeds: 78% (after 12 h) and 85% (after 24 h).

In a second trial, neonates were tested in a binary choice assay (Figure 21). As the first assay already showed larvae preferred natural food sources over artificial diet, we asked how larvae would decide if only two natural diets were offered. The percentage of neonates that had chosen a diet within 24 hours changed over time, but was the same for all species. At timepoints one and two, all species had chosen differently. After 12 hours, *C. subflexa* and hybrid larvae showed a higher preference for *Physalis* fruits than for cottonseeds. *C. virescens* did not preferably select a diet by this time point. At

time point three, *C. subflexa*s' choice behaviour differed significantly from that of the hybrids and *C. virescens*. *C. subflexa* larvae clearly preferred *Physalis* fruits to cottonseeds, while larvae of the hybrids and *C. virescens* did not show a clear preference for either diet.

Freshly hatched neonates are fragile, and their transfer to plastic cup might have slowed their decision making. Hence, more robust second instar larvae were also tested in a ternary choice assay (Figure 22). A 27% higher proportion of them (compared to neonates) had chosen a diet after one hour. Statistical analyses showed that the ratio of non-choosing larvae to larvae that made a choice depended on the treatment and species at time points one and two. It was then asked if larvae preferred a particular diet at each time point. *C. subflexa* larvae significantly preferred *Physalis* fruits to the other two food sources at all time points. Larvae of both, *C. virescens* and hybrids showed no statistically significant preference for any particular diet at time point one. However, *C. virescens* larvae were found significantly less often on *Physalis* fruits than on cottonseed or artificial diet after 12 hours; and after 24 hours *C. virescens* larvae were detected less on *Physalis* fruits, but the larvae did not differ significantly in their choice between cottonseeds and artificial diet. The hybrids preferred *Physalis* fruits to cottonseeds after 12 hours, but not cottonseeds to artificial diet. Hybrid larvae preferred *Physalis* fruits to the other diets at time point three.

In summary, *C. subflexa* larvae fed on non-host food sources less often than *C. virescens* larvae. Usually less than 18% of all individuals changed their diet between time points. Interestingly, after first feeding on a food source outside of their host range, *C. subflexa* larvae were more likely to move to another diet than *C. virescens* or the hybrids. Although food choice differed between larvae of the two *Chloridea* species, hybrids did not display a clear pattern. Though second instar larvae fed on the artificial diet immediately after hatching, no increased preference for this food option compared to the natural food sources offered was seen in later assays.

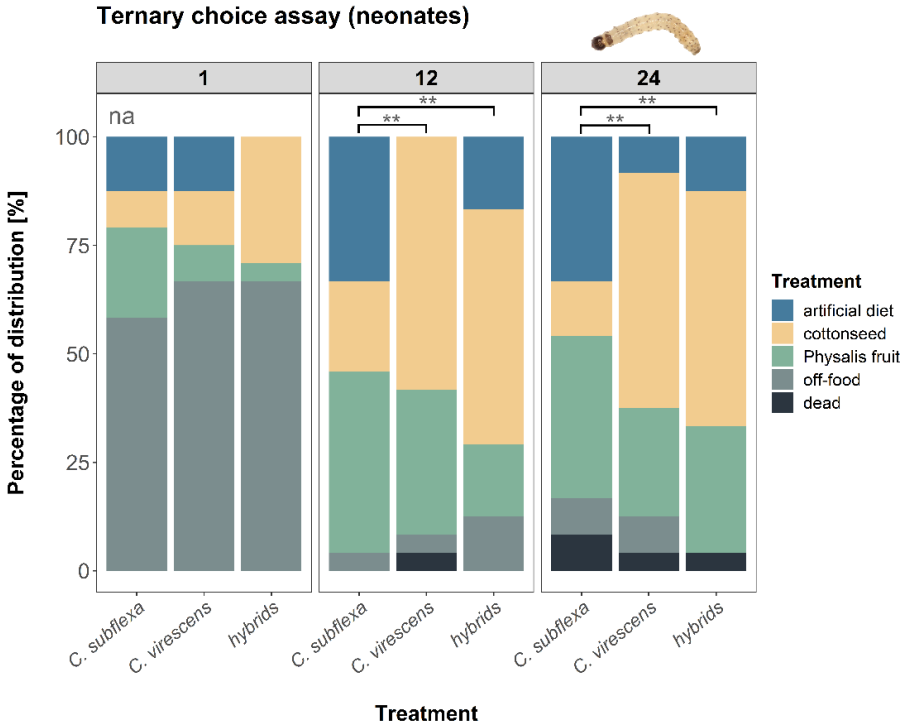

Figure 20: Ternary choice assay of *Chloridea* and hybrid neonates. The larval position in the cup was monitored after 1, 12 and 24 h. Significant differences in the choice behaviour between species are indicated by asterisks ($p < 0.01$**; n = 24). Time point one was not analysed (na).

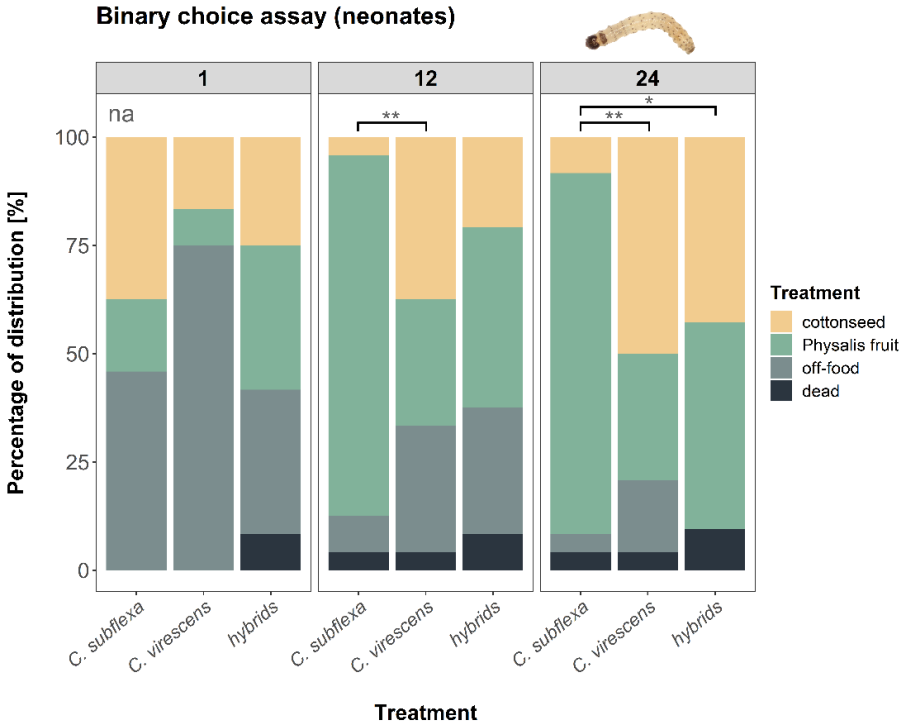

Figure 21: Binary choice assay of second instar *Chloridea* and hybrids. The larval choice was monitored after 1, 12 and 24 h. Significant differences in the choice behaviour are indicated by asterisks ($p < 0.001$**, $p < 0.05$*; n = 24). Na = not analysed.

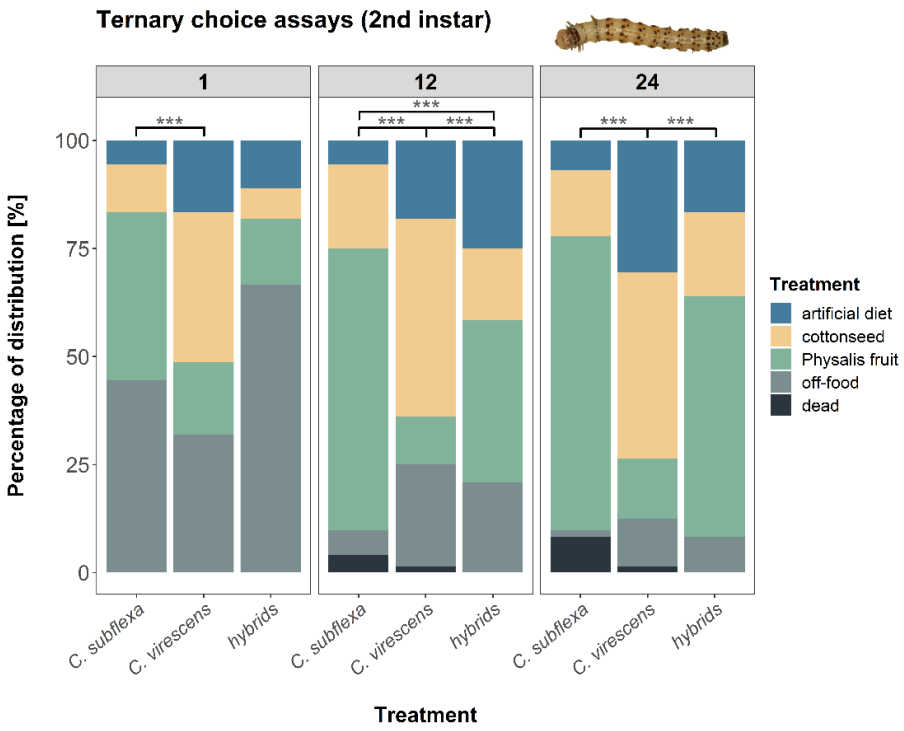

Figure 22: Ternary choice assay of second instar *C. subflexa*, *C. virescens* and hybrids. The position of the larvae in the cup was monitored after 1, 12 and 24 h. Significant differences in the choice behaviour are indicated by asterisks ($p < 0.001$***; n = 72).

4. Hole-boring efficiency of neonate larvae

Low weight gains and survival rates may result not only from food rejection but also from the larvae's inability to establish a feeding site on *Physalis* fruits. Early work found that *C. virescens* and backcross generations of *C. subflexa* and *C. virescens* hybrids lack the ability to "establish a feeding site on the viscous surface of the fruits" when exposing them to *P. angulata* fruits (Laster *et al.*, 1982). The authors observed that small larvae were trapped and later died, whereas larger larvae, in contrast, were able to move around and bore holes into the fruits. This section presents observational as well as experimental data with the goal of shedding light on the question of whether neonate larvae of Noctuidea vary in their ability to establish feeding sites on *Physalis* fruits.

In an initial survey (**Figure 23A**), freshly hatched neonate larvae of four related Noctuidae moth species (+ hybrids) were placed directly on *Physalis* fruits, and the number of holes was counted after 12 hours. All species managed to establish characteristic feeding sites on the fruit's surface (**Figure 23D**). However, the number of holes created differed significantly between the test organisms. Larvae of *C. subflexa* bored significantly more holes into the fruit compared to the other three noctuid species, including *C. virescens* (**Figure 23A**). Intriguingly, the number of holes bored did not differ significantly between *C. subflexa* and the hybrids. In total, hybrid larvae were able to produce

74 holes overnight and larvae from *C. subflexa*, 63 holes. These numbers differ dramatically from the number of holes bored by larvae of *C. virescens* (20 holes), *H. zea* (11 holes) and *H. armigera* (18 holes) (total counts: Suppl. Figure 50A). During the ripening process, the amount of the sticky film increases and later covers the whole fruit surface, causing ripe fruits to appear stickier than unripe ones. Based on this observation, larvae of *C. virescens* and *C. subflexa* were additionally tested for their ability to bore holes in ripe and unripe berries (Figure 23B). Though the number of holes differed interspecifically, there was no significant difference between the two diets in either species (total counts: Suppl. Figure 50B). It therefore seems that fruit ripeness does not prevent larvae of *Chloridea* species from feeding on *Physalis* fruits. However, several larvae of *C. virescens* became immobilised by the sticky coating of the fruit. All neonates trapped were found at the pedicel where the fruit is connected to the calyx. Here, the sticky film is thickest and accumulates in a sort of natural groove on the fruit (Figure 23C). It was observed that the film accumulated on the insect body. Trichomes and substances produced in the plant's glands might affect the larval feeding performance. Glandular trichomes have specialised cells on their tip that exude a variety of specialised metabolites (including acylsugars, flavonoids and methyl ketones); these may prevent larvae from feeding (Glas *et al.*, 2012). The most abundant metabolites produced are acylsugars, which are predominantly polyesters of glucose or sucrose (Schilmiller *et al.*, 2010). These sticky compounds, which are known to have antimicrobial (Chortyk *et al.*, 1993), oviposition-inhibitory (Leckie *et al.*, 2016) and anti-herbivory activities may trap aphids, other small insects and spider mites (Dias *et al.*, 2016; Resende *et al.*, 2002). As the passage through the fruit's groove needs to be mastered by all larvae that do not use silk to lower themselves down to the fruit, another experiment was conducted in order to test if the film represent a barrier to non-adapted larvae. Larvae of *H. zea* and *H. armigera* were found to be trapped frequently, but in contrast to larvae of *C. virescens*, those of both heliothines generally appeared to be less willing to crawl around and to start chewing holes. Therefore, only *C. virescens* and *C. subflexa* larvae were directly compared for their efficiency to bore holes. Instead of placing the neonates directly onto the fruit, larvae were positioned on a sort of platform of remaining calyx material on top of the fruit (compare Figure 23C & D). In this setup, the larvae were forced to cross the groove before accessing the food. Again, after a time span of 12 hours, the fruits were inspected for the number of holes larvae produced as well as for the position of the larvae in the cup. The results (Figure 24A & Suppl. Figure 51A) clearly show that compared to generalist larvae, *C. subflexa* larvae bored significantly more holes into the fruits when placed on top of the fruits. To examine a potentially defensive function of the sticky coating, experiments were repeated with fruits that were manipulated by wiping their surface and that of the remaining calyx material with moist cotton buds. Larvae were placed again on the calyx platform. As previous experiments indicated that *C. virescens* neonates bored fewer holes because of the coating

barrier, it was expected to see an increased hole count once the coating was removed. Interestingly, however, the absence of the coating did not significantly enhance hole boring by larvae of either *C. virescens* or *C. subflexa* (**Figure 24B**). In contrast, total count numbers indicate that fewer holes were bored when the coating was absent (Suppl. **Figure 51B**).

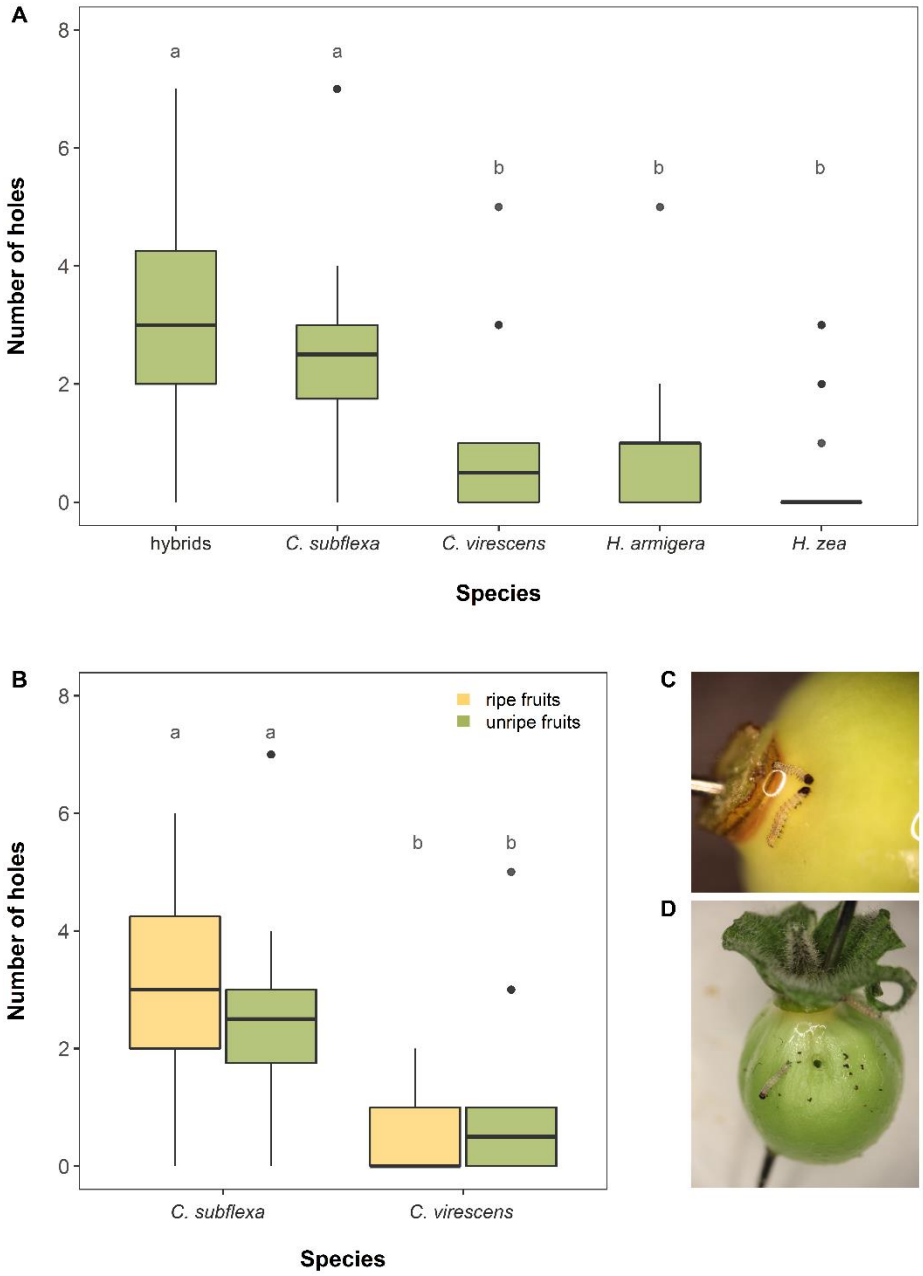

Figure 23: Number of holes created by neonate Noctuidea larvae on *Physalis* fruits. **A)** The number of holes from four Noctuidea species and *Chloridea* hybrids. **B)** Direct comparison of hole counts in *C. subflexa* and *C. virescens* neonates on ripe/unripe *Physalis* fruits. **C)** *C. virescens* larvae trapped in the sticky film (calyx removed). **D)** Example of a typical hole in an unripe *Physalis* fruit (calyx partly left). In both plots, the letters indicate significant differences according to a *post hoc* multiple comparison of estimated means using Tukey contrasts (GLM, $p < 0.001$; n = 24).

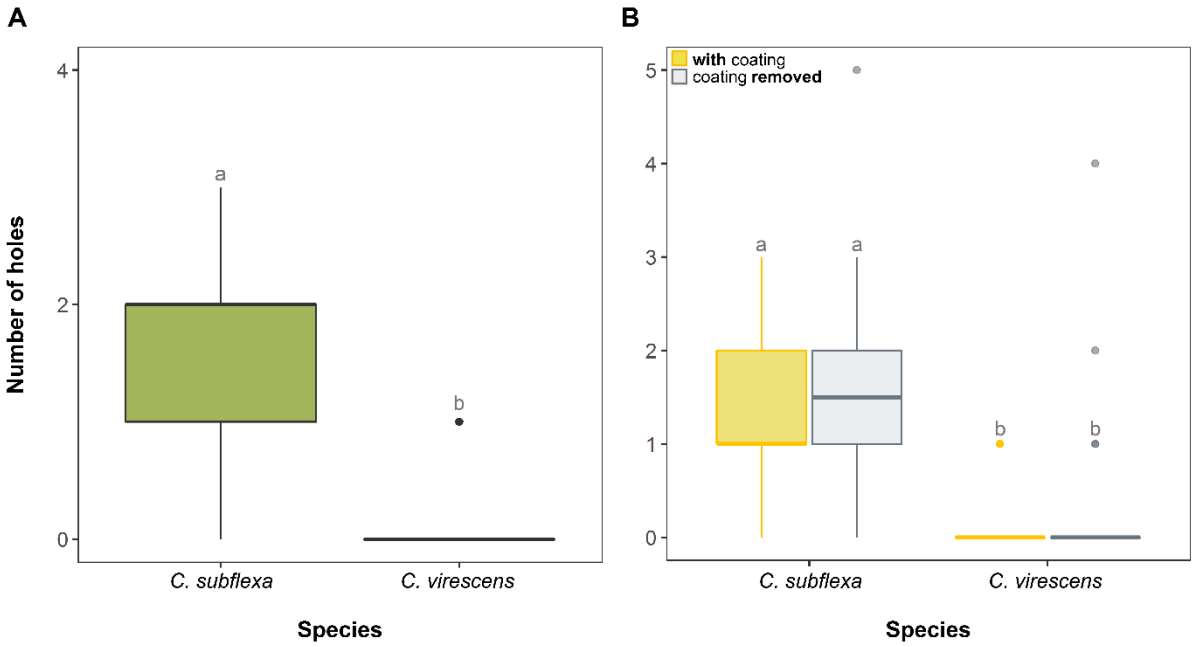

Figure 24: Number of holes created by neonate *C. subflexa* and *C. virescens* on unripe *Physalis* fruits: A) Differences in the number of holes when larvae were placed on the calyx platform of the fruit. **B)** Differences in the number of holes on *Physalis* fruits with sticky coating and on fruits without the coating. In both plots the letters indicate significant differences according to a *post hoc* multiple comparison of estimated means using Tukey contrasts (GLM, $p < 0.001$; n = 24).

The analysis of the position of larvae in the cups (**Figure 25**) showed different distribution patterns among the species. Independent of the presence of the fruit coating, more *C. subflexa* larvae were located directly on the fruit compared to larvae of *C. virescens.* Although 5% of *C. virescens* larvae were trapped by the fruit's sticky coating, none of the *C. subflexa* larvae got caught. Interestingly, in both species the larval proportion on fruits was higher when the coating was present (*Cs* = 30%/*Cv* = 11% higher compared to fruits without coating), and the proportion of larvae that stayed on the calyx platform increased when the fruit coating was removed (*Cs* = 42%/*Cv* = 18% more larvae). Statistical analysis using the total numbers of larvae on the fruit (directly on the fruit or on the calyx) showed that significantly more larvae were found on the calyx when the fruit coating was removed compared to the number of larvae on the calyx in the absence of the coating (χ^2-test, *Cs*: $p < 0.01$; *Cv*: $p < 0.05$). The proportion of larvae on the calyx in relation to the number of larvae on the fruit also differed significantly between species when the coating was present (χ^2-test, coating present: $p < 0.01$; coating removed: $p > 0.05$). Interestingly, it was observed that *C. subflexa* larvae moved their mandibles more frequently compared to *C. virescens* when crossing the fruit coating and the larvae also showed some sort of swimming behaviour. Apart from this, no specific differences in larval behaviour, which might point to different strategies in locomotion and feeding were observed.

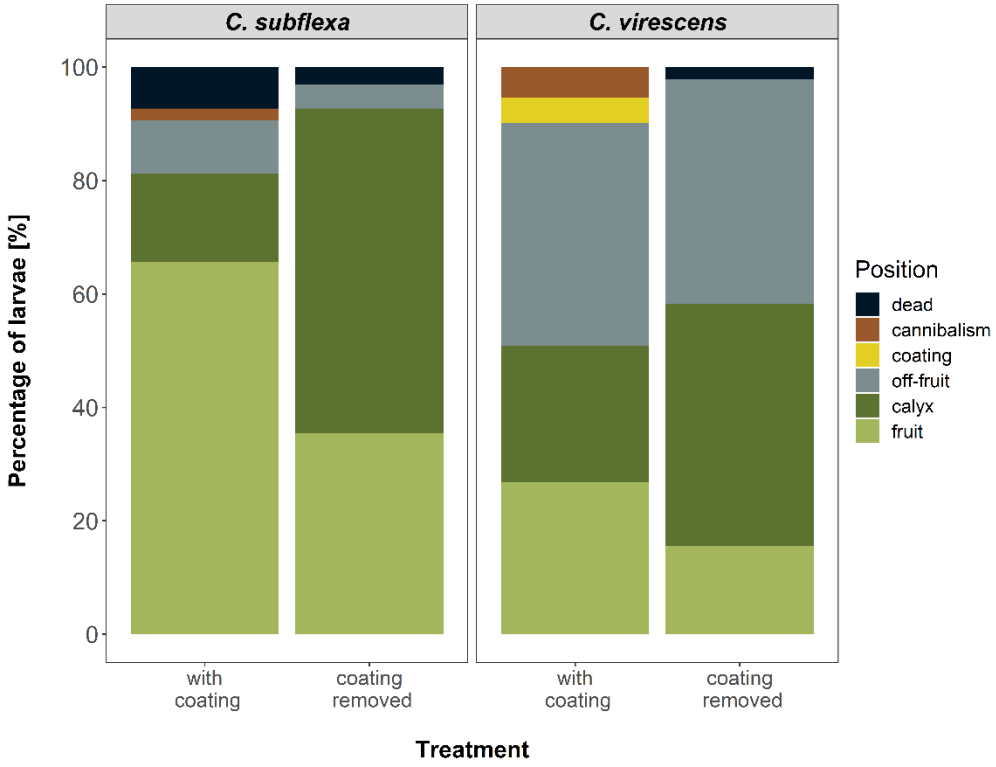

Figure 25: Distribution of *C. subflexa* and *C. virescens* neonates on *Physalis* fruits in the presence or absence of the fruit coating. Larvae were found lifeless [dead], missing [cannibalism] or immobilised (usually dead) in the sticky fruit coating [coating].

4.1 Oviposition behaviour in *Chloridea* moths

Larvae can locate food plants by oriented movements, but in most lepidopterans, the female predetermines feeding sites through her choice of oviposition site. For many specialist insects such as *C. subflex*a, a single plant species often provides food and shelter during their entire life span. The female insect can maximise offspring fitness by laying her eggs on the host plant that is most appropriate for larval development. The capacity to identify the desired host plant prior to egg deposition is an important fitness component on which selection may act. It was previously observed that oviposition is stimulated in *C. subflexa* when ripe *P. peruviana* fruit halves were added on top of the egg cloths covering the mating containers. It was investigated how *C. virescens* females respond to the presence of the fruits (**Figure 26**). These tests were included as side experiments in standard insect rearing. In a first trial, the female oviposition behaviour of the two species against ripe and unripe *Physalis* fruits was tested. Typically, the number of eggs varies strongly and ranges from a few to many. The supplementary **Figure 52A** shows examples of typical egg-laying patterns without the addition of any fruit. In contrast to *C. virescens*, a considerable proportion of *C. subflexa* moths (28%) laid eggs directly underneath or in close proximity to the ripe fruits (Suppl. **Figure 52C**). No such patterns were detected when no fruits or unripe fruits were applied. Most remarkably, several

C. virescens females (16%) clearly avoided ovipositioning close to the ripe fruits and displayed unusual patterns of egg-laying, (Suppl. **Figure 52D**) often laying eggs into the mating cup rather than attached to the egg cloths. No divergent oviposition patterns were detected when no fruits were added or when unripe berries wer used. Whereas in *C. subflexa* moths, egg-laying appeared to be slightly stimulated when fruits were present, the proportion of egg-laying events in *C. virescens* moths was lower compared to in normal matings (without fruits). A second moth generation showed identical patterns, and the data were pooled with previous data. However, although a substantial proportion of moths of both species demonstrated attractant/repellent responses to ripe fruits, most females either laid no eggs (*Cv*: 43%/*Cs*: 25%) or oviposited in a typical random manner when fruits were present (*Cv*: 31%/*Cs*: 47%) (**Figure 52B**).

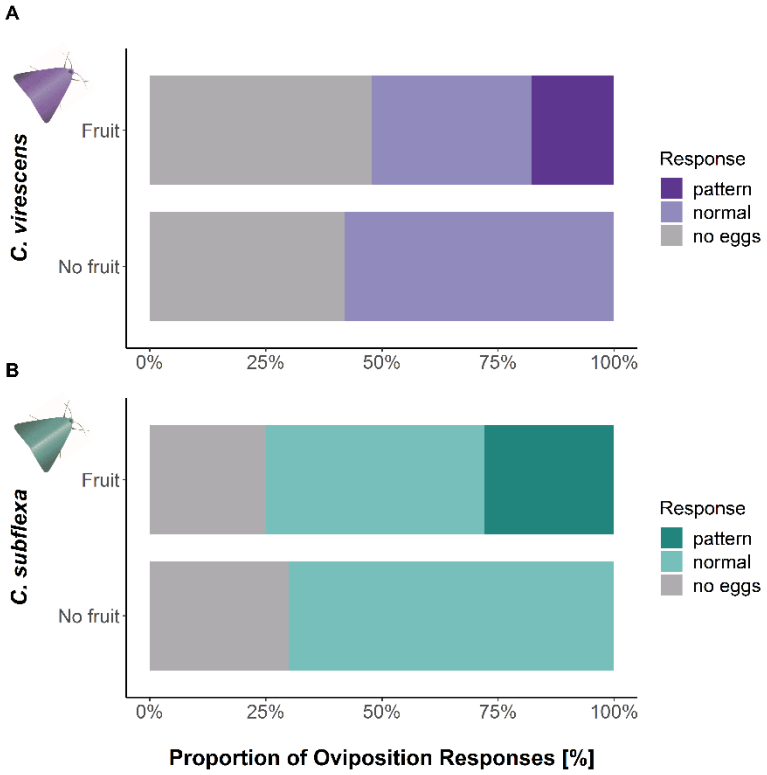

Figure 26: Oviposition response in female *Chloridea* moths in the presence or absence of ripe *Physalis* fruits. Response is divided into: no oviposition event (no eggs), normal egg-laying (normal) or specific oviposition patterns (pattern). Specific patterns in **A)** *C. virescens* refer to repellence while **B)** *C. subflexa* moths were attracted (n = 48). The colour code (right) represents the different responses of the moths.

Previous research indicated that oviposition behaviour of both species is partly influenced by visual signals (Petzold *et al.*, 2009). It was therefore tested if colours corresponding to fruit ripeness influenced female choice or whether their behaviour is primarily guided by olfactorial cues (n = 48). It was expected that ripe fruits emit stronger olfactorial signals, which could explain the differences

in moths' oviposition response to the presence of fruits at two different stages of ripeness. Paper-based fruit imitations in green (corresponding to unripe fruits) and orange (mimicking ripe fruits) were placed on top of the egg cloths. However, neither of the two species responded to the fruit dummies with specific oviposition patterns (Suppl. Figure 52E). The proportion of moths ovipositing was not different between treatments without fruits or with fruit dummies (no oviposition: *Cv*: 39%/*Cs*: 31%; oviposition: *Cv*: 61%/*Cs*: 69%). At first glance, these experiments demonstrated that the presence of ripe *Physalis* fruits has different effects on the ovipositing behaviour of both *Chloridea* moths. Furthermore, results indicate that olfactorial cues rather than visual signals influence oviposition site choice by female moths.

Discussion & Outlook

Previously observed beneficial effects of withanolides on *C. subflexa* are not reproducible

Experiments in chapter I aimed to better understand the previously observed physiological and behavioural effects of *Chloridea* larvae feeding on *P. peruviana* fruits and/or withanolide-containing diet. Feeding assays were conducted to validate the previously observed beneficial effect of withanolide-spiked diet on the weight gain of early third instar *C. subflexa* larvae. However, the beneficial effects described in Barthel *et al.* (2016) could not be reproduced in any trials under similar laboratory conditions. Neither of the test agents (*Physalis* withanolide extracts or purified withaferin A) significantly affected the growth rate of the larvae. However, consistent with Barthel *et al.* (2016), the addition of withanolides did not negatively affect growth rates in *C. virescens*. Experiments were repeated with several modifications. A controlled experimental setup requires careful consideration of external factors that may influence the variation in the experimental outcome. Because the feeding study of Barthel *et al.* (2016) was performed only once, it is possible that any positive impact on *C. subflexa* might have been a random effect in only one particular moth generation. Additionally, average starting weights differed between both species, suggesting that different larval instars may have been compared. However, the direct comparison of two different *C. virescens* instars underlines the importance of synchronising larval starting weight and instar. Thus, all assays in this thesis were performed using larvae with very similar starting weights and of the same instar. Barthel *et al.* (2016) fed each of the two species on a different diet in experiments separated by two years: whereas *C. virescens* was reared on a pinto bean-based diet containing an antibiotic, *C. subflexa* was fed on artificial GPL diet without any antibiotic supplementation. In this doctoral thesis, GPL diet was used in all feeding assays to ensure consistency and to control for

potential effects of the diet. Additionally, synchronised insect rearing prevented large time gaps from occurring between experiments. In summary, the optimisation steps to confirm the findings of Barthel *et al.* (2016) have not been successful. Collectively, these results cast doubt on whether beneficial effect on the specialist's weight gain can be directly attributed to withanolides. As previous results on artificial diet were not reproducible in this study, larvae were subsequently fed on their natural diet (*P. peruviana* fruits) in most follow-up experiments. Using whole fruits in feeding assays reflects a more natural scenario. No significant differences in weight gain were detected between and across larvae on *Physalis* fruits and cottonseed diet in a first trial. Significant differences, however, occurred in the combined feeding/survival assays in which *C. subflexa* performed better on *Physalis* fruits - its natural diet. Overall, variance was most homogenous when weight gain was measured in larvae fed on a natural diet.

Feeding on *Physalis* fruits seems to affect larval development and moulting processes

Although withanolide-uptake and *Physalis* fruit feeding did not robustly affect the performance of either species, developmental analyses revealed delayed development in the larvae of *C. virescens* and hybrids compared to *C. subflexa*. In addition, *C. virescens* larvae experienced supernumerary moulting steps when fed on a *Physalis* diet, especially those that did not survive. Personal observation showed that none of the larvae differed visibly either in their feeding activity or in the amount of food eaten. However, it remains difficult to disentangle if starvation was the ultimate reason of death in *C. virescens* or if certain components of *Physalis* fruits disrupted essential moulting processes. Insect steroidal hormones regulate the timing of important developmental steps. Those processes are known to respond sensitively to environmental conditions and nutritional factors (Kannangara *et al.*, 2021, and references therein). Before larvae moult, their ecdysone production increases, which in turn activates the expression of genes regulating moulting (Yamanaka *et al.*, 2013). In *D. melanogaster* third instar larvae, three ecdysone pulses are detected; the first mediates a critical weight threshold, which is considered a "developmental checkpoint". Reaching this critical threshold initiates metamorphosis, which can no longer be prevented by any further periods of starvation (Rewitz *et al.*, 2013). A critical larval weight has also been demonstrated for lepidopteran *Chilo partellus*, *Cnephasia jactatana* and *M. sexta* (Nijhout, 1975; Ochieng'-Odero *et al.*, 1994). Likewise, in some *C. virescens*, although larvae were not obviously starving, their weight might not have passed this threshold, which resulted in supernumerary moulting steps and retarded development. Larval growth probably continues until this particular checkpoint is reached and pupation is initiated. Some *C. virescens* larvae may later catch up with the relative weight gain of *C. subflexa* and it may well be that the two species differ only temporarily in their weight gain. In

other words, the rate of weight gain might have differed even though the species ended up gaining the same relative amount of weight. Earlier work also described a prolonged duration of larval stages in *C. virescens* and hybrid backcrosses feeding on *P. peruviana* fruits, but did not mention additional moulting events (Laster *et al.*, 1982).

Further investigation is required to investigate if large fractions of *C. virescens* reach a critical weight later as a direct cause of lower nutrient uptake (starvation) or because of negative post-ingestive effects such as hormonal abnormalities. In fact, the results do not point to a moult-inhibitory effect given that the moulting procedure itself takes place, albeit occurring more often. On the other hand, the developmental timing may have been disturbed by stimulated hormonal responses, which then resulted in repeated moulting events. Any additional moulting event may impair larval fitness, which in turn might explain increased death rates in larvae feeding on fruits. In general, *Physalis*-reared *C. virescens* appeared less fit than conspecific individuals feeding on artificial diet. Potential causes of moulting disorders are further analysed and discussed in chapter II.

Larval weight gain and survivability on *Physalis* fruits correlate with the degree of relatedeness to the genus *Chloridea*

This chapter has shown that noctuid and heliothine moth caterpillars outside of the *Chloridea* genus have significantly higher mortality when feeding on *Physalis* fruits than *Chloridea* moth caterpillars, and that this likely reflects their unwillingness to feed on available *Physalis* fruits. Both polyphagous *Spodoptera* species clearly rejected the fruit as a food source. Though the two *Helicoverpa* generalists showed intermediate feeding activity, their larval weight gain was as low as that of the *Spodoptera* species. Previous experiments showed delays in development and a significantly lower proportion of successful pupations in larvae fed on a *Physalis* fruit diet; however, the survival rate of *C. virescens* larvae appeared to be relatively stable. Although they differed significantly in weight gain, no visual differences in feeding activity and survival occurred in the *Chloridea* group (+ hybrids). It is important to note that the two members of monophyletic *Chloridea* larvae grouped together with the hybrids, whereas in comparison, the other two heliothine moths showed significantly lower survival rates on the fruits. The more distantly related *S. littoralis* and *S. frugiperda* died quickly within a few days and exhibited the lowest survival rates. It requires further testing to investigate if the ability to feed and thrive on *Physalis* plants can be traced back to specific evolutionary innovations. Except in *S. littoralis*, the survival rate did not drop immediately after fruit consumption. Death might have been caused by various reasons, but this experimental set-up does not allow us to distinguish between death by starvation or death by any post-digestive effects (for instance by secondary plant compounds). It remains unknown which factors have evoked an aversive feeding

response in the two *Spodoptera* species. Although the consumption of withanolide-containing fruits does not seem to be acutely toxic or elicit immediate repellent responses, there may still be long-term costs associated with chronic withanolide uptake.

Behavioural aspects of *Physalis* fruit feeding

Oppenheim and Gould (2002) hypothesised that *C. virescens*'s failure to efficiently feed on *Physalis* fruits might be partly attributed to differences in behaviour, rather than differences in physiological traits. Therefore, this chapter also asks whether behavioural factors contribute to successful host-plant use and to the circumvention of *Physalis* defences. Behavioural aspects of host-plant use include oviposition site choice, larval food selection and the establishment of feeding sites.

