

Trichoplusia Ni Transcriptomic Responses to the Phytosaponin Aglycone Hederagenin: Sex-related Differences

YINTING CHEN

McGill University

CHRISTINE LAFLEUR

McGill University

RYAN J. SMITH

McGill University

DILJOT KAUR

McGill University

BRIAN T. DRISCOLL

McGill University

JACQUELINE C. BEDE

`jacqueline.bede@mcgill.ca`

McGill University

Research Article

Keywords: Antifeedant, Cytochrome P450, Hederagenin, Saponin, Sex-specific, Transcriptomics, Toxicity.

Posted Date: October 4th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-3356648/v1>

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Journal of Chemical Ecology on March 5th, 2024. See the published version at <https://doi.org/10.1007/s10886-024-01482-1>.

Abstract

Many plant species, particularly legumes, protect themselves with saponins. Previously, a correlation was observed between levels of oleanolic acid-derived saponins, such as hederagenin, in the legume *Medicago truncatula* and caterpillar deterrence. Using concentrations that reflect the foliar levels, hederagenin did not have a toxic effect on 4th instar caterpillars of the cabbage looper *Trichoplusia ni* nor did it act as a feeding deterrent. However, female caterpillars consumed more diet than males, presumably to obtain the additional nutrients required for oogenesis, and are, thus, exposed to higher hederagenin levels. When fed diet with lower hederagenin levels, male caterpillars expressed more genes encoding trypsin-like (LOC113500509, LOC113501951, LOC113501953, LOC113501966, LOC113501965, LOC113499659, LOC113501950, LOC113501948, LOC113501957, LOC113501962, LOC113497819, LOC113501946, LOC113503910) and the cytochrome P450 6B2-like (LOC113493761). When fed the high hederagenin, male caterpillars expressed stress-responsive genes (LOC113503484, LOC113505107). In contrast, female caterpillars expressed high levels of the cytochrome P450 6B7-like (LOC113492289). Bioinformatic tools predict that cytochrome P450s could catalyze the oxygenation of hederagenin which would increase the hydrophilicity of the compound. Expression of the MFS transporter (LOC113492899) showed a hederagenin dose-dependent increase in gene expression suggesting that this transporter may be involved in saponin efflux. These sex-related differences in feeding and detoxification should be taken into consideration in insecticide evaluations to minimize pesticide resistance.

INTRODUCTION

Saponins are plant specialized metabolites important for defense against pathogens and insect herbivores, particularly in legume crops (Hussain et al., 2019; Roopashree and Naik, 2019; Zaynab et al., 2021). Saponin levels, specifically oleanolic acid-derived saponins, have been correlated to resistance of the barrel medic, *Medicago truncatula*, and related plant species to caterpillar herbivory (Adel et al., 2000; Agrell et al., 2003; Cai et al., 2017). This research was performed to determine if the oleanolic acid-derived saponin aglycone hederagenin acted as a toxin or antifeedant and contributed to this resistance. In addition, caterpillar counter-measures to detoxify hederagenin were investigated through a transcriptomic study with special attention paid to sex-related differences in the expression of genes encoding detoxification enzymes.

The barrel medic *Medicago truncatula*, a forage crop in Australia and Mediterranean countries (Crawford et al., 1989; Howieson et al., 2000), has morphological and chemical defenses to protect itself against insect herbivores of which triterpenoid saponins are key defensive plant specialized metabolites (Bede, 2020). From the precursor b-amyrin, triterpenoid saponins can be classified into groups depending on the aglycone: soyasaponin, hederagenin, bayogenin, medicagenic acid, of which the last three are derived from oleanolic acid (Vincken et al., 2007; Moses et al., 2014). Cai et al. (2017) compared different *M. truncatula* ecotypes for resistance against herbivory by caterpillars of the beet armyworm, *Spodoptera exigua* (Hübner). Ecotypes with higher oleanolic acid-derived saponins were more resistant to caterpillar herbivory which appeared to reflect antifeedant activity.

