ABSTRACTS:

Insect Physiological Responses to Plant Toxins: Oral Presentations

In programme order

Session 20 (Part I)
Session 23 (Part II)

Evolutionary Physiology of Insect-Plant Interactions on a Tritrophic Scale

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Plants produce a tremendous variety of toxic compounds to protect themselves against herbivores. Remarkably, many herbivorous insects not only cope with plant toxins but also store them in their bodies as a defense against predators and parasitoids (sequestration). Since sequestering insects exploit plants in at least two ways, as a dietary resource and as a source of defensive compounds, understanding the evolution of their host plant relationships require the integration of both traits. We study the physiological mechanisms underlying insect resistance to host plant toxins in the milkweed butterflies (Lepidoptera: Danainae) and found that resistance and sequestration can be intertwined. Specifically, sequestration of plant toxins requires resistance mechanisms different from those needed to cope with toxins in the diet. Hence, predators selecting for sequestration can spur the coevolutionary arms race between insects and plants. Moreover, in the milkweed bugs (Heteroptera: Lygaeinae) it seems likely that acquisition of plant toxins for defense directed specific associations with certain host plants. Although sequestration is common among herbivorous insects, the underlying mechanisms and the physiological constraints of sequestration are largely unknown. Using mechanistic approaches in a comparative evolutionary framework, our research revealed first insights how quantitative differences between sequestering species and non-sequestering relatives are mediated. Furthermore, we found that dietary exposure to toxins causes different physiological effects in closely related sequestering species suggesting that physiological constraints are highly context dependent. Based on our findings, we propose that detailed physiological analyses are required to understand the evolutionary forces directing plant-insect coevolution in sequestering insects.

Keywords: cardenolide; milkweed bug; milkweed butterflies; resistance; sequestration

The role of quinolizidine alkaloids from *Genista* plants (Fabaceae) in aphid-plant interactions

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The pea aphid (*Acyrthosiphon pisum*), native to legume plants, comprises at least 15 genetically different biotypes, each adapted to just one or a very few different host plants. Only on its respective host plant, an aphid biotype can perform well. One of these biotypes is specialized on *Genista* plants, known to contain quinolizidine alkaloids (QAs). These compounds normally stimulate and paralyze the central nervous system of insects, ultimately leading to their deaths. This raises the question what enables *Genista* biotype aphids to perform on this chemically defended plant? Before answering this question, we needed to identify the most abundant QAs within extracts of *Genista tinctoria*. This was done using NMR spectroscopy and high-resolution mass spectrometry. Eight QAs were found, one of which new for *Genista* species and one previously unknown. To investigate whether the aphids are exposed to these, we evaluated the compounds' distribution within the aphid-plant system. The identified QAs were quantified by LC-MS/MS in *G. tinctoria* phloem sap (the aphids' food), leaf and stem extracts, as well as in *Genista* biotype aphid tissue and honeydew. Additionally, cross-sections of stems and leaves from *G. tinctoria* were investigated by MALDI-MS supported by microscopy and histochemical staining. Only a few of the identified QAs are present within the *G. tinctoria* phloem sap and, in turn, in the aphids and their honeydew. Additionally, we observed translocation of particular compounds into the aphids' hemolymph. These findings provide the basis for further investigations how aphids of the *Genista* biotype are able to avoid poisoning by QAs.

Keywords: *Acyrthosiphon pisum; alkaloids; chemical defense; Genista tinctoria; sequestration*

Functional characterization of Na,K-ATPase subunit combinations reveals the adaptive strategy of cardenolide-resistant large milkweed bugs