Results from a preliminary experiment indicated that female *C. subflexa* and *C. virescens* moths are sensitive to the presence of ripe *P. peruviana* fruits in their choice of suitable oviposition sites. In both species, females recognised and responded either positively (*Cs*) or negatively (*Cv*) to the presence of ripe fruits. Although some *C. virescens* females avoided egg-laying in close proximity to the fruits, several *C. subflexa* moths oviposited directly underneath or close to them. Interestingly, unripe fruits did not elicit distinct egg deposition patterns. That only a few females responded to the presence of fruits in the mating cups might be due to the limited availability of surface area on which to oviposit. The cups used to set up matings do not reflect natural oviposition conditions and may prevent the moths from responding robustly towards the fruits. It is also possible that the results reflect the natural tendency of *C. subflexa* moths to lay eggs on non-host plants (Benda *et al.*, 2011). Preliminary results showed that colour dummies did not noticeably affect oviposition patterns. It was thus assumed that the moths oviposited according to chemical cues. Odours emitted during the ripening process of *Physalis* fruits may play a role in host selection. In earlier work, methanolic homogenates of *P. angulata* leaves increased oviposition activity on treated non-host plants compared to controls (Oppenheim and Gould, 2002). In binary choice wind tunnel experiments, female *C. subflexa* moths were attracted to odour-stimuli from *P. angulata*, but did not respond to the volatiles from non-hosts such as tobacco, cotton and *Desmodium tortuosum* (Tingle *et al.*, 1990). Compared to mated females, male moths and virgin females responded significantly less to *P. angulata* methanolic extracts, indicating that plants are visited specifically for oviposition (Tingle *et al.*, 1989). When offered a choice between soybean, cotton, tobacco and *P. angulata* plants, *C. subflexa* moths preferred to oviposit on *P. angulata* shrubs*;* in contrast*, C. virescens* and the hybrids preferred tobacco plants. Following up on this, it would be interesting to investigate which *Physalis* fruits components are recognised and explore whether moths can discriminate between single compounds. However, little information is available on *Physalis* plant volatiles, and identifying

specific odour components can be challenging as they occur in complex compound mixes. Pursuing tests with isolated *P. peruviana* volatiles in elaborate experimental settings such as wind tunnels was beyond the scope of this doctoral thesis.

Host selection is also determined by larval food acceptance. Information on a plant's suitability as a food source can be obtained from the insect's taste and odour receptors. Depending on the metabolite content, insects may respond either positively (attraction) or negatively (rejection). Often, plant compounds serve as specific recognition cues that play a significant role in host-plant identification (Wink, 2018). Bernays and Chapman (1994) hypothesised that specialists are generally less sensitive to secondary metabolites of their host plants and that differences in chemoreception sensitivity may have originally contributed to host-plant switches. A comparative study on *C. virescens* and *C. subflexa* further supports the theory that specialists have greater taste sensitivity towards feeding deterrents (Bernays *et al.*, 2000). It is believed that a higher detoxification capacity in generalists allows a reduced chemoreception sensitivity to plant defence compounds. Specialists may have a higher degree of host selectivity due to their lower toxin tolerance. If they are to survive, specialists must have the ability to locate appropriate food efficiently within a narrow host-plant range.

Binary and ternary choice experiments show that *P. peruviana* plants did not elicit a strong repellent response in *C. virescens* larvae, but that a large proportion of *C. virescens* expressed a preference for their natural host plant (cotton) when they had the choice. With a clear attraction towards *Physalis* fruits in the choice assays, *C. subflexa* larvae exhibited less plasticity in feeding preference. Hence, it can be concluded that some degree of food recognition occurred. In order to exclude the possibility that handling of neonates had influenced larval foraging behaviour, more robust second instars were additionally tested. However, the experience of one food source may largely affect what an insect feeds on next. Since second instar larvae had fed on artificial diet prior to the experiment, it was interesting to see that natural food sources were preferred to artificial diet. The results may support the theory that generalist herbivores make less accurate decisions in host selection than specialists do. It remains to be determined if the two species differ in their capacity to detect (detrimental) plant compounds or whether larvae chosen their diet due to genetically manifested adaptation patterns.

The sticky coating of *Physalis* fruits may strongly influence the successful establishment of feeding sites by *Chloridea* larvae

The assays testing the ability to bore holes into *P. peruviana* fruits clearly show that the larvae of *C. subflexa* and the hybrids were most efficient in boring holes. Similar to observations in Laster *et al.* (1982), some *C. virescens* were found to be caught on the fruit surface when they were placed on the calyx platform. No trapped larvae of other species were detected when they were placed directly on the fruit and did not have to cross the pedicel groove in which the coating is thickest. In line with this, the number of holes differed significantly between *C. virescens* larvae placed directly on the fruit or on the calyx platform. However, the starting position of the larvae also significantly influenced the number of feeding sites in *C. subflexa* larvae. At a first glance, these findings suggest that especially the pedicel groove poses an efficient barrier to larvae and so reduces feeding damage.

Acylsugars have been detected in several *Physalis* plants including *P. peruviana* (Maldonado *et al.*, 2006, and references therein; Franco *et al.*, 2014). The fact that these sugars cover the fruit and interior tissues of the calyx, but appear to be rare in other tissues, indicates they have a protective function. In the case of *Physalis* plants*,* acylsugar films may influence larval feeding in multiple ways. First of all, the viscous film might cause the mouthparts to stick together, so the larvae are prevented from chewing and eventually starve. Aggravated locomotion on the sticky fruit surface might also contribute to reduced feeding rates in non-adapted larvae. Previous studies discovered that the function of trichomes and exudates can be very instar-specific (Kariyat *et al.*, 2018; Feng *et al.*, 2021). In support of this, the current experiments showed that only freshly hatched neonates *C. virescens* were trapped; whereas late first instars, in contrast, could cross the sticky secretions accumulating on top of the fruit and their movements appeared less hindered. The described insecticidal activity of acylsugars might be caused by their physical properties. Puterka *et al.* (2003) proposed that due to the wetting nature of the acylsugars, spiracles on the larval body may be covered, which in turn leads to suffocation. Secondly, the authors suggested that the fatty acid moiety of acylsugars could disrupt cellular membranes underneath the cuticle, which may cause desiccation of the insect. After having crossed the pedicel groove, all heliothine species tested showed trails of viscous film behind them. The fruit coating was found to accumulate on the neonates, often covering the whole body. Sugar ester coatings may also cause adverse effects by direct chemical toxicity. Synthetic acylsugars, for instance, display high toxicity against small insects such as whiteflies, the Asian citrus psyllids and aphids (Feng *et al*., 2021). A high sugar content may also counteract feeding-inhibitory effects of otherwise bitter-tasting compounds. Preliminary results indicate that the production of acylsugars is positively correlated with the production of withanolides (Frederico Roda Fornaguera, personal communication). This link raises the question of whether the fruit coating contains (toxic) anti-insect

compounds such as withanolides. The actual cause of death of trapped small insects and early instar lepidopteran larvae on *Physalis* fruits needs to be investigated in future studies.

Based on what is known about the *Physalis* fruit coating, it was hypothesised that the coating not only constrains larval mobility but also serves as a feeding deterrent in non-adapted species. It was therefore expected to see an increased number of holes on washed fruits in *C. virescens*. However, the absence of the coating led to reduced larval mortality but did not enhance feeding in either species. Thus, the sticky film does not seem to act as a strong feeding barrier. Interestingly, however, the assessment of the larval position on the fruit revealed that larvae preferred to stay on the calyx after the coating was removed. This suggests instead that the coating stimulates feeding.

Different acylsugar secretions could have multiple effects on insect species. It is therefore also conceivable that the sugar-rich fruit coating is of nutritive value to some species. Quite surprisingly, it has been shown that trichome secretions were the first meal for the four Lepidoptera species *M. sexta*, *Manduca quinquemaculata, S. littoralis* and *S. exigua* after hatching on *N. attenuata* plants (Weinhold and Baldwin, 2011). Subsequent analysis demonstrated that neither specialist nor generalist larvae experienced adverse effects on acylsugar-free plant materials. The authors concluded that both, trichomes and exudates provide a "sugary first meal" rather than fulfilling a defensive function. However, it was observed that the secretions might also function as an indirect resistance trait. After consuming *N. attenuata* secretions, larvae are marked through a distinctive body and frass odour; this odour allows predatory *Pogonomyrmex rugosus* ants to better locate them on the plant (Weinhold and Baldwin, 2011). *C. subflexa* larvae hiding inside the calyx of the *Physalis* plants escape any potential odour tagging. *C. virescens* larvae do not use the calyx as an enemy-free space, stay longer outside of the calyx before initiating feeding and do not enter the calyx with their whole body (if at all) (Sisterson and Gould, 1999; Oppenheim and Gould, 2002). Any tagging effects as described in Weinhold and Baldwin (2011) would likely lead to more predation/parasitism on the larvae of *C. virescens* than on the larvae of *C. subflexa*.

Although its efficacy varies, the sticky coating likely obstructs access to fruits even for specialists and delays movement and feeding. However, *C. subflexa* neonates seemed less susceptible to the viscous surface than do *C. virescens* neonates. Any trait or behaviour that eases locomotion on sticky surfaces might be highly beneficial and could be under strong selection in this system. *C. subflexa* larvae appeared to be efficient enough in freeing themselves from the coating that no larvae remained entrapped during the experiments. Furthermore, their mandibular function did not seem to be noticeably constrained in the specialist; in fact, *C. subflexa* larvae moved their mandibles more actively while crossing the sticky coating than larvae of *C. virescens* did. In this way, the larvae may

twist themselves free by masticatory movements. However, separating the effects of behaviour from physiological effects remains challenging.

In Feng *et al.* (2021), knockout mutant lines of *N. benthamiana* showed that the absence of acylsugars has led to decreased resistance to six generalist herbivore species, including the three Noctuidea species: *H. zea*, *C. virescens* and *Trichoplusia ni*. The establishment of acylsugar-free knockout *Physalis* plants would further our understanding of the role of acylsugars in plant defences and provides a more efficient *in vivo* manipulation of acylsugar concentrations compared to mechanical washing.

CHAPTER II

Chapter II: Larval Moulting Disorders and Transcriptome Analyses

Background

The two main hormones that regulate developmental processes and growth in insects are juvenile hormones (sesquiterpenoids) and the steroid hormone 20E. As described in the introduction, the core structure of withanolides resembles 20E as well as phytoecdysteroidal cucurbitacin B and D (Figure 2). Plant-derived secondary metabolites with (de)-regulatory function of herbivore hormonal processes are widely distributed among plant taxa (Lafont *et al.*, 2021). Those phytoecdysteroids mimic steroid hormones and can interfere with arthropod ecdysis (Dinan, 2009). Over 500 ecdysteroid analogues have been detected, of which 20E is the most frequently found compound (Lafont *et al.*, 2021). Because phytoecdysteroids have been reported to exert insect deterrent activity, they may help plants avoid feeding damage (Mithöfer and Boland, 2012). The compounds were observed to cause disruptive effects on larval development and growth, inducing cannibalism and supernumerary larval instars. Spinach plants (*Spinacia oleracea*), for instance, are known to produce 20E, which affects the development in the larvae of *B. mori* and *Pectinophora gossypiella* (Kubo *et al.*, 1983). Detrimental effects of phytoecdysteroids were also reported on larvae of *Plodia interpunctella* (Rharrabe *et al.*, 2010) and *S. frugiperda* (Kubo *et al.*, 1981). Since 20E controls key processes in insect development, any disruptions of the hormonal pathways may also have downstream effects on immunity and larval fitness. However, studies investigating a potential role for withanolides as phytoecdysteroids are rare. Two studies examined the topical application of *W. somnifera* acetone extracts on lepidopteran *P. ricini and S. litura* larvae or on freshly pupated individuals, which resulted in most cases in non-viable intermediate stages and adultoid forms (Gaur and Kumar, 2010; Gaur and Kumar, 2018). These findings indicate that development-suppressing properties of certain withanolides may be caused by substantial interference with hormonal pathways.

Whereas the first chapter aimed to evaluate how and to what extent the performance of the specialist and non-adapted species differ when larvae are fed on *Physalis* fruits or withanolide-supplemented diet, this chapter focusses on potential molecular aspects associated with the adaptation to *Physalis* plants. More specifically, it focusses on the observed moulting disorders and developmental delays in *C. virescens* larvae and investigates whether differences in the ecdysone receptor (EcR) of *C. virescens* and *C. subflexa* may influence the response to withanolide consumption. In addition, transcriptomes of the *Chloridea* species and hybrids were analysed to gain

insight into the differentially expressed genes found in larvae fed on withanolide-containing diet and on *Physalis* fruits.

Results

1. Moulting disorders upon *Physalis* fruit feeding

Moulting disorders were first observed as a side effect in *C. virescens* larvae that were about to moult from the third into the fourth instar during initial feeding assays using *Physalis* fruits. As exemplarily shown in the photos (Figure 27), some larvae were unable to shed their old cuticle or the head capsule during the moulting process, so that they remained attached to the larval body. When the old cuticle could not be shed, larvae usually died within one or two days. In all other treatments (larvae that fed on artificial diet with/without withanolides and on a cottonseed diet), no such failure of moulting was observed. No moulting problems have ever been detected in feeding assays with *C. subflexa* and hybrid larvae. As described in chapter I, some larvae did not only fail to complete ecdysis to the fourth larval instar, but also showed supernumerary moulting steps, which may occur as a consequence of low larval weight gain but could also have hormonal causes.

Figure 27: Moulting abnormalities in *C. virescens* larvae. The shown third instar larvae were unable to shed the old cuticle, which remained attached to the abdomen and/or anterior body parts.

1.1 Moulting disorders in *Chloridea* larvae

In order to estimate the proportion of *C. virescens* larvae with moulting disorders, four different generations of *C. virescens* and *C. subflexa* were reared on *Physalis* fruits. No moulting abnormalities were observed in *C. subflexa*. A relatively low percentage of *C. virescens* failed to moult: 4% of all tested larvae in June 2019, June 2020 and August 2020 (n = 24) experienced moulting problems. In August 2019, 12% of the larvae showed moulting disorders (n = 72). These numbers were generally lower than expected from the high number of cases occurring in initial feeding studies on *Physalis* fruits (cf. 13% in the year 2018; n = 24).

1.2 Comparative analysis of *Chloridea* ecdysone receptor sequences

In order to compare nucleotide sequences, the missing full-length sequence of the *C. subflexa* EcR was first completed via RACE-PCR (Suppl. **Figure 53**). An alignment against the *C. virescens* EcR sequence published in GenBank revealed that the two sequences of the EcR open reading frame (ORF) were almost identical and encoded proteins differed only in three positions within the last 20 amino acids. The *C. subflexa* EcR DNA sequence was cloned in *E. coli*, and a total of 96 clones were sequenced to discover putative receptor variants (isoforms). Four different *C. subflexa* EcR isoforms were detected showing two different indels (**Figure 28** & Suppl. **Figure 54**). The EcR complex includes four independent functional domains: the N-terminal A/B domain is highly variable and believed to include a motif that mediates ligand-independent transcription. In contrast, the DNA-binding domain (C domain) with the linker domain D represents the most conserved part. The fourth domain (domain E) is a multifunctional domain that seems to control ligand binding (Mello *et al.*, 2014). The domain F at the C-terminus is not present in all receptors. One indel was located outside of the binding pocket (domain C/D), and the second one was detected in the anterior part of the binding domain (Suppl. **Figure 55**). Within the 96 sequenced clones of *C. subflexa*, isoforms were distributed as follows: EcR isoform-1 = 6% (+ I -), predominantly expressed EcR isoform-2 = 45% (- I +), EcR isoform-3 = 26% (+ I +) and EcR isoform-4 = 23% (- I -). Despite several optimisation steps, the amplification of the *C. virescens* EcR DNA sequence was not successful. Thus, no data on the proportion of the four isoforms can be provided.

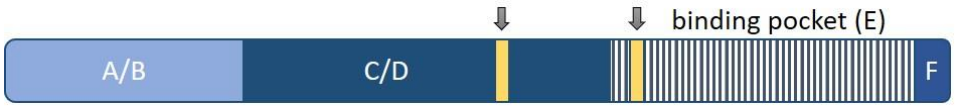

Figure 28: Schematic description of the *Chloridea* EcR sequence showing the six functional domains from the N-terminus (left) to the C-terminal region (right). Grey arrows indicate the position of the two microindels (yellow). The second indel is located in the ligand-binding pocket.

2. Gene expression analyses

Upon ingestion, withanolides may induce a detoxification program in the larvae that involves specific transcriptional responses. In order to analyse *Chloridea* and hybrid RNA-Seq data in response to feeding on *P. peruviana* fruits or artificial diet supplemented with either *Physalis* withanolide extracts or withaferin A, larvae were allowed to feed on the diet for 72 hours. Afterwards, RNA was extracted from whole guts and pooled.

2.1 Principal component analysis

A principal component analysis (PCA) plot was generated in order to get an impression of the similarity of the transciptome samples and of how the data are structured based on transcriptome-wide signatures in *C. subflexa*, *C. virescens* and hybrid larvae fed on *Physalis* fruits and on artificial diet (**Figure 29**). Each component was interpreted as a transcriptomic profile for genes having a similar pattern of expression variation in all the samples analysed. Those with similar expression profiles are clustered together. The essence of the data is captured in a few PCs, which themselves convey the most variation in the dataset. The PCA illustrates the relatively high variance in the five replicates in all species and across all treatments. The highest overlap between transcriptomes was detected among the two *C. virescens* datasets, which did not separate well in the analysis. Clusters of hybrids and *C. subflexa* are more similar to each other than clusters of *C. virescens* and the hybrids. In addition, it was interesting to see that hybrids display their own transcriptional signature in this projection.

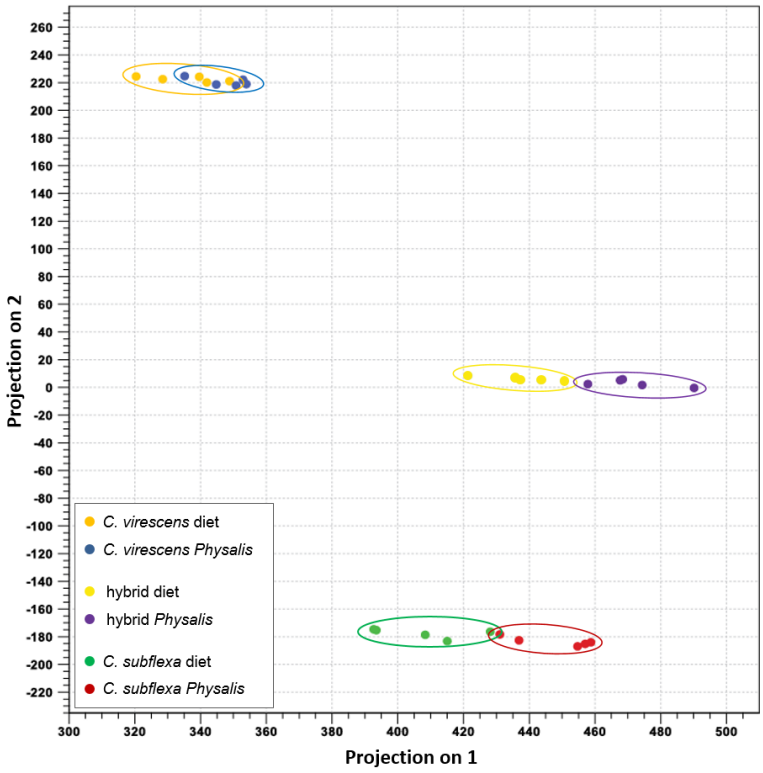

Figure 29: Principal component analysis of the transcriptomic data of *C. virescens*, *C. subflexa* and the hybrids fed on either *Physalis* fruits or on artificial diet. Different datasets per species/diet are represented by the colour code described in the legend (bottom left). Each dot represents a sample pool per treatment/species (n = 5).

2.2 Analyses of the general gene expression activity

The numbers of significantly differentially expressed genes ($p < 0.05$) relative to larvae of the same species of the control group (artificial diet) indicated that there was in total less downregulation on gene expression levels in all samples of *C. subflexa* compared to *C. virescens* and hybrid samples (**Table 2**). The highest number of differentially regulated genes relative to the control was found in hybrid samples. Clearly more genes were differentially regulated after larvae fed on *P. peruviana* fruits, compared to after larvae fed on artificial diet supplemented with withanolide compounds. This is not surprising as the fruits contain a large amount of plant metabolites that may elicit a response in the larvae. With the exception of the transcriptome from larvae fed on diet supplemented with withaferin A diet, most significantly regulated genes were upregulated in *C. subflexa* larvae. In contrast, more genes were downregulated than upregulated (except in larvae fed on *Physalis* fruit treatment) in *C. virescens* larvae. Although the two sister species did not differ notably in the number of upregulated genes in all three treatments, the number of downregulated genes was clearly higher in the transcriptomes of larvae of *C. virescens* compared to those of *C. subflexa*. Interestingly, *C. virescens* larvae that fed on *P. peruviana* accumulated several genes associated with a general stress response to insecticides and to plant-derived defence compounds such as CYPs, UDP-glucuronosyltransferases (UGTs), glutathione-S-transferases (GSTs) and (carboxyl-) esterases. Some of those genes were also detected among downregulated genes, but in fewer numbers. Most interestingly, only a few stress-responsive genes were found in the two transcriptomes of *C. virescens* larvae feeding on artificial diet spiked with withanolides. With more downregulated than upregulated genes (except for the *Physalis* transcriptome), the hybrid transcriptional activity followed the gene expression trend in *C. virescens*.

Treatment	Species	upregulated ↑	downregulated ↓
Physalis fruits	*C. virescens*	3884	2587
	C. subflexa	3685	658
	hybrids	***Cv*** 5214	***Cv*** 1399
		Cs 1293	***Cs*** 1026
Physalis withanolide extracts	*C. virescens*	201	1397
	C. subflexa	177	115
	hybrids	***Cv*** 318	***Cv*** 1360
		Cs 393	***Cs*** 975
withaferin A	*C. virescens*	606	1043
	C. subflexa	210	426
	hybrids	***Cv*** 241	***Cv*** 360
		Cs 218	***Cs*** 345

Table 2: Total numbers of differentially expressed genes in *Chloridea* species and hybrid transcriptomes across the three diet treatments. The list comprises significantly regulated genes regardless of the annotation status. Hybrid gene counts were split into the two parental alleles (highlighted in purple (*Cv*) and green (*Cs*).

Venn diagrams of annotated and differentially regulated genes of *C. subflexa* and *C. virescens* larvae across diet treatments are shown in **Figure 30**. Interestingly, the figure illustrates that differentially regulated genes do not overlap strongly across all transcriptomes within each species. Among overlapping genes in the Venn diagrams, no expression patterns were detected that may indicate a specific response in larvae towards the uptake of withanolide compounds. In contrast to those of *C. subflexa* larvae, however, Venn diagrams of *C. virescens* larvae transcriptomes shared some upregulated genes associated with detoxification mechanisms and insecticide resistance. Larvae fed on withaferin A and *P. peruviana* fruits shared four CYPs and seven esterases. Larvae fed on withanolides and withaferin A shared four CYPs and a UGT. A CYP enzyme and a juvenile hormone esterase-like gene was upregulated in all three transcriptomes. Except for a differentially downregulated CYP gene shared between the *C. virescens Physalis*/withaferin A transcriptomes, no such gene annotations were detected in *C. subflexa* transcriptomes and among differentially downregulated genes in *C. virescens*.

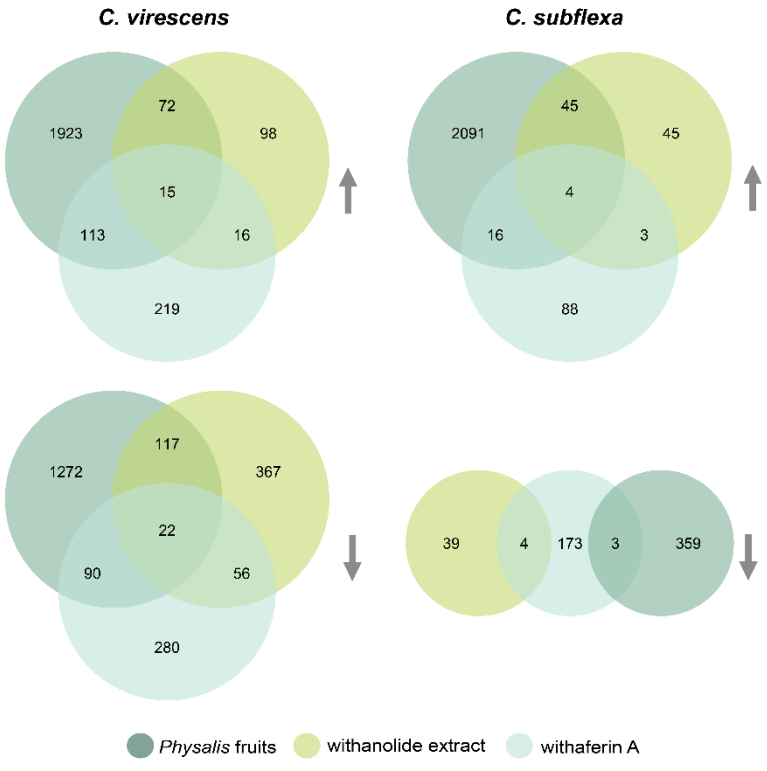

Figure 30: Venn diagrams showing differentially regulated and annotated genes in *C. virescens* and *C. subflexa* larvae. The colour code below represents the diet treatments. Grey arrows indicate a significantly ($p < 0.05$) up- or downregulated gene expression relative to the control (artificial diet).

2.3 GO term enrichment analysis in transcriptomes of *Physalis*-fed larvae

A gene ontology (GO) term enrichment analysis was performed for differentially expressed genes in transcriptome of *C. subflexa* and *C. virescens* larvae fed on *Physalis* fruits. With regard to their associated biological characteristics, enriched GO terms were found to be of basic molecular and cellular functions (corresponding word clouds are attached to the Supplementary Data; **Figure 56** & **Figure 57**). Except for *C. subflexa* downregulated genes ("cytoplasmic translation"), "anatomical structure development" was the most overrepresented functional term in all gene sets. Interestingly, "hydrolase activity" and "oxidoreductase activity" were overrepresented terms among downregulated *C. subflexa* genes, but were not found among upregulated ones. In contrast, those GO terms were not present in *C. virescens* datasets. However, the species are not model organisms and full-genome annotations are not yet available, which may strongly affect the quality of the GO term classification. Thus, the explanatory power of the GO term enrichment analysis should not be overrated.

2.4 Osiris gene expression patterns

The only gene expression pattern that stood out in the transcriptomic analyses is the gene expression activity of the insect-specific Osiris gene cluster. Due to their association with insect cuticle formation and development, this gene family was analysed more closely. For all four diet treatments, 27 Osiris genes (six paralogues of Osiris 9) were detected in the transcriptomes of the two *Chloridea* species and the hybrids. True orthologous genes were identified by manual annotations from draft genomes of the corresponding species. Osiris genes from *B. mori* were used as a query (Suppl. **Figure 58**). Overall, the relatively low reads per kilobase per million mapped reads (RPKM) values indicated a generally low expression of Osiris genes in the third larval instar. Osiris gene expression was not significantly different between *C. subflexa* larvae that fed on *Physalis* fruits, withanolide-diets and those that fed on the control diet (**Figure 31**). In the *Physalis* fruits dataset, most Osiris genes were upregulated (16 up | 8 down), while in the dataset of larvae that fed on diet with *Physalis* withanolide extracts (10 up | 14 down) and withaferin A (4 up | 16 down) the majority of genes were conversely downregulated. Opposite to the *C. subflexa* transcriptomes, a general trend towards the downregulation of Osiris genes is clearly visible in the *C. virescens* transcriptomes (**Figure 31**). In the dataset of larvae fed on *Physalis* fruits, four Osiris genes were moderately upregulated while 20 Osiris genes were downregulated. Similarly, most Osiris genes were also downregulated in the larvae that fed on *Physalis* withanolide extracts (2 up | 22 down) and on withaferin A (0 up | 24 down). However, only one gene (Osiris 16a) was significantly downregulated in the transcriptome of *C. virescens* larvae.

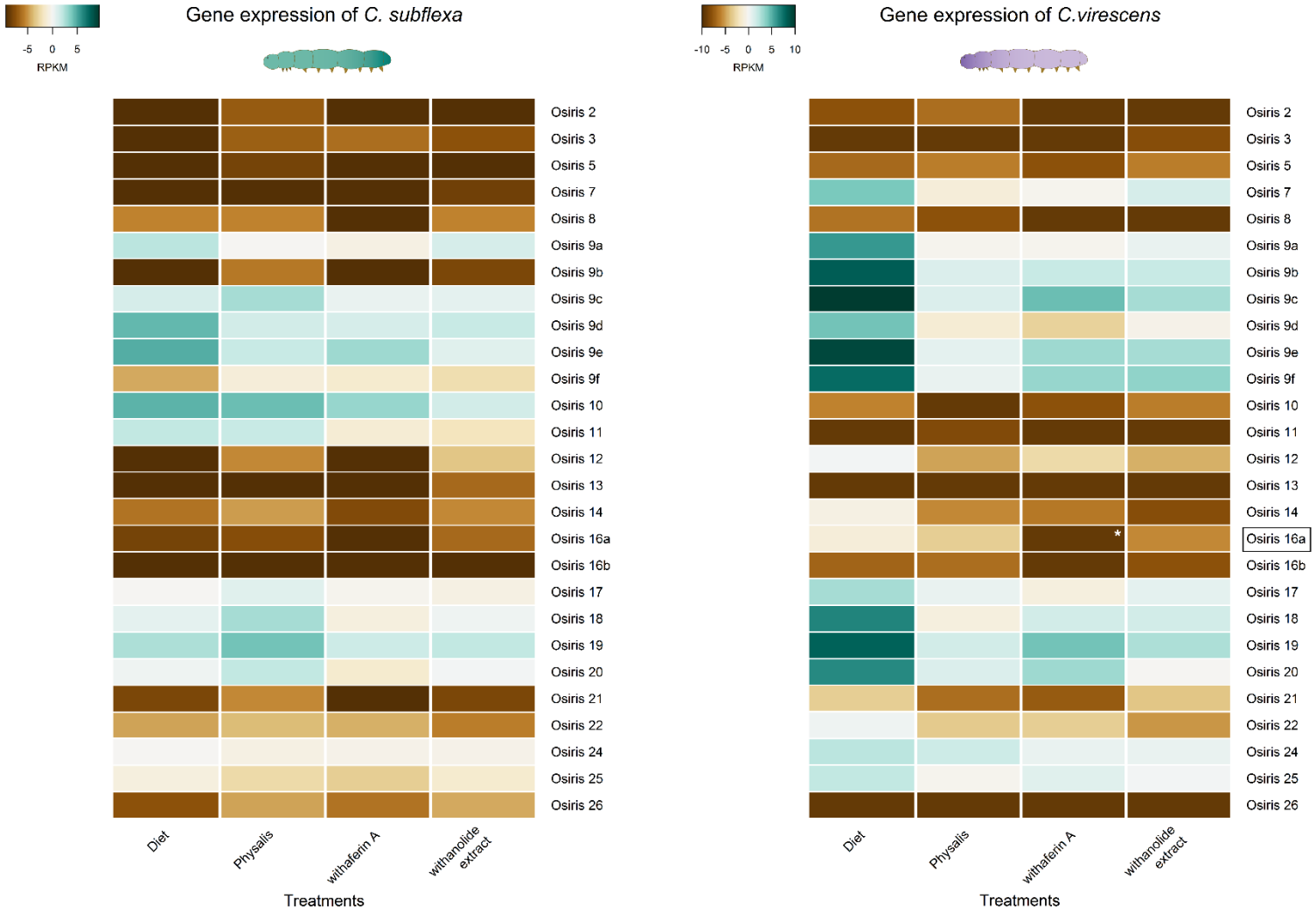

Figure 31: Heatmaps of Osiris gene expression profiles in *C. subflexa* and *C. virescens* after larvae fed on artificial diet, *Physalis* fruits or artificial diet supplemented with withaferin A or *Physalis* withanolide extracts (100 µg/ml). Shown are the log2-transformed RPKM values. An asterisk (*) indicates a statistically significant gene expression ($p < 0.05$, moderated t-test). Relative expression levels are represented in a colour code as shown in the charts.

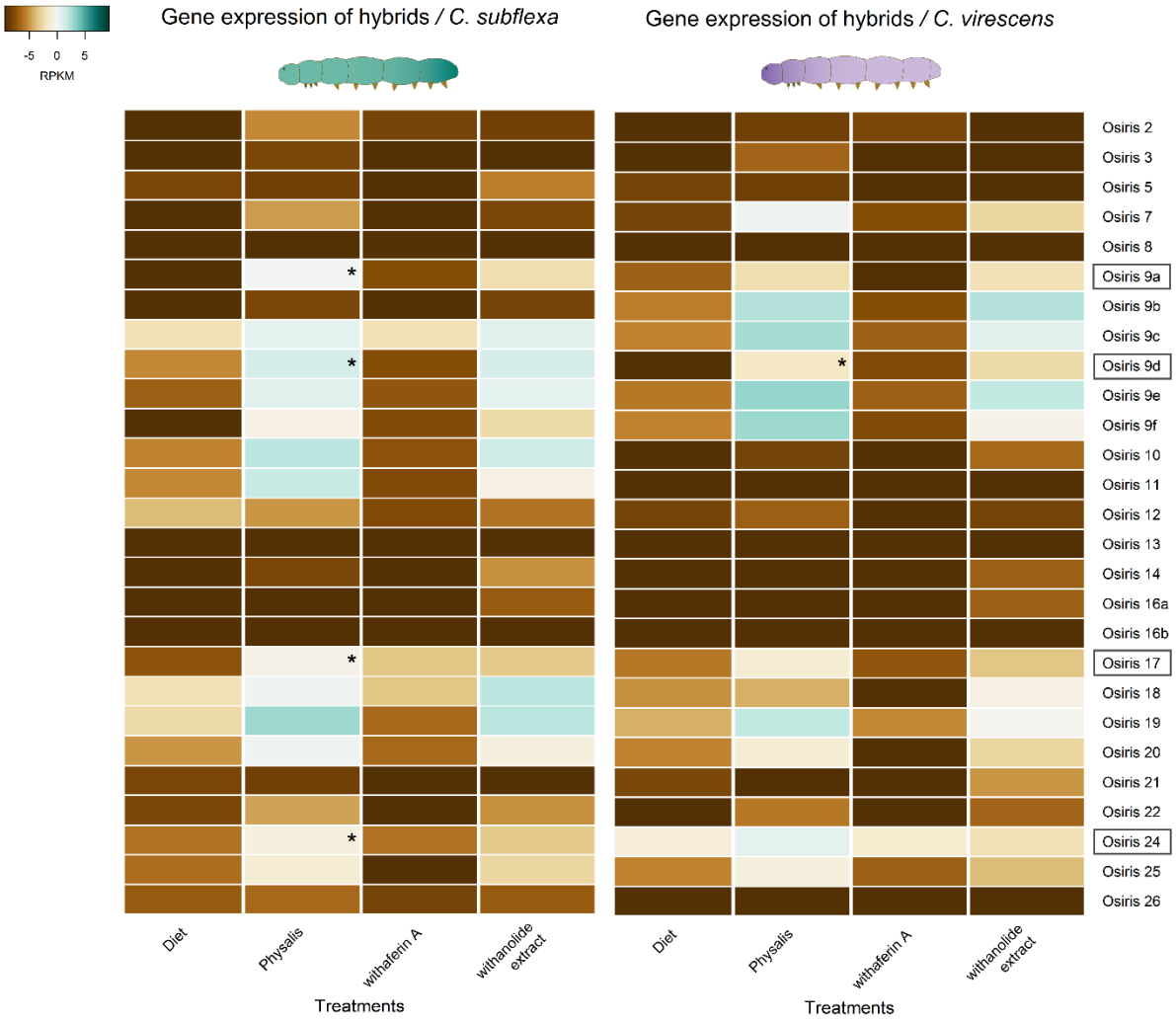

Figure 32: Heatmaps of Osiris gene expression profiles in *Chloridea* hybrids after larvae fed on artificial diet, *Physalis* fruits or artificial diet supplemented with withaferin A or *Physalis* withanolide extracts (100 µg/ml). Shown are the log2-transformed RPKM values. An asterisk (*) indicates a statistically significant gene expression ($p < 0.05$, moderated t-test). Relative expression levels are represented in colours as shown in the corresponding colour bar on the left side.

Although the PCA (**Figure 29**) suggests a distinctive transcriptional response in the hybrid larvae, the Osiris gene expression profile followed the patterns of the parental *C. subflexa* profile: in the *C. subflexa* allele set of hybrids fed on *Physalis* fruits most genes were upregulated (16 up | 3 down), but the upregulation of only four Osiris genes was statistically significant. In *C. virescens* alleles (17 up | 2 down), only the upregulation of the Osiris 9d paralogue was significant. This gene did not show an allele-specific expression and was upregulated in both parental allele datasets. In larvae fed on diet with *Physalis* withanolide extracts, most genes were upregulated (*Cs:* 18 up | 2 down/ *Cv:* 21 up | 2 down), while in contrast the majority of them were downregulated when larvae fed on diet with withaferin A (*Cs:* 3 up | 16 down/*Cv:* 3 up | 14 down). The extrinsic transcriptional signature of *C. subflexa* was still detectable in the hybrids, and, interestingly the gene expression activity of parental *C. virescens* alleles followed this signature.

2.5 Gene expression of immune-related genes and phenoloxidase activity

As a previous gene expression analysis showed that immune-system related genes were differentially expressed in *C. subflexa* (upregulated) and *C. virescens* (not affected or downregulated) larvae after they fed on diet supplemented with withanolide extracts (Barthel *et al.*, 2016), all datasets were screened for similar expression patterns. However, a comparative analysis of the expression levels of immunity-related genes did not reveal any outstanding patterns in the expression profiles. Additional analysis of the PO activity was conducted to confirm the previously described increase in PO activity in *C. subflexa* larvae (Barthel *et al.*, 2016). A positive effect on the PO activity of *C. subflexa* feeding on withanolide-containing diet or *Physalis* fruits could not be verified (Suppl. Figure 59 & Figure 60).