Saponins can negatively affect insect herbivores in different ways (De Geyter et al., 2007). Haemolytic-type saponins, for example oleanolic acid-derivatives, can form pores in the lining of the insect gut, leading to fluid loss and eventually death (Voutquenne et al., 2002; Gauthier et al., 2009; De Geyter et al., 2012). Less haemolytic saponins, for example medicagenic acid-derivatives, form micelles that can prevent the absorption of hydrophobic nutrients, such as sterols. Since insects cannot synthesize steroidal compounds and rely on obtaining them from their diet (Behmer and Nes, 2003; Lavrynenko et al., 2015), reduction of dietary lipids could impact levels of sterol-derived ecdysteroid hormones that are crucial for insect development. Saponins may also non-specifically inhibit insect gut-associated digestive enzymes through protein-saponin interactions (Ishaaya, 1986).

Saponins may also act as antifeedants (Jian and Trpathi, 1991; Koul, 2008; Zalucki and Furlong, 2017). Thus, when insects detect the compounds, they will not feed on the plant. The triterpenoid saponin, 3-O-[O- β -D-glucopyranosyl-(1 $\rightarrow$ 4)- β -D-glucopyranosyl]-hederagenin, found in bittercress *Barbarea vulgaris*, is a strong feeding deterrent to caterpillars of the diamondback moth, *Plutella xylostella* (Shinoda et al., 2002). Wound-induced saponins, particularly soyasaponin- and medicagenin-type saponins, are proposed to act as antifeedants to Egyptian cotton leafworm, *Spodoptera littoralis*, caterpillars (Agrell et al., 2003).

How oleanolic-derived saponins protect *M. truncatula*, as toxins or antifeedants, is unclear. To understand this, we used caterpillars of the cabbage looper, *Trichoplusia ni*. These insects are adaptive specialists; they can feed on a broad range of plant hosts but prefer Brassicaceous plants, making them aggressive pests of cruciferous crops, particularly in greenhouses (Capinera, 2003; Cervantes et al., 2011). Brassicaceous plants, such as those in the *Barbarea* genus, protect themselves with saponins, particularly hederagenin-derived compounds (Shinoda et al., 2002). As explained above, these compounds can act as antifeedants and modify caterpillar feeding behaviour or may have direct noxious effects on the insect. However, insects have multiple strategies to reduce the impact of toxins, such as reducing penetration, target site modification and detoxification enzymes (Siddiqui et al., 2023). In particular, caterpillars often upregulate expression of gut- and Malpighian tubule-associated detoxification genes when exposed to xenobiotics (Amezian et al., 2021).

After uptake, xenobiotic detoxifications involve three phases: functionalization, conjugation and excretion (Amezian et al., 2021; Esteves et al., 2021). In phase 1, enzymes, such as esterases or cytochrome P₄₅₀S, modify the xenobiotic either catabolizing it or making it more hydrophilic for excretion. In phase 2, molecules, such as glutathione or glycosides, can be added either directly to the xenobiotic or to the modified xenobiotic, by enzymes such as glutathione *S*-transferases (GSTs) or UDP-glycosyl transferases (UGTs), respectively, again, often to enhance excretion. In the *T. ni* genome, 199 putative detoxification genes have been identified: 108 cytochrome P₄₅₀S, 34 GSTs and 57 α -carboxylesterases (Fu et al., 2018). Our understanding of which of these enzymes are involved in the detoxification of plant specialized metabolites and insecticides as well as the regulation of their expression is still relatively preliminary (Amezian et al., 2021).

Male and female insects may have different abilities to detoxify xenobiotics. Female caterpillars need to acquire sufficient protein at the larval stage for ovarian development and yolk production (Wheeler, 1996; Wheeler et al., 2000). This is reflected in their feeding and nutrient utilization. Female *Spodoptera litura* caterpillars select a more protein-rich diet than males and utilize these proteins for growth more efficiently (Lee, 2010). Considering this difference in nutrient requirements, females may invest less in detoxification enzymes prioritizing egg production over detoxification or may invest more in detoxification to ensure overall fitness. As well, more detoxification genes are associated with female sex-linked genes than males as evidenced by the higher levels of *CYP* and *GST* genes on the W chromosome of the diamondback moth, *Plutella xylostella* (You et al., 2013).