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The evolutionary arms race between plants and specialized herbivorous insects has been widely studied but the myriad mechanisms involved in this race still pose fascinating riddles. Here, we present one facet of coevolution from the insect perspective: the evolution of three Na,K-ATPase α-subunit gene copies with strongly differing resistance towards cardenolides in the large milkweed bug (*Oncopeltus fasciatus*). The large milkweed bug ingests high concentrations of cardenolides by sucking the seeds of *Asclepias* plants, and does not suffer any harm in the process. We identified different amino acid substitutions in the cardenolide binding site of three α-paralogs (A,B,C), and we found that each paralog had altered enzymatic activity and resistance. We further identified four different β-subunits that modulate the Na,K-ATPases' behavior. We heterologously expressed nine possible α/β-subunit combinations of *O. fasciatus* Na,K-ATPases in *Sf*9 cells with the baculovirus expression system. The recombinant enzymes were exposed to increasing concentrations of two cardenolides, which differ in source, structure, and steric conformation: ouabain and calotropin. Calotropin, synthesized in the bug's host plants, showed a much stronger inhibitory effect on the Na,K-ATPase than ouabain. The conserved and most enzymatically active αC-copy was completely inhibited at high cardenolide concentrations, whereas αB maintained moderate residual activity at the same concentrations. We found evidence that specific α/β-combinations can enhance the activity (αCβ3) or resistance to cardenolides (αAβ1) of the Na,K-ATPase. Our results thus reveal that adaptation to the host plant toxins tuned the duplicated paralogs to trade-off the pleiotropic effects of resistance versus ion transport.

Keywords: recombinant enzymes; inhibition; calotropin; ouabain; α/β-subunit combinations

Convergent recruitment of detoxification enzymes: The flavin-dependent monooxygenases of *Longitarsus jacobaeae*

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Toxic secondary plant compounds impose a strong selective pressure on herbivores for their tolerance or detoxification. Convergently evolved strategies to deal with plant toxins are emerging across an increasing number of species. In this study we focus on the physiological adaptations of insects to detoxify pyrrolizidine alkaloids (PAs). The flea beetle *Longitarsus jacobaeae* is one of only a few animals specialized to feed on the tansy ragwort (*Senecio jacobaea*). This common wild flower is highly toxic, containing pyrrolizidine alkaloids, which function as a defense mechanism against mammalian and insect herbivores. Transcriptomic analyses of *L. jacobaeae* revealed two genes similar to known flavin-dependent monooxygenases (FMOs) and highly conserved in their predicted crystal structure. These enzymes have been recruited in two other groups of herbivorous insects as PA-specific detoxification enzymes that reconvert the alkaloids to non-toxic *N*-oxides. In our functional tests using heterologously expressed proteins of *L. jacobaeae* FMOs we tested the ability of both FMOs to *N*-oxidize senecionine, the common PA of *S. jacobaea*. Tissue-specific gene expression analyses suggest that FMO 1 is responsible for primary tissue-protection throughout the entire body, whereas FMO 2 is restricted to the intestinal and nervous tissue. Phylogenetic analyses support that, unlike the situation in arctiid moths, the gene duplication in the genus *Longitarsus* predates the adaptation to PA plants and that both genes were recruited as detoxification genes in the beetles.

Dietary cardenolides enhance growth and change the direction of the fecundity-longevity trade-off in specialized sequestering milkweed bugs (Heteroptera: Lygaeinae)