Discussion & Outlook

As studies on the mode of action of withanolides are rare, it is as yet unknown if these compounds can act at the hormonal level. Since withanolides resemble in structure the natural EcR-ligand 20E, the EcR is a promising candidate to interact with. The EcR of *C. virescens* has been shown to adapt its structure to different steroidal ligands, but also to agonistic non-steroidal ligands, indicating a generally high flexibility of the binding pocket (Billas *et al.*, 2003; Browning *et al.*, 2021). The adverse impact of non-steroidal ligands on hormonal processes seems to depend primarily on the binding affinity (Minakuchi *et al.*, 2003). So far, no direct binding has been confirmed despite the observed antagonistic effect of withanolides. However, radioligand binding assays demonstrated the docking of cucurbitactin B and D (structurally very similar to withanolides) to the EcR complex (Dinan *et al.*, 1997). In spectrometric microplate assays, withanolides exhibited antagonistic activity to the action of 20E in *D. melanogaster* BII cell lines carrying the EcR (Dinan *et al.*, 1996). The authors surveyed 16 different withanolide isolates from Solanaceous *Iochroma gesnerioides* shrubs against ecdysteroid-responsive *D. melanogaster* BII cell lines. Although nine of the tested compounds showed antagonistic activity, none of the extracts were found to be agonistically active. In another study, the authors screened 128 plants and found only weak antagonistic activity (Savchenko *et al.*, 2000). This may indicate that the observed activity is either very specific or that the actual effect of withanolides *in vivo* is overrated.

The presence of different withanolides and their derivatives in one single plant is suggestive of their different molecular target points to act on. If the ligand-binding domain of the molecular target has been changed through point mutations in such a way that the binding of ecdysteroid analogues is

aggravated, insects could likely tolerate high concentrations of otherwise development-disruptive compounds. In this regard, stable genetic modifications of the EcR may confer a permanent adaptation, preventing the extra costs of producing e.g. decoy proteins or of expressing excess ecdysone to compensate for blocked hormone receptors. However, target-site mutations are generally rare, possibly because novel genetic modifications often undermine the original functions of the specific gene (Després *et al.*, 2007).

Nucleotide sequence analysis of the *Chloridea* ecdysone receptor

To test the assumption that the EcRs of the two *Chloridea* species differ genetically, the missing full-length DNA sequence of *C. subflexa* was amplified via RACE-PCR. Subsequent nucleotide alignment revealed that the receptor sequences of both species were almost identical. Therefore, it was considered unlikely that differences in moulting success can be attributed to genetic differences of the ecdysone receptor alone. Further screening of additional GenBank entries revealed that the four detected receptor isoforms in *Chloridea* are shared with other lepidopterans. In insects, several EcR isoforms commonly coexist with variants specific to sub-species (Watanabe *et al.*, 2010). In Lepidoptera there seems to be only one receptor gene that encodes four different isoforms; no *C. subflexa* specific receptor variant was discovered. Previous studies have shown temporal and tissue-specific isoform expression patterns in *D. melanogaster* (Talbot *et al.*, 1993), *Apis mellifera* (Mello *et al.*, 2014) and in *Tribolium castaneum* (Tan and Palli, 2008). Because distinct expression profiles of the four different receptor isoforms have not been analysed in *C. virescens* or other lepidopterans, it cannot be ruled out that *C. subflexa* exhibits a unique isoform signature.

Although the basic function of EcRs may be highly conserved, isoforms seem to control different regulatory functions (Nakagawa and Henrich, 2009). Likewise, the individual EcR isoforms detected in *Chloridea* might also be regulated independently and according to current developmental stage. Typically, isoforms displayed a high degree of similarity in both the DNA and amino acid sequences in most EcR functional domains and differed only in the N-terminal region that interacts with different transcription factors (Watanabe *et al.*, 2010; Gauthier *et al.*, 2012). The N-terminal regions of *Chloridea* EcR sequences did not differ. Though there might still be isoform-specific functions, the location of the binding pocket indel in a crystal structure of EcR suggests that the maintainance of the docking function remains unaffected; in other words, this position is not expected to be involved in ligand binding (Billas *et al.*, 2003). As neither the ligand-binding pocket nor the N-terminal region seem to be affected, it was considered unlikely that the binding affinity is substantially affected by the different sequence variants within and between species. However, whether the functions of other domains are influenced by the indels remains unknown. Clearly more research is required to

understand putative isoform-specific functions in moulting and metamorphosis as well as the potentially different tissue and instar-specific expression patterns of *Chloridea* isoforms. Additionally, there is no concrete evidence of direct binding of withanolides to insect ecdysone receptor sites.

In silico molecular modelling would be a good strategy to simulate the docking of different withanolide compounds to *Chloridea* EcR crystal structures. However, early work also showed that computer models failed to predict the highly adaptive binding pocket formation (Billas *et al.*, 2003). Binding studies using overexpressed EcR ligand-binding domains followed by crystallisation provide a better option but were beyond the scope of this thesis.

Withanolides could also interfere with enzymes that synthesise or degrade 20E and thus indirectly affect moulting. Withanolides may be able to block the active site of, for example, the last enzyme in the biosynthetic pathway or the first enzyme in the degradative pathway of 20E. The 20E titres could be decreased or increased respectively compared to their normal values. This hypothesis could either be tested indirectly by modelling enzyme structures *in silico* and docking withanolides into the active site or directly by measuring ecdysone titres in larvae fed with artificial diet with or without withanolides.

In addition to non-specific differences in EcR structure, the present feeding studies investigating the moulting performance of *C. virescens* larvae fed on withanolide-containing *Physalis* fruits found only a small proportion had moulting disorders, suggesting diet has as rather weak effect on the development. A high/low degree of sensitivity in a particular moth generation or seasonal variation of plant compounds in *P. peruviana* may explain the observed variable impact on the larval development. Although generalist larvae fed on *Physalis* fruits performed poorly in feeding/survival assays (described in chapter I), moulting disorders were observed only in assays involving *C. virescens* larvae. This discrepancy might be explained by the low number of tests using other species; and in addition, most of those larvae died relatively quickly before they could go through supernumary moulting steps and some even failed to moult sucessfully. Interestingly, no moulting disorders or additional moulting steps have ever been observed in *C. virescens* larvae fed on artificial diet containing withanolide extracts or withaferin A. Therefore, the observed effects cannot be directly associated with withanolide ingestion. As already discussed in chapter I, it was hypothesised that because the rich artificial diet used in those experiments compensated for potential negative effects, no such moulting disorders occurred when withanolides were added. However, in withanolide-feeding assays using low nutrient diet, no abnormal moulting occurred. In addition, withanolides may act synergistically and take full effect only in combination with certain other *Physalis* constituents, which are absent in the withanolide-supplemented artificial diet.

If the EcR complex is not affected by withanolide-binding, it is also possible that high levels of ingested withanolides act directly on the larval gut morphology, which could also explain the worse performance of *C. virescens* larvae on withanolide-producing plants compared to the specialist. Two different studies demonstrated that the ingestion of 20E caused damage to midgut epithelial cells of lepidopteran *B. mori* (Tanaka and Yukuhiro, 1999) and *P. interpunctella* larvae (Rharrabe *et al.*, 2009). The potentially cytotoxic effect of withanolides on insect (epithelial) cells should be confirmed in future work. Some studies on noctuid moths (for example, *C. virescens*), indicate that metabolic transformations via esterification mediate high tolerance to orally ingested ecdysteroids. Interestingly, esterified ecdysteroids were observed to be significantly less active than unmodified ecdysteroids (Duan *et al.*, 2020, and references therein). The fast and efficient metabolisation of those compounds might be a potent resistance strategy. Potential metabolic conversions of withanolides are further discussed in chapter III.

Osiris genes are differentially regulated in *Chloridea* larvae in response to *Physalis* fruit feeding and withanolide uptake

The EcR complex regulates the expression of a range of ecdysteroid-responsive genes by binding to their promoters (Nakagawa and Henrich, 2009). Though the mode of action of withanolides may not involve direct interaction with the receptor, these compounds might affect genes transactivated in the ecdysis-signalling cascades or other genetic networks. Therefore, transcriptomic data were explicitly screened for interesting patterns in the expression of ecdysis-associated genes, such as ecdysteroid kinases or chitinases. Insects have been shown to increase or decrease their production of detoxification enzymes after a prolonged exposure to xenobiotic compounds (Glendinning and Slansky, 1995; Zhou *et al.*, 2019). Therefore, I searched for transcriptionally enriched genes associated with metabolic detoxification mechanisms, insecticide resistance and stress response. Several of these genes were upregulated in the transcriptome of *C. virescens* larvae fed on *Physalis* fruits. That only a few were significantly upregulated across *C. subflexa* transcriptomes may indicate that the generalist boosts its metabolic defence system in response to the uptake of certain plant compounds. However, as the expression of such genes was not noticeably induced in *C. virescens* larvae fed on withaferin A or on *Physalis* withanolide extracts supplementing the experimental diet, a direct connection to withanolides is difficult to make.

The only pattern that clearly stood out in the transcriptomes was the differential expression profile of Osiris genes. The Osiris genes form a highly conserved insect-specific gene family. Though poorly characterised, this large multigene family is thought to have played an important role in insect evolution and adaptation processes (Smith *et al.*, 2018). A cluster of 23 Osiris genes was first

discovered in *D. melanogaster* (Dorer *et al.*, 2003) and later reported in 11 other *Drosophila* species (Bhutkar *et al.*, 2008). Current knowledge indicates that insect genomes encompass 20 - 25 orthologous Osiris genes with highly conserved synteny and sequence homology across different species (Shah *et al.*, 2012; Coolon *et al.*, 2019). Shah *et al.* (2012) predicted that Osiris genes evolved by gene duplication early in the evolution of insects. Further gene duplications have also occurred in some lineages. There are, for example, multiple paralogues of Osiris 9 in *B. mori* and other lepidopterans (Nallu *et al.*, 2018). Six copies of Osiris 9 were also discovered in the *Chloridea* transcriptomes. Osiris 9 copies appear to be unique to lepidopterans and seem to be involved in silk production (Shah *et al.*, 2012; Coolon *et al.*, 2019). In response to feeding on *Physalis* fruits and artificial diet containing withaferin A or withanolide extracts, *C. virescens* larvae downregulated most Osiris genes, but no distinct patterns were detected in response to feeding of larvae of *C. subflexa* and the hybrids. It seemed, however, that the transcriptional regulation in the hybrids was more efficient in parental *C. subflexa* alleles as they followed the expression patterns in *C. subflexa*. The results suggest a biologically relevant response to the consumption of *Physalis* fruits and withanolides.

Osiris genes, which are thought to have multiple essential functions in insect development and phenotypic plasticity, are also linked to detoxification processes and immune responses (Smith *et al.*, 2018). With regard to the observed moulting disorders, it is important to note that these genes are thought to play a crucial role in cuticle formation in embryos as well as in pupae. No GO terms are available for the molecular function of these genes. However, Smith *et al.* (2018) found genes co-expressed with the Osiris cluster enriched in gene ontology terms associated with chitin/cuticle activity. The GO term analysis for the present *C. subflexa* and *C. virescens* datasets did not reveal any such associations.

During larval moulting periods, the expression of several Osiris genes is upregulated when a new cuticle layer is produced. Also, the midgut structures are shed and rebuilt (Hakim *et al.*, 2010). Furthermore, knocking down Osiris genes in *T. castaneum* beetles, especially Osiris 7, Osiris 17 and Osiris 24, led to increased lethality and changes in metamorphosis (Schmitt-Engel *et al.*, 2015).
In *D. melanogaster*, the CRISPR/Cas9-mediated suppression of Osiris 23 resulted in impaired epidermal cuticle secretion (Ando *et al.*, 2019). Furthermore, the authors detected that Osiris genes are primarily expressed in embryonic cuticle-secreting tissues at developmental stages in which the secretion is usually stimulated. Yang *et al.* (2016) injected 20E into fourth instar *B. mori* and analysed transcripts of larval midgut samples taken at different time points during moulting. Interestingly, the injection of 20E seemed to induce the expression of Osiris-related genes in the midgut. At 24 h after injection, all Osiris genes were upregulated, and the highest levels of expression of were observed

during the early moulting stage. Smith *et al.* (2018) claimed that because chitin is part of an important infection barrier (peritrophic membrane), Osiris genes (which are thought to be involved in regulating chitin) might play a role in insect immunity and detoxification. Barthel *et al.* (2016) described an upregulated immune-system activity of *C. subflexa* in response to withanolide-feeding. However, in the present thesis, such an effect could not be confirmed either by comparative transcriptomics or by PO activity assays.

Osiris genes appear to have conserved functions in the adaptation to plant defence compounds, as observed in the dietary specialists *Drosophila sechellia* and *Drosophila yakuba*. Both species feed specifically on fruits of their host plant (*Morinda citrifolia*), which contain insecticidal octanoic acid (Yassin *et al.*, 2016). Subsequent RNAi-knockdowns of Osiris 6 - 8 led to the loss of resistance in *D. sechellia* larvae and adult (Lanno *et al.*, 2019). The two genetically isolated species adapted independently to the toxic fruits, suggesting the Osiris gene cluster forms the genetic basis of toxin tolerance (Andrade López *et al.*, 2017). Similarly, Osiris genes seem to play a role in the response of the drosophilid fly *Scaptomyza flava* to their glucosinolate-containing host-plant order (Brassicales). Several Osiris genes were upregulated in larvae that fed on wildtype *Arabidopsis* compared to those that fed on glucosinolate-knockout plants (Whiteman *et al.*, 2012). Dennis *et al.* (2020) showed that in the parasitoid wasp *Lysiphlebus fabarum,* Osiris genes were more expressed in larvae (that are at the active feeding stage) than in adults, supporting a conserved role for these genes in insect immunity and the metabolism of xenobiotic compounds.

Interestingly, Osiris genes were not expressed predictably (Smith *et al.*, 2018). The Osiris 6 gene, for example, was found upregulated in *D. melanogaster* larvae challenged with caffeine and heavy metals (Smith *et al.*, 2018, and references therein), but was significantly downregulated in *Apis mellifera* larvae that had been exposed to an insect-pathogenic fungus (Aronstein *et al.*, 2010). The expression in two *Chloridea* moth species appears to be differentially regulated in response to *Physalis* fruits and withanolide-feeding. Additional transcriptomic approaches in other insects fed on *Physalis*/withanolides would likely yield interesting insights in the general responsiveness of this gene family. Overall, the relatively low RPKM values of the transcripts indicated a generally low expression of Osiris genes in the third instar of *Chloridea*. It is thought that Osiris genes are expressed in different tissues, but not exclusively in the gut (Shah *et al.*, 2012). In addition, the expression of Osiris genes peaks across developmental stages. Smith *et al.* (2018) monitored the expression of four Osiris genes in different nymph and adult stages of *Blattella germanica* cockroaches and detected high peaks only in the last nymphal stage. In lepidopteran *M. sexta*, expression peaks twice, in embryos and second instar larvae (Cao and Jiang, 2017). The results not only pointed to a conserved timing of gene expression at certain stages but also revealed very low detectability in others. The RNA-Seq analysis

of *Chloridea* third instar gut samples may have covered a developmental stage in which Osiris expression is generally low. Therefore, it would be interesting to explore the temporal dynamics of Osiris gene expression across different developmental stages of *Chloridea.* In addition, in order to shed light on the function of this understudied gene family in *Chloridea* and its potential involvement in moulting disorders, researchers should consider silencing individual genes via CRISPR/Cas9.

General remarks on the gene expression activity in *Chloridea* larvae and hybrids fed on *Physalis* fruits and withanolide-containing diet

Although the number of downregulated genes was lower than that of upregulated ones after the exposure to *Physalis* fruits, *C. subflexa* transcriptomes displayed notably fewer downregulated genes than *C. virescens*. These distinct responses indicate that *C. virescens* downregulated a considerable proportion of genes while *C. subflexa* did not decrease gene activity of not that many genes. In contrast to the transcriptomic data of larvae feeding on diet supplemented with withanolides, an upregulation of a larger number of genes was recorded on larvae fed on *Physalis* fruits. Since the fruits contain a broad spectrum of different plant metabolites, larvae were expected to respond in a stronger manner. The fact that the uptake of withanolides did not elicit a strong transcriptional response in either species and that no clear regulatory patterns could be detected, do not point to a specific mechanism of processing those compounds upon ingestion. Several upregulated genes associated with general detoxification responses were detected in *C. virescens* transcriptomes, indicating that the uptake of withanolides may have stimulated the deployment of broad defence mechanisms as a sort of stress reaction. However, the broadly decreased gene activity in *C. virescens* leads to the assumption that withanolide-feeding rather points to a general inhibitory mode of action in non-adapted species as demonstrated for instance in the Osiris gene cluster.

As neither of the two species are common model organisms, annotations can only give a hint and do not provide reliable data. Many (up to 50%) significantly expressed genes could not be annotated properly. In addition, as shown *i.a.* in the PCA, the relatively high biological variance between replicates of the same species/treatment combinations might have additionally affected the statistical power.

CHAPTER III

Chapter III: Analysis of a Potential Withanolide Metabolism in *Chloridea* Larvae

Background

So far, nothing is known about the fate of withanolides after ingestion by insects. However, the ability to minimise or prevent potentially detrimental effects of withanolides upon ingestion might be an essential adaptive advantage. Dinan *et al.* (1996) suggested that withanolides need to be activated first to exert antagonistic activity to the structurally similar 20E. The precursor molecule for ecdysteroid synthesis - cholesterol - is converted into ecdysone and its active metabolite 20E through a series of oxidation and hydroxylation steps. Members of the cytochrome P450 enzyme family (CYPs) catalyse this enzymatic conversion (Festucci-Buselli *et al.*, 2008). As shown schematically in **Figure 33**, the highly reactive unsaturated carbonyl system may pose a conceivable target point for CYP enzymes.

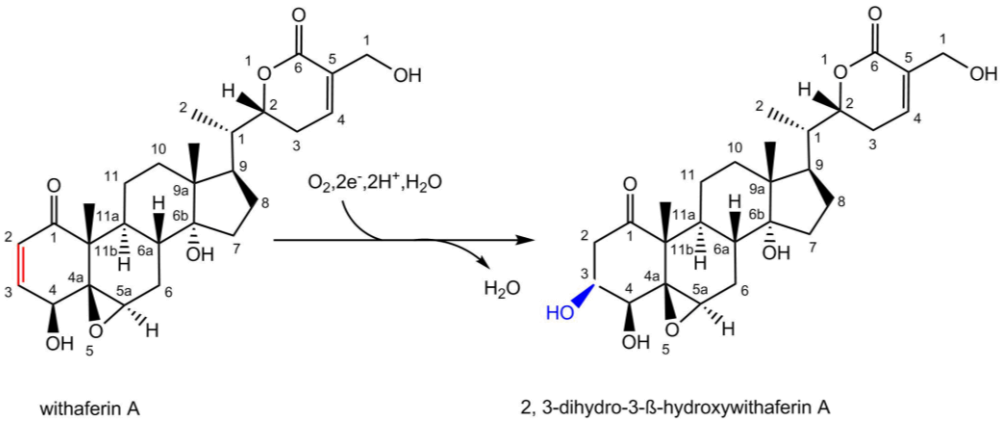

Figure 33: Potential metabolisation of withanolides by CYP enzymes upon ingestion. Hydroxylation reactions could lead to the conversion of withanolides (exemplary shown for withaferin A) into hydroxyl- or methoxywithanolides.

On this account, microsomal CYPs were concentrated after extraction from guts of *C. virescens* and *C. subflexa* larvae. The samples were compared via *in vitro* enzyme assays to identify potential differences in their metabolic properties. Many herbivorous insect species have been shown to have an efficient digestion; absorbing nutrients and eliminating potentially toxic contaminants through rapid excretion (Heckel, 2014). However, it is often not understood how adapted species metabolically circumvent the effect of defensive host-plant compounds. This chapter addresses the hypothesis that different metabolic strategies after withanolide uptake contribute to differences in the larval performance and host preference of the two *Chloridea* species.

Results

1. *In vitro* microsome assays

To test the metabolic stability of withaferin A upon exposure to CYPs, microsomes from *C. subflexa* and *C. virescens* were incubated with the test compound withaferin A and NADPH as cofactor. The resulting extracts were analysed via LC-MS/MS. Prior to testing, the integrity of the microsome was confirmed by SDS PAGE (Suppl. Figure 61). Non-target compounds with the molecular weight (MW) 472 g/mol ($C_{28}H_{40}O6$) and 474 g/mol ($C_{28}H_{42}O6$) (Table 6) were detected in the mass spectra of both species (Suppl. Figure 62 & Figure 64). An increase in the molecular mass of the parent compound by 2 and 4 g/mol indicates that hydrogenation may have occurred. These two compounds are most likely metabolites of withaferin A. However, NMR analysis to determine the chemical structure of the metabolites has not yet been performed. The fact that neither compound could be detected in the absence of microsomes strongly suggests that this conversion is mediated by microsomal CYPs (Suppl. Figure 63 & Figure 65). The MS chromatograms and peak areas did not differ notably between the two species, suggesting that withaferin A is converted in the same way in *C. virescens* and *C. subflexa* larvae (Suppl. Table 4 & Table 5).

2. *In vivo* analysis of a potential withanolide metabolism in *Chloridea* larvae

Physalis plants are rich in chemical compounds and it can be difficult to isolate sufficient amounts of withanolides from raw plant materials. In addition to the necessity of separating withanolides from other plant components such as lipids, sugars, amino acids, pigments and chlorophyll, individual withanolides must also be separated from other co-occurring withanolides and structurally related steroids. Therefore, greenhouse *P. peruviana* plants were isotopically labelled using a method previously established at the Max Planck Institute for Chemical Ecology (Feistel *et al.*, 2018). A ^{13}C-labelled withanolide compound was extracted and fed to *C. virescens* and *C. subflexa* larvae. Frass samples were analysed by LC-MS/MS and NMR to identify potential species-specific metabolic transformations.

2.1 Identification of ^{13}C-labelled withanolides in *P. peruviana* leaf extracts

Extracts from the leaf material of *P. peruviana* were fractionated and screened for compounds showing the characteristic withanolide MS fragmentation pattern (Suppl. Figure 66). A list of all detected compounds and the corresponding MS spectra are attached to the Supplementary Data (Suppl. Figure 67 & Table 6). Although the pruned *P. peruviana* plants showed excellent growth

during a preliminary test series in the labelling chambers, they did not grow well during the actual labelling experiment. Therefore, the yield of newly grown (labelled) plant material was relatively low. However, one of the two main compounds of *P. peruviana* - 4β-hydroxywithanolide E ($C_{28}H_{38}O_8$) - was identified in the fractions and sufficient labelled material could be isolated for the feeding experiment (**Figure 34**). The compound's structure was confirmed by NMR analysis (Suppl. **Figure 73**). However, neither the second main compound withanolide E nor other labelled withanolides could be obtained from the leaf extract fractions. To investigate the metabolic pathways of 4β-hydroxywithanolide E, the compound was added to the artificial diet and fed to *C. virescens* and *C. subflexa* last instar larvae.

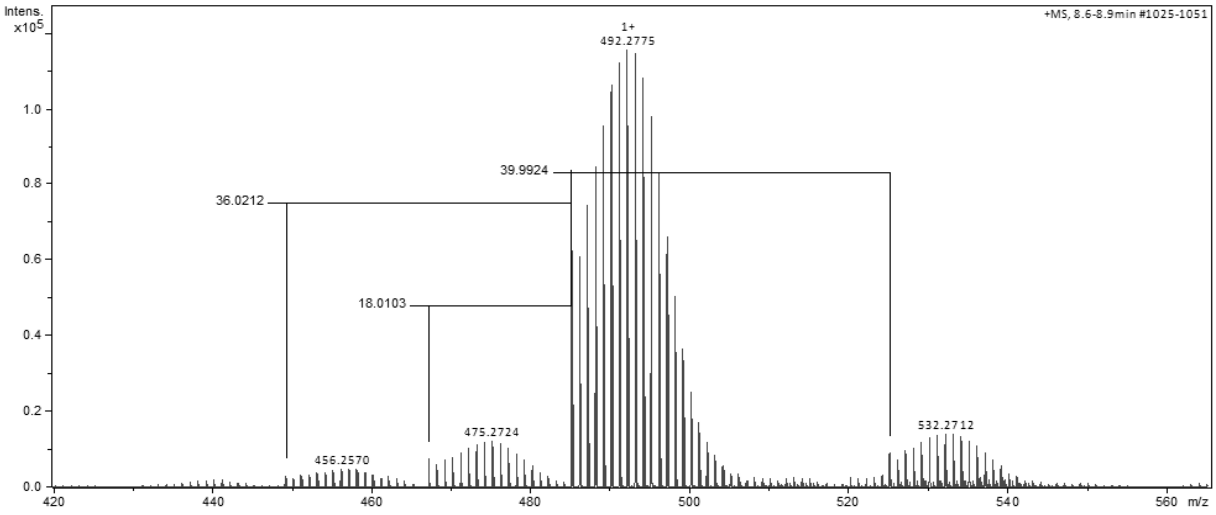

Figure 34: Characteristic isotopologue signature of [U-^{13}C]-labelled 4β-hydroxywithanolide E from *P. peruviana*. The extracted mass spectrum shows the labelled fragments at a retention time of 8.6 - 8.9 min (measured in pos. ionisation mode).

2.2 Analyses of *Chloridea* frass samples

Mass spectrometric analysis of the frass did not detect measurable amounts of the parent compound 4β-hydroxywithanolide E within the expected retention time range of 8.6 - 8.8 min (Suppl. **Figure 68** & **Figure 71**). Instead, the search for potential break-down products in the MS spectra of both species revealed the presence of two ^{13}C-labelled compounds exhibiting the characteristic isotopologue pattern. The first metabolite ($C_{28}H_{38}O_8$), with a retention time range of 8.3 - 8.4 min showed a characteristic peak at *m/z* 547.26 (Suppl. **Figure 71** & **Figure 72**). The second metabolite ($C_{28}H_{40}O_8$) showed strong signals at 8.4 - 8.5 min *m/z* 549.27. This compound was identified as the main transformant in both *Chloridea* species (**Figure 35** & **Figure 36**). Most importantly, almost identical metabolic profiles were found, suggesting that 4β-hydroxywithanolide E is not metabolised differently in the two species. Since the frass extracts of the *Chloridea* species were qualitatively similar, both frass samples were combined to obtain sufficient material for NMR analysis.

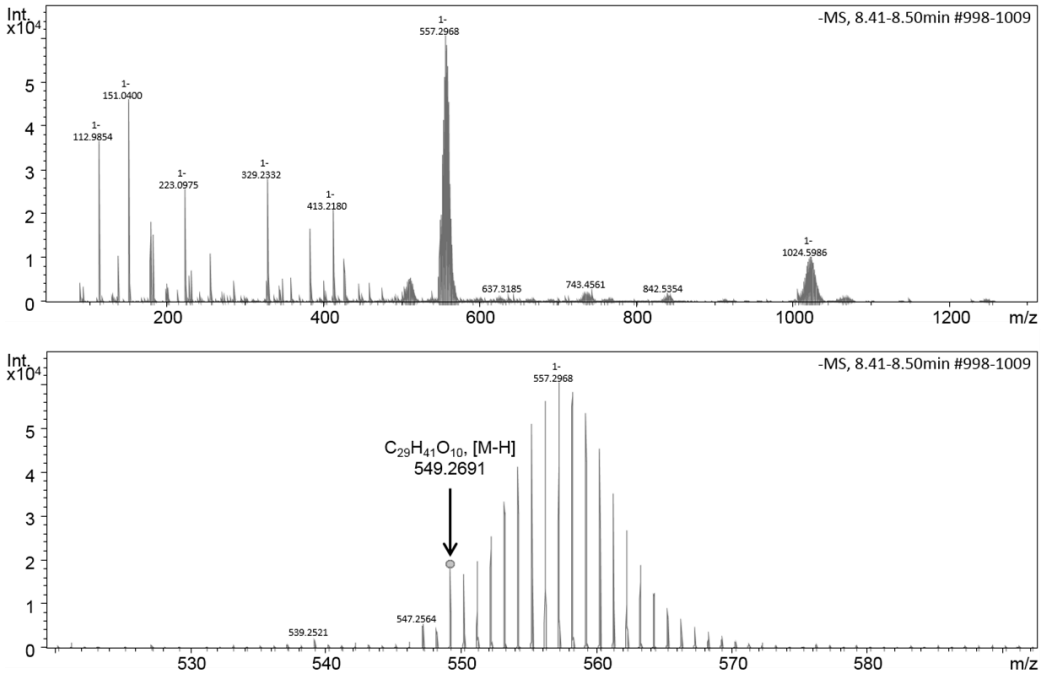

Figure 35: Extracted MS spectra from *C. virescens* frass showing the main transformant ($C_{28}H_{40}O_8$) of ^{13}C-labelled 4β-hydroxywithanolide E (formate adduct) at a retention time range of 8.4 - 8.5 min [measured in neg. ionisation mode].

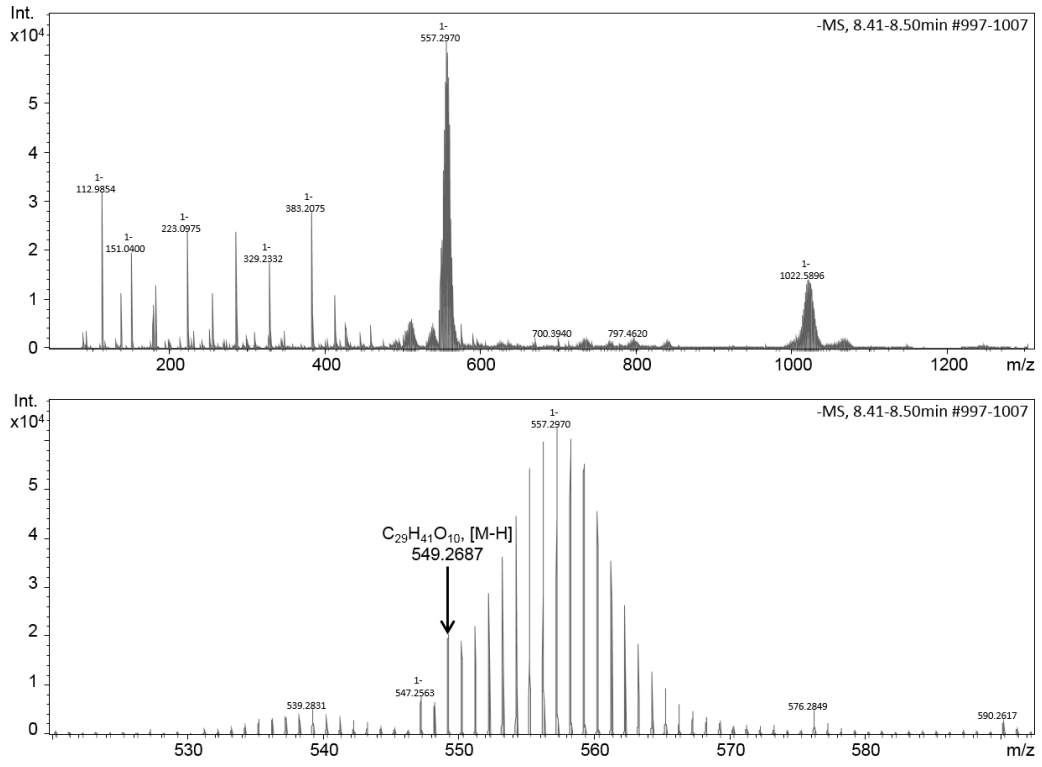

Figure 36: Extracted MS spectra from *C. subflexa* frass showing the main transformant ($C_{28}H_{40}O_8$) of ^{13}C-labelled 4β-hydroxywithanolide E (formate adduct) at a retention time range of 8.4 - 8.5 min [measured in neg. ionisation mode].

NMR structure identification revealed that the first withanolide peak detected was the starting product 4β-hydroxywithanolide E (Suppl. **Figure 74**). The main transformant was identified as withanolide S (**Figure 37A** & Suppl. **Figure 75**). These results indicate that the conversion of 4β-hydroxywithanolide occurs via a reductive chemical reaction. The predicted mechanism for this conversion is shown in **Figure 37C**. The fact that the product formed is a pure diastereomer (5α-hydroxylation) suggests a concerted mechanism. The change in substitution at position 5 (β -> α) leads to a change in spatial structure (Suppl. **Figure 76**). However, it is important to note that traces of withanolide S were also present as a minor contaminant in the original plant extract fed to the insects. However, relative to 4β-hydroxywithanolide E, withanolide S accounted for only about 7 % of the withanolides detected in the plant extract, whereas in the frass extracts about 72 % were identified as withanolide S and 28 % as 4β-hydroxywithanolide E. This is also reflected in the intensity of the compounds's peaks in the MS spectra. These results are clear evidence that 4β-hydroxywithanolide E is transformed in both *Chloridea* species after oral ingestion.

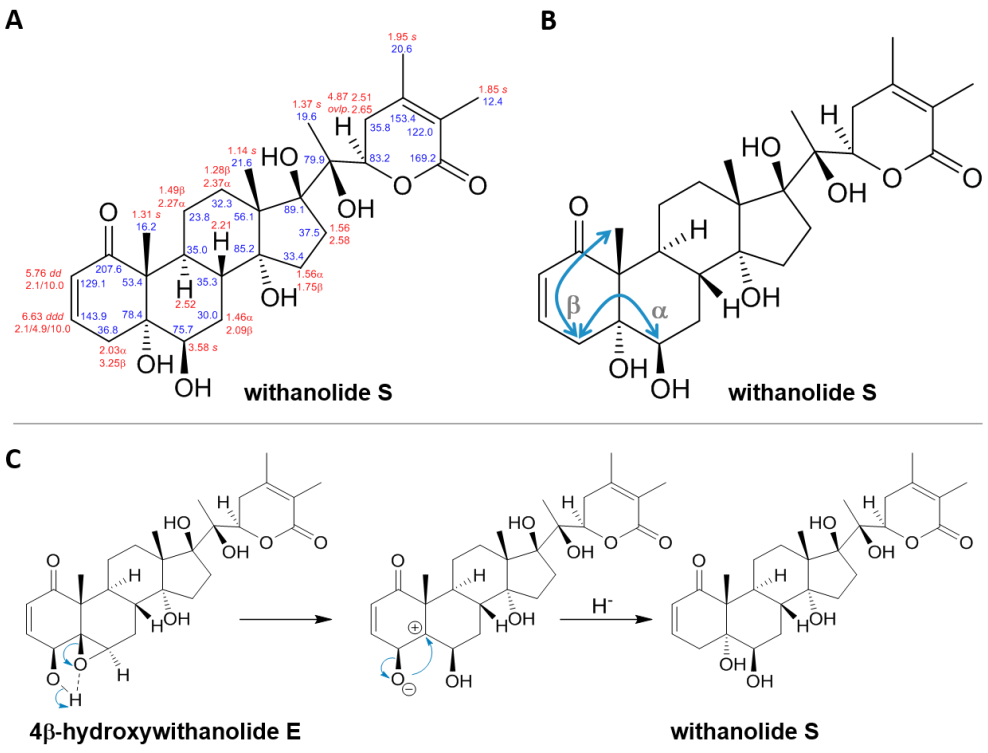

Figure 37: A) Structure of [U-^{13}C]withanolide S isolated from pooled frass of *C. virescens* and *C. subflexa* larvae after feeding on artificial diet supplemented with [U-^{13}C]4β-hydroxywithanolide E. ^{1}H chemical shifts (δ ppm) and multiplicities together with relative orientation to the structural plane are shown in red, ^{13}C chemical shifts (δ ppm) are shown in blue. **B) Important NOE correlations for structure determination of ring A and B of withanolide S** as revealed by ^{1}H-^{1}H ROESY. Blue arrows show the spatial proximity of H-4α/H-6α and H-4β and H-19$_{Me}$. **C) Predicted mechanism for the formation of withanolide S from 4β-hydroxywithanolide E.** Enzymatic catalysis and/or deprotonation of the 4β-hydroxy group may cause the opening of the 5β-epoxide. Nucleophilic attack on the resulting carbocation and subsequent hydride transfer may lead to the formation of withanolide S.

Discussion & Outlook

Since their first detection in *P. peruviana*, many studies have reported the discovery of novel withanolides and derivative forms. The list of known withanolides includes physalins, withaperuvins, physagulides and many others, of which withanolide E and 4β-hydroxywithanolide E are the major components (Baumann and Meier, 1993; Huang *et al.*, 2020). A complete and structured metabolic profile of *P. peruviana* does not exist. In the present thesis, several different compounds were found that exhibited the typical withanolide fragmentation pattern in spectroscopic analysis. However, these compounds were not further characterised, as the identification of the two main components withanolide E and 4β-hydroxywithanolide E was the main focus.

So far, the fate of withanolides after *in vivo* uptake has only been studied in mammals. Liu *et al.* (2018) identified the chemical structure of two sulfonate metabolites of physalin A from *P. alkekengi* var. *franchetii* in rat faeces. The authors speculated that sulfonation is an important step for the rapid elimination of this compound. Previously, Feng *et al.* (2017) had detected a total of 22 metabolites of physalin A in the faeces of rats. According to their findings, the main metabolic pathways for physalin A appear to be sulfonation, reduction and hydroxylation. Dai *et al.* (2019) analysed the metabolites of withaferin A *in vitro* using human liver and rat microsomes. The authors observed rapid elimination of withaferin A and discovered seven major metabolites, three of which were detected only in human liver microsome assays. Using multiple reaction monitoring information-dependent acquisition-enhanced production (MRM-IDA-EPI) scanning, hydrogenation, hydroxylation and hydrolysis were confirmed as the main metabolic pathways. Interestingly, this study also examined the metabolites of withaferin A formed upon exposure to extracts from the intestinal microflora of humans and rats. Although the depletion of the compound was relatively slow and inefficient, measurable metabolic conversion still occurred. A potential involvement of gut microbes in withanolide degradation is further discussed in chapter IV.