Saponins are involved in protection of many plant species against pathogens and insect herbivores (Hussain et al., 2019; Roopashree and Naik, 2019; Bede, 2020; Zaynab et al., 2021). Foliar levels of oleanolic acid-derived saponins, such as the aglycone hederagenin, are correlated with caterpillar deterrence (Cai et al., 2017); however, whether this compound acts as a toxin or antifeedant is unclear. Toxin and antifeedant assays were performed with caterpillars of the cabbage looper, *T. ni*. In parallel, transcriptomic experiments were conducted to understand possible mechanisms of detoxification and if there is a sex-specific difference in detoxification-related gene expression.

METHODS AND MATERIALS

Chemicals

Hederagenin was purchased from Cayman Chemical Company. All other chemicals, including ethanol, RNAlater™ Solution etc., were purchased from ThermoFisher Scientific.

Insect colony

From *Trichoplusia ni* eggs initially obtained from the Canadian Forest Service – Great Lakes Forestry Service (CFS-GLFS), caterpillars were reared on a modified McMorran-Grisdale diet and kept at 28.5°C, 28–40% humidity, 16:8 light to dark cycle (Cai et al., 2017). Pupae were moved to an aquarium to allow adult moths to emerge and mate. Eggs were collected from a sterile mesh hung from the roof of the aquarium and used to maintain the colony. Adults were provided with a 10% honey solution (Ebling and Dedes, 2015). For the experiments, when insects were third instars, males and females were separated based on the presence of yellow gonads in the males. Fourth instar caterpillars were used in the experiments.

Determination of hederagenin concentration to use in bioassays

Hederagenin levels range from 0.018 to 0.2 mg/g DW in *M. truncatula* (Huhman et al., 2005; Tava and Pecetti 2012; D'Addabbo et al. 2020). Based on the assumptions that *M. truncatula* leaves are approximately 80% water and caterpillars (*Spodoptera exigua*) normally eat ~ 25 mg DW of leaf material

in a day (Cai et al. 2017), a caterpillar should be exposed to 25 x the measured foliar saponin (DW) levels. Therefore, 8 µg and 80 µg hederagenin added to the diet approximate the constitutive and induced levels that a caterpillar would be exposed to while feeding on *M. truncatula* plants.

Experiment 1. Hederagenin toxicity

Hederagenin (concentration range from 2 to 80 µg) or ethanol was added to a small piece of artificial diet (~ 0.1 gm) to ensure that it would be consumed in 24 hr. The concentration range of hederagenin tested was reflective of levels in *M. truncatula* leaves and limited by the solubility of the compound in ethanol (D'Addabbo et al. 2020; Tava and Pecetti 2012; Huhman et al., 2005). The diet was added to the centre of a Petri dish (60 mm) and the male or female caterpillar added and allowed to feed. If the caterpillar consumed all the diet, artificial diet alone was added to ensure that the mortality was due to hederagenin and not lack of food. Mortality was recorded at 24 and 48 hr. Mortality was determined by lack of movement when the insect was prodded with forceps. For each sex and concentration, 10 caterpillars were tested. The experiment was repeated with no toxicity observed over the concentration range tested.

Experiment 2. Hederagenin antifeedant activity

In no-choice studies, 4th instar *T. ni* caterpillars were given artificial diet spiked with either ethanol or hederagenin and the amount of diet eaten in 24 hours determined. In a 60 mm Petri dish, a large piece of pre-weighed artificial diet (0.5 gm ~ 0.6 gm) was placed in the middle and ethanol or 8 µg or 80 µg hederagenin added. Male or female caterpillars were added to the Petri dish at 16:00 and allowed to feed for 18 hours in the growth chamber with settings: 28.5°C, 28–40% humidity, 16 hours light and 8 hours dark cycle. At that time, any diet left in the Petri dish was collected into labeled envelopes, followed by drying in the oven at 50°C for 3 days and then weighed in preweighed envelopes. In addition, a control without caterpillars was performed to determine the fresh weight (FW) to dry weight (DW) conversion ratio of the diet: $R_{(FW/DW)} = FW/DW$. Four temporal replicates were performed with 10 caterpillars in each treatment (ethanol control or two hederagenin concentrations, male or female).

The amount of diet consumed was analyzed by two-factor analysis of variance (2-way ANOVA)(Factors: sex, concentration)(SPSS statistics ver. 28) followed by a Tukey HSD *post-hoc* test.

Experiment 3. Effect of hederagenin on Trichoplusia ni detoxification gene expression.