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Milkweed bugs (Heteroptera: Lygaeinae) sequester high amounts of plant-derived cardenolides as a defense against predators. Although being inhibitors of the ubiquitous animal enzyme Na^+/K^+-ATPase, milkweed bugs can tolerate cardenolides by means of resistant Na^+/K^+-ATPases. Both adaptations, resistance and sequestration, are ancestral traits shared by most species of the Lygaeinae. Using four milkweed bug species and the related European firebug *Pyrrhocoris apterus* showing different combinations of the traits 'cardenolide resistance' and 'cardenolide sequestration', we tested how the two traits affect larval growth upon exposure to dietary cardenolides in an artificial diet system. While cardenolides impaired the growth of *P. apterus* nymphs neither possessing a resistant Na^+/K^+-ATPase nor sequestering cardenolides, growth was not affected in the non-sequestering milkweed bug *Arocatus longiceps,* which possesses a resistant Na^+/K^+-ATPase. Remarkably, cardenolides increased growth in the sequestering specialists *Caenocoris nerii* and *Oncopeltus fasciatus* but not in the sequestering generalist *Spilostethus pandurus,* which all possess resistant Na^+/K^+-ATPases. Furthermore, we investigated the effect of dietary cardenolides on life-history traits in *O. fasciatus*. Interestingly, nymphs developed faster and lived longer as adults. However, adults raised on cardenolide-containing diet produced less offspring when maintained on the same diet, while no effect was observed when adults were transferred to sunflower seeds. We speculate that the resistant Na^+/K^+-ATPase of milkweed bugs is selected for working optimally in a 'toxic environment', i.e. when cardenolides are sequestered in the body tissues. Our results indicate a trade-off between longevity and fecundity, whose direction can be altered by the availability of cardenolides in the diet.

Keywords: Fitness costs; Life-history traits; Na^+/K^+-ATPase; Sequestration; Trade-offs

The horseradish flea beetle selectively absorbs glucosinolates across the foregut

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The ability to accumulate (sequester) plant defense compounds for protection from predators is widespread in herbivorous insects. Yet, the absorption mechanisms for polar defense compounds that cannot passively diffuse through cell membranes are not well understood. We investigated the uptake of glucosinolates, the characteristic defense compounds in Brassicaceae, from the gut lumen into the hemolymph of the horseradish flea beetle (*Phyllotreta armoracia*e). By allowing beetles to ingest an aqueous mixture of different plant glucosides (including glucosinolates and non-host glucosides), we demonstrate a selective absorption of glucosinolates across the gut. In addition, beetles accumulated sinigrin, the major glucosinolate in their natural host plant horseradish, at a higher rate than other glucosinolates. Together, these findings show that glucosinolate absorption in *P. armoraciae* is highly selective. We next determined where exactly glucosinolates are absorbed from the gut lumen, and detected a much higher uptake rate across the foregut compared to the midgut. Since the foregut is of ectodermal origin and, therefore, lined with a chitin-containing cuticle, we hypothesized that morphological adaptations are necessary for glucosinolate uptake to occur. Indeed, we observed a massive reduction of the procuticle thickness and chitin content of the *P. armoraciae* crop in comparison to a non-sequestering leaf beetle. In summary, we show that physiological and morphological adaptations enable *P. armoraciae* to sequester plant defense compounds. Our results lay the groundwork for the identification of glucosinolate-specific transporters in the foregut.

Keywords: adaptation, cuticle, plant defense, sequestration, transporter

Re-igniting the mustard oil bomb: knocking out key host plant adaptive genes in *Pieris* butterfly larvae

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Pieris butterfly larvae feed on plants containing diverse glucosinolates (GLS) with redirecting toxic breakdown products of GLSs to less toxic metabolites using gut-expressed nitrile-specifier proteins (*NSP*s). *NSP* is considered an evolutionary key innovation for Pieridae that enabled these butterflies to colonize GLS containing plants and allowed subsequent diversification. However, we still do not fully understand the roles of both *NSP* and its sister gene, major allergen (*MA*), in overcoming the wide range of host plant GLSs larvae encounter in the field. Here, we tested the ecological relevance of *NSP* and *MA* for *Pieris* larvae by knocking out both genes in *Pieris brassicae* using the CRISPR/Cas9 genome editing technique. We found that gut protein extracts of *NSP*/*MA* double KO larvae completely lost their activity against all GLSs tested. Moreover, we also found that *NSP* KOs and *MA* KOs showed lower performance on host plants with different GLS profiles, whereas *NSP*/*MA* KOs could not survive on host plants with higher GLS concentration. These results clearly suggest that both *NSP* and *MA* have different but complementary roles in defusing the mustard oil bomb in *Pieris* larvae, and that both genes are crucial for *Pieris* in overcoming their host plants´ major chemical defense.