Microsomal enzymes from *Chloridea* larvae are able to convert withaferin A

Insect CYP enzymes initiate a wide range of different chemical reactions, including hydroxylation, oxidation, epoxidation, dealkylation and numerous others (Feyereisen, 2012). CYPs have been hypothesised as conceivable candidates for catalysing the withanolides metabolism, and the present results suggest that *Chloridea* CYPs do indeed affect the metabolic stability of withaferin A. Based on the observed mass shifts in the MS-spectra, it can be speculated that hydration may take place e.g. at the double-bonds of the steroid backbone. However, this needs to be proven by further investigations of the chemical structures of the metabolites using NMR.

Plant-derived compounds have been shown to induce the transcription of CYP genes (Feyereisen, 2012). However, the existing transcriptome data from *Chloridea* larvae feeding on diet supplemented with withaferin A or with *P. peruviana* withanolide extracts did not reveal any outstanding patterns in the CYP gene expression within and between species. To confirm that withanolides are substrates of lepidopteran CYPs, it would be interesting to express candidates of microsomal CYP genes in insect cell lines to test their activity in future enzyme assay.

Larvae of *C. virescens* and *C. subflexa* do not seem to differ in the metabolic processing of 4β-hydroxywithanolide E

LC-MS/MS screening of the frass products showed that 4β-hydroxywithanolide E was converted by a reductive chemical reaction (addition of hydrogen). The spectroscopic analysis of metabolites from frass provides a reliable method for detecting the actual end products of the metabolic pathways. In contrast, metabolites detected in microsome assays may only reflect intermediate products of the metabolic pathways. It is therefore possible that metabolic phase I enzymes such as CYPs are primarily involved in the initial steps, but that the compounds are further processed through a series of biochemical transformations until they are finally converted to the excreted metabolites. Usually, phase I reactions are mediated by esterases and CYPs and facilitate excretion or promote phase II metabolism by increasing the compound's polarity (Yu, 2008). Phase II reactions include a wide range of other metabolic processes such as the conjugation of functional groups like sulfates, sugar moieties or glutathione. The observed sulfonation of physalin A in rats represents a typical phase II biotransformation (Liu *et al.*, 2018). However, the observed mass shifts of withaferin A and 4β-hydroxywithanolide E detected in the frass do not point to massive substrate modifications. Instead, the predicted chemical reduction suggests a typical phase I reaction. Since withanolide S was detected not only in the frass but also in the leaf extracts, it is possible that this conversion may also take place in the plant. It remains unclear whether this conversion is catalysed by enzymes (e.g. CYPs) or whether other factors such as the highly alkaline pH value in the larval midgut lumen may lead to this reaction. As only one labelled compound of the many withanolides of *P. peruviana* could be isolated in sufficient quantities for use in feeding assays, it is important to improve the yield of further interesting candidates for future experiments.

Is a metabolic conversion of withanolides required to feed on *Physalis* plants with impunity?

As discussed in chapter II, withanolides resemble in structure insect hormones and defensive plant-derived hormone analogues. Although a direct phytoecdysteroidal function has not yet been

confirmed, withanolides may well act in a similar way. Interestingly however, previous studies have reported high resistance to phytoecdysteroids particularly in some Noctuidea species (Duan *et al.*, 2020). Follow-up studies on the metabolic pathways of ingested exogenous ecdysteroids revealed that this high resistance is primarily achieved by the metabolic conversion of these compounds in the insect gut to 22-long-chain-fatty-acyl esters via ecdysteroid-22-O-acyltransferases (Duan *et al.*, 2020, and references therein). Since esterified 20E is significantly less active in *C. virescens* than 20E, this conversion is considered an essential step in the detoxification mechanisms of such compounds (Zhang and Kubo, 1992). In this thesis, however, no characteristic peaks were discovered in the MS spectra that would indicate an esterification reaction of the test compounds.
Besides an esterification-guided metabolism, ecdysone-20 monooxygenases may affect withanolides that are structurally-related to 20E. Furthermore, there are several CYP enzymes with ecdysone-20 monooxygenase activity (Weirich, 1997; Feyereisen, 2012). Differential metabolic modification of potentially phytoecdysteroidal-active withanolides could theoretically explain why moulting disorders were only observed in *C. virescens*. However, it is important to note that the current results do not point to a species-specific metabolic conversion, as *C. subflexa* and *C. virescens* did not differ in their metabolic profiles in either assay.

To date, there is no clear evidence that metabolic conversion of withanolides is required to feed unscathed on withanolide-producing plants. From the present results, it cannot be concluded whether the metabolic conversion of withaferin A or 4β-hydroxywithanolide E contributes to rapid excretion and/or conversion to non-toxic or low toxic compounds. Therefore, this conversion may not be of biological relevance. The results do not suggest rapid elimination of the tested withanolides as described e.g. for phytoecdysteroids (esterification) or physalin A (sulfonation). The predicted conversion of withaferin A and 4β-hydroxywithanolide E does not appear to make the compounds notably more polar, so they are not necessarily more easily excreted. However, changes in the spatial arrangement (stereochemistry) of the molecules after conversion may affect the binding affinity of withanolides (e.g to hormonal receptors or to the active site of enzymes). It is therefore conceivable that withanolides are activated and only take full effect after metabolic conversions.

Interestingly, CYP enzymes can generate metabolites with significantly higher toxicity than the original parental compound (Feyereisen, 2012). However, little is known about the effect of withanolides on insect cells. Clear evidence of a toxic mode of action would help to assess the effect of ingested withanolides and their metabolites on herbivorous insects. In addition, quantitative frass analyses of the two *Chloridea* species could show whether the larvae differ in the amount of labelled compounds excreted. Although there is no indication in the literature on a potential withanolide

sequestration, it is not yet clear whether the same amount of withanolides is excreted as was ingested. Therefore, absorption of withanolides via the larval gut epithelium cannot be excluded.

As mentioned above, the close relationship between *C. virescens* and *C. subflexa* may pose a problem in identifying potentially harmful effects of withanolide uptake. Because the two species are so closely related, they may not differ in their metabolic processing of withanolide compounds. Therefore, it would be very interesting to include more distantly related Lepidoptera, where harmful effects of withanolides are more pronounced than in *C. virescens*. In brief, the preliminary data analysing a potential withanolide metabolism do not suggest that the two species process withanolides differently. However, this thesis clearly shows that withanolides can be transformed *in vivo* upon ingestion of *Chloridea* larvae and also after *in vitro* exposure to *Chloridea* microsomes. The results indicate that reduction (e.g. hydrogenation) is a plausible metabolic pathway of withanolides.

CHAPTER IV

Chapter IV: Antimicrobial Activity of Withanolides and their Effect on the Gut Microbiome of *C. virescens* and *C. subflexa* larvae

Background

Plant metabolites may strongly contribute to shaping the plant microflora by selectively preventing the growth of certain microorganisms and favouring the establishment of others. Each plant species may therefore harbour a specific core microbiota based on its individual chemical profile and habitat. Beyond their own chemical defence arsenal, plant responses against herbivory and pathogenic infections have been shown to be substantially supported by mutualistic microorganisms (van Loon *et al.*, 1998; Sugio *et al.*, 2015). Recent studies, for instance, demonstrated that several bacterial isolates from *P. peruviana* inhibit the growth of the major *Physalis* pathogen *F. oxysporum* (Urrea *et al.*, 2011; Toloza-Moreno *et al.*, 2020).

The search for novel sources of antibiotics and medicinally relevant plant metabolites has been the subject of numerous recent studies (reviewed in Compean, 2014). While many compounds clearly exhibit potent antimicrobial activity, their mode of action is often poorly understood. This is also true for withanolides that are without controversy antimicrobially active (reviewed in Sell *et al.*, 2021). Withanolides have been reported to act primarily against Gram-positive bacteria and showed no or only moderate activity against the Gram-negative type. Opposite to Gram-positive bacteria, Gram-negative bacteria possess an outer membrane, which is the main reason for their resistance to a wide range of antibiotics (Exner *et al.*, 2017). By including both Gram-types and different bacterial classes in inhibition zone (IHZ) assays in this thesis, I aimed to confirm the observed strong effect on Gram-positive bacteria and to discover potential patterns in morphological features from which resistance against withanolides may have arisen.

Plant diet and plant defence compounds not only affect physiological processes in insects, but probably also influence the composition of the larval gut microbiome. A recent study on the generalist Noctuidea larvae of *Trichoplusia ni* showed that the microbial composition strongly depends on the host plant it is reared on for several generations (Leite-Mondin *et al.*, 2021). This example indicates that there can be shifts in the microbiota in response to the diet, so that, for example, some taxa with low abundance become dominant and, *vice versa*, that the proportion of previously dominant taxa decreases. There is also an increasing number of studies that seek to understand how microorganisms may be involved in insect adaptations to plants. Some microbial taxa contribute to the degradation of plant materials through cellulolytic, xylanolytic as well as

pectinolytic activity (Anand *et al.*, 2010; Suen *et al.*, 2010). Apart from digestive support, symbionts can also provide nutrients and protect their hosts from pathogens (Paniagua Voirol *et al.*, 2018; Compant *et al.*, 2019). The insect digestive system is the site for dietary intake and detoxification processes commonly targeted by plant defences through physical and functional disruption (Mason *et al.*, 2019). Any damage to gut tissues can adversely alter their protective functions allowing pathogenic microbes to invade the body cavity. Bacterial communities of herbivorous insects vary strongly depending on the insects' host-plant diet, but some studies also report a stable core community even across different food sources (Ceja-Navarro *et al.*, 2015; Jones *et al.*, 2019; Leite-Mondin *et al.*, 2021). These microbial communities may considerably influence host traits associated, for example, with host-plant use and pathogen protection (Oliver *et al.*, 2010; Hansen and Moran, 2014). Barthel *et al.* (2016), for example, showed that *P. peruviana* withanolide extracts exert antimicrobial activity against the spores of the insect pathogen *B. thuringiensis*. The presence of antibiotic withanolides may change the abundance and taxonomic diversity of microbes in insects. This would apply in particular to insects such as the specialist *C. subflexa*, which are exposed to withanolide-producing plants over a long period because they use them as their only food source. Therefore, the second part of this chapter addresses the question of whether the bacterial composition changes in *C. virescens* and *C. subflexa* when larvae are fed on withanolide-spiked diet. Data for this part of chapter IV were obtained and analysed as part of a master thesis project by Dries Amezian under the supervision of Marie Pauline Sell (Amezian, 2018). The results are here revised and re-interpreted with consideration of novel research findings. Dries Amezian kindly gave permission to publish the results of his master thesis as part of this dissertation.

Results

1. Antimicrobial activity of withanolides

IHZ assays were performed to assess the susceptibility of selected bacteria and three fungal strains to withaferin A and different fractions of *P. peruviana* leaf extracts (see page 22, section 2.1.) The test organisms included the Gram-negative plant pathogens *A. tumefaciens*, *P. syringae*, *P. ananatis*, two pathovars of *X. campestris*, and the phytopathogenic fungi *Alternaria* spp. and *F. brachygibbosum*. Pathogens strongly associated with nightshade hosts were represented by Gram-positive *R. fascians*, *C. m.* subsp. *michiganensis*, Gram-negative *R. solanacearum* and *F. oxysporum* f. sp. *lycopersici*.

1.1 Plate inhibition zone assays

The antimicrobial activity of the withanolide extracts was qualitatively evaluated by inspecting the degree of inhibition in agar diffusion tests (IHZ assays). 40% methanol was used as a negative control and bacteria-specific antibiotics were applied for positive testing. Withaferin A, the fresh extract (fraction III) and the old extract showed moderate to strong inhibitory activity against Gram-positive plant pathogens *R. fascians, C. m.* subsp. *michiganensis* (Figure 38A & B) and the model bacterium *B. subtilis* (Figure 39E). As predicted in the literature, no growth inhibition was observed for all test compounds against the Gram-negative plant pathogenic *P. syringae* and *A. tumefaciens* (Figure 38G & H) and Gram-negative *E. coli* (Figure 39F). However, withaferin A and both *Physalis* extracts (fresh & old) exhibited antibacterial activity against the Gram-negative species *P. ananatis, R. solanacearum* and *X. campestris* pv. *horticola* (Figure 38C, D & F). Interestingly, only withaferin A, but none of the other test compounds inhibited the growth of the pathotype *X. campestris* pv. *campestris* (Figure 38E). This result indicates a remarkably high degree of specificity even at sub-specific levels. Interestingly, the most sensitive phytopathogen appeared to be *C. m.* subsp. *michiganensis*, as it was the only bacterium inhibited even by crude leaf extracts (Figure 38B [2]). In addition to the plant pathogens, two insect-associated Gram-positive bacteria (+ spores) were included in the tests (Figure 39B - D). Whereas the growth of both vegetative and sporulated *Bt* cells was inhibited by all withanolide treatments, only withaferin A displayed antibiotic activity against the common insect gut inhabitant *E. mundtii*. Withaferin A, fraction III and the old *Physalis* extracts exhibited clear inhibition zones in *Alternaria* spp. inoculated plates (Figure 39A). The compounds were also tested against two *Fusarium* strains, but as several different positive controls did not yield an appropriate result, this test was excluded from the analysis. As expected, purified and commercially available withaferin A generally displayed stronger activity than multi-component *Physalis* extracts.

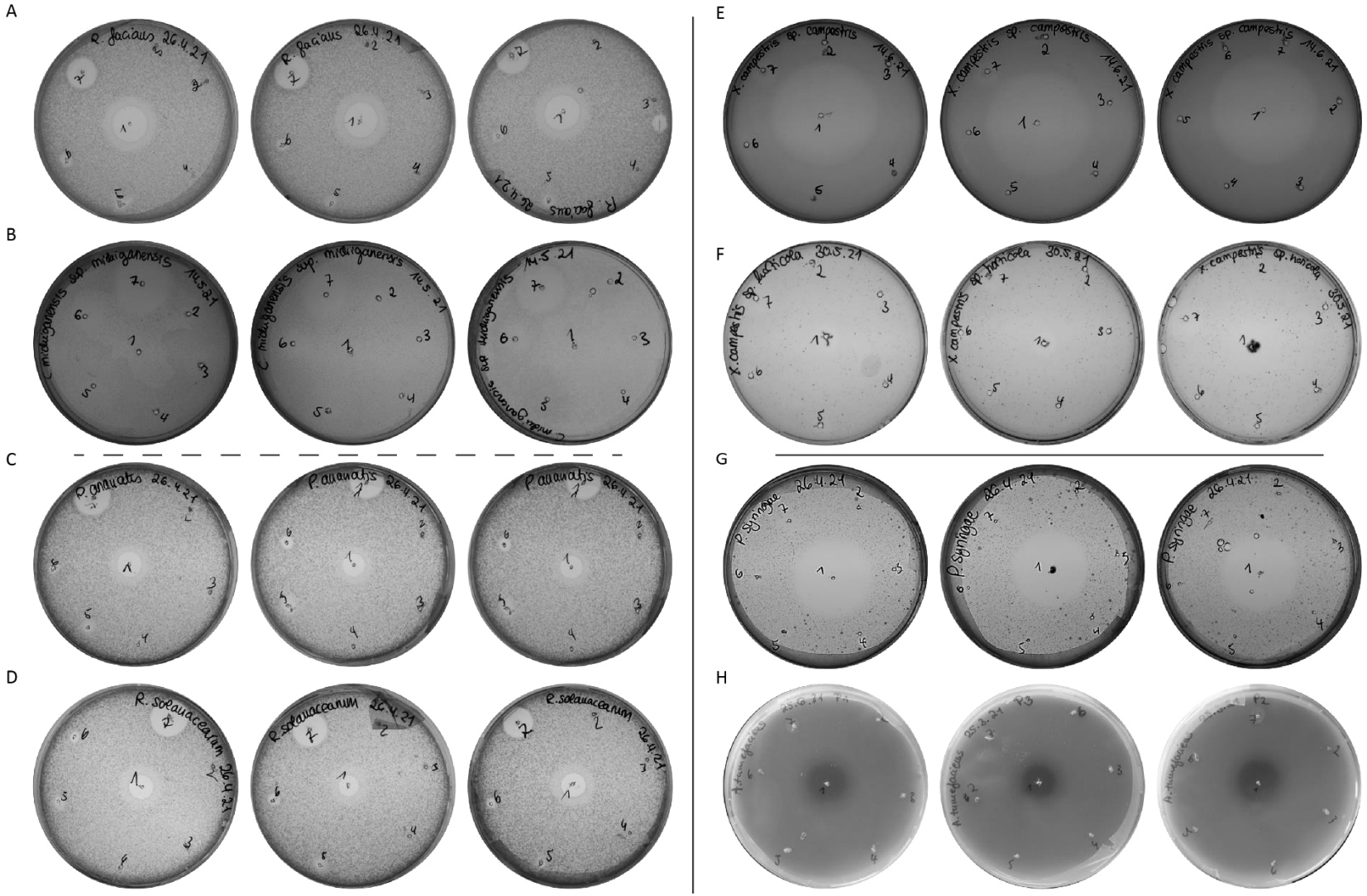

Figure 38: Inhibition zone assays I of common plant pathogens: Gram-positive **A)** *R. fascians* and **B)** *C. michiganensis* pv. *michiganensis*. Gram-negative **C)** *P. ananatis,* **D)** *R. solanacearum*, **E)** *X. camprestris* pv. *campestris*, **F)** *X. campestris* pv. *horticola*, **G)** *P. syringae* and **H)** *A. tumefaciens.* 1) antibiotic, 2) crushed *Physalis* leaves, 3 - 5) fraction I - III of fresh *Physalis* extracts, 6) old *Physalis* extracts and 7) withaferin A.

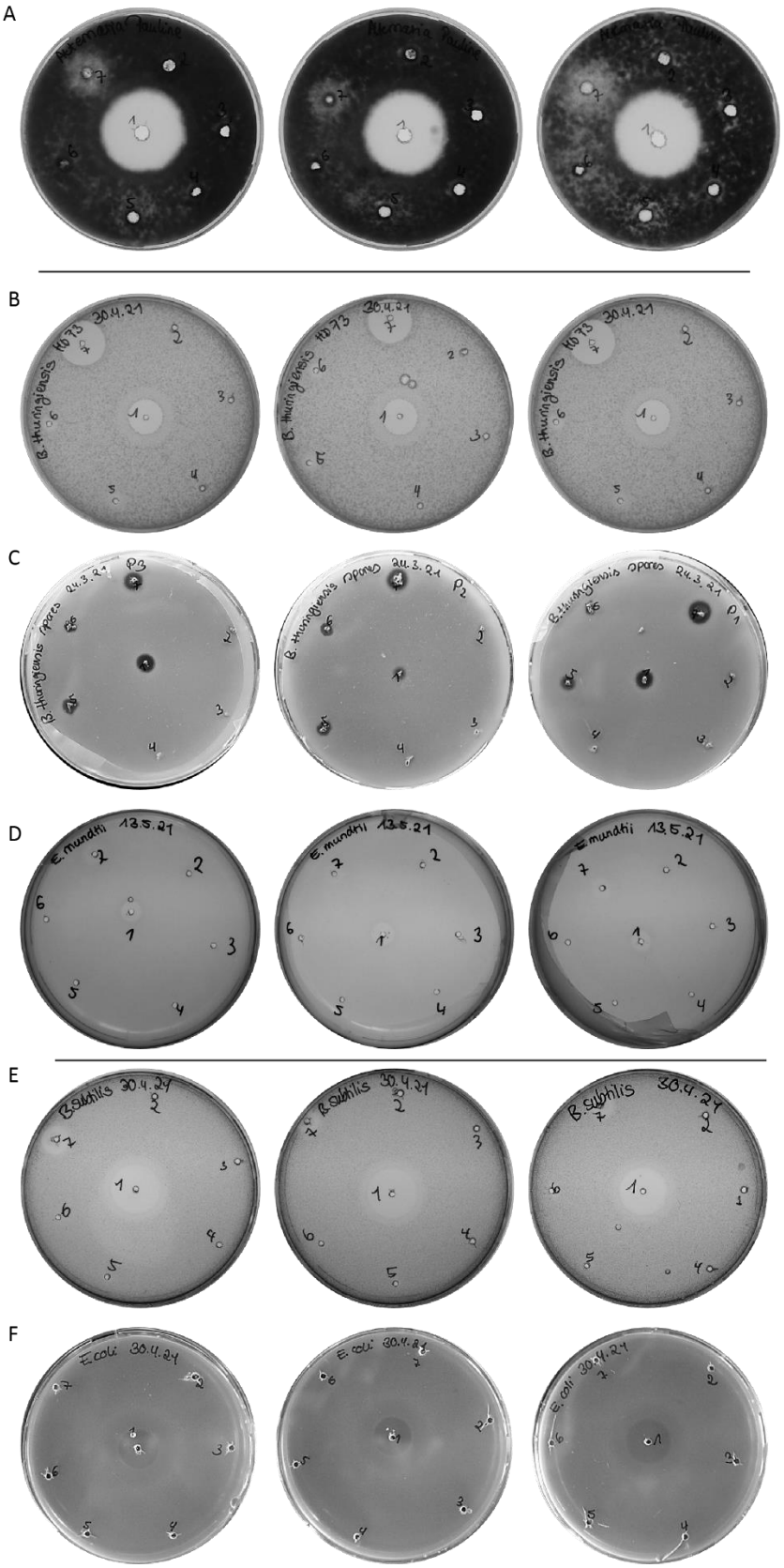

Figure 39: Inhibition zone assays II with **A)** the fungus *Alternaria* spp., the insect-associated **B)** *Bt* cells, **C)** *Bt* spores and **D)** *E. mundtii*. The Gram-positive bacteria **E)** *B. subtilis* and **F)** Gram-negative *E. coli* represent common model bacteria. 1) antibiotic, 2) crushed *Physalis* leaves, 3-5) fraction I-III of fresh *Physalis* extracts, 6) old *Physalis* extracts and 7) withaferin A.

1.2 Dose response curve

In a previous study by Barthel *et al.* (2016), *P. peruviana* extracts showed high antimicrobial activity against vegetative *Bt* cells and *Bt* spores. This effect could also be demonstrated in the above shown IHZ assays (**Figure 39B**). The authors speculated that withanolide-uptake might confer protection against common insect pathogens. Easy to cultivate *Bt* were used exemplarily to determine the dose levels at which a response was elicited by withaferin A and *Physalis* extracts. A dose response model was generated to estimate the concentration required to inhibit the growth of 50% of *Bt* cells (ED_{50}) after an exposure time of 24 hours in liquid media (**Figure 40**). The amount of withaferin A required for half of the maximal effective response was ~22 µg/ml (~6 µg/ml for gentamicin), corresponding to a molar concentration of ~46.75 µM (~8.45 µM gentamicin). As expected, a higher dose of the less purified *Physalis* extracts was required to elicit a response. An exact molar concentration cannot be determined because this mixture contains different withanolide compounds and their actual concentration is not known. *Physalis* extracts were nevertheless included in the model to better illustrate the differences in the inhibitory potency compared to withaferin A, as shown already in IHZ assays.

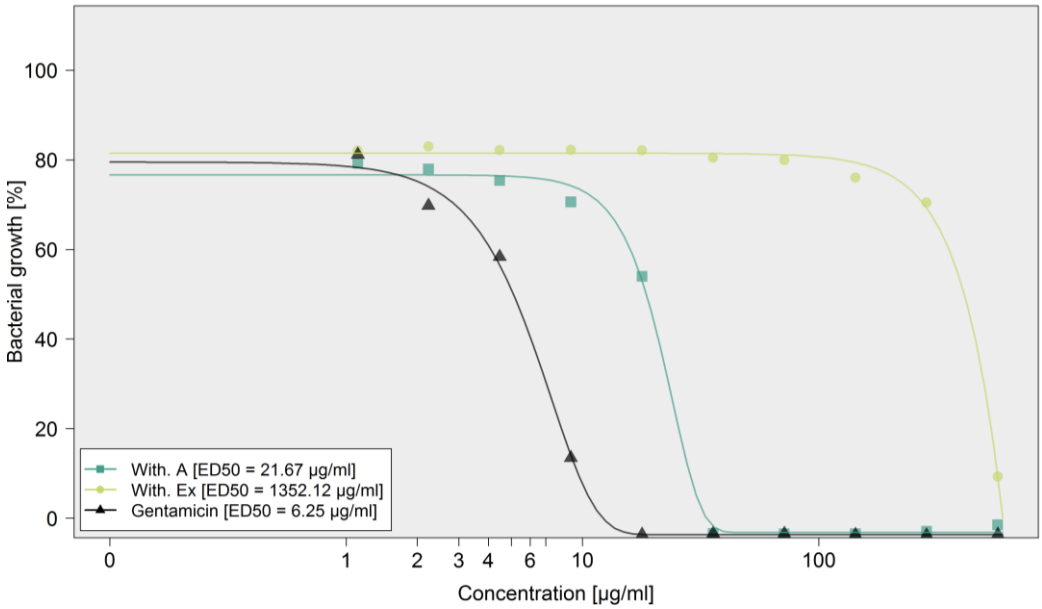

Figure 40: Effective dose estimation. Separate downward sigmoidal dose response curves of withaferin A (With. A), *Physalis* withanolide extracts (With. Ex) and gentamicin against *Bt* cells. Decreasing bacterial growth is plotted against the concentration of the test solution. The estimated ED_{50} are shown in the legend. A fitted dose response model was generated using the drc package in R.

2. Microbiome analyses of laboratory-reared *Chloridea* larvae

Metagenomic sequencing was performed to infer the relative abundance of bacterial genera and families in the gut of the two *Chloridea* species. Based on bacterial 16S rRNA amplicon sequencing, sequence-based differentiated taxa or operational taxonomic units (OTUs) were assessed in the gut microbiome of larvae to determine whether diet affects the composition of the microbial communities. Larvae were fed on either control diets or diets containing withaferin A or *Physalis* extracts. In this metagenomic study, the gut bacterial communities of laboratory-reared *C. subflexa* and *C. virescens* were compared interspecifically and intraspecifically across the three different diet groups. For *C. virescens* the total number of counts per sample ranged from 22687 to 33617, whereas for *C. subflexa* six out of 15 samples had less than 1000 counts (lowest count: 388).

2.1 General assessment of bacterial communities at family level

Analysis of the composition of the major gut bacteria revealed that Enterococcaceae was the dominant family in both species with 99.89% (*C. virescens*) and 84.57% (*C. subflexa*) (**Figure 41**). With the exception of *E. cecorum* in *C. virescens* (0.03%) and *E. casseliflavus* in *C. subflexa* (0.11%), most *Enteroccoccus* spp. could not be classified down to the species level. With a proportion of 12.29%, Bacillaceae formed the second largest group in the specialist species. Only five different families were detected in *C. virescens*, whereas *C. subflexa* guts contained 70 different families of which 60 families alone represented a proportion less than 0.01% of the entire family abundance.

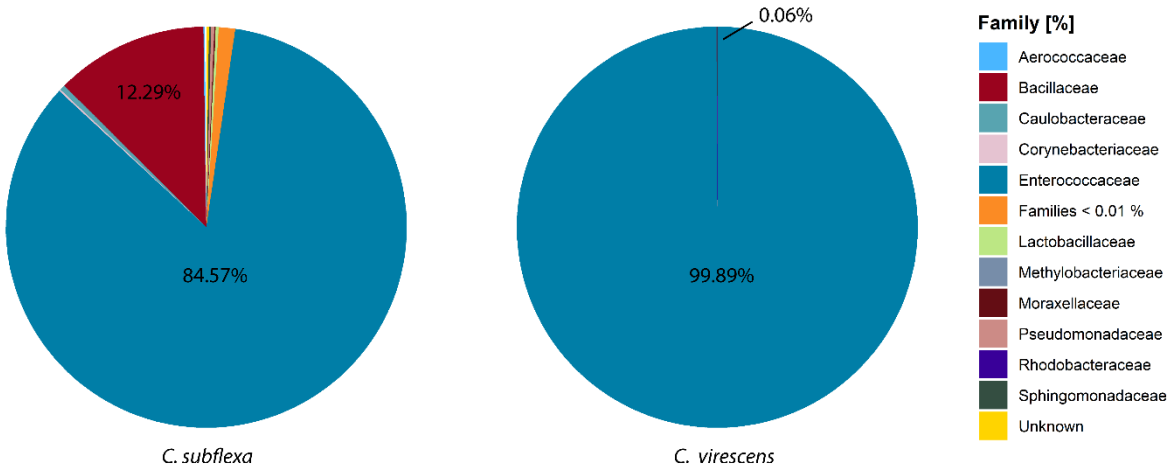

Figure 41: Microbial community structures represented at the family level. This figure was created based on data from the master thesis of Dries Amezian (Amezian, 2018). Data from all diet test groups (withaferin A, withanolide extracts and the control diet) was combined for this analysis.

2.2 Relative abundance of the bacterial composition

The relative abundance of bacterial taxa (genus or family) in all pools (per species and per treatment) is shown in a heatmap diagram (**Figure 42**). This figure illustrates the strong dominance of *Enteroccoccus* species in all diet treatments and general differences in taxon richness between the two moth species.

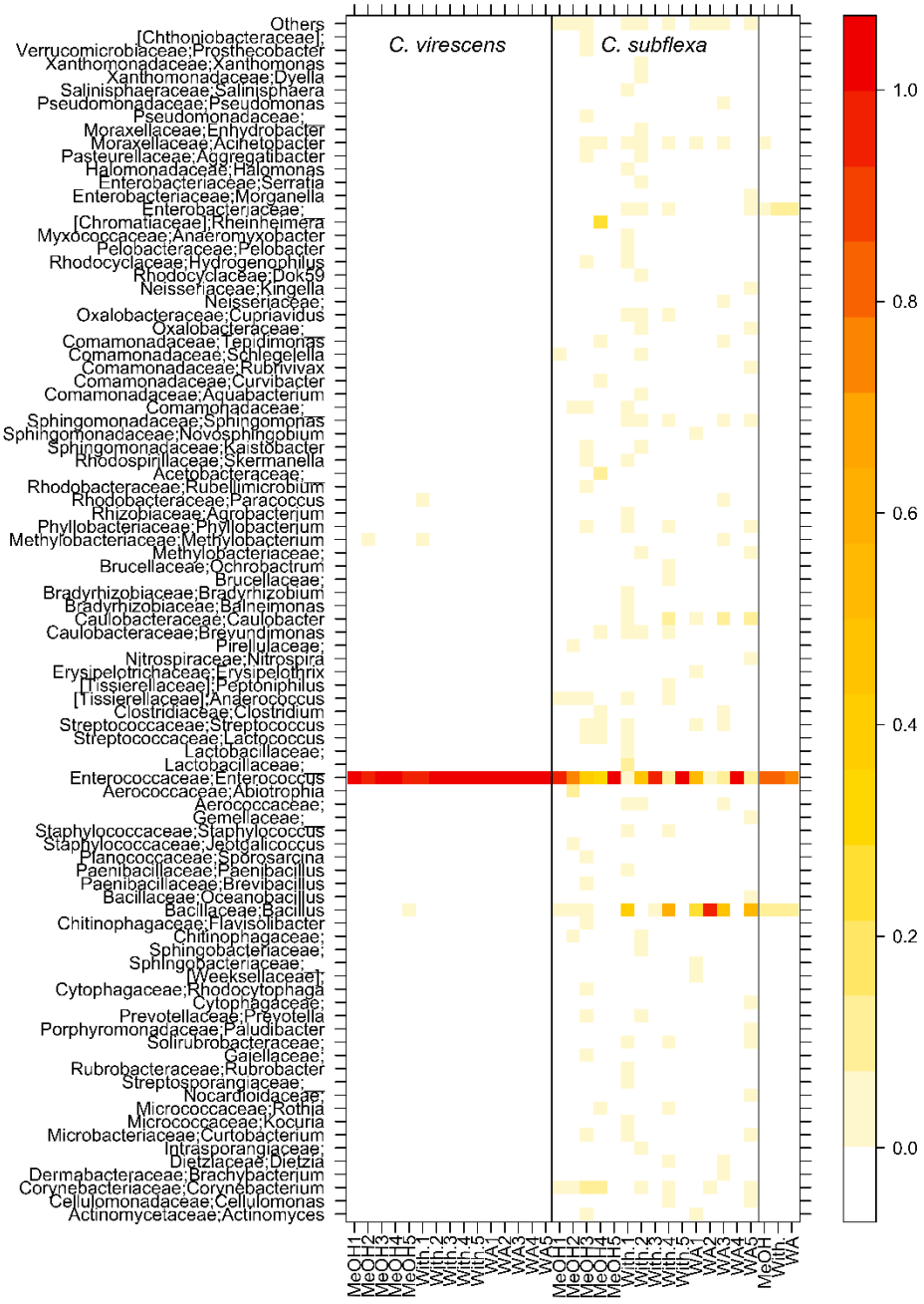

Figure 42: Bacterial composition profiles in gut sample of *C. virescens* and *C. subflexa* identified by 16S rRNA amplicon sequencing**.** Shown is the relative abundance (0 - 1) of each genus or family when the best classification was only possible up to that taxonomic level. Subset samples 1 - 15 *C. virescens* and 16 - 30 *C. subflexa* larvae. The last three columns show the negative controls (blank) for each diet treatment.

In contrast to the combined analysis of pooled samples (**Figure 41**), the heatmap better reflects the variance in species abundance between pools of the same treatment and between the two species. Especially the samples of *C. subflexa* guts vary greatly within replicates of the same diet treatment. However, apart from the high variability in individual pools, no significant changes in bacterial community profiles were detected between the different treatments of both species. Furthermore, the heatmap highlights the low species diversity in *C. virescens*, as already shown in **Figure 41**. Overall, all *C. subflexa* pools show higher taxonomic diversity compared to *C. virescens*. Besides the predominant occurrence of *Enterococcaceae* species in both species, *Bacillaceae* bacteria were the second most abundant group in *C. subflexa*. Both taxonomic groups were also detected in the negative controls; artificial diet treated with either withaferin A, *Physalis* extracts or MeOH (control).

2.3 Alpha-diversity and observed richness at family level

The Shannon-Wiener index was calculated to describe the α-diversity between different treatments. In addition, taxonomic richness for each treatment was defined as the mean in richness for each gut pool. In *C. subflexa* guts, neither α-diversity nor species richness were significantly affected by withanolides compared to the control (**Figure 43**). It is important to note that all treatments in *C. subflexa* exhibited a high variance in richness within the pooled samples and especially in α-diversity compared to its sister species. As with *C. subflexa*, no significant intraspecific differences in α-diversity or richness were observed in *C. virescens*.

In the interspecific comparison, significant differences in α-diversity ($p < 0.005$) and species richness ($p < 0.001$) were assessed between the two species using a Wilcoxon rank sum test. The species differed significantly in both categories. In addition, it was investigated whether the composition of the different bacterial orders changed in the control diet treatment and the treatments containing withanolides. Only in samples of *C. subflexa* larvae fed with withaferin A-containing diet, shifts were documented in the abundance of the order of Bacillales bacteria (**Figure 44**). Whereas in the control samples a proportion of only 0.27% consisted of Bacillales, in the larvae feeding on withaferin A this family accounted for 31% of the entire gut microbiota. The proportion of Bacillales increased only slightly in larvae fed on diet with withanolide extracts (+ 3.73%). These differences likely occurred because of the different concentrations of withanolides in the test agents. As already shown in the IHZ and in the calculations of the effective dose, pure withaferin A was more efficient in inhibiting bacterial growth than multi-component *Physalis* extracts. This was also true for the inhibition of *E. mundtii* growth (Lactobacillales) (**Figure 39D**).

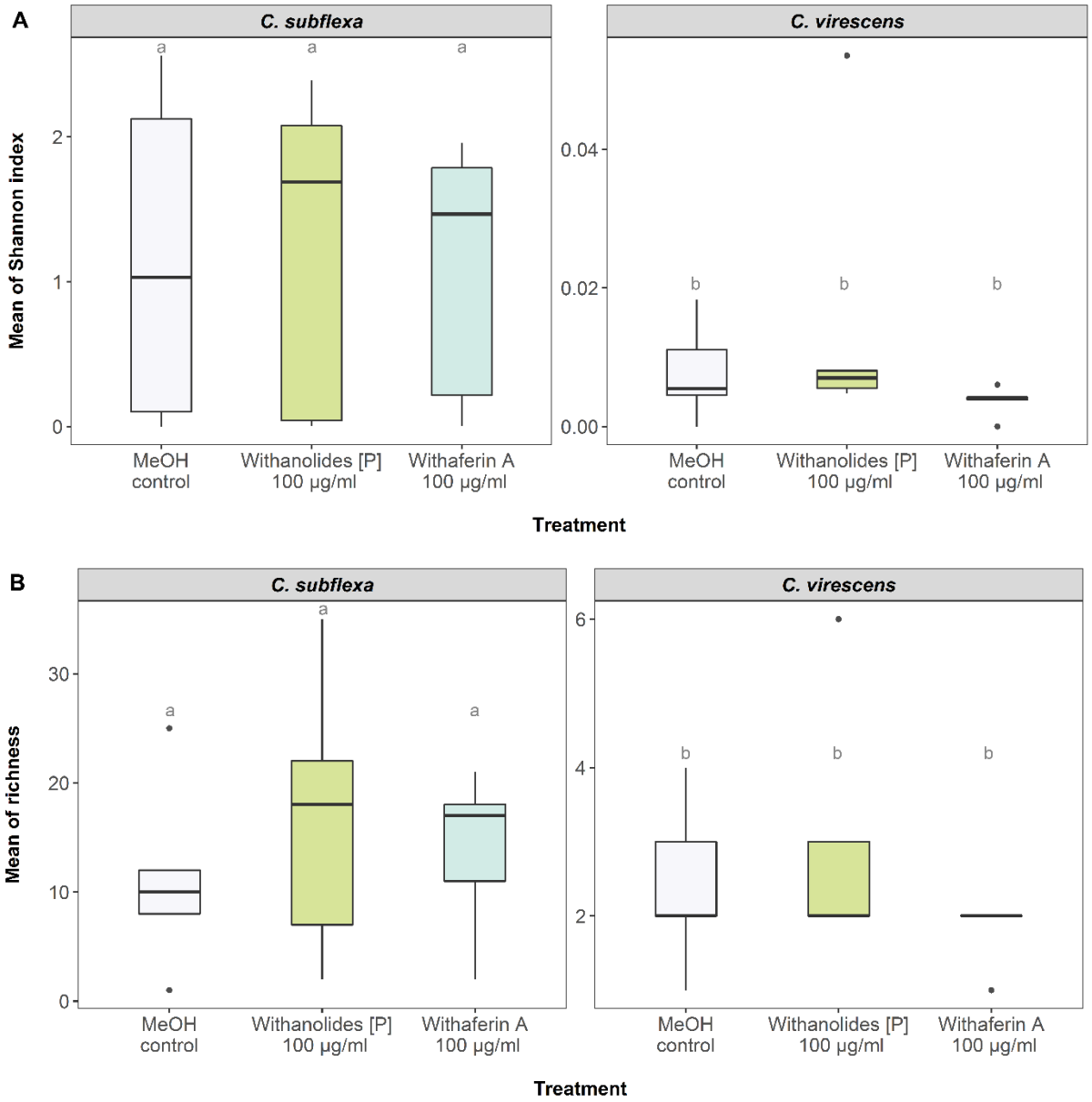

Figure 43: A) α-diversity and B) observed richness of OTUs in guts of *C. subflexa* and *C. virescens* larvae feeding on a control diet or on artificial diet supplemented with 100 µg/ml *Physalis* extracts or withaferin A. The scale on the y-axis is adapted to the species. Differences in richness ($p < 0.001$) and α-diversity ($p < 0.005$) between the two species were assessed using a Wilcoxon rank sum test. The effect of the diet treatment on diversity and richness was analysed using a Kruskal-Wallis test. Letters indicate statistically significant differences between and among species. Both plots were modified from data of the master thesis of Dries Amezian (Amezian, 2018).