The experimental set-up is as described in Experiment 2. The whole gut with attached Malpighian tubules were dissected from caterpillars, rinsed in sterile lepidopteran saline (0.25 g NaCl, 0.768 g KCl, 1.7 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.63 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.476 g HEPES, 6.48 g glucose dissolved in 400 mL distilled water, pH 7.2 (Christensen et al., 1991)), placed in RNA later and stored at -20°C until RNA extraction. A food bolus in the gut was not observed for any samples. Three temporal replicates were performed with 1 caterpillar per treatment (ethanol control or two hederagenin concentrations, male or female).

RNA extraction and next-generation sequencing (RNA-Seq)

Total RNA was extracted from *T. ni* samples using Qiagen RNeasy Mini Kit following the manufacturer's protocol. The gut and attached Malpighian tubules were removed from the RNA later and placed in a 1.5 mL Eppendorf tube containing lysis buffer (600 μ L of 1:100 μ L β -mercaptoethanol:Buffer RLT) and kept at room temperature for 3 minutes. Tissues were disrupted by homogenization (ProCulture™ Cordless Homogenizer Unit) using a sterile grinder that was changed between samples. Homogenized samples were centrifuged at 16,000 x *g* for 3 min to separate phases. The supernatant containing the total RNA was carefully moved into another Eppendorf tube to which ethanol (70%, 600 μ L) was added followed by mixing the lysate by pipetting. The sample (600 μ L) was transferred onto a RNeasy Mini spin column in a collection tube (2 mL) and centrifuged at 16,000 x *g* for 15 seconds. RW1 buffer (350 μ L) was added to each column and centrifuged again for 15 seconds at the same speed and the flow-through discarded. On column DNase digestion was conducted to avoid potential DNA contamination in the sample. Samples were incubated at room temperature for 15 minutes. After adding RW1 buffer (350 μ L) to the column, samples were centrifuged at 16,000 x *g* for 15 seconds. Similarly, RPE Buffer (500 μ L) was added to each column and samples were centrifuged at 16,000 x *g* for 15 seconds. After discarding the flow-through, Buffer RPE (500 μ L) was added again and samples were centrifuged at 16,000 x *g* for 2 minutes. Columns were placed in new collection tube and total RNA was eluted by adding RNase-free water (50 μ L) followed by centrifugation at 16,000 x *g* for 1 minute. To ensure a high RNA yield, the collection step was repeated twice.

Total RNA samples were submitted to Genome Québec (Montreal, Canada) who prepared libraries (NEB) followed by sequencing (Illumina NovaSeq6000 S4 PE100). The obtained reads were analyzed following the pipeline outlined in Kolosov et al. (2019).

RNA-Seq data analysis

Paired-end reads were analyzed on the Unix-based server Narval provided by the Digital Research Alliance of Canada. Quality check for raw reads data was performed using FastQC (Andrews, 2010). Raw reads were processed through fastp version 0.20.1 which removed adapters, low quality bases (Phred score < 20) and reads shorter than 25 bp from the 3' end (Chen et al., 2018a). Reads were mapped against the *Trichoplusi ni* reference genome GCF_003590095.1 (National Center for Biotechnology Information) using STARv2.7.9a (Dobin et al., 2013; Fu et al., 2018; Liu and Li, 2019). FeatureCounts was used to obtain the raw gene counts (Supplemental Table 3). Only genes with expression counts greater than 15 in at least one sample were considered. Differentially expressed genes were identified using DESeq2 assuming a negative binomial (gamma-Poisson) distribution and using a criteria of log₂-fold change (logFC) (Love et al. 2014). Differentially expressed genes were identified by adjusting for false discovery rate *p* value (*padj*) and using a ≥ 2 or ≤ 0.5 fold change (Supplemental Table 2). Data visualization was performed using MetaboAnalyst and ExpressAnalyst platforms (Pang et al., 2020; Liu et al., 2023). [Transcriptomic reads will be deposited in the NCBI database upon acceptance of this manuscript].

Bioinformatic prediction of hederagenin modification by cytochrome P₄₅₀

To identify the putative target site of cytochrome P₄₅₀ enzymes on hederagenin, the simplified molecular input line entry system (SMILES) format for hederagenin was deposited in three bioinformatic sites: Xenosite (<https://xenosite.org>), Way2Drug (<http://www.way2drug.com/somp/>) and SMART Cyp (https://smartcyp.sund.ku.dk/mol_to_som) (Rydberg et al., 2010; Dang et al., 2020). These computational softwares are designed to identify targets of human cytochrome P₄₅₀ enzymes.