Keywords: Nitrile-specifier protein; Glucosinolate; counter-adaptation; genome-editing; substrate specificity

Diabrotica virgifera virgifera females can sequester multiple plant toxins to protect their eggs against natural enemies

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Various herbivorous insects sequester defensive secondary metabolites from their host plant and use them against their natural enemies. The known examples of sequestration involve one specific type of chemical defense and this is well documented for insect larvae and adults. However, it is still rarely shown that metabolites can be transferred to eggs and to our knowledge there are no examples of insects sequestering multiple types of defense metabolites. In this study, we: i) investigate sequestration by *Diabrotica virgifera virgifera* beetles, ii) whether sequestered metabolites are transferred to beetle eggs, and iii) whether those metabolites serve defensive functions. *Diabrotica* beetles feed on various host plants, including maize, cucumber and beans, containing defensive benzoxazinoids, cucurbitacins, and cyanogenic glucosides, respectively. Chemical analyses revealed that *D. v. virgifera* beetles sequester and transfer benzoxazinoids and cucurbitacins to their eggs, but not cyanogenic glucosides. To test whether the two sequestered toxins protect eggs against predators, we fed *D. v. virgifera* beetles with toxin-free or toxin-containing plants, and offered their eggs to the rove beetle *Atheta coriaria* and the minute pirate bug *Orius laevigatus*. In choice experiments, both predators consumed more toxin-free eggs than toxin-containing eggs. Moreover, survival assays confirmed the toxic effects of benzoxazinoid-containing eggs on the predators, but, surprisingly, cucurbitacins had no apparent effect. Our results reveal a unique ability of *D. v. virgifera* to use multiple plant defensive chemicals against higher trophic levels, which may in part explain the extraordinary success of this invasive pest.

Keywords: biological control; maize; predators; progeny; *western corn rootworm*.

Rapid specialization of counter defenses enables two-spotted spider mite to adapt to novel plant hosts

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Genetic adaptation, occurring over a long evolutionary time, enables host-specialized herbivores to develop novel resistance traits and to efficiently counteract defenses of a narrow range of host plants. In contrast, physiological acclimation, leading to the suppression and/or detoxification of host defenses is hypothesized to enable broad-generalists to shift between plant hosts. However, the host adaptation mechanisms used by generalists composed of host-adapted populations are not known. *Tetranychus urticae* is an extreme generalist herbivore whose individual populations perform well only on a subset of potential hosts. We combined experimental evolution, *Arabidopsis* genetics, mite reverse genetics, and pharmacological approaches to examine mite host adaptation upon the shift of a bean-adapted population to *Arabidopsis thaliana*. We showed that cytochrome P450 monooxygenases are required for mite adaptation to *Arabidopsis*. We identified activities of two tiers of P450s: general xenobiotic-responsive P450s that have a limited contribution to mite adaptation to *Arabidopsis* and adaptation-associated P450s that efficiently counteract *Arabidopsis* defenses. In ≈25 generations of mite selection on *Arabidopsis* plants, mites evolved highly efficient detoxification-based adaptation, characteristic of specialist herbivores. This demonstrates that specialization to plant resistance traits can occur within the ecological timescale, enabling the two-spotted spider mite to shift to novel plant hosts.

Chemical & Physical Defenses of Conifers and Insect Counter Strategies

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Conifers have evolved complex oleoresin-based chemical defenses against herbivores and pathogens. In co-evolved bark beetles, the terpene components of the conifer oleoresin also serve various chemo-ecological functions as pheromone precursors, chemical barcodes for host identification, or nutrients for insect-associated microbiomes. This presentation highlights the genomic, molecular and biochemical underpinnings of the large chemical space of oleoresin terpenes produced by trees. While oleoresin terpenoid defenses have contributed much to the evolutionary success of conifers, under new conditions of climate change, these chemical defenses may become inconsequential against range-expanding forest pests. Another line of conifer defense against insects involves the deposition of stone cells as a physical barrier against stem feeding insect larvae. Physical stone cell defenses can provide a durable resistance, that may be difficult to overcome by insects.