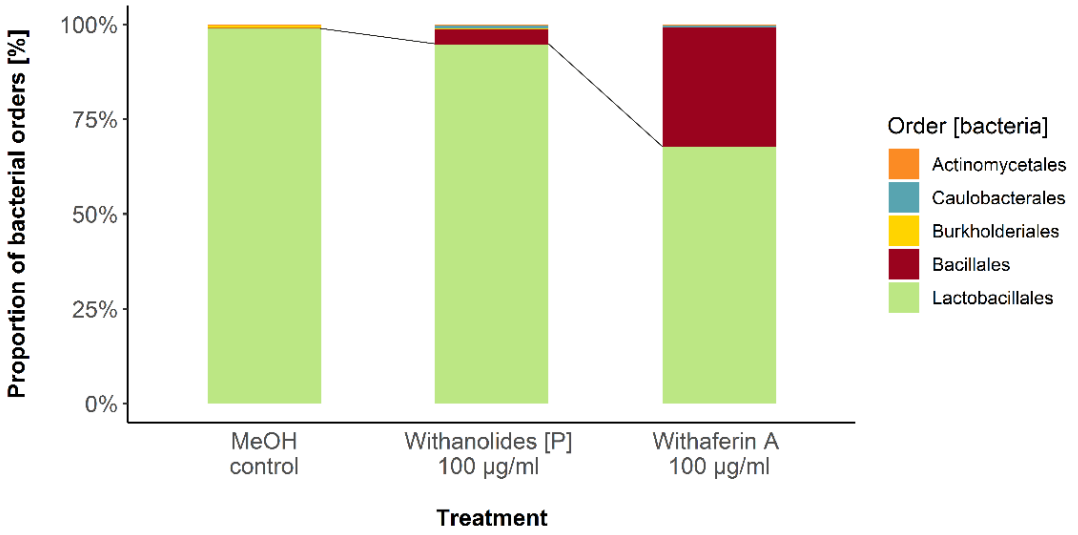

Figure 44: Proportion of bacterial orders in the guts of *C. subflexa* across pooled samples of the control diet and artificial diet supplemented with 100 µg/ml *Physalis* extracts or withaferin A. This figure was created based on the data from the master thesis of Dries Amezian (Amezian, 2018).

Discussion & Outlook

Withanolides show potent inhibitory activity against Gram-negative bacteria

The present experiments particularly aimed to test the susceptibility of different pathogens relevant to plant fitness. IHZ assays demonstrated that, contrary to the prevailing hypotheses about the spectrum efficacy of withanolides, at least two withanolide isolates from two different plant species are able to efficiently inhibit the growth of Gram-negative bacteria. Six common plant pathogens were inhibited by withanolides. These results point to a potential role of withanolides in protecting the plant from infections. The observed differences in the inhibitory efficacy of withanolides against the two *Xanthomonas* pathovars indicate a high degree of specificity.

The antibacterial triterpenoid zeylasterone from the Celastraceae plant *Maytenus blepharodes*, which shares structural similarity with withanolides, has been shown to block macromolecular synthesis and to disrupt the cytoplasmic membrane in Gram-positive *B. subtilis* and *Staphylococcus aureus* (de León *et al.*, 2005). It is possible that withanolides act in a similar manner. However, since withanolides are not exclusively active against Gram-positive bacteria, conclusions about the mode of action cannot be drawn based on Gram classification alone. A screening study by Chatterjee and Chakraborti (1980) found that the antibacterial activity in the assays varied with the substitution patterns of the test compounds. Further research is required to understand the exact mode of action of withanolides.

Whereas the antimicrobial activity of multi-component *P. peruviana* leaf extracts was only moderate in all assays, highly purified withaferin A showed the strongest activity; it was often as effective as the positive-control antibiotics. For less purified multi-compound extracts, it remains unclear which of the chemicals present exhibit antimicrobial activity. Therefore, future research approaches should expand efforts to isolate and test individual compounds of *Physalis* withanolides. However, plants usually possess more than one type of withanolides (Glotter, 1991). In nature, withanolides may therefore act synergistically with each other and/or in combination with other (antimicrobially active) compound classes (Compean, 2014). Future screenings should take this into account and therefore also investigate putative additive antimicrobial effects by combining different withanolides and other interesting compound candidates from *Physalis* plants.

Unfortunately, no samples of *F. oxysporum* f. sp. *physali* were available for IHZ experiments. Vascular wilt caused by this fungal strain is considered the main disease of *Physalis* plants (Osorio-Guarín *et al.*, 2016; Simbaqueba *et al.*, 2021). Instead, two other *F. oxysporum* ff. spp. were included, but have not been successfully tested so far. Future research should also also focus on further optimising these fungal IHZ assays and to obtaining additional samples of *Physalis*-specific bacteria and fungi. Although simple and effective, it is important to note that IHZ assays are subject to many factors that can influence the experimental outcome and should therefore only be used as qualitative measures. A more informative measure is the determination of the effective dose using liquid growth assays. Dose response curves of individual withanolides are essential for understanding antibiotic efficiency and dosing strategies for future antimicrobial testing. As discussed in chapter III, the actual withanolide content in *Physalis* plant tissues is only sparsely described. Additional research on natural withanolide concentrations and a broader understanding of the dosages required to elicit an inhibitory response will provide a more comprehensive picture of whether a plant's withanolide content can affect bacterial growth in insect guts as well as in the plant itself.

The gut microbial composition in *Chloridea* larvae does not change dramatically upon short exposure to dietary withanolides

The results of the present bacterial 16S rRNA amplicon sequencing demonstrate that the gut community in laboratory populations of *C. subflexa* is more diverse than in *C. virescens*. However, the variance between pooled gut samples of *C. subflexa* was surprisingly high, and the total read numbers ranged from very low quantities (388) up to counts equally high as in *C. virescens* (30695). The differences in count numbers can not be attributed to individual rearing families, as they were evenly distributed across the pooled samples. Although there was a slight tendency for proportional shifts of Bacillales in the specialist species, overall no statistically significant effect on the taxonomic

diversity and abundance of species in the treatments was observed. One could speculate that Lactobacillales species are inhibited by the uptake of withaferin A in *C. subflexa* guts, which in turn may have promoted the growth of some tolerant and otherwise outcompeted Bacillales. The two taxonomic orders of Lactobacillales and Bacillales are interesting candidates for future metagenomic analyses. Both taxa comprise Gram-positive bacteria, including the insect-associated genus *Enteroccocus* (Lactobacillales) and several entomopathogenic species such as *B. thuringiensis, Lysinibacillus sphaericus* and *Paenibacillus popilliae* (Bacillales).

The occurrence of bacterial sequences per sample was highly divergent between the two species, and there was also a high heterogeneity within the samples of *C. subflexa*. While none of the *C. virescens* samples had fewer than 22687 counts, there were six samples in *C. subflexa* with fewer than 1000 counts. However, this is consistent with other studies showing generally high inter- and intraspecific variability in taxonomic abundance and composition in caterpillar gut microbiomes (Hammer *et al.*, 2017). From previous studies, it appears that herbivorous insect guts are dominated by only a few microbial taxa and generally possess low species diversity (Sugio *et al.*, 2015). As field samples were not available, a clear limitation of the present analysis is that only laboratory-reared species were used to assess the core microbiota and a potential effect of withanolide ingestion on the bacterial composition. Not only the diet and host-plant metabolites, but also the ecological niche and life-history of individual herbivores affect the composition of the gut flora. Recent metagenomic analyses revealed that bacterial communities differ greatly between larvae collected in the laboratory and in the field (Staudacher *et al.*, 2016). Including field-collected gut samples into the analyses would clearly be more informative and better reflect actual natural conditions. Furthermore, the analyses of the *Physalis* microbiome on leaves and fruits compared to the microbial profile of the specialised and of non-adapted sister species would be interesting follow-up experiments to the present data. Furthermore, it would be revealing to look for common patterns in the microbial profiles of insects that naturally feed on withanolide-producing plants.

However, this metagenomic investigation was not primarily aimed at analysing the intrinsic natural microbial composition in *C. subflexa* and *C. virescens* larvae. Previous experiments with laboratory colonies of *C. subflexa* larvae, which showed increased weight gains on a withanolide-spiked diet in combination with the antimicrobial activities of withanolides, originally initiated the investigation of the gut composition (Barthel *et al.*, 2016). Based on these findings, the main goal was to examine what effects a withanolides-supplemented diet might have on the gut flora and whether adapted and non-adapted species were affected differently. A major advantage of using laboratory stocks is that the experiments can be conducted under controlled conditions with less variable background noise in the sequencing data. Thus, while larvae in the field are exposed to varying environmental

microbes from different sources, laboratory colonies constantly encounter the same bacterial strains typically occurring in laboratories. A presumably high variance in field-collected samples, together with differences in the amount of withanolides ingested, would make it difficult to disentangle a general, direct effect of withanolides on the bacterial composition.

In the feeding assays, the larvae were exposed to withanolides for a relatively short time. In total, larvae were allowed to feed on the diets for 48 hours before moulting into the next instar would have taken place. During their life cycle, lepidopterans undergo a complete metamorphosis and moult several times before they emerge as adults. Any larva-to-larva ecdysis can radically affect the internal microbial communities as the insects shed the exoskeletal layers of the fore- and hindgut. Previous studies have shown that the bacterial composition varies substantially between developmental stages in *S. littoralis* and *Grapholita molesta* (Chen *et al*., 2016; Wang *et al.*, 2020). In these studies, pupae of *S. littoralis* harboured the lowest number of taxa, indicating that bacterial communities are reducing over the course of development and that not all bacteria persist moulting from egg to pupation. Therefore, third instar larvae were prevented from moulting before gut samples were collected. Expanding the sample collection to different larval stages should be considered in future studies that seek to characterise distinct microbial profiles. Although the broad-spectrum antibiotic tetracycline was not present in the experimental diets, it is usually added to pinto bean-based diet when rearing *C. virescens*. This might explain the generally low bacterial diversity compared to *C. subflexa* stocks. There is clearly a trade-off between controlling microbial uptake to maintain a healthy Lepidoptera lab rearing and designing meaningful experiments.

Enterococcaceae dominate the gut microbiome of lab-reared *Chloridea* larvae

Both low bacterial quantities and low species diversity were observed in several other studies on lepidopterans (Sugio *et al.*, 2015; Staudacher *et al.*, 2016; Hammer *et al.*, 2017). This brought up the much-debated question of whether lepidopterans have a resident gut flora at all. Hammer *et al*. 2017, for example, screened a broad variety of field-collected caterpillar species and found a generally low bacterial abundance and the absence of host-specific and resident gut communities in all samples. Furthermore, the authors found that antibiotic suppression of bacterial growth in *M. sexta* had no effect on larval survival and growth (Hammer *et al.*, 2017). It was further argued that the digestive tract of lepidopterans does not provide optimal conditions to promote the establishment and growth of a rich and resident gut community. As the main feeding stage, the gut architecture of caterpillars is simple and functional (Jones *et al.*, 2019). Ingested food passes through the gut quickly, and morphological structures such as invaginations that would allow microbial colonisation and biofilm formation are usually absent (Hammer *et al.*, 2017). Furthermore, the high

alkalinity of the midgut (pH > 10) in Lepidoptera may additionally eliminate all species that are sensitive to high and varying pH conditions (Dow, 1992; Funke *et al.*, 2008). However, there are also studies that contradict the work of Hammer *et al*. 2017. A metagenomic study comparing the microbiome of two specialist moth larvae feeding exclusively on latex-producing spurge (*Euphorbia* spp.) and alkaloid-rich sea daffodils (*Pancratium maritimum*) found that *Enterococcus* species were by far the most abundant in last instar larvae. Scanning electron microscopy also confirmed that the microbiota was able to form biofilms in the hindgut. The authors suggest a putative role of *Enterococcus* strains in the adaptation to latex-/ alkaloid-producing plants (Vilanova *et al.*, 2016). As only one instar was evaluated in that study, nothing is known about how the bacterial composition may change when monitored across different developmental stages. A stable core microbiota has also been described in polyphagous *S. littoralis* and *H. armigera* larvae (Tang *et al.*, 2012). Even though lepidopterans have a rather simple and limited species variety compared to other insect taxa, several studies have shown that certain bacterial groups are able to persist moulting and the harsh environmental conditions in the gut (Vallet-Gely *et al.*, 2008; Paniagua Voirol *et al.*, 2018). In the present microbiome analysis, *Enterococcus* was found to be the dominant genus in laboratory-bred *C. subflexa* and *C. virescens*. This is in line with other studies showing a close association of Enterobacteriaceae with herbivorous insects (Sugio *et al.*, 2015). The remarkably high association of *Enterococcus* species in particular with laboratory-reared lepidopterans is striking. The work of Staudacher *et al.* (2016) showed that the *Enterococcus* genus was largely dominant in laboratory populations of *C. virescens* (91.5% - 97.8%), but absent from larvae collected in the field. However, *Enterococcus* was the most abundant genus in two other field-collected Lepidoptera species, contradicting the assumption that *Enterococcus* colonisation only reflects laboratory artefacts (Vilanova *et al.*, 2016). A recent study on the mechanisms of survival strategies of *E. mundtii* - the dominant symbiotic species of lepidopteran *S. littoralis* - outlines the dynamics by which gut-associated bacteria can indeed adapt to adverse gut conditions and illustrates the close association of *Enteroccocus* with their Lepidoptera hosts (Mazumdar *et al.*, 2021). Most interestingly, *E. mundtii* has been shown to actively release an antibacterial substance called mundticin KS, which has been shown to act against bacterial invaders but not against the resident host microbiota of *S. littoralis* larvae (Shao *et al.*, 2017). These findings may not only explain the dominance of this family in some lepidopterans, but also indicate that these insects may have a natural interest in accumulating Enteroccoceae in their guts.

Several cases have been described in the literature in which mutualistic gut bacteria suppress the effect of toxic plant metabolites (Chung *et al.*, 2013; Ceja-Navarro *et al.*, 2015; Welte *et al.*, 2015; Francoeur *et al.*, 2020). For example, the bacteria in the oral secretions of Colorado potato beetle

larvae (*Leptinotarsa decemlineata*) have been shown to block insecticidal responses of the host plant (Chung *et al.*, 2013). In the gut of the coffee berry borer (*Hypothenemus hampei*), bacterial demethylases help to degrade caffeine, which serves as an antifeedant for the coffee plant. It was found that the degradation activity of caffeine was inactive in beetle larvae treated with antibiotics and reactivated when bacterial inoculates were added (Ceja-Navarro *et al.*, 2015).

While a few studies have already analysed the microbiome of economically important pest species such as *C. virescens* and *H. zea* (Staudacher *et al.*, 2016; Deguenon *et al.*, 2021), no information on the intestinal flora of *C. subflexa* can be found in the literature. Interestingly, the above-described case of the coffee berry borer showed that a a specialist species can have a characteristic and stable microbial core community (Ceja-Navarro *et al.*, 2015). Specialised feeding might likely result in a gut flora that was shaped in a characteristic way by a constant, uniform food intake. This raises the question of whether a specific core community can be identified for the specialist *C. subflex*a, and if so, whether it has functions to degrade withanolides or other *Physalis*-associated phytochemicals. Interestingly, previous studies have shown that withaferin A is metabolised by several microbial species. Cultures of *Arthrobacter simplex* are capable of dehydrogenating withaferin A (Fuska e*t al.*, 1985). Fuska *et al.* (1987) also demonstrated the conversion of 4,27-O-acetyl-withaferin A into various derivatives mediated by *A. simplex*. The fungus *Cunninghamella elegans*, known to metabolise a broad range of xenobiotics, has been shown to convert withaferin A into two major metabolites; one of which has been identified as 14-α-hydroxywithaferin A (Rosazza *et al.*, 1978). A recent study investigated the metabolic activity of the intestinal gut flora extracted from faecal samples of rats and humans towards withaferin A (Dai *et al.*, 2019). The authors observed a slow microbe-mediated conversion of the compound, indicating a rather minor contribution of the gut flora to the degradation of withaferin A.

Symbiotic bacteria are thought to have paved the way in the expansion of host-plant ranges and speciation in insects (Chung *et al.*, 2013; Sugio e*t al.*, 2015). Surprisingly enough, though, that there are still few reports dealing with this topic. It would therefore be promising to investigate a) whether a (shared) core microbiota exists in natural *C. subflexa* populations as well as in other *Physalis*-associated herbivores and b) whether such a resident gut community might have contributed to a host-plant shift in the last common ancestor of *Chloridea* species. Further sequencing approaches are needed to gain a deeper understanding of the association of microorganisms with natural and laboratory populations of Lepidoptera and their potential biological role. This is relevant to understand the mechanisms by which microbes might affect host traits, which in turn could drive adaptation and host shift processes.

GENERAL DISCUSSION

General Discussion

The questions why specialists limit their diet to only a few host plants and how they have evolved to overcome plant defences are major topics in evolutionary ecology. The specialised adaptation of *C. subflexa* is an interesting example as this is the only known insect species so tightly associated with the *Physalis* genus. Even though *C. virescens* larvae are able to develop on *Physalis* plants, it is thought that these plants pose poor hosts for them and that *C. virescens* rather experiences deleterious effects of ingested chemicals, which is why they exclude those plants from their host range. By comparing the specialist with a generalist species that is usually repelled by *Physalis* plants, I aimed to identify adaptive key strategies and the underlying mechanisms that may have contributed to specialisation processes.

Evaluation of previously described effects of withanolide uptake and *Physalis* fruit feeding on *Chloridea* larvae

Barthel *et al.* (2016) hypothesised that *C. subflexa* larvae may have the capability to tolerate *Physalis* plant defences and also benefit from withanolide uptake. However, in the course of this doctoral thesis, those previously observed beneficial effects (an upregulated immune system and increased weight gain) could be not reproduced in *C. subflexa* larvae via feeding assays, PO activity assays or RNA-Seq analysis. Because any measurable differences between *C. subflexa* and *C. virescens* occurred only when larvae were reared on *Physalis* fruits, not on diet supplemented with withanolide extracts, it is difficult to attribute any observed effects directly to withanolide consumption.

Chapter I addresses the adverse or beneficial effects of feeding on withanolide-containing *Physalis* fruits on basic biological parameters in *Chloridea* larvae. The consequences of *Physalis* fruit feeding were found to be moderate in *C. virescens* (no acute deleterious effects were detected). Surprisingly, the two species did not differ significantly in their survival rates when fed on *Physalis* fruits. Though prior work described poor growth in *C. virescens* larvae fed on a *Physalis*-based diet (Oppenheim and Gould, 2002), this was not observed consistently in the present study. In this thesis, it was observed that the exposure to *P. peruviana* fruits did not lead to a visible disinclination to feed in *C. virescens* larvae. Most remarkably, body weights did not differ significantly between species even when they were fed on immature cottonseeds. The absence of clear noxious effects of feeding on this plant genus makes it difficult to understand *C. virescens's* strict exclusion of *Physalis* plants from its host range. Since both species exploit distinct ecological niches in which they usually have to cope with very different plant compounds, it was expected to see more pronounced differences in their overall performance. However, even though it may well appear that there are no acute

harmful effects on *C. virescens* after feeding on *Physalis* fruits, *Physalis* defence compounds may affect *C. virescens*, but this effect is not sufficient to arrest feeding. *Physalis* compounds may function in a rather long-term mode of action, and many subsignificant adverse effects may add up to make plants of this genus a poor host for *C. virescens*.

Effects of *Physalis* fruit feeding on the development of *Chloridea* larvae and the putative function of withanolides as phytoecdysteroids

Clear differences between the two moth species seem to appear primarily during development of the larvae. Additional moulting steps, prolonged development and fewer pupation events were shown in *C. virescens* larvae reared on *P. peruviana* fruits. Whether such effects would have also occurred in other Noctuidea test species is unknown, as most larvae did not live long enough during the experiments to initiate moulting. Broadening feeding experiments to include other insect taxa would clearly indicate whether *Physalis* fruit components on herbivorous insects interfere with moulting. As discussed already in chapter I, observed moulting disorders and delayed development of *C. virescens* larvae might have occurred only as a side effect of additional moulting steps when failing to reach the required mass checkpoint because of less efficient food utilisation or food rejection. This assumption, however, awaits further validation, as weight gain, supernumerary moulting steps and moulting disorders were not correlated. An alternative hypothesis posits that development was disrupted by the interference of *Physalis* compounds with insect hormonal regulators. However, withanolides have not been proven to bind to insect EcRs. In addition, the genetic differences between the EcR in *C. virescens* and *C. subflexa* do not point to functional differences in the binding affinity to 20E analogues.

It is also important to note that in previous studies many polyphagous noctuid moths strongly resisted exposure to phytoecdysteroids, which indicates a generally high degree of genetic adaptability. Previous work demonstrated a high tolerance of polyphagous *H. armigera* larvae to artificial diet supplemented with 20E (Robinson *et al.*, 1987; Duan *et al.*, 2020). Other species that can tolerate high amounts of ingested ecdysteroids are *H. zea* and *C. virescens* (Kubo e*t al.*, 1981; Kubo *et al.*, 1987). *Helicoverpa* larvae showed only moderate feeding activity on *P. peruviana* fruits, and the probability of their survival as well as their relative weight gain was significantly lower than that of *C. virescens* larvae. The number of induced feeding sites in *P. peruviana* fruits was the same among neonates of *C. virescens* and *Helicoverpa*, but significantly higher in *C. subflexa* and the hybrids. Although *S. frugiperda* is highly susceptible to phytoecdysteroids (Kubo *et al.*, 1981), closely related *S. littoralis* larvae displayed high resistance to ingested ecdysone, 20E and phytoecdysteroids from *Chenopodium album* (Blackford *et al.*, 1996). This is especially interesting in the light of the fact

that *S. littoralis* showed the lowest survival rate when larvae fed on *P. peruviana* fruits. Therefore, these findings further support the hypothesis that either food rejection or the inability to create feeding sites, rather than detrimental hormonal factors, caused the high mortality rate in non-adapted Noctuidea species feeding on *P. peruviana* fruits.

Investigation of species-specific transcriptional and metabolic strategies upon withanolide uptake in *Chloridea* larvae

With exception of the differential regulation of the Osiris gene cluster, there was no indication at gene regulatory level for potential key strategies of *C. subflexa* to better cope with ingested *P. peruviana* fruit materials or withanolides than *C. virescens*. The predicted involvement of Osiris genes in cuticle formation, development and detoxification makes them interesting candidates to direct future research on. In *C. virescens*, some genes associated with general detoxification responses were significantly upregulated, especially in larvae fed on *P. peruviana* fruits. Few such genes were detected among significantly downregulated *C. virescens* genes. This discrepancy between up- and downregulated genes was not found in *C. subflexa* larvae, indicating that *C. virescens* may respond to *Physalis* plant compounds with a moderate stress reaction. As this response was only a trend in larvae fed on withanolide-spiked diet, it cannot be concluded that withanolides act as the main stressor to the larvae. It is also conceivable that fitness differences between *C. subflexa* and *C. virescens* larvae may occur because of a delayed metabolic response in *C. virescens*. The non-adapted generalist may induce the production of enzymes required for detoxification or metabolic breakdown processes only after having ingested the plant material over time. Therefore, it would be interesting to compare the transcriptomes of larvae fed on a withanolide-containing diet or on a *Physalis* fruit diet for a long time with the already available transcriptomes of larvae exposed to those diets for a relatively short time.

The present data on the analysis of a potential withanolide metabolism do not suggest that the two species metabolise withanolides differently. However, withanolides are clearly metabolised as only traces of the parent compound were detected in the insect frass. The LC-MS/MS screening of frass products revealed that 4β-hydroxywithanolide E is converted via a reductive chemical reaction to withanolide S. These data represent the first time that an individual ^{13}C-labelled withanolide compound has been generated, isolated and fed to insects. Since *Physalis* plants are compound-rich and the compounds are difficult to separate, the isotopic labelling proved to be a powerful tool to investigate the fate of single withanolides upon ingestion. To validate the theory that *C. subflexa* and *C. virescens* larvae do not differ in their metabolic profile, it would be interesting to isolate and feed additional labelled *Physalis* withanolides in future experiments.

In short, the transcriptomic data as well as first results from metabolite analyses do not support the theory that the specialist has an integrative strategy to contend with withanolide challenges. In other words, their ability to feed on *Physalis* plants without obvious fitness repercussions may not require strategies of metabolic elimination or detoxification (of withanolides) as e.g. shown in insect species specialised on glucosinolate- or alkaloid-containing plants (Hartmann *et al.*, 2005; Jeschke *et al.*, 2016). However, again, the close genetic similarity between *C. virescens* and *C. subflexa* larvae may mask an intrinsic response signature of withanolide-adapted species. Therefore, it might be instructive to include more distantly related species in future analyses.

The selection of the developmental stage seems to play an important role in comparative studies using Lepidoptera larvae

Differences in the relative larval weight gain of two different *C. virescens* instars feeding on artificial diet underline the importance of thoroughly synchronising developmental stages and starting weights when comparing different species. In addition, the present findings indicate that successfull feeding may strongly depend on the developmental stage of *C. virescens* larvae. Experiments assessing the number of holes bored into *Physalis* fruits, for example, indicate that *Chloridea* neonates differ in the ability or willingness to establish feeding sites in fruits. The bigger the larvae, the more freely they feed. In some lepidopterans, first instar larvae are unable to detoxify plant defence compounds, whereas later instars develop the ability to circumvent harmful effects (Lahtinen *et al.*, 2006). Based on these considerations, food utilisation may well be challenging in early instars of *C. virescens*, but less so in later instars, as measured by larval fitness. This hypothesis, however, awaits further validation.

Newly hatched neonates, the most important larval stage, are the first to colonise the host plant and to initiate feeding. If neonates experience suboptimal conditions on the host plant, the survival, reproduction and future generations may be affected. Due to difficulties in handling vulnerable neonates, in this thesis third instar larvae were mostly used. However, to get a comprehensive picture of the consequences of withanolide uptake, it might be interesting to compare the effects also between developmental stages of the same species. Therefore, future experiments should consider investigating the effects of withanolides on all instars separately.

Behavioural traits may be key factors in the host specificity of *C. subflexa*

Although the adverse physiological and molecular effects of withanolide uptake and *Physalis* fruit feeding were not obvious in *C. virescens*, behavioural assays pointed to characteristic differences between *C. virescens* and *C. subflexa*. Efficient host-plant utilisation may require larvae to make physiological adaptations, and may depend on specific behaviours including host localisation and the willingness to feed on the plant. In addition to withanolides, *Physalis* plants possess secretory trichomes that hinder the locomotion of small insects, reducing its attractiveness as a feeding site. In addition, the unique fruit architecture with the calyx may also prevent the fruit from being colonised. As described in the introduction, the literature reported several behavioural traits in *C. subflexa* that differ from *C. virescens* and may be subject to selection. In short, *C. subflexa* showed a quick colonisation of the fruits and entered the calyx with its entire body. This speeds access to nutrition and to the interior of the calyx, where larvae are protected from predators and parasites. *C. virescens* larvae, in contrast, often feed with part of their bodies outside of the calyx and take longer to initiate feeding. Oppenheim and Gould (2002) postulated that the use of the calyx as an enemy-free space with low food competition poses the main benefit for *C. subflexa*. Using *Physalis* plants as a host seems to be genetically fixed in *C. subflexa* (Oppenheim *et al.*, 2012). Besides rapid fruit colonisation, a crucial mechanism larvae use to exploit *Physalis* fruits as a food source may be *C. subflexa*'s ability to establish feeding sites. Earlier work demonstrated that *C. virescens* larvae were occasionally trapped in the fruit's sticky coating and suggested that they were less efficient in creating holes than were larvae of *C. subflexa* (Laster *et al.*, 1982). This thesis showed that *C. subflexa* (+ hybrids) are more efficient in hole boring compared to two other Heliothine moth species. Although only a few *C. virescens* neonates were trapped and died, and bigger larvae appeared less affected, the viscous coating may well contribute to prevent herbivorous attack of small insects; restricting the use of insect mouthparts and respiratory systems as well as immobilising them on the fruit surface. Non-adapted species might have learned to associate contents of the coating with adverse feeding conditions and responded by creating fewer feeding sites. The discrepancy in the number of feeding sites might be caused by feeding repellence (in non-adapted species) or feeding stimulation (in the specialist), by physical obstructions or by toxic effects of plant compounds. However, separating behavioural effects from physiological effects remains difficult. Therefore, it cannot be assessed conclusively whether *C. subflexa* and hybrid larvae were generally more encouraged to feed or simply less hindered to create feeding sites.

Another key aspect of host-plant use is the perception and acceptance of the plant for oviposition and feeding. Alterations in behaviour play a key role in host-plant expansions, and the acquisition of new hosts, in turn, can be an important driver for the formation of novel species (Henniges-Janssen

et al., 2014). Besides the finding that *C. virescens* larvae are in general only moderately affected by *Physalis* feeding, the results of the present feeding studies may also suggest that plant deterrents cannot be perceived and recognised by *C. virescens* larvae and did therefore not prevent further feeding (other than e.g. in *S. littoralis*). In complete contrast, the choice experiments as well as the oviposition assays indicate that *C. virescens* larvae and adults can identify plants outside of their host range and actively decide to reject them as food sources or egg-laying location.

Based on a report from a collaborating research group, it was tested if ovipositing females respond differently in the presence of *P. peruviana* fruits. If plant recognition takes place in *C. subflexa* moths only, no avoidance behaviour would have been observed in *C. virescens*. However, a considerable proportion of ovipositing *C. virescens* females clearly avoided the surrounding area of the fruit. These results are partly in line with previous wind-tunnel experiments showing a significant preference of fertilised *C. subflexa* females for *Physalis* volatiles (Tingle *et al.*, 1990). On the other hand, the study of Tingle *et al.* (1990) also showed that *C. virescens* moths responded positively not only to its own hosts but also to *Physalis* volatiles. A positive response of *C. virescens* females was not detected in the present thesis. In conclusion, a combination of larval choice and female oviposition preference may underlie the host specialisation of *C. subflexa*. However, the effects of behaviour and physiology remain challenging to untangle. Odour-induced attraction/repellence may be an important factor in the divergent development of the two species. The outcome of the choice assays, the oviposition behaviour as well as result from hole-boring experiments can be interpreted as an attraction of *C. subflexa*; in contrast, *C. virescens* may possess a moderate, learned aversion and prefers its own hosts to *Physalis* species. The specialist's behaviour may have become fixed in the course of adaptation.

Summary of the performance of *Chloridea* hybrids across experiments

Generalists and specialists that can be hybridised provide an interesting genetic tool for studying adaptation, speciation and host shifts. In this thesis, hybrids of *C. subflexa* and *C. virescens* were included in most experiments to gain insight into the genetic factors of the larvae that are affected by the uptake of withanolides relative to the effect on their parents. The data collected suggest that hybrids mainly resemble *C. subflexa* in behaviour and development. Though a PCA suggests hybrids have a different transcriptional response, the expression profile of the Osiris genes resembled the parental *C. subflexa* profile. In contrast to *C. virescens* larvae, hybrids did not fail to moult and in only a few cases were supernumerary moulting steps detected. The duration of larval stages until pupation was between that of their parents. The survival rates of larvae feeding on *P. peruviana* fruits did not differ within the *Chloridea* group. In choice assays, neonate larvae of hybrids chose different

diets compared to *C. subflexa* larvae, but chose similar diets compared to *C. virescens* larvae; second-instar larvae differed in their choice of diet from *C. virescens* but not from *C. subflexa*. Neonates preferred cottonseeds to *P. peruviana* fruits and artificial diet, while second instar larvae showed a higher preference for the fruits. Interestingly, larvae of hybrids gained either as much as or significantly more weight when they fed on the fruits compared to larvae of *C. subflexa*. Larval weight gain on cottonseeds was significantly higher than that of either parental species. In addition, hybrid larvae established more feeding sites in *P. peruviana* fruits than did larvae of parental *C. subflexa*. This slightly increased performance over either of its parents could be due to heterosis (hybrid vigour). Heterosis refers to advantageous traits that arise from the combination of genetic contributions from the hybrid's parents (Shull, 1948). Heterosis sometimes includes that deleterious and undesirable recessive alleles from one parent are masked by dominant alleles from the other (Birchler *et al.*, 2006). Sheck and Gould (1993) used F1 hybrids and a backcross to *C. subflexa* to study performance and mortality rate relative to parental performance on soybean, tobacco, cotton (*C. virescens's* hosts) and *P. pubescens*. *C. subflexa* genes were found to be overdominant for larval survival and also dominant for weight gain on *Physalis* plants. Their results showed that at least one major genetic locus is likely involved in feeding preference between the two host plants and that preference for the *Physalis* genus is a dominant trait.

Hybridisation in insect pest species may have severe consequences since interspecific gene transfer between species and the exchange of adaptive traits such as insecticide resistance and host use can strongly improve insects' fitness and survival (Corrêa e*t al.*, 2019). If new hybrid species suddenly become favoured by natural selection, this may also have consequences for natural populations and pest management. Hybridisation events may lead to pest outbreaks especially in invasive species. Introgressive hybridisations (events that occur between invasive and native pests) may impact the evolution of the host range and so require new strategies to manage resistance. In light of host-range expansion, it was therefore interesting to see that *Chloridea* hybrids seem to have the potential for novel host acquisition within the *Physalis* genus. This observation raises the question of why there are no *Chloridea* hybrids in the field. Though *C. virescens* and *C. subflexa* produce viable offspring, the male and female F1 hybrids have very low fertility (Laster, 1972). In addition, *C. subflexa* and *C. virescens* moths differ in the composition of their sex pheromones; as a result males of each species accept only their own pheromone blend and significantly prefer conspecific females (Wyatt, 2003). Differences in pheromone quality and quantity lower the likelihood that *Chloridea* hybrids will be found in the field (Groot *et al.*, 2006).

Remarks on the suitability of the *Chloridea* model system and the use of artificial diet in feeding assays

Considering that both species can grow on each other's host plant when no alternative was available, and that the consumption of *Physalis* fruits and withanolides had a relatively low impact on *C. virescens*, suggests that they still share a high degree of genetic similarity (despite ongoing speciation). For this reason, other generalist Noctuidea species were included in some experiments. Interestingly, more distantly related species showed more differences than did the sister species: arrested feeding, lower weight gain and higher mortality rates. In addition, the rejection of food was generally more pronounced when the degree of relationship to *Chloridea* was lower. Based on these findings, one can speculate that genetic prerequisites, which permit *C. subflexa*, *C. virescens* and the hybrids to feed on *Physalis*, have already been present in their last common ancestor. To explore this hypothesis further, future feeding assays might test the performance of *C. tergeminae,* a possible third member of the *C. virescens* group. Whether there are taxonomic aspects of host selection (genetic prerequisites) would be interesting to investigate. Research on *C. subflexa* and *C. virescens* will widen our understanding of how recent speciation and specialisation processes have evolved. However, because of their close genetic similarity, *C. subflexa* and *C. virescens* may not be the best choice to shed light on the mechanistic underpinnings of the effects of larvae feeding on a diet containing withanolides; more distinctly related species may yield more insight.

Initially, artificial diets were planned for all feeding experiments in order to control for the many possible confounding variables when using plant parts instead. However, rich artificial diets may compensate for potentially interesting (negative) effects of test compounds. In addition, the present datasets of larvae feeding on artificial diet showed a high degree of variance. Interestingly, a high degree of variance was also reflected in both phenoloxidase enzyme assays using artificial diet. The variance in the data might affect the statistical power of the data and obscure the weak effects of less potent test compounds. Interestingly, the variance was lowest on the natural food sources cottonseeds and *Physalis* fruits. Because the suitability of (rich) artificial diets for Lepidoptera larvae to mimick natural conditions in the lab might be questionable - especially when food-sensitive specialists are tested - future experiments should reconsider their use.