RESULTS

Experiment 1. Hederagenin toxicity

Fourth instar *T. ni* caterpillars were fed on diet containing ethanol or hederagenin (2–80 µg) for 48 hours. Mortality was not observed over the hederagenin levels tested. Even though mortality was not observed, at the higher concentrations, some caterpillars did not eat all the diet.

Experiment 2. Hederagenin antifeedant activity

Fourth instar *T. ni* caterpillars fed on artificial diet containing ethanol or hederagenin (8 µg and 80 µg) for 18 hours. Hederagenin concentration did not affect caterpillar feeding behaviour (Hederagenin: $F_{(2,218)} = 0.413$; $p = 0.662$) (Fig. 1). However, female caterpillars consumed about 7% more diet than males (Sex: $F_{(1,218)} = 5.628$; $p = 0.019$).

Experiment 3. Effect of hederagenin on Trichoplusia ni detoxification gene expression.

For each sample, an average of 35.2 million total 100 bp reads were obtained (Supplemental Table 1). After processing to remove adaptor sequences and poor quality reads, the sample average was 35.3 million reads with a Phred score ≥ 20 . The high quality reads were aligned to the *T. ni* genome (NCBI) with a mapping efficiency of 81.9%.

In total, 12,109 genes were expressed in *T. ni* gut and Malpighian tubule tissues. As expected, > 99% of the genes were constitutively expressed between treatments and only 73 genes were found to be differentially expressed (Supplemental Tables 2,3). Regardless of diet or caterpillar sex, most detoxification genes were constitutively expressed (as an example see Fig. 5A); approximately 17 *UDP-glycosyl transferases*, 48 *esterases*, 23 *GST* and 62 *CYP* genes were not affected by hederagenin diet or sex-specific. Many of these genes are likely involved in metabolic processes related to cellular homeostasis and do not necessarily play a detoxification role, but there may be some generic detoxification genes constitutively upregulated when the caterpillar is feeding to enable the insect to cope with the myriad of plant compounds that may be in its diet.

Female Trichoplusia ni caterpillars: effect of diet on gene expression

In female *T. ni* caterpillars fed artificial diet spiked with the hederagenin, there was little difference in gut/Malpighian tubule gene expression compared to the ethanol control (Fig. 2A,B). At low hederagenin concentration (8 µg), only two genes were differentially expressed: *uncharacterized LOC113496501* and

lachesin-like (LOC113502562) which are down- and upregulated, respectively, and there was no difference in gene expression in insects fed the higher hederagenin diet (80 µg).

Male Trichoplusia ni caterpillars: effect of diet on gene expression

In contrast to female caterpillars, male caterpillars had more differentially expressed genes when fed the hederagenin-spiked diets compared to the ethanol control. At low hederagenin concentration (8 µg); 20 genes were upregulated and 2 genes were downregulated (Fig. 2C). Many of the gut/MT-associated genes upregulated in response to hederagenin were trypsin-like proteins that are involved in protein digestion (differentially expressed trypsin genes: *LOC113500509*, *LOC113501951*, *LOC113501953*, *LOC113501966*, *LOC113501965*, *LOC113499659*, *LOC113501950*, *LOC113501948*, *LOC113501957*, *LOC113501962*, *LOC113497819*, *LOC113501946*, *LOC113503910*) (Fig. 3A).

In males fed on artificial diet spiked with the higher hederagenin concentration (80 µg), 21 genes were upregulated and 1 gene was downregulated (Fig. 2D). In particular, the stress-responsive gene encoding the glycine-rich, antimicrobial peptide putative holotricin-3-like protein (LOC113503484) was upregulated in male caterpillars facing hederagenin challenge (Bulet et al., 1999; Tatić et al., 2023)(Fig. 3B); this is more likely a gloverin-type peptide which are found in Lepidoptera and share sequence similarity with coleopteran holotricins.