Keywords: Terpenes; oleoresin; stone cells, bark beetle; weevil

ABSTRACTS:

Insect Physiological Responses to Plant Toxins: Poster Presentations

In programme order

Poster Session 4

Preference of the bronze bug *Thaumastocoris peregrinus* (Hemiptera: Thaumastocoridae) for eucalyptus species: correlation with its secondary chemistry

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Plants are constantly attacked by several enemies, including insect herbivores. The diversity of plant secondary chemistry has been partially linked, to the coevolution triggered by competition between plants and insects. Some plant secondary compounds have evolved as plant against herbivory. In turn, insect herbivores have evolved adaptations to these defenses, including the capacity of host choice and avoidance. Food-plant choice is mediated by visual, tactile, and chemical cues. The later include volatiles detectable at distances, and non-volatile compounds detected after contacting the plant. Here, we studied secondary metabolites (volatiles and fixed) from leaves of different eucalyptus species and correlated these chemical profiles with the feeding preference of *Thaumastocoris peregrinus*. A two-choice preference bioassay (t-test, $P<0.001$) resulted in 4 eucalyptus species classified in 3 categories (preferred, medium, and non-preferred). GCMS data showed that one of the most preferred species (*E. tereticornis*) produces the highest content of p-cimene ($p=0.025$), but that was not the case for the other preferred species (*E. grandis*) and that the 1,8-cineol amount did not differ between the most preferred and the non-preferred species (*E. globulus*, $p>0.05$). These two volatiles have been previously identified as potential biomarkers for the bug preference. Non-volatile putative chemical markers were then identified by multivariate analyses based on GCMS analyses (derivatized aqueous extracts) as 2-hydroxy-propionic acid, glucuronic acid, as well as mixtures of hydroxyaldehydes, monosaccharides and disaccharides. Preferred eucalyptus species present some metabolites in higher concentrations, possibly acting as feeding stimulants, while non-preferred species have higher concentrations of D-xylulose, a potential feeding deterrent.

Keywords: metabolomics; feeding preference; biomarker; feeding stimulant; feeding deterrent

Effect of essential oils on young honeybee gustatory response

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During their life cycle, honeybees are constantly exposed to xenobiotics from the environment and from in-hive products applied as pesticides. Many of these products do not produce honeybee mortality at the applied doses, however their sublethal effects in physiological processes such as gustatory responsiveness are not often evaluated. Among other potential new acaricides for honeybee colonies, plant essential oils (EO) have been found effective and safe for honeybees. However, EO intake can produce sublethal effects as has been shown for cuticular hydrocarbon profiles. To further characterize the potential chronic effects of the exposure of honeybees to EO, we here studied the effect of the consumption of EO from *Eupatorium buniifolium* (Asteraceae), a known acaricide, on the sucrose sensitivity of young honeybees. We characterized the sucrose response threshold (lowest concentration to which honeybees extend their proboscis) of young bees at 2/3, 5/6 and 9/10 days old, using imidacloprid as a positive control given its known effect on the gustatory response of honeybees. Our results showed that the intake of *E. buniifolium* EO does not reduce sensitivity to a sucrose reward at any age (one-way ANOVA, p=0.526, p=0.985, p=0.645, respectively for each age group). Contrarily, the intake of imidacloprid in our experimental setup reduced the sensitivity on bees at 5/6 and 9/10 days old (one-way ANOVA, p=0.034, p=0.044, respectively). These results suggest that the use of EO as acaricides may be safer than other products, and highlights the need for testing sublethal effects of potential sanitary products for honeybees.

Keywords: Asteraceae; *Eupatorium buniifolium*; Imidacloprid; Sublethal effect; Xenobiotics