Final conclusions

In contrast to early views, the strongest benefit of *Physalis*-feeding is probably that it ensures food competition is low and protection from predators and parasites is high. This thesis presents results that support the theory that behavioural mechanisms are key drivers in the dietary specialisation of

C. subflexa and in the divergent development between the two *Chloridea* species. If behavioural modifications had originally paved the way for a host-plant shift, further minor (as yet unknown) adaptations might have followed in the course of specialisation. Oppenheim and Gould (2002) speculated that since the two species remain closely related, *C. virescens* larvae could habituate to *Physalis* plants under high selective pressure. *C. virescens* would likely benefit from the enemy-free space this plant offers if its larvae were able to reach the fruit efficiently and use the calyx as shelter. However, there does not seem to be strong selection on the broad generalist to expand its host range, because it can theoretically feed and thrive on the fruit but does not in practice. In this thesis, factors that may contribute to making *Physalis* plants unattractive hosts for *C. virescens* are identified. Several of those subsignificant factors may add up and influence behaviour and fitness. Yet these factors may not be sufficient to explain why *C. virescens* is not at least occasionally found on *Physalis* plants. As Oppenheim and Gould (2002) argue, the scarcity of *Physalis* plants compared to natural host plants may partially explain the insect's absence. This moth species seems to be particularly successful within its exisiting host range. Why *Physalis* plants are not part of this food spectrum of *C. virescens* larvae remains unknown.

In order to relate to the title of this thesis, the predominant use of whole *P. peruviana* fruits instead of withanolide extracts makes it challenging to attribute the observed effects to single compounds alone. Though withanolides seem to play a role in plant defences, how they affect the specialisation of *C. subflexa* on *Physalis* plants is still not fully understood. Given the current state of knowledge, many aspects of the adaptation to *Physalis* plants seem complex and closely intertwined. There is clearly much to be learned about the ecological role of withanolides to understand the evolution of their diverse structures and their broad activity spectrum.

Future Perspectives

Apart from the fact that withanolides aid in herbivore protection, their function in the plant itself is only barely understood. However, secondary metabolites are often multifunctional and may also fulfill diverse other functions in plants in which they occur (Erb and Kliebenstein, 2020). So far, it is still unknown if withanolides have a role in plant metabolic pathways, growth and development. This makes it difficult to discuss the ecological role of these understudied compounds in a broader context. The complete biosynthetic pathways are not fully decoded, and information on the plant's individual withanolide composition and content is incomplete. Basic research on withanolides will likely advance our understanding of how these compounds interact with insect herbivores and will also clarify how genetic manipulation might proceed. Promising experiments on mutant *P. grisea* plants in which withanolides were knocked out, for example, showed reduced plant growth (Joyce van Eck, Boyce Thompson Institute, personal communication). This example underlines those deeper insights in withanolide functionality are crucial for future experiments.

In this thesis, the antimicrobial efficacy of withanolide compounds is addressed and widened to include a range of bacteria from both Gram-positive and Gram-negative classes; compounds were particularly tested against phytopathogenic microbes. There is now convincing evidence that the antimicrobial activity is more potent and specific than originally thought. Besides their function as insect-repellents, their antimicrobial properties against plant pathogens attribute some considerable role to the protection of the plant. This hypothesis, however, demands further research.

Our knowledge of bacterial associates in Lepidoptera is still limited. Some symbiotic gut microbes are capable of degrading (toxic) plant compounds in their herbivore host (Anand *et al.*, 2010; Shukla and Beran, 2020). Whether there are *Physalis*-associated microorganisms and whether they are involved in degradation processes or nutrient provisioning is an open question that needs to be addressed experimentally. Knowledge of the microbial profile of withanolide-producing plants and of the gut flora in their associated herbivores may clarify any involvement by microbes. The system of the specialist *C. subflexa*, its related generalist sister and *Physalis* plants thereby furnishes excellent opportunities for comparative research approaches. Although the mechanism underlying withanolides' antimicrobial action is not understood, the discovery of novel sources for antibiotic production is experiencing increasing interest. The overuse of antibiotics in agriculture and medicine has led to multidrug-resistant microorganisms, which have become a major global health threat. Because many existing drugs are no longer effective, screening plants for alternative agents with promising medicinal properties may help combat the rapid emergence of resistant strains. Similarly,

the increased rate of resistance to insecticides in common pest populations has become an urgent worldwide problem. Botanical insecticides are seen as alternatives to industrial and synthetic pesticides.

It remains unknown whether withanolides are directly responsible for the observed differences in survival probability, feeding deterrence and developmental abnormalities in the Noctuidea larvae tested in this doctoral thesis. A first attempt to address this problem could involve the topical application of purified isolates on naturally withanolide-free host plants of insects that are strongly deterred by *Physalis* plants. Alternatively, the genetic alteration of withanolide-free plants via gain-of-function knock-ins is another promising approach. If herbivorous enemies exposed to modified *Physalis* plants are damaging less than those exposed to unmodified plants, or if the modified plants are less repellant for the specialist than the wildtype plants, withanolides may be implicated.

The use of artificial diet in experiments makes it difficult to attribute observed effects to single compounds. However, in order to characterise the effects of particular plant compounds it is necessary to examine them individually. Therefore, future research efforts should be directed toward generating CRISPR/Cas9-mediated withanolide-free *Physalis* mutants. The creation of loss-of-expression *Physalis* mutants provides an ideal opportunity to test the direct effects of withanolides. Differences in the performance and survival of adapted and non-adapted species on mutant and wildtype plants would reveal much about the role of withanolides. Based on recent advances in the identification of withanolide biosynthetic pathways (Knoch *et al.,* 2018), mutant lines of *P. grisea* and *P. peruviana* were established to knockout genes involved in withanolide synthesis using CRISPR/Cas9 (Joyce van Eck, personal communication). These mutant plants have been evaluated for withanolide content and are currently being investigated for their impact on *C. subflexa* and *Lima daturaphila*. Within the frame of the *Physalis* Improvement Project, this work mainly aims to create insect-resistance plants and improve their growth in future breeding programs (Van Eck, 2022).

In addition to withanolides, the sticky coating of *Physalis* fruits and the acylsugars contained therein may be involved in plant protection, but more research needs to be done to understand precisely how. Generating acylsugar-free *Physalis* mutants with the CRISPR/Cas9 method to study the ability of larvae to forage on the fruits' surface would clarify factors that may contribute to *Physalis* defence.

Research on the understudied Osiris gene family is a growing but relatively young field in science. These insect-specific genes seem to be involved in many important functions and regulatory networks. It is to be expected that novel research approaches are currently on the way and will contribute to advance our understanding of the exact function of Osiris genes. Whether *Chloridea*

Osiris genes are responsive to withanolide uptake still needs to be proven. *Physalis* mutants whose withanolide expression is altered by CRISPR/Cas9 will be useful in future experiments.

Despite great attention to their medically relevant properties, research on withanolides remains a small niche. However, because of the need for robust and naturally resistant crop plants, as well as for alternative insecticides for sustainable agriculture, interest in functionally characterising withanolides will clearly increase in the future.

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Appendix

Supplementary data for chapter I

Experimental diets used for feeding assays

The factor diet strongly affects the accuracy of measured biological parameters in insects such as growth, viability, reproduction and, consequently, the experimental validity. With the aim to choose the best experimental diet available for *Chloridea* larvae, initial feeding trials were carried out prior to main feeding assays with withanolide compounds. To do so, the effect of different types of diet, including pinto bean-based, chickpea-based and commercially available Lepidoptera artificial diet (GPL) on larval weight gain (**Figure 45**) and pupal weight on the day of pupation (**Figure 46**) of both species was evaluated. *C. subflexa* larvae showed a significantly higher weight gain on GPL diet compared to the pinto bean diet, but not in comparison with the chickpea diet. Interspecific differences were detected within the pinto bean diet test group and, for the sake of completeness, also between the larval weight gain of GPL-reared *C. virescens* and *C. subflexa* feeding on pinto bean diet. Within the three diet treatments, there was no statistically significant influence of diet on the weight gain in *C. virescens*.

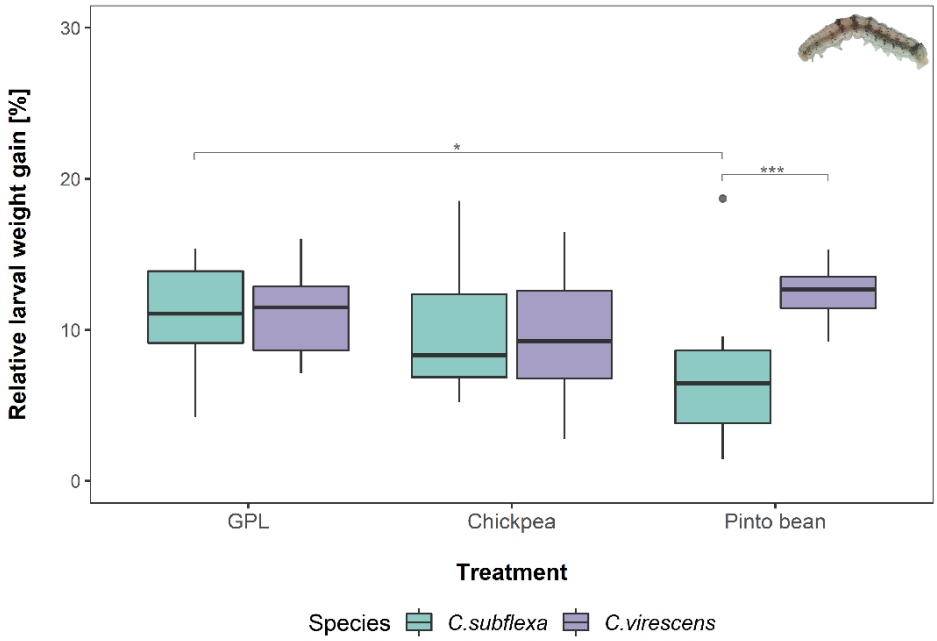

Figure 45: Relative larval weight gain in *C. subflexa* and *C. virescens* third instar larvae. Weight gain was assessed after seven days of exposure to GPL, chickpea or pinto bean diet. According to a two-way ANOVA, the interaction between diet and species ($f(2) = 7.200$; $p < 0.01$; $n = 15$) was significantly different as well as the mean relative weight gain between the two species ($f(1) = 5.851$; $p < 0.05$). Important statistical differences are marked by asterisks ($p < 0.001$***, $p < 0.05$*, Tukey *post hoc* test).

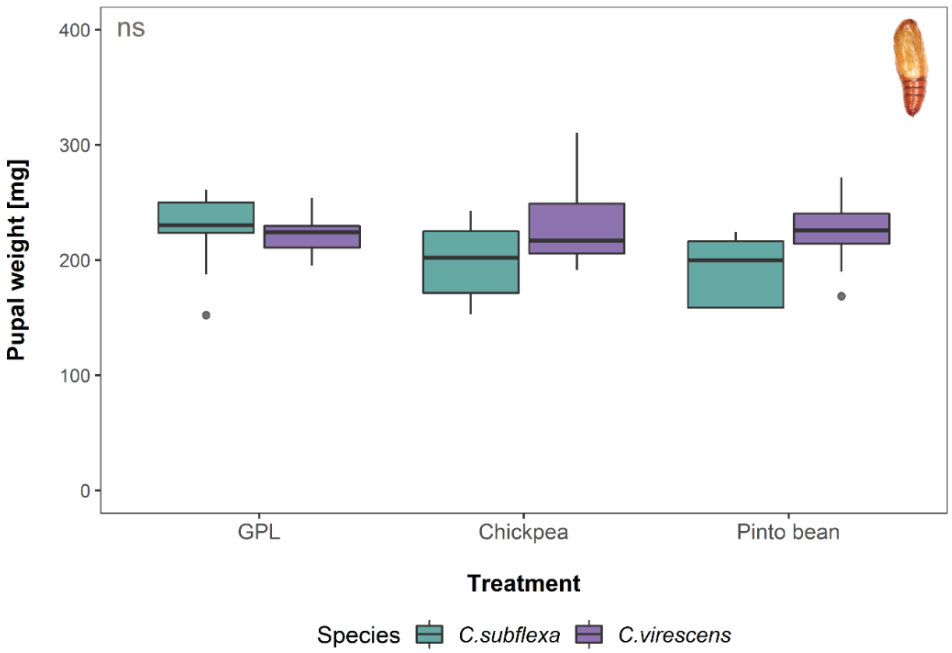

Figure 46: Pupal weight of *C. subflexa* and *C. virescens*. Larvae were either reared on GPL, chickpea or pinto bean diet until pupation. Das Gewicht der Puppen unterschieden sich zwischen Diet und Spezies (two-way ANOVA; species × diet: f(2) = 3.231, $p = 0.046$ / species: f(1) = 7.587, $p = 0.007$; n = 15). A Tukey *post hoc* test did not recover statistically significant differences (ns) between treatments and between species ($p > 0.05$).

In addition, no statistically significant differences were found in the average pupal weight (Figure 46). Per treatment, all larvae survived the experimental time and pupated successfully. Although the generalist showed a slight trend towards a better performance on pinto bean-based diet, the diet treatment had no significant influence on the pupal weight. As expected from the dietary specialist, *C. subflexa* responded sensitively to different diets. Since the pinto bean diet mix contains an antibiotic, and due to the higher homogeneity of the species in weight gain and pupal weight on GPL diet, this mix was chosen as experimental diet.

Optimisation of feeding assays

Because previously discovered effects on larval weight gain (Barthel *et al.*, 2016) could not be reproduced at a concentration of 100 µg/ml *Physalis* withanolide extracts, higher concentrations were tested against both species (Figure 47). These concentrations correspond to naturally detected withanolide concentrations of *P. peruviana* plants (Baumann and Meier, 1993). Statistical analysis revealed that the relative larval weight did not differ significantly across three different withanolide concentrations and between the species. This again contrasts with Barthel *et al.* (2016), who discovered interspecies differences (higher weight gain in *C. virescens*) as well as differences across the different withanolide concentrations compared to the MeOH control.

In order to verify the biological activity of *P. peruviana* withanolide extracts, compounds were frequently tested in IHZ assays. However, to exclude that older compounds were degraded, new extracts were freshly prepared as described in **section 8.2** and tested again in feeding assays with third instar larvae (**Figure 48**). No statistically significant differences were found between species and between the two withanolide treatments. This shows that freshly prepared withanolide isolates and older extracts do not differ noticeably in their effect on larval weight gain when added to their diet. To investigate whether a rich artificial GPL diet could have compensated for any negative effects of withanolides, *C. subflexa* and *C. virescens* were additionally tested on a minimal diet mix (**Figure 49**). In a first test, about 40% of larvae of the control diet died within the first days. Therefore, acetone was used as a solvent to replace MeOH, which seems to react with components of the minimal diet mix (see **page 143**). No unusually high rates of mortality were observed in acetone treatments. The finding that the use of the minimal diet did not cause any statistically significant differences in larval weight gain compared to the other standard artificial diet, does not support the hypothesis that rich diet might cover any negative or beneficial effects. The combined results of the feeding experiments together with the applied optimisation strategies lead to the conclusion that the effect of withanolide-spiked diet on the relative larval weight gain is not as strong as originally thought.

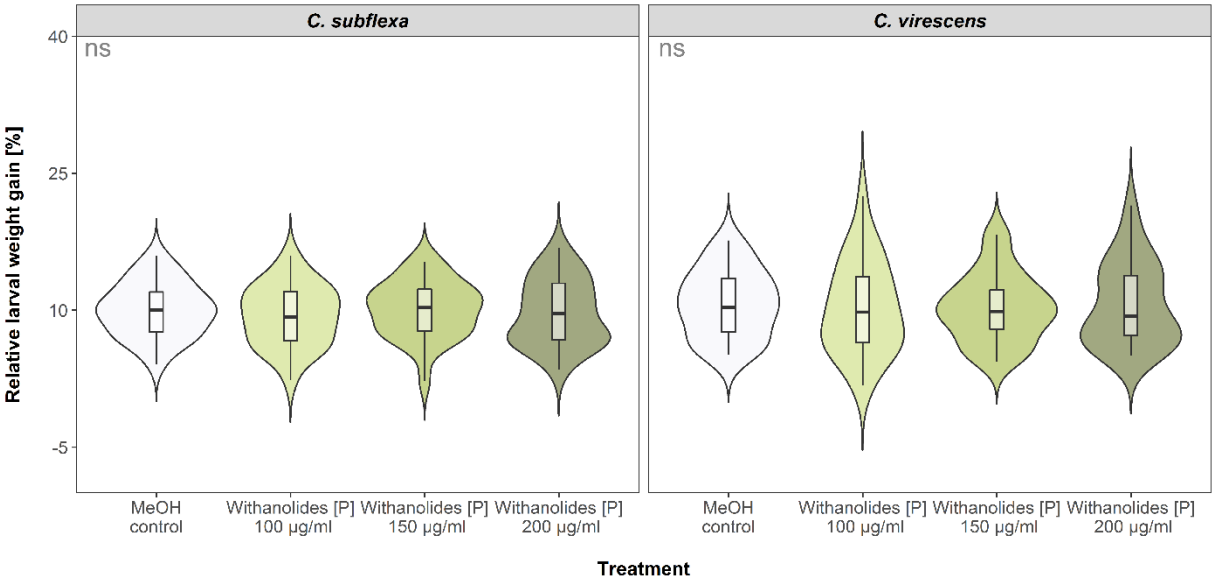

Figure 47: Relative larval weight gain of third instar *C. subflexa* and *C. virescens* larvae that were fed either on three different concentrations of *Physalis* withanolide extracts or on control diet (for seven days). Statistical analysis revealed that neither the factor species nor the diet treatment had a significant influence (ns) on the weight gain (GLS; $p > 0.05$; n = 28).

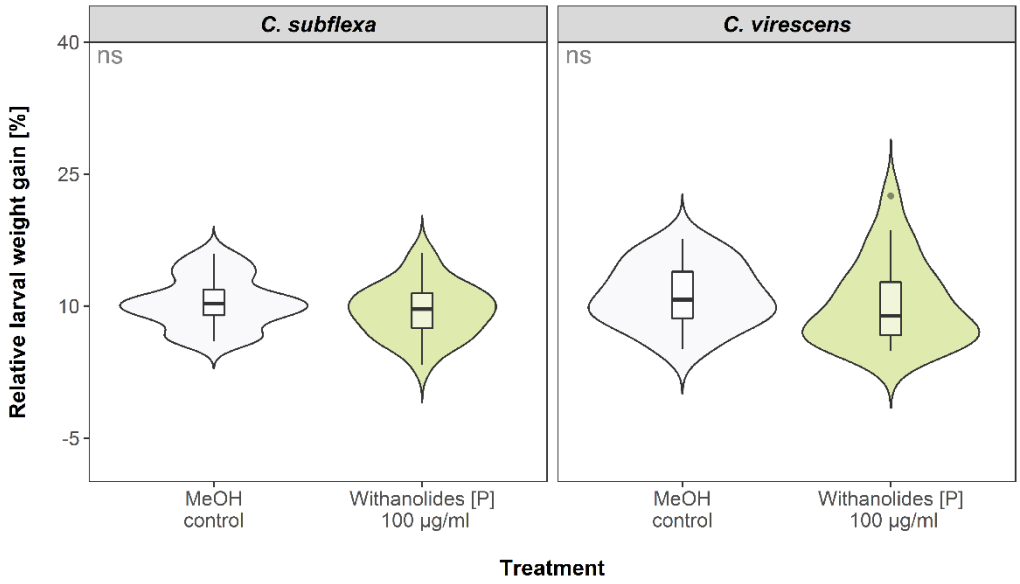

Figure 48: Relative larval weight gain in third instar *C. subflexa* and *C. virescens* larvae fed on diet supplemented with freshly prepared 100 µg/ml *Physalis* withanolide extracts. Differences in weight gain are not statistically significant (ns) (GLS; $p > 0.05$; n = 24).

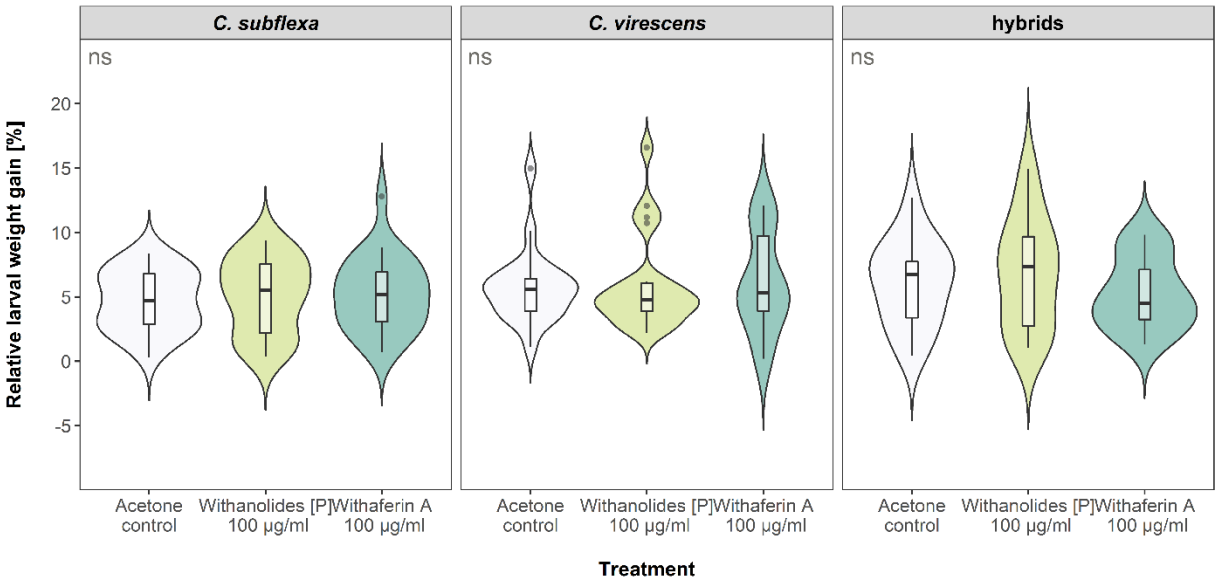

Figure 49: Relative larval weight gain in third instar *C. subflexa*, *C. virescens* and hybrid larvae fed on low-nutrient diet (minimal diet). Larvae were reared for seven days on a control diet (+ acetone), on diet supplemented with 100 µg/ml *Physalis* withanolide extracts or with 100 µg/ml withaferin A. Differences in weight gain are not statistically significant (ns) within and between species (GLS; $p > 0.05$; n = 18 - 24).

Formulas for additional experimental diets

Caterpillars were reared on three additional types of artificial diet with the following ingredient list:

A) Pinto bean-based diet

35 g agar (Roth®)
125 g pinto bean (Frontier Scientific)
100 g wheat germ (Frontier Scientific)
50 g soy protein (Frontier Scientific)
50 g casein (Fisher Scientific)
62.5 g torula yeast (Frontier Scientific)
10 g Vanderzant vitamin mix (Frontier Scientific)
6 g ascorbic acid (Frontier Scientific)
5 g methyl paraben (Frontier Scientific)
3 g sorbic acid (Frontier Scientific)
0.25 g tetracycline (Sigma-Aldrich)
per 1.1 l of distilled H_2O

B) Chickpea-based diet

Chickpea artificial diet was produced like pinto bean diet, but pinto bean flour was replaced by chickpea flour (Seyfrieds).

C) Minimal diet*

30 g agar (Roth®)
40 g casein (Fisher Scientific)
1 g L-cysteine (Sigma-Aldrich)
40 g sucrose (Roth®)
50 g cellulose (Roth®)
2.5 g methyl paraben (Frontier Scientific)
1.5 g sorbic acid (Frontier Scientific)
10 g Wesson salt mixture (MP Biomedicals)
25 µl cobalt chloride (Merck)
25 µl sodium molybdate (Sigma-Aldrich)
50 µl zinc acetate (Sigma-Aldrich)
3 ml 10 M potassium hydroxide (Sigma-Aldrich)
5 g oil (sunflower seed)
0.5 g cholesterol (Sigma-Aldrich)
0.25 g Beta-carotene (Sigma-Aldrich)
0.005 g Vitamin D3 (cholecalciferol)
10 g Vanderzant vitamin mix (Frontier Scientific)
0.005 g menadione (Sigma-Aldrich)
10 ml EtOH (Roth®)
per 800 ml of distilled H_2O

*(Vanderzant, 1968)

Hole-boring efficiency (total counts)

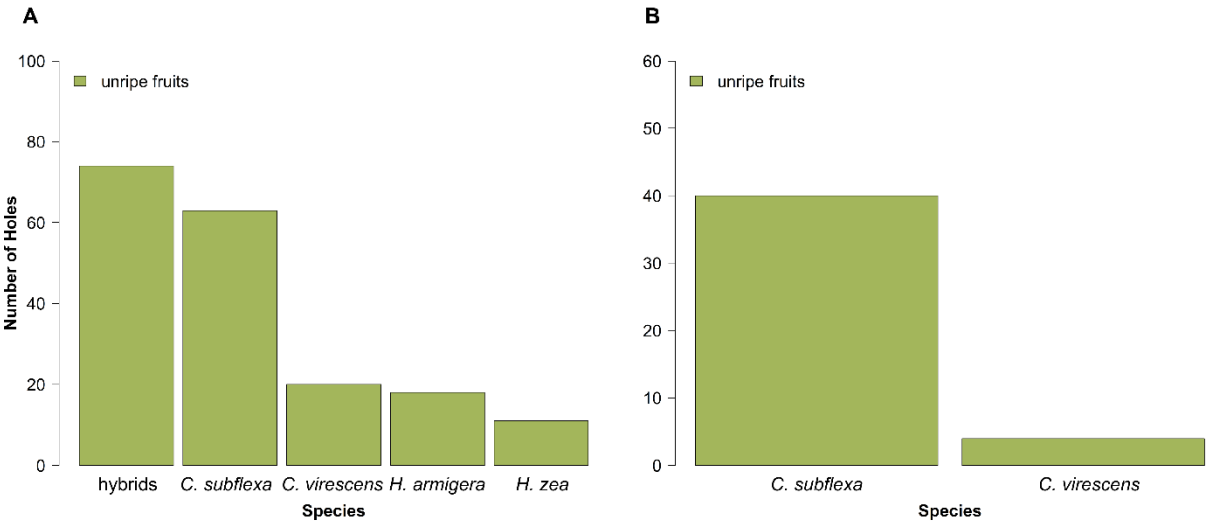

Figure 50: Total number of holes: A) of four Heliothine neonates and *Chloridea* hybrids placed directly on unripe *Physalis* fruits. **B)** Numbers of holes of *C. subflexa* and *C. virescens* neonates placed on the calyx platform. In both experiments, the neonates were allowed to feed on the fruits for 12 h.

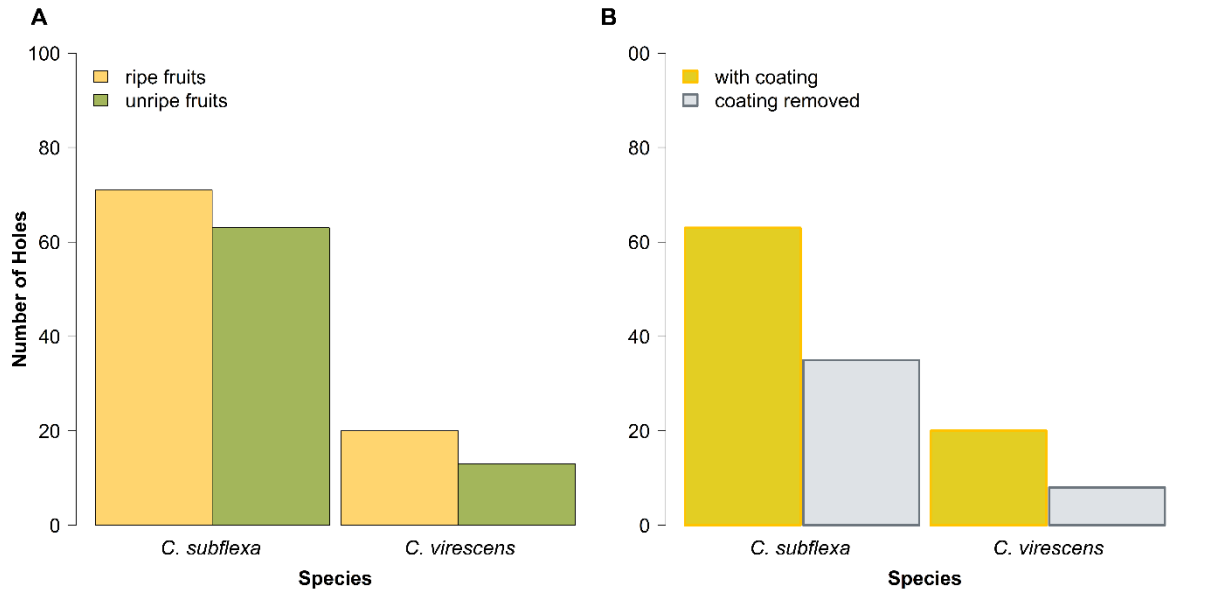

Figure 51: Total number of holes: A) of *C. subflexa* and *C. virescens* neonates feeding on either unripe or on ripe *Physalis* fruits. **B)** Direct comparison of total numbers of holes of *C. subflexa* and *C. virescens* neonates feeding on immature *Physalis* fruits with or without fruit coating. In all experiments, neonates were placed on the calyx platform and then allowed to feed on the fruits for 12 h.

Oviposition behaviour in *Chloridea* moths

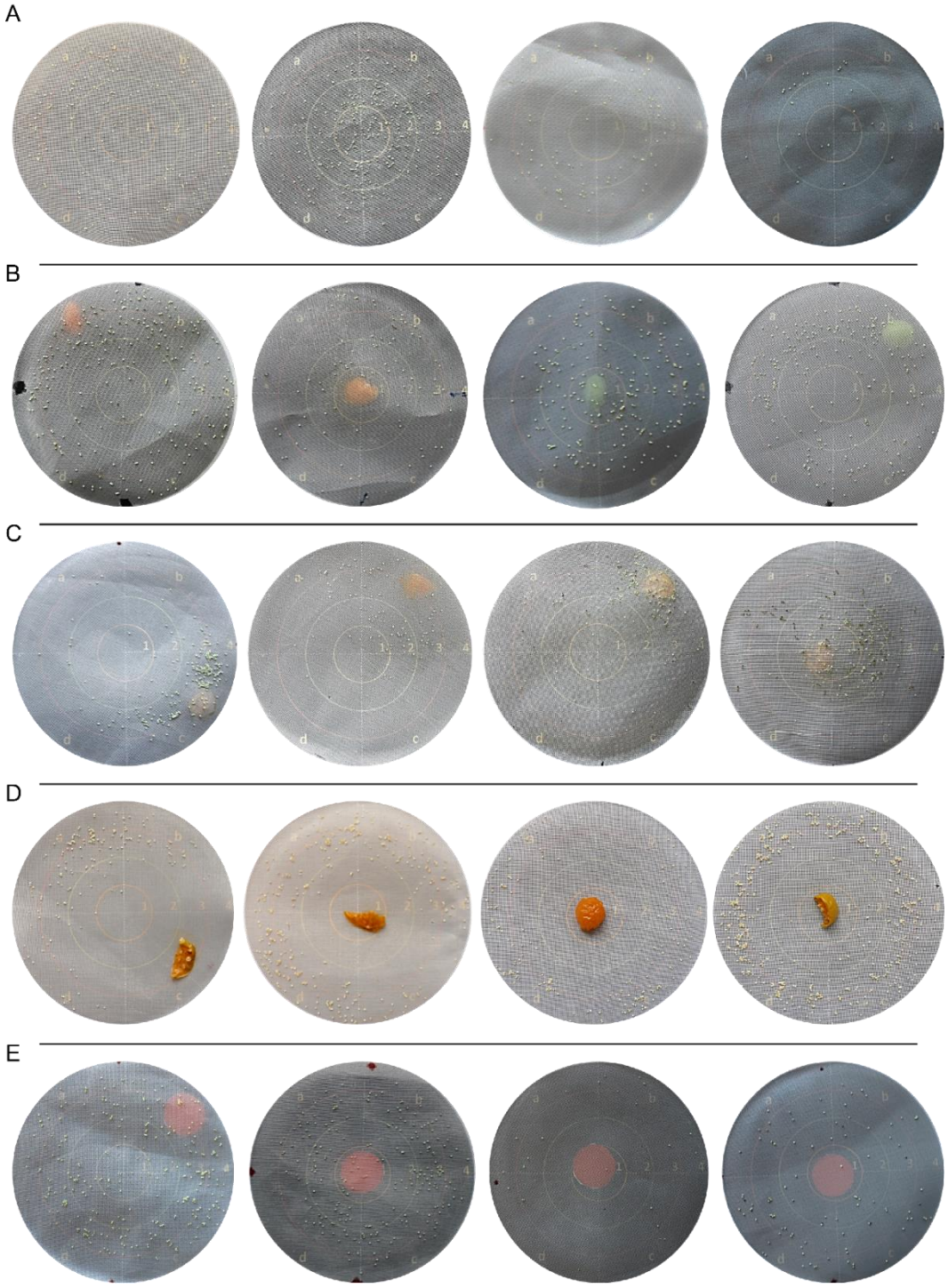

Figure 52: Representative examples of oviposition patterns in *C. subflexa* and *C. virescens* females. A) Typical egg-laying patterns: *C. virescens* (1 & 4) and *C. subflexa* (2 & 3). **B)** Examples of females not responding to the presence of ripe/unripe *Physalis* fruits: *C. virescens* (1 & 4) and *C. subflexa* (2 & 3). **C)** Positive response of *C. subflexa* females to ripe *Physalis* fruits in contrast to **D)** negative response of *C. virescens*. **E)** Neither *C. virescens* (1 & 4) nor *C. subflexa* (2 & 3) were repelled by or attracted to colour dummies.

Supplementary data for chapter II

C. subflexa ecdysone receptor ORF

***C. subflexa* Ecdysone Receptor** (ORF nucleotide)

```
ATGTCCATAGAGTCGCGTTTAGATAGTTTAGTGCGAGGAAAAAGTGAAGTGAAAGCCTTCCTCGGAGGAT
GTCCCTCGGCGCTCGTGGATACCGGAGCGTGTGACACGCTCGCAGACATGAGACGCCGCTGGTATAACAA
CGGAGGATTCCAGACGCTGCGAATGCTCGAGGAGAGCTCGTCTGAGGTGACGTCGTCTTCAGCACTGGGC
CTGCCACCGGCAATGGTGATGTCCCCGGAGTCGCTCGCGTCGCCCGAGTACGGCGGCCTGGAGCTGTGGG
GCTACGACGATGGCATTACTTACAGCATGGCACAGTCGCTGGGCACCTGCACCATGGAGCAGCAGCAGCC
CCAGCCGCAGCAGCAGCCGCAGCAGACACAACCCCTACCTTCCATGCCGTTACCAATGCCACCGACAACA
CCTAAATCAGAAAACGAGTCAATGTCATCAGGTCGTGAGGAATTGTCTCCAGCTTCGAGTGTAAACGGCT
GCAGCACAGATGGCGAGGCGAGGCGGCAGAAGAAAGGCCCAGCGCCGAGGCAGCAAGAAGAGCTATGTCT
TGTCTGCGGCGACAGAGCCTCCGGATATCACTACAACGCGCTCACATGTGAAGGGTGTAAAGGTTTCTTC
AGGCGGAGTGTAACCAAAAATGCAGTGTACATATGCAAATTCGGCCATGCTTGCGAAATGGATATGTATA
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GCCGGAGAACCAGTGTGCAATGAAACGGAAAGAGAAGAAGGCGCAGAGGGAAAAAGACAAATTGCCCGTT
AGCACGACGACAGTAGACGATCACATGCCTCCCATCATGCAATGTGACCCTCCGCCCCCGGAGGCCGCTA
GAATTCTGGAATGTTTGCAGCACGAGGTGGTGCCGCGATTCCTGAATGAGAAGCTAATGGAACAGAACAG
ATTGAAGAACGTGCCCCCCCTCACTGCCAATCAGAAGTCGTTGATCGCAAGGCTCGTGTGGTACCAGGAA
GGCTATGAACAACCTTCCGAGGAAGACCTGAAGAGGGTTACACAGACCTGGCAGTCGGACGAGGACGACG
AAGACTCGGATATGCCGTTCCGTCAGATAACCGAGATGACGATTCTCACAGTGCAGCTCATCGTAGAATT
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ACCAGGCGTACACTCGCGACAACTACCGCAAGGCGGGCATGGCGTACGTCATCGAGGACCTGCTGCACTT
CTGTCGGTGCATGTACTCCATGATGATGGATAACGTGCATTATGCGCTGCTTACAGCCATTGTCATCTTC
TCAGACCGGCCCGGGCTTGAGCAACCCCTGTTGGTGGAGGAGATCCAGAGATATTACCTGAACACGCTAC
GGGTGTACATCCTGAACCAGAACAGCGCGTCGCCCCGCTGCGCCGTCATCTTCGGCAAGATCCTGGGCAT
ACTGACGGAGATCCGCACGCTGGGCATGCAGAACTCCAACATGTGCATCTCCCTCAAGCTGAAGAACAGG
AAGCTGCCGCCGTTCCTCGAGGAGATCTGGGACGTGGCGGACGTGTCGACGACGGCGACGCCGGTGGTGG
CGGACGCGCCGGCGCTCTAG
```

Figure 53: Full-length nucleotide sequence of the *C. subflexa* EcR ORF. The DNA sequence was completed via RACE-PCR.