Even though in *Drosophila melanogaster*, the Ca^{2+} -binding protein plays a role in Ca^{2+} buffering in photoreceptors (Wiess et al., 2012), calphotin in other insect Orders appears to be regulated by stress. This gene was downregulated in *H. armigera* caterpillars fed the plant protein defensin and also in *P. xylostella* caterpillars infected with the entomopathogenic fungi *Beauveria bassiana* (Wang et al., 2021; Mulla and Tamhane, 2023). In our study, *calphotin-like* (LOC113505107) was upregulated in male *T. ni* caterpillars exposed to the lower hederagenin dose (Fig. 3C), which supports other studies which found that this gene was upregulated in Western corn root, *Diabrotica virgifera virgifera*, larvae exposed to Bt-corn, thrips *Thrips palmi* infected by groundnut bud necrosis virus, rickettsia-infected whitefly *Bemisia tabaci*, (Brumin et al., 2019; Mahanta et al., 2022; Pingault et al., 2022), which suggests that these homologues have broader functions in stress responses likely related to their Ca^{2+} binding activity.

The major facilitator superfamily (MFS) transporter is a single polypeptide transporter involved in the transport of endogenous metabolites or xenobiotics (Reddy et al., 2012). The expression of this gene (LOC113492899) increased with hederagenin dosage and in caterpillars fed 8 µg hederagenin, a slight increase in gene expression is observed in male caterpillars (Fig. 3D). In the generalist two-spotted spider mite, *Tetranychus urticae*, strong upregulation of genes in this family was associated with pesticide resistance or exposure to plant specialized metabolites (Dermauw et al., 2012).

Male vs female Trichoplusia ni caterpillar gene expression

Male and female caterpillars show some distinct gene expression patterns (Fig. 4). In particular, genes encoding trypsin (*LOC113500509*, *LOC113501951*, *LOC113501953*, *LOC113501966*, *LOC113501950*,

LOC113501948, LOC113501957, LOC113501962, LOC113497819, LOC113501946, LOC113503910) and cytochrome P₄₅₀ 6B2-like (LOC113493761) were higher in males exposed to hederagenin than females (Fig. 2C, 3A, 4B, 5B); in insects fed artificial diet with 80 µg hederagenin, *cytochrome P₄₅₀ 6B2-like* (LOC113493761) expression was twice as high in males compared to female caterpillars. In contrast, genes encoding doublesex-like (LOC113508204) and the cytochrome P₄₅₀ 6B7-like (LOC113492289) were more highly expressed in females (Fig. 4A,C, Fig. 5C, 6A). Doublesex is involved in sex-differentiation and different alternative splice variants are seen in males vs females (Suzuki et al., 2003). Cytochrome P₄₅₀ enzymes are involved in xenobiotic detoxification, particularly those associated with the subfamily 6B (Hlavica, 2011). Transcript levels of cytochrome P₄₅₀ 6B7-like (LOC113492289) in males were basal while expression levels in females were ~ 90 times higher.

Computational identification of the potential site of cytochrome P₄₅₀-mediated hederagenin modification

The computational sites used to identify target site modification by cytochrome P₄₅₀s were designed to identify drug target sites by human cytochrome P₄₅₀ enzymes. The sites most consistently identified for oxygenation of hederagenin by cytochrome P₄₅₀s were C23, C31, C4 and C19 (Supplemental Table S4), however, it must be recognized that this reflects human enzymes in a different class than the insect ones upregulated in our study. However, the consistent ranking does suggest that these carbons may be promising sites of modification.

DISCUSSION

In response to hederagenin-spiked diet, extremely few genes were differentially regulated in female caterpillars (Fig. 2A,B). In comparison, > 40 genes were upregulated in response to hederagenin in male caterpillars (Fig. 2C,D). At the lower exposure, many of the upregulated genes in male caterpillars encoded trypsin digestive enzymes (Fig. 2C, 3A, 4B). Saponins can nonspecifically interact and inhibit digestive enzymes (Ishaay, 1986); this increase in *trypsin-like* expression may be a mechanism to counteract this. Moreover, in male caterpillars, the presence of genes encoding stress-related proteins were observed (Fig. 2C,D, 3B,C)(Wang et al., 2021; Brumin et al., 2019, Pingault et al., 2022; Mahanta et al., 2022; Tatić et al., 2023; Mulla and Tamhane, 2023). This was not observed in female caterpillars, suggesting