Ecdysone receptor isoforms

```
          1                    2                    3                    4
MSIESRLDSLVRGKSEVKAFLGGCPSALVDTG MSIESRLDSLVRGKSEVKAFLGGCPSALVDTG MSIESRLDSLVRGKSEVKAFLGGCPSALVDTG MSIESRLDSLVRGKSEVKAFLGGCPSALVDTG
ACDTLADMRRRWYNNGGFQTLRMLEESSSEVT ACDTLADMRRRWYNNGGFQTLRMLEESSSEVT ACDTLADMRRRWYNNGGFQTLRMLEESSSEVT ACDTLADMRRRWYNNGGFQTLRMLEESSSEVT
SSSALGLPPAMVMSPESLASPEYGGLELWGYD SSSALGLPPAMVMSPESLASPEYGGLELWGYD SSSALGLPPAMVMSPESLASPEYGGLELWGYD SSSALGLPPAMVMSPESLASPEYGGLELWGYD
DGITYSMAQSLGTCTMEQQQPQPQQQPQQTQP DGITYSMAQSLGTCTMEQQQPQPQQQPQQTQP DGITYSMAQSLGTCTMEQQQPQPQQQPQQTQP DGITYSMAQSLGTCTMEQQQPQPQQQPQQTQP
LPSMPLPMPPTTPKSENESMSSGREELSPASS LPSMPLPMPPTTPKSENESMSSGREELSPASS LPSMPLPMPPTTPKSENESMSSGREELSPASS LPSMPLPMPPTTPKSENESMSSGREELSPASS
VNGCSTDGEARRQKKGPAPRQQEELCLVCGDR VNGCSTDGEARRQKKGPAPRQQEELCLVCGDR VNGCSTDGEARRQKKGPAPRQQEELCLVCGDR VNGCSTDGEARRQKKGPAPRQQEELCLVCGDR
ASGYHYNALTCEGCKGFFRRSVTKNAVYICKF ASGYHYNALTCEGCKGFFRRSVTKNAVYICKF ASGYHYNALTCEGCKGFFRRSVTKNAVYICKF ASGYHYNALTCEGCKGFFRRSVTKNAVYICKF
GHACEMDMYMRRKCQECRLKKCLAVGMRPECV GHACEMDMYMRRKCQECRLKKCLAVGMRPECV GHACEMDMYMRRKCQECRLKKCLAVGMRPECV GHACEMDMYMRRKCQECRLKKCLAVGMRPECV
VPENQCAMKRKEKKAQREKDKLPVSTTTVDDH VPENQCAMKRKEKKAQREKDKLPVSTTTVDDH VPENQCAMKRKEKKAQREKDKLPVSTTTVDDH VPENQCAMKRKEKKAQREKDKLPVSTTTVDDH
MPPIMQCDPPPPEAARILECLQHEVVPRFLNE MPPIMQCDPPPPEAARI-----HEVVPRFLNE MPPIMQCDPPPPEAARILECLQHEVVPRFLNE MPPIMQCDPPPPEAARI-----HEVVPRFLNE
KLMEQNRLKNVPPLTANQKSLIARLVWYQEGY KLMEQNRLKNVPPLTANQKSLIARLVWYQEGY KLMEQNRLKNVPPLTANQKSLIARLVWYQEGY KLMEQNRLKNVPPLTANQKSLIARLVWYQEGY
EQPSEEDLKRVTQ---SDEDDEDSDMPFRQIT EQPSEEDLKRVTQTWQSDEDDEDSDMPFRQIT EQPSEEDLKRVTQTWQSDEDDEDSDMPFRQIT EQPSEEDLKRVTQ---SDEDDEDSDMPFRQIT
EMTILTVQLIVEFAKGLPGFAKISQSDQITLL EMTILTVQLIVEFAKGLPGFAKISQSDQITLL EMTILTVQLIVEFAKGLPGFAKISQSDQITLL EMTILTVQLIVEFAKGLPGFAKISQSDQITLL
KACSSEVMMLRVARRYDAATDSVLFANNQAYT KACSSEVMMLRVARRYDAATDSVLFANNQAYT KACSSEVMMLRVARRYDAATDSVLFANNQAYT KACSSEVMMLRVARRYDAATDSVLFANNQAYT
RDNYRKAGMAYVIEDLLHFCRCMYSMMMDNVH RDNYRKAGMAYVIEDLLHFCRCMYSMMMDNVH RDNYRKAGMAYVIEDLLHFCRCMYSMMMDNVH RDNYRKAGMAYVIEDLLHFCRCMYSMMMDNVH
YALLTAIVIFSDRPGLEQPLLVEEIQRYYLNT YALLTAIVIFSDRPGLEQPLLVEEIQRYYLNT YALLTAIVIFSDRPGLEQPLLVEEIQRYYLNT YALLTAIVIFSDRPGLEQPLLVEEIQRYYLNT
LRVYILNQNSASPRCAVIFGKILGILTEIRTL LRVYILNQNSASPRCAVIFGKILGILTEIRTL LRVYILNQNSASPRCAVIFGKILGILTEIRTL LRVYILNQNSASPRCAVIFGKILGILTEIRTL
GMQNSNMCISLKLKNRKLPPFLEEIWDVADVS GMQNSNMCISLKLKNRKLPPFLEEIWDVADVS GMQNSNMCISLKLKNRKLPPFLEEIWDVADVS GMQNSNMCISLKLKNRKLPPFLEEIWDVADVS
TTATPVVADAPAL                    TTATPVVADAPAL                    TTATPVVADAPAL                    TTATPVVADAPAL
```

Figure 54: Isoforms of the *Chloridea* EcR amino acid sequence. Shown are the four receptor variants based on GenBank entries and sequencing approaches. Indels (insertions/deletions) are highlighted in grey.

Binding pocket sequence

pdb|7BJV|E **Chain E, Ecdysone Receptor**
GSHMASMTGGQQMGRDPLKNVPPLTANQKSLIARLVYYQEGYEQPSEEDLKRVTQ**TWQ**SDEDDEDSDMPF
RQITEMTILTVQLIVEFAKGLPGFSKISQSDQITLLKACSSEVMMLRVARRYDAATDSVLFANNQAYTRD
NYRKAGMAYVIEDLLHFCRCMYSMMMDNVHYALLTAIVIFSDRPGLEQPSLVEEIQRYYLNTLRVYILNQ
NSASPRSAVIFGKILGILTEIRTLGMQNSNMCISLKLKNRKLPPFLEEIWDVADVA

Figure 55: Ligand-binding domain in the amino acid sequence of the *C. virescens* ecdysone receptor (domain E) containing the indel -TWQ- (highlighted in grey). The sequence was extracted from GenBank: accession no. 7BJV_E; after Browning *et al*. (2021).

GO-term enriched word clouds

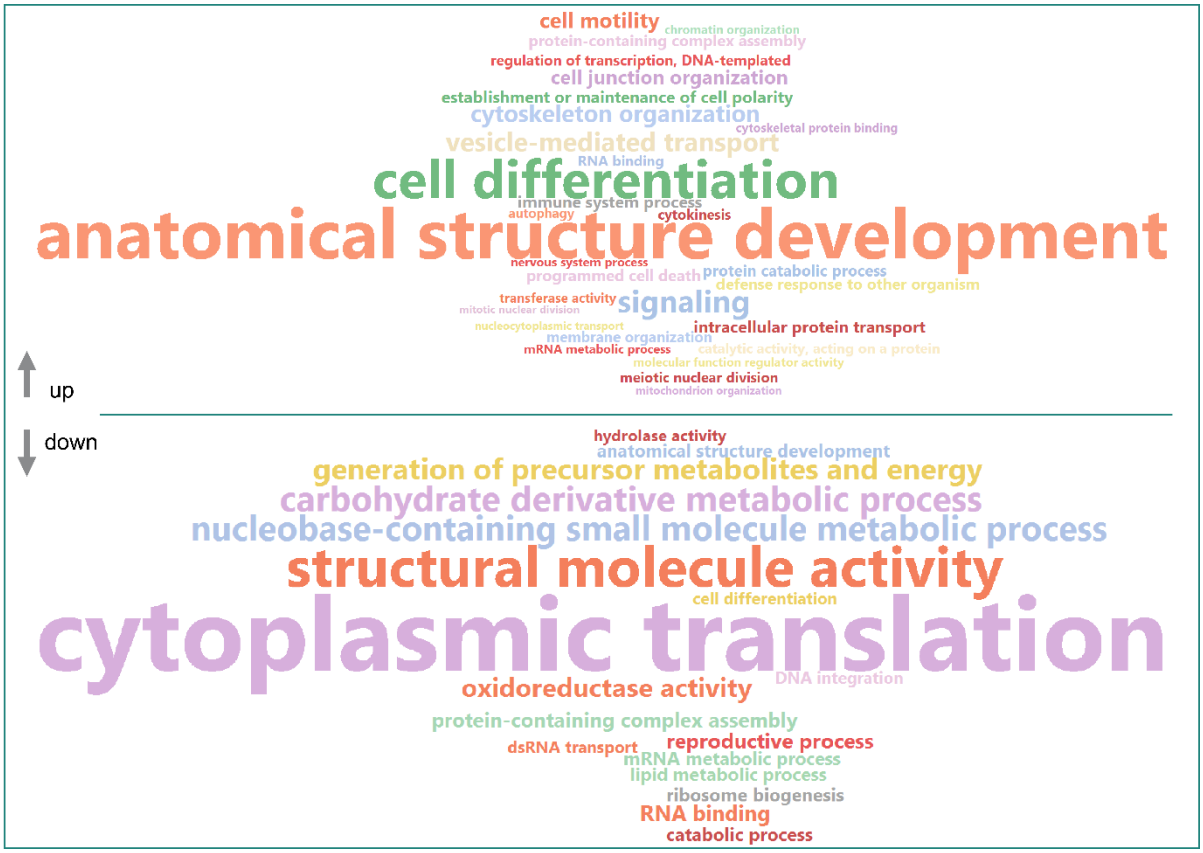

Figure 56: Word clouds showing over- and underrepresented GO terms among lists of differentially expressed genes in *C. subflexa* larvae feeding on *Physalis* fruits. The upper cloud represents GO terms among significantly upregulated genes; the lower cloud shows GO terms of significantly downregulated genes. The GO-enriched word clouds were simplified to display only the most specific GO terms. A GO term was considered significantly enriched if the *p*-value corrected by FDR control was < 0.01.

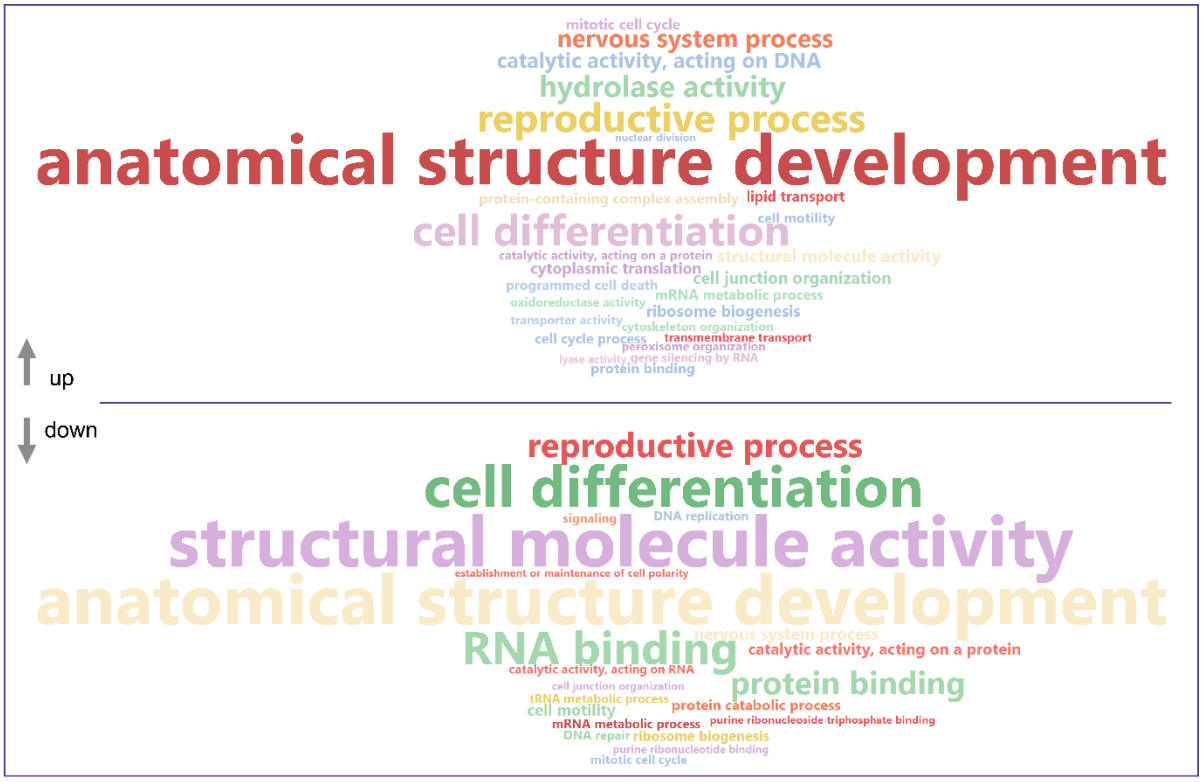

Figure 57: Word clouds showing over- and underrepresented GO terms among lists of differentially expressed genes in *C. virescens* larvae feeding on *Physalis* fruits. The upper cloud represents GO terms among significantly upregulated genes; the lower cloud shows GO terms of significantly downregulated genes. The GO-enriched word clouds were simplified to display only the most specific GO terms. A GO term was considered significantly enriched if the *p*-value corrected by FDR control was < 0.01.

Detection of Osiris true orthologues

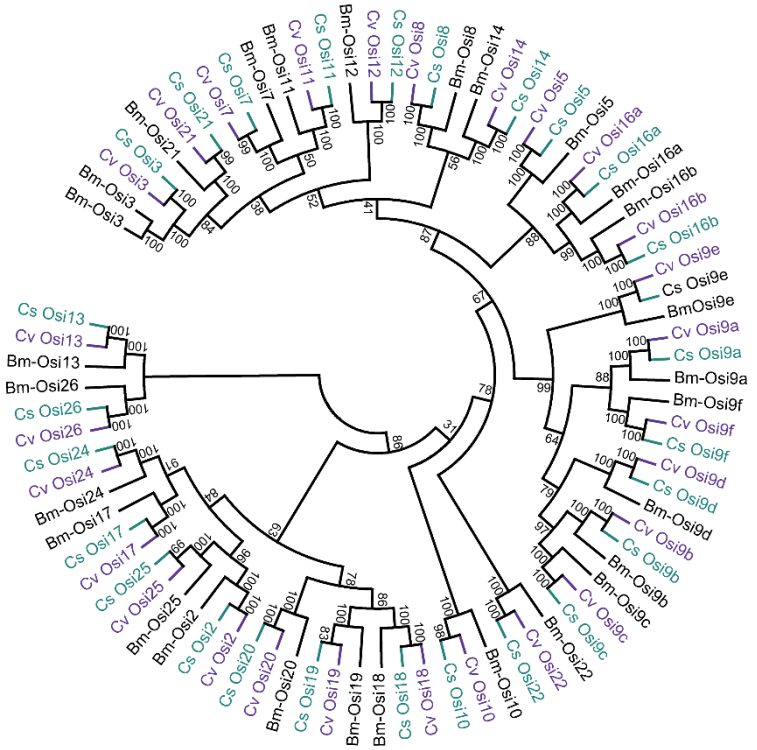

Figure 58: Phylogeny of Osiris genes annotated from draft genomes of *C. virescens* and *C. subflexa*. The Osiris genes were manually annotated from draft genomes of both species using Osiris genes from *B. mori* as a query. The resulting amino acid sequences were aligned using MAFFT and the resulting alignment was used to perform a maximum likelihood analysis in IQ-TREE. The best-fit substitution model for this dataset was that of Le and Gascuel (LG) with a FreeRate model (4 categories; +R4). Ultrafast bootstrap values are given at corresponding branches.

List of *B. mori* Osiris Gene Accession Numbers

Gene	GenBank Accession number	Gene	GenBank Accession number
Bm-Osi 2	XP021203628.2	**Bm-Osi 13**	chr2611851853BGIBMGA000049
Bm-Osi 3	X1-X 012545769.1/X2-XP004926109.1	**Bm-Osi 14**	BGIBMGA000050
Bm-Osi 5	XP037876593.1	**Bm-Osi 16a**	BGIBMGA000051
Bm-Osi 7	chr2611671970 BGIBMGA000044	**Bm-Osi 16b**	XP004926138.2
Bm-Osi 8	chr2611783 044 BGIBMGA000009	**Bm-Osi 17**	XP004926122.1
Bm-Osi 9a	chr2611770 526 BGIBMGA000010	**Bm-Osi 18**	chr2611955367BGIBMGA000006
Bm-Osi 9b	chr2611751 437 BGIBMGA000011	**Bm-Osi 19**	19chr263999391BGIBMGA010716
Bm-Osi 9c	XP004926115.1	**Bm-Osi 20**	chr263990078BGIBMGA010717
Bm-Osi 9d	XP004926114.2	**Bm-Osi21**	chr04-NP001129358.1
Bm-Osi 9e	chr2611719128BGIBMGA000045	**Bm-Osi 22**	chr1212127368BGIBMGA010678
Bm-Osi 9f	chr2611684676BGIBMGA000013	**Bm-Osi 24**	chr2611223134BGIBMGA000025
Bm-Osi 10	chr2611802171BGIBMGA000046	**Bm-Osi 25**	XP037876616.1
Bm-Osi 11	chr2611813656BGIBMGA000008	**Bm-Osi 26**	chr2611893869BGIBMGA000053-mod-ACY06909.1
Bm-Osi 12	XP004926118.1		

Table 1: GenBank (NCBI) accession numbers of *B. mori* Osiris genes used for the generation of the phylogenetic tree of true orthologous Osiris genes in *Chloridea* (see **Figure 58**).

Effects of withanolides and *Physalis* fruits on immune system activity in *Chloridea* larvae

IS activity was measured as the enzymatic activity of the phenoloxidases (PO) in the haemolymph. The extracellular PO cascade is a conserved defence system in invertebrates that initiates the formation of melanin upon wounding or pathogen infections. As shown in previous studies, the PO activity of third instar *C. subflexa* larvae was upregulated after a seven-day exposure to *Physalis* withanolide extracts added to the artificial diet (Barthel *et al.*, 2016). To confirm the observed effects on PO activity of *C. subflexa* larvae, experiments were repeated under the same conditions with withaferin A as an additional treatment (Figure 59). The effect of withanolide compounds on larval PO activity was not statistically significant.

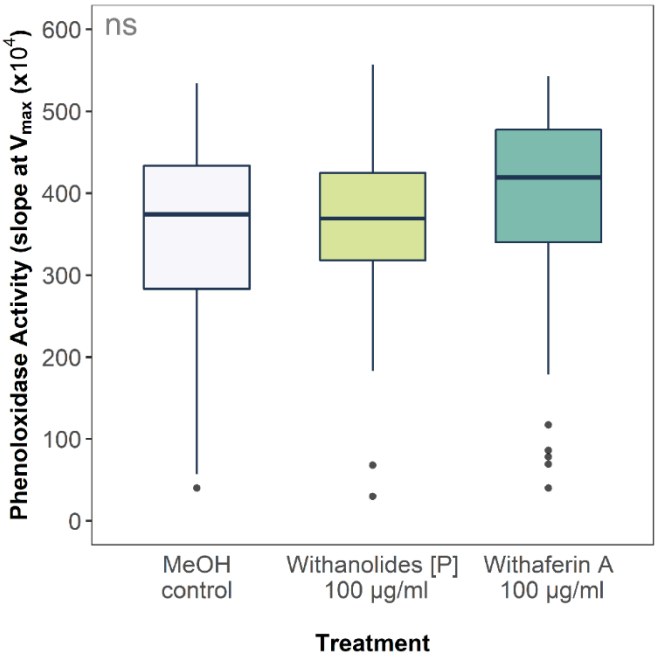

Figure 59: PO activity in third instar *C. subflexa* larvae after feeding for seven days on the control diet or on artificial diet supplemented with 100 µg/ml *Physalis* withanolides extracts or 100 µg/ml withaferin A. The treatment had no statistically significant (ns) effect on the PO activity (Kruskal-Wallis test; $\chi^2 = 3.204$, df= 2 $p = 0.202$; n = 26 - 29).

In order to measure the PO activity in response to two natural food sources, haemolymph samples were additionally collected from individuals feeding on the artificial diet, *Physalis* fruits or cottonseeds. *C. virescens* larvae and hybrids were included in the experiments (Figure 60). Enzymatic activity did not differ significantly between *Physalis* fruit and cottonseed treatments, and within and between species (with exception of hybrids and *C. subflexa* fed on *Physalis* fruits; $p < 0.05$).

However, PO activity was significantly different in all test groups feeding on artificial diet compared to those exposed to natural food sources (cottonseeds: $p < 0.001$; *Physalis* fruits: $p < 0.05$). Within the artificial diet treatment, PO activity differed significantly in *C. subflexa* from that of *C. virescens* and the hybrids ($p < 0.001$). While variance in *C. subflexa* was generally lowest and surprisingly low in larvae fed on natural food sources, both the hybrids and *C. virescens* larvae displayed a high degree of variance in the *Physalis* fruit treatment groups as well as on artificial diet.

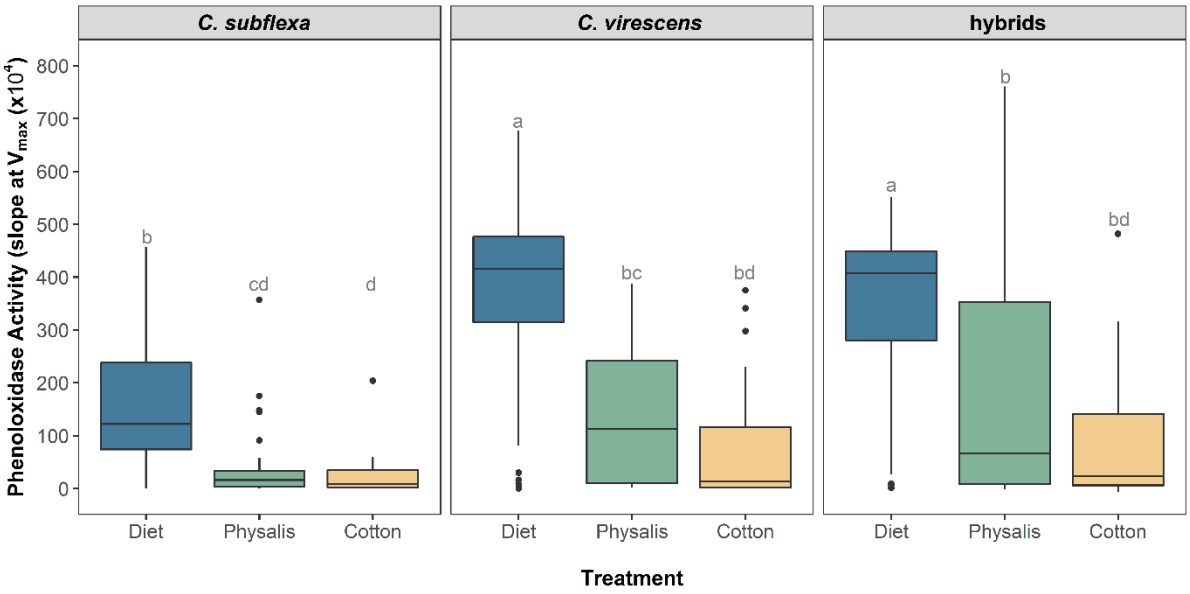

Figure 60: PO activity in third instar *C. subflexa*, *C. virescens* and hybrid larvae. Larvae were fed either artificial diet, *Physalis* fruits or cottonseeds for seven days. Species and diet treatment had a statistically significant influence on enzyme activity (GLS; $p < 0.001$; n = 18 - 38). Letters indicate significant differences between treatment/species combinations as determined by a *post hoc* multiple comparison of estimated means using Tukey contrasts.

As observed in previous feeding assays (Figure 12), data varied particularly widely when larvae were reared on artificial diet compared to data from larvae reared on natural food sources. Since individuals with the same parent combination generally differed strongly in all feeding assays and also in the present PO assays, the high variance in the datasets could probably not be explained by family level. Neither the consumption of withanolide-containing *Physalis* fruits, nor ingestion of single compounds caused an upregulation of PO activity in *C. subflexa*. In fact, the enzyme activity did not differ significantly from that of larvae feeding on non-host cottonseeds. Together with the results of the digital gene expression analysis, these findings contradict the hypothesis that *C. subflexa* might benefit from *Physalis*-feeding through a stimulated immune system.

Supplementary data for chapter III

SDS PAGE of microsome samples

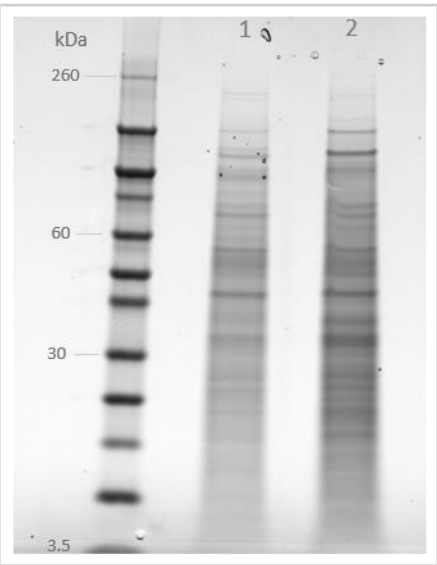

Figure 61: SDS PAGE of microsome extracts of *C. virescens* (1) and *C. subflexa* (2). Novex™ sharp (Thermo Fisher Scientific™) protein standard 3.5 - 260 kDa.

Overview of the accurate masses of detected metabolites (microsome assays)

Compound	MW g/mol	Sum formula	rt (min)	Signal neg. ion m/z	Ion
withaferin A	470	$C_{28}H_{38}O6$	8.9	515.2647	formiate adduct
unknown metabolite 1	472	$C_{28}H_{40}O6$	8.7	517.2808	formiate adduct
unknown metabolite 2	474	$C_{28}H_{42}O6$	8.5	519.2948	formiate adduct
Compound	**MW g/mol**	**Sum formula**	**rt (min)**	**Signal pos. ion m/z**	**Ion**
withaferin A	470	$C_{28}H_{38}O6$	8.9	471.2743	[M+H]+
unknown metabolite 1	472	$C_{28}H_{40}O6$	8.7	473.2904	[M+H]+
unknown metabolite 2	474	$C_{28}H_{42}O6$	8.5	475.305	[M+H]+

Table 3: Accurate masses of withaferin A and the two detected metabolite compounds measured via LC-MS/MS. Samples were measured in pos. ion and neg. ionisation modes.

Microsome assay: mass spectrum chromatograms

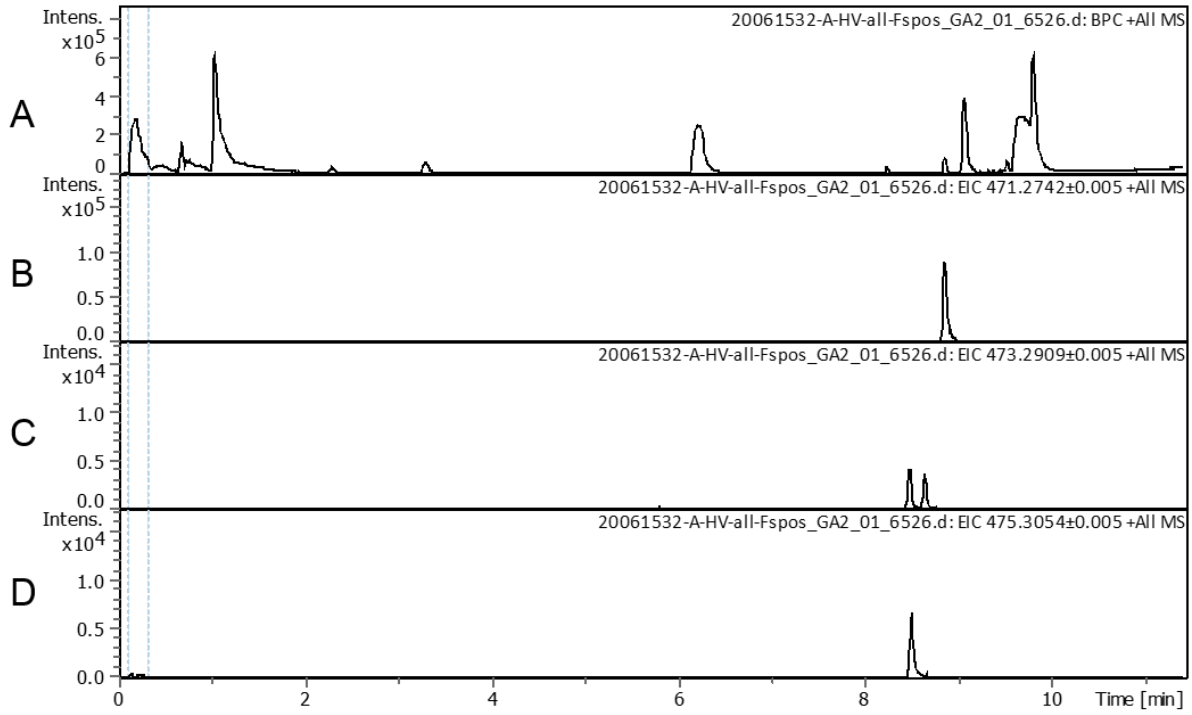

Figure 62: Chromatograms for the *C. virescens* microsome assay: A) Basepeak chromatogram, B) withaferin A, C) metabolite 1 and D) metabolite 2 measured in pos. ionisation mode.

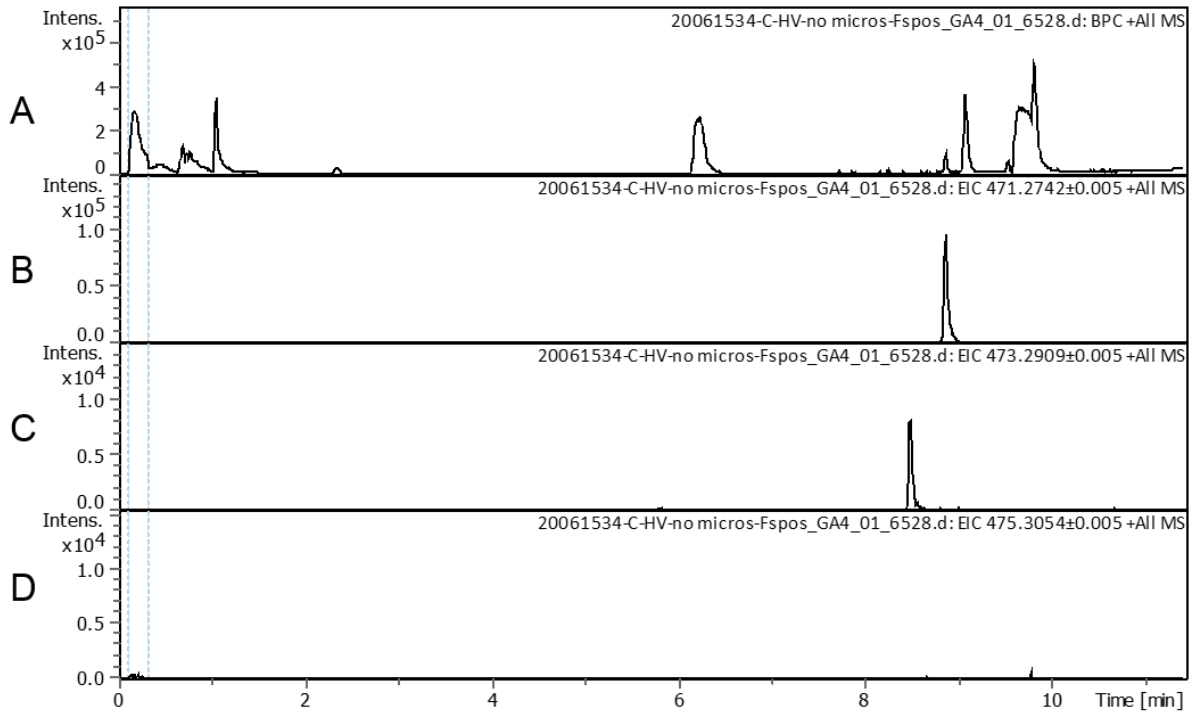

Figure 63: Chromatograms for the *C. virescens* microsome assay (control): A) Basepeak chromatogram and B) withaferin A. C) and D) missing peaks for metabolite 1 and metabolite 2. [Pos. ionisation mode].

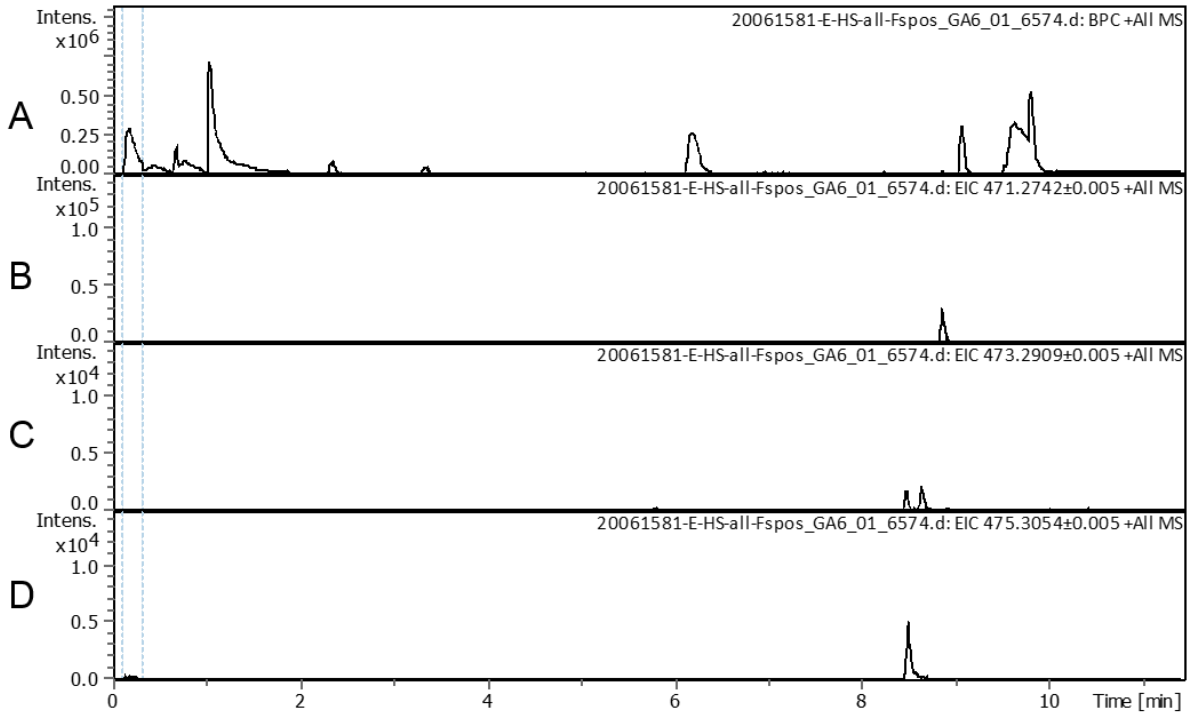

Figure 64: Chromatograms for the *C. subflexa* microsome assay: A) Basepeak chromatogram, **B)** withaferin A, **C)** metabolite 1 and **D)** metabolite 2 measured in pos. ionisation mode.

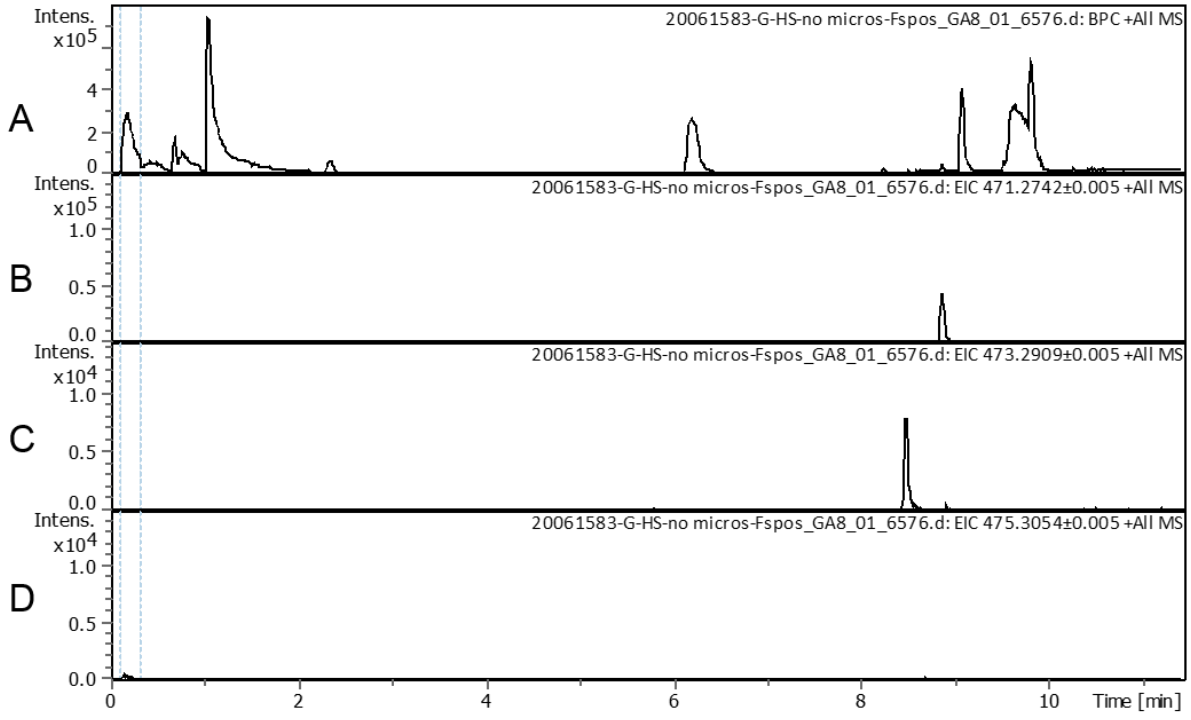

Figure 65: Chromatograms for the *C. subflexa* microsome assay (control): A) Basepeak chromatogram and **B)** withaferin A. **C)** and **D)** missing peaks for metabolite 1 and metabolite 2 [pos. ionisation mode].

Detected peak areas (microsome assay)

C. virescens	MW 470	MW 472	MW 474
	EIC 471.2743±0.005 +All MS	EIC 473.2904±0.005 +All MS	EIC 475.3050±0.005 +All MS
Sample	**Area Target Cmpd**	**Area Target Cmpd**	**Area Target Cmpd**
	withaferin A	unknown metabolite 1	unknown metabolite 2
A-CV-all-Fspos	325170	9358	17793
A-CV-all-Fspos	290777	11193	19868
A-CV-all-Fspos	269571	10935	17428
B-CV-no with a-Fspos	49754	0	0
B-CV-no with a-Fspos	41958	0	0
B-CV-no with a-Fspos	38438	0	0
C-CV-no micros-Fspos	352149	0	0
C-CV-no micros-Fspos	315316	0	0
C-CV-no micros-Fspos	300267	0	0
D-CV-no NADPH-Fspos	74697	0	0

Table 4: Measured peak areas of parental compound withaferin A and two potential withaferin A metabolites (MW 472 & MW 474) detected in *C. virescens* microsome assays. Triplicates of A) the complete enzyme assay; and the controls B) without withaferin A, C) without microsomes and D) without NADPH. All samples were measured in pos. ion mode (LC-MS).

C. subflexa	MW 470	MW 472	MW 474
	EIC 471.2743±0.005 +All MS	EIC 473.2904±0.005 +All MS	EIC 475.3050±0.005 +All MS
Sample	**Area Target Cmpd**	**Area Target Cmpd**	**Area Target Cmpd**
	withaferin A	unknown metabolite 1	unknown metabolite 2
E-CS-all-Fspos	248524	9253	17874
E-CS-all-Fspos	90807	7055	15064
E-CS-all-Fspos	88337	7684	15217
F-CS-no with a-Fspos	2962	0	0
F-CS-no with a-Fspos	2487	0	0
F-CS-no with a-Fspos	2505	0	0
G-CS-no micros-Fspos	354415	0	0
G-CS-no micros-Fspos	142793	0	0
G-CS-no micros-Fspos	136675	0	0
H-CS-no NADPH-Fspos	63771	0	0
Additional controls			
I-meoh--Fspos	20921	0	0
J-withaf-proced--Fspos	668898	0	0
K-withaf--Fspos	2824338	0	0

Table 5: Measured peak areas of parental compound withaferin A and two potential withaferin A metabolites (MW 472 & MW 474) detected in *C. subflexa* microsome assays. Triplicates of E) the complete assay; and the controls F) without withaferin A, G) without microsomes and H) without NADPH. Additional controls: I) MeOH (the solvent), J) withaferin A used to test for the recovery rate upon the extraction procedure and K) reference withaferin A. All samples were measured in pos. ion mode (LC-MS).

Characteristic fragmentation pattern of withanolides and potential withanolide candidates detected in *P. peruviana* leaves

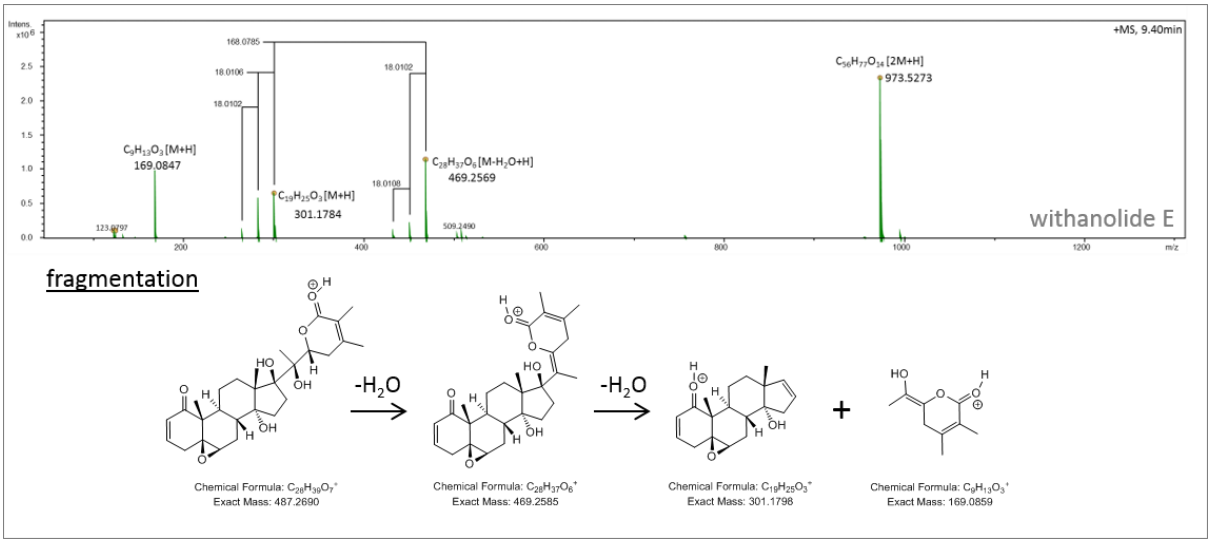

Figure 66: Characteristic MS fragmentation pattern of the withanolides measured in the pos. ionisation mode. The MS fragmentation pattern is exemplary shown for withanolide E.

Predicted withanolide candidates detected in *P. peruviana* leaf exracts

Compound	Sum formula neg. mode	Retention time (*m/z*)	Predicted compound
W1	$C_{28}H_{38}O_9$	7.89	
W2	$C_{28}H_{40}O_9$	8.07	
W3	$C_{28}H_{38}O_8$	8.35	4β-hydroxywithanolide E
W4	$C_{28}H_{40}O_8$	8.41	
W5	$C_{28}H_{40}O_8$/$C_{28}H_{38}O_8$	8.59	
W6	$C_{28}H_{38}O_7$	8.68	
W7	$C_{28}H_{40}O_8$	8.77	
W8	$C_{28}H_{38}O_7$	8.87	
W8a	$C_{28}H_{38}O_7$	8.97	
W9	$C_{28}H_{40}O_7$	9.07	
W10	$C_{28}H_{39}O_7Cl$	9.15	withanolide C?
W11	$C_{28}H_{38}O_7$	9.25	
W12	$C_{28}H_{38}O_7$	9.37	withanolide E
Compound	**Pos. mode**	**Retention time (*m/z*)**	**Predicted compound**
W1	$C_{28}H_{38}O_9$	7.89	27-hydroxy-4β-hydroxywithanolide E isomer
W2	$C_{28}H_{40}O_9$	8.07	2,3-dihydro-27-hydroxy-4β-hydroxywithanolide E
W3	$C_{28}H_{40}O_9$	8.07	isomer hydroxylated 4β-hydroxywithanolide E
W4	$C_{28}H_{38}O_8$	8.35	4β-hydroxywithanolide E
W5	$C_{28}H_{40}O_8$	8.41	2,3-dihydro-17,27-hydroxylated withanolide D derivative
W6	?	8.59	chlorinated withanolide?
W7	$C_{28}H_{42}O_5$	8.80	dunawithagenin
W8	$C_{28}H_{38}O_7$	8.90	physangulatin A or B
W9	?	9.18	withanolide C
W10	$C_{28}H_{38}O_7$	9.40	withanolide E

Table 6: Major fragments with sum formula and retention time of predicted withanolides and derivatives in leaf extracts from unlabelled *P. peruviana*. All compounds listed displayed the characteristic fragmentation pattern for withanolides in the positive and negative ionisation mode.

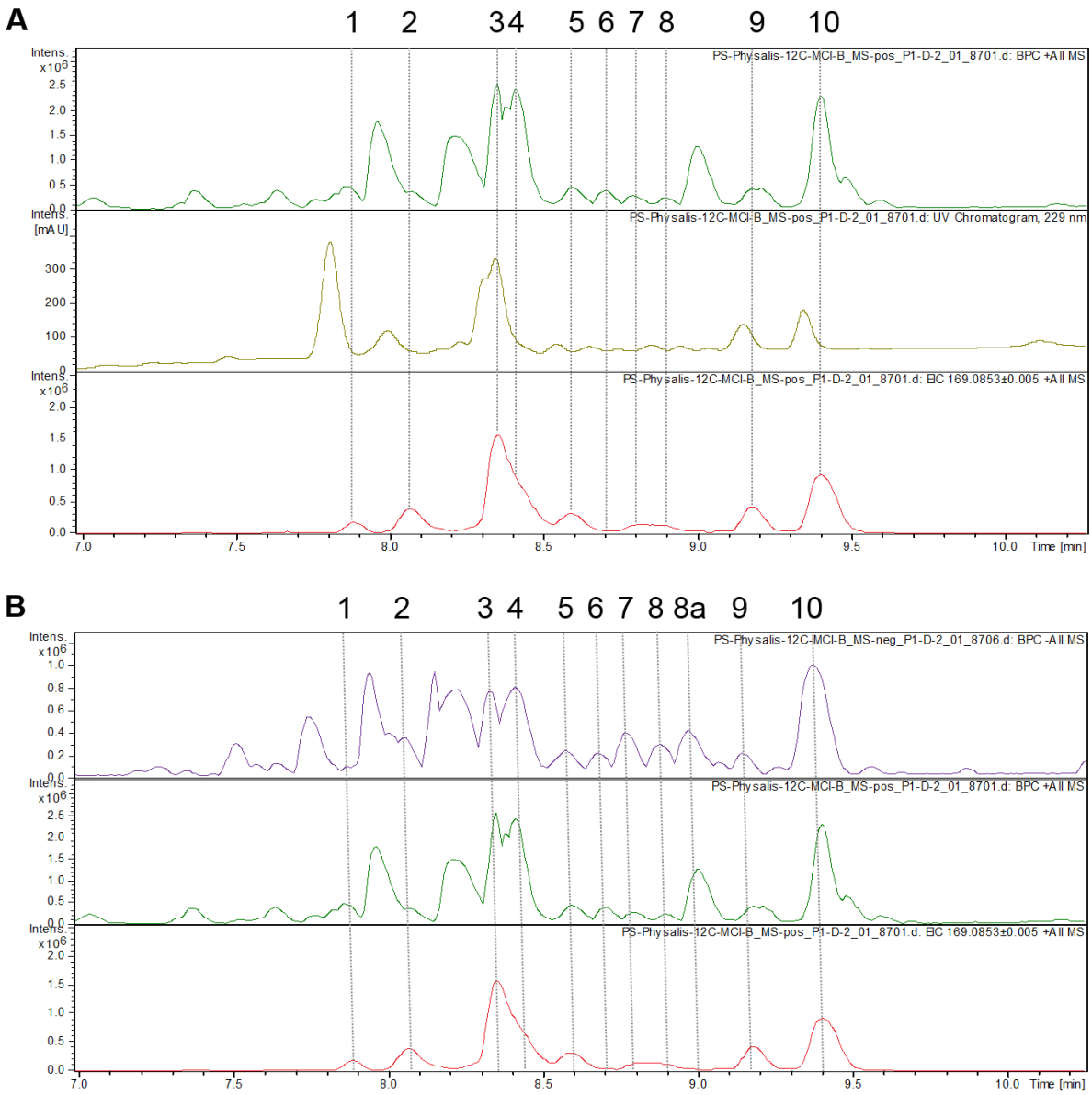

Figure 67: Extracted MS spectra in *P. peruviana* leaf extracts. A) Base peak MS chromatograms of compounds with fragment *m/z* 169.0853 measured in pos. ionisation mode and the corresponding UV chromatogram. **B)** Base peak MS chromatograms of these compounds measured in neg. ionisation mode. The numbers refer to potential withanolide candidates showing the characteristic fragmentation pattern (cf. **Table 6**).

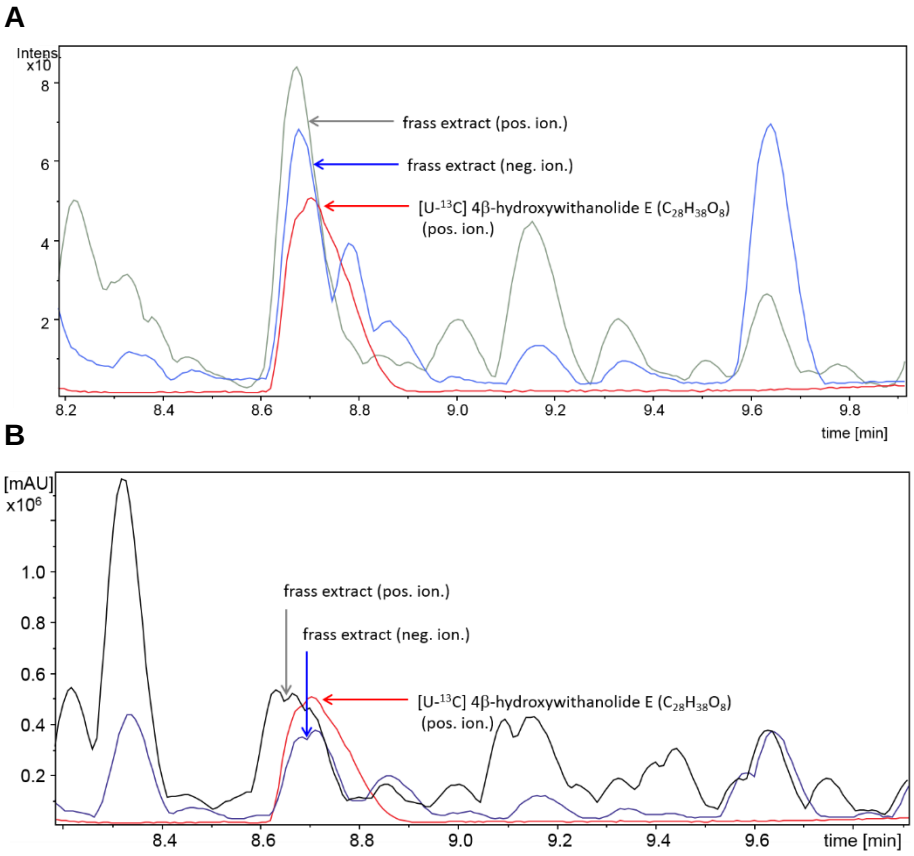

Figure 68: Base peak MS chromatograms of the *Chloridea* frass extracts from **A)** *C. virescens* and **B)** from *C. subflexa* measured in pos. (black) and neg. (blue) ionisation modes. The reference spectrum for [U-^{13}C]-4β-hydroxywithanolide E is shown in red (pos. ionisation mode).

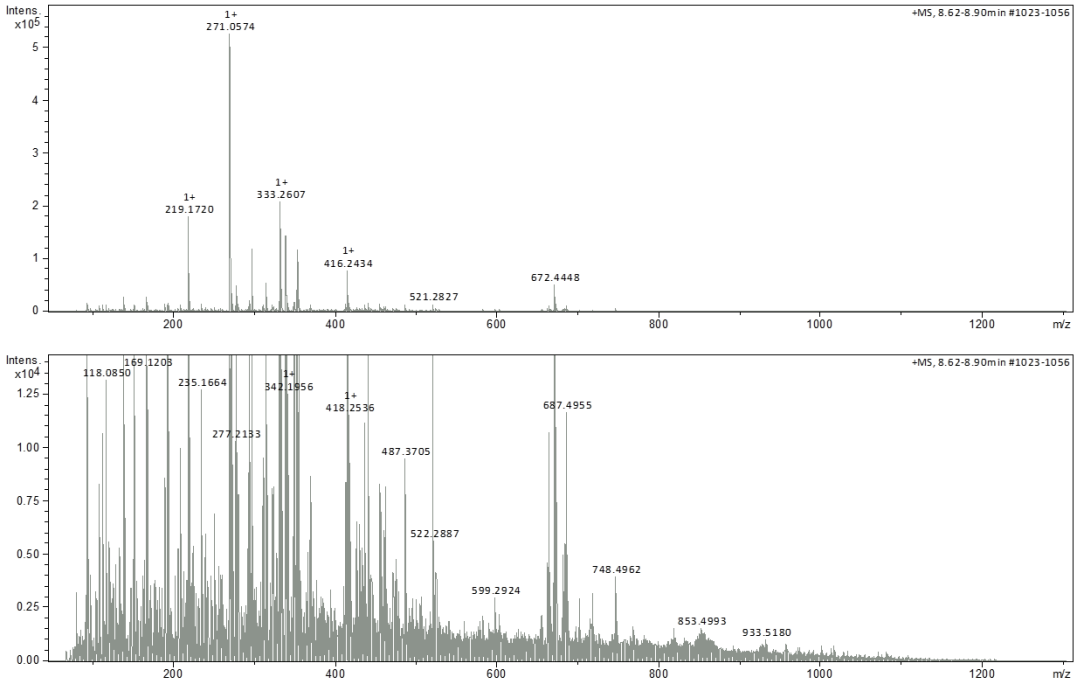

Figure 69: Extracted MS spectra of *C. virescens* frass samples in the retention time range of 4β-hydroxywithanolide E. Peaks occurring at the expected retention time do not show the typical isotopologue pattern. The parental compound could not be recovered in the frass.

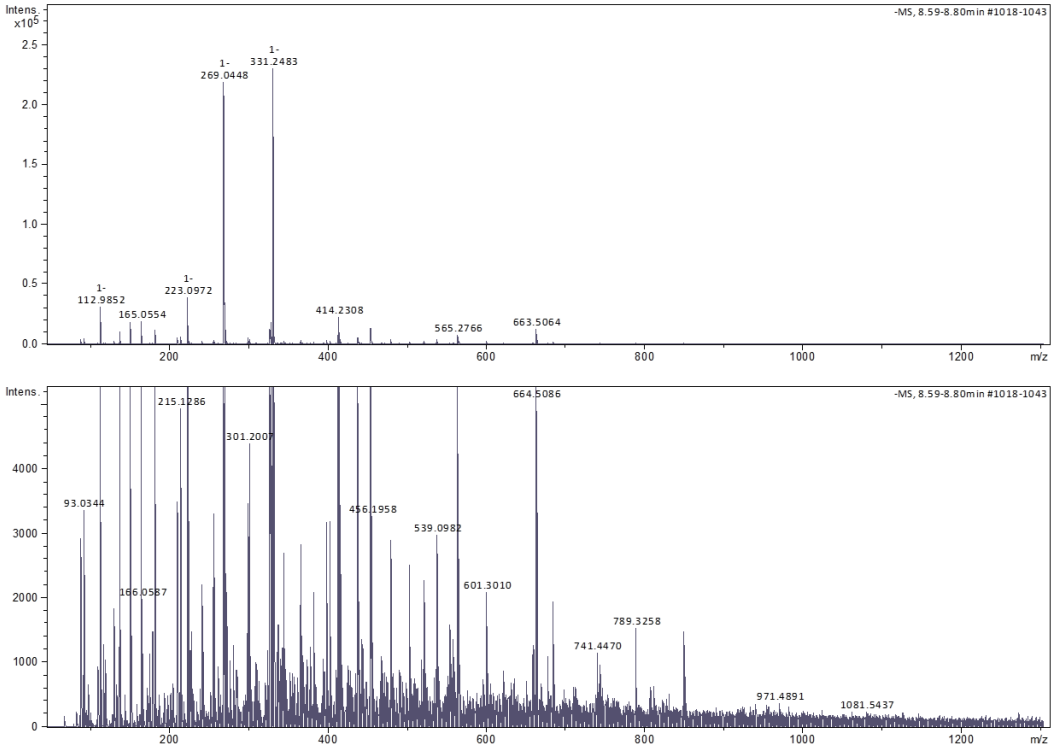

Figure 70: Extracted MS spectra of *C. subflexa* frass samples at the retention time of 4β-hydroxywithanolide E. Peaks occurring at the expected retention time do not show the typical isotopologue pattern. The parental compound could not be recovered in the frass.

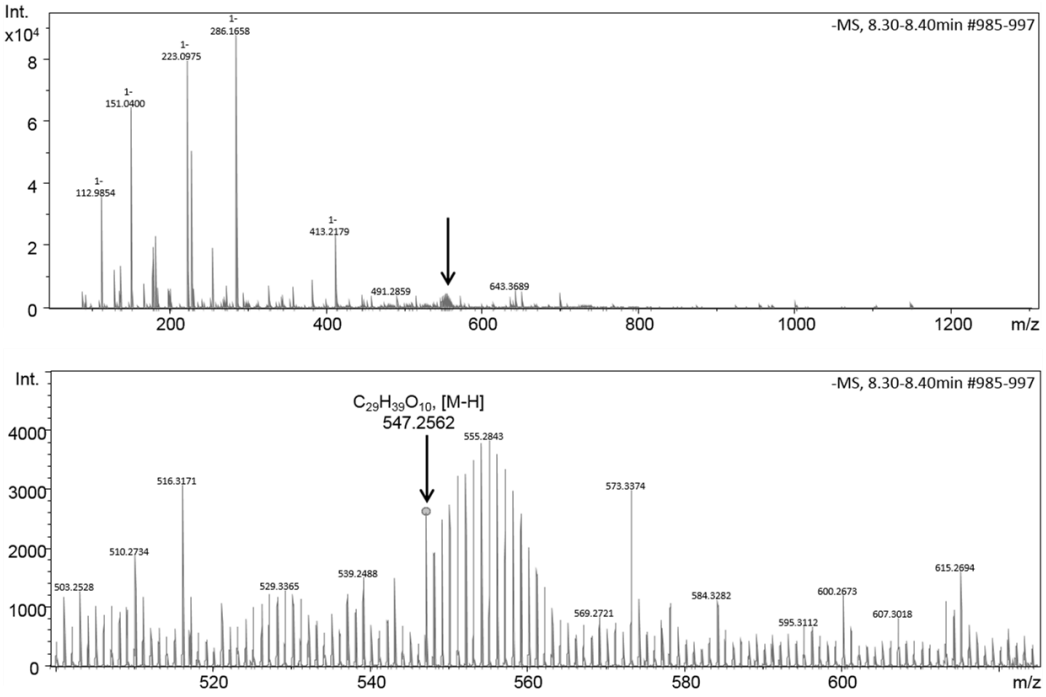

Figure 71: Extracted MS spectra of *C. virescens* frass samples showing the transformant ($C_{28}H_{38}O_8$) of ^{13}C-labelled 4β-hydroxywithanolide E (formate adduct) at a retention time range of 8.3 - 8.4 min [measured in neg. ionisation mode].

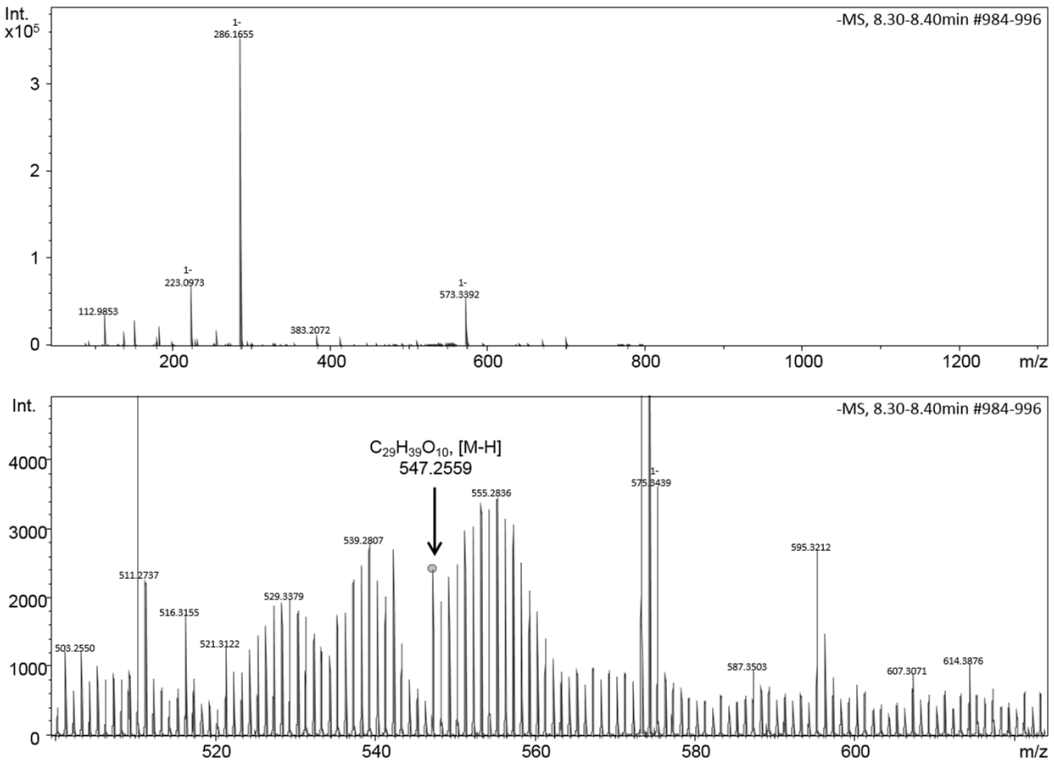

Figure 72: Extracted MS spectra of *C. subflexa* frass samples showing the main transformant ($C_{28}H_{38}O_8$) of ^{13}C-labelled 4β-hydroxywithanolide E (formate adduct) at a retention time range of 8.3 - 8.4 min [measured in neg. ionisation mode].

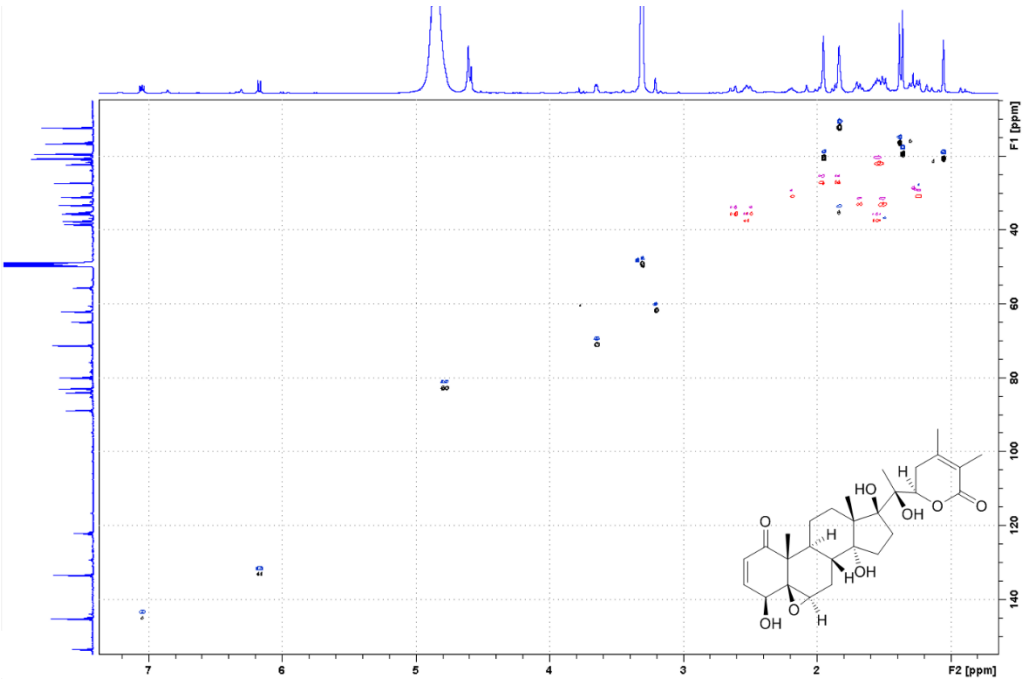

Figure 73: Superimposed ^{1}H-^{13}C HSQC spectra of [U-^{13}C]-4β-hydroxywithanolide E (black and red signals), isolated from ^{13}C-labelled plant material of *P. peruviana* and a 4β-hydroxywithanolide E reference (blue and magenta signals). For clarity, the reference signals have been shifted upwards; the compounds are identical. The F2 and F1 projections show ^{1}H- and ^{13}C-NMR spectra of [U-^{13}C]-4β-hydroxywithanolide E.

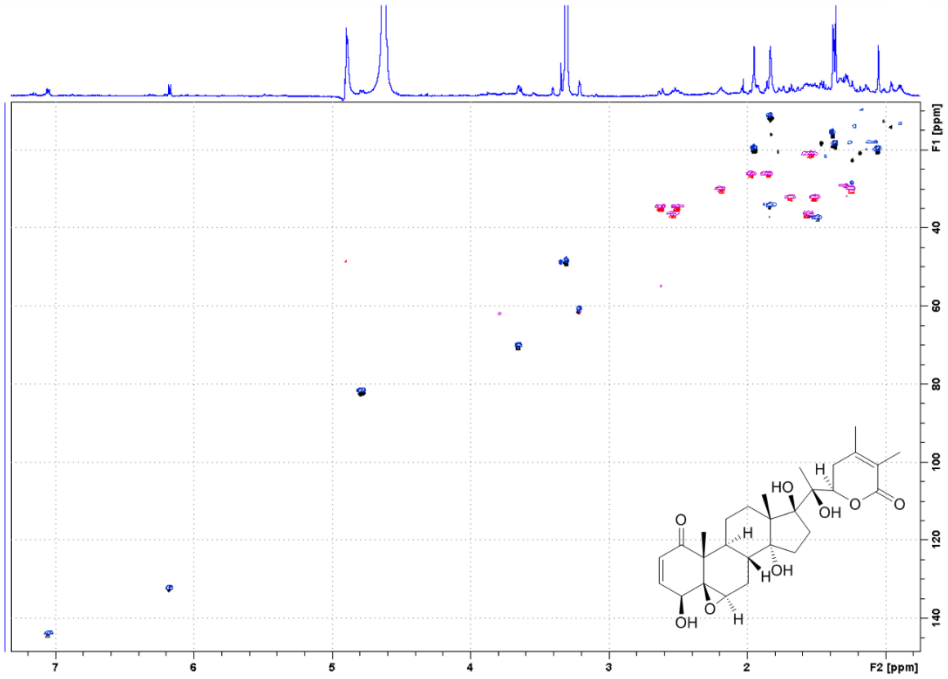

Figure 74: Superimposed ^{1}H-^{13}C HSQC spectra of [U-^{13}C]-4β-hydroxywithanolide E (black and red signals), recovered from the combined frass samples of *C. virescens* and *C. subflexa* , and a 4β-hydroxywithanolide E reference (blue and magenta signals). For clarity, the reference signals have been shifted upwards; the compounds are identical. The F2-projection shows the ^{1}H-NMR spectrum of [U-^{13}C]-4β-hydroxywithanolide E isolated from the frass samples.

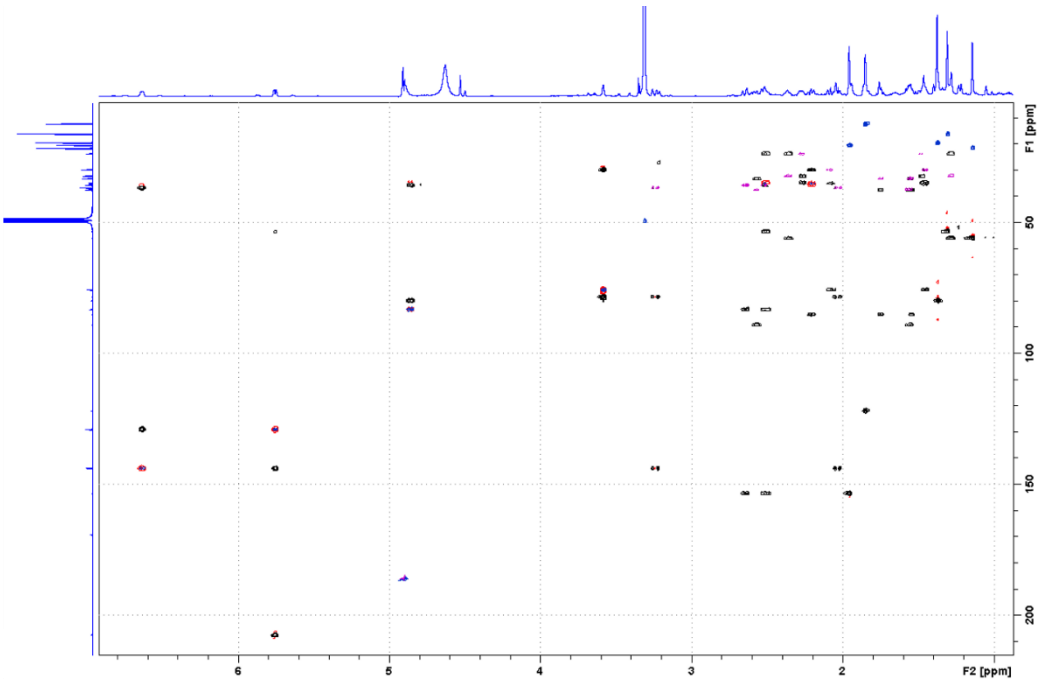

Figure 75: Superimposed spectra of [U-^{13}C]-withanolide S (^{1}H-^{13}C ADEQUATE (refocussed, black and red signals) and ^{1}H-^{13}C HSQC (blue and magenta signals)) recovered from the combined frass samples of *C. subflexa* and *C. virescens*. Red signals in ^{1}H-^{13}C ADEQUATE represent $^{1}J_{CH}$ correlations, black signals stand for $^{2}J_{CH}$ correlations arising from direct ^{13}C-^{13}C coupling. The present data allow for unequivocal assignment of the structure.

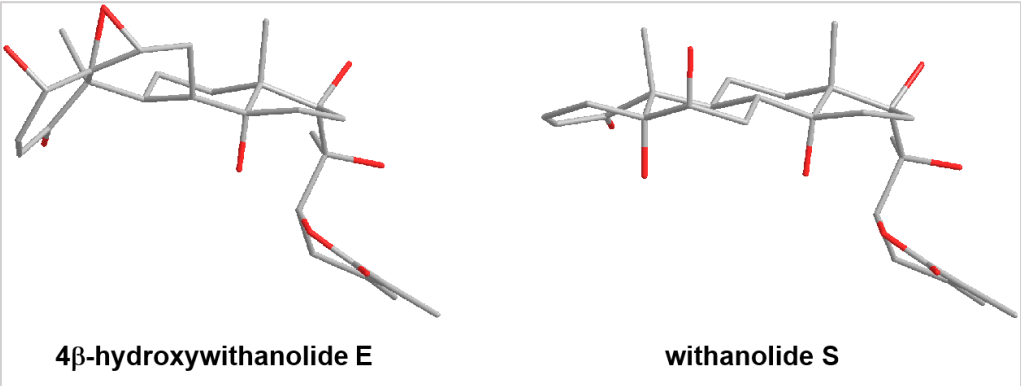

Figure 76: Force-field (MM2) optimised structures of 4β-hydroxywithanolide E and withanolide S. Compared to the geometry of starting product 4β-hydroxywithanolide E that of the metabolite withanolide S has changed considerably.

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List of Publications

Peer-reviewed Publications in Scientific Journals

- Sell, M. P., Amezian, D., Heckel, D. G., Pauchet, Y. (2021). Biological function of solanaceous withanolides and their effects on herbivorous insects. Annual Plant Reviews, 4(2), 625-648.
- Schulz, N. K. E., Sell, M. P., Ferro, K., Kleinhölting, N., Kurtz, J. (2019). Transgenerational developmental effects of immune priming in the red flour beetle *Tribolium castaneum*. Frontiers in Physiology, 10:98.
- Futo, M., Sell, M. P., Kutzer, M. A. M., Kurtz, J. (2017). Specificity of oral immune priming in the red flour beetle *Tribolium castaneum*. Biology Letters, 13(12): 20170632.

Conference Contributions (selection)

Talks

Sell, M. P. (2018). Effects of the main secondary metabolites of *Physalis* plants on a specialist and a generalist species of Lepidoptera. Talk presented at the 10th International Workshop on Molecular Biology and Genetics of the Lepidoptera, Kolympari, Crete, GRC

Posters

Sell, M. P., Pauchet, Y., Heckel, D. G. (2020). How to adapt to feeding on the main secondary metabolites of *Physalis* plants? Ask a specialist and a generalist species of Lepidoptera! 18TH IMPRS SYMPOSIUM, INTERNATIONAL MAX PLANCK RESEARCH SCHOOL, Dornburg, DE

Sell, M. P., Pauchet, Y., Heckel, D. G. (2019). How to adapt to feeding on the main secondary metabolites of *Physalis* plants? Ask a specialist and a generalist species of Lepidoptera! 112TH ANNUAL CONFERENCE OF THE GERMAN ZOOLOGICAL SOCIETY, Jena, DE

Sell, M. P., Pauchet, Y., Heckel, D. G. (2018). Effects of the main secondary metabolites of *Physalis* plants on a specialist and a generalist species of Lepidoptera. XI EUROPEAN CONGRESS OF ENTOMOLOGY, Naples, IT

Sell, M. P., Barthel, A., Pauchet, Y., Heckel D. G. (2018). Effects of the main secondary metabolites of *Physalis* plants on a specialist and a generalist species of Lepidoptera. Poster presented at 17TH IMPRS SYMPOSIUM, INTERNATIONAL MAX PLANCK RESEARCH SCHOOL, Dornburg, DE

Sell, M. P., Futo, M., Kutzer, M. A. M., Kurtz, J. (2017). Specificity in oral immune priming in *T. castaneum*. PHD STUDENT MEETING "Conflict and Cooperation - Bridging Evolution, Ecology and Immunology", Bautzen, DE

Sell, M. P., Futo, M., Kutzer, M. A. M., Kurtz, J. (2016). Specificity in oral immune priming in *T. castaneum* 109TH ANNUAL CONFERENCE OF THE GERMAN ZOOLOGICAL SOCIETY, Kiel, DE

Supervision of Students

Amezian, Dries (2018). Effect of withanolide-containing diet on gut microbial communities and AMP expression in two closely related Lepidoptera species: *Heliothis subflexa* and *Heliothis virescens*. Master thesis, National Institute of Further Education, Montpellier, FRA

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Des Weiteren versichere ich, dass ich weder die Hilfe eines Promotionsberaters noch einer kommerziellen Promotionsvermittlung in Anspruch genommen habe und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir im Zusammenhang mit Inhalten dieser Dissertation erhalten haben. Die vorliegende Dissertation oder eine in wesentlichen Teilen ähnliche Abhandlung wurde zu keinem früheren Zeitpunkt an einer anderen Hochschule oder Fakultät als Dissertation eingereicht.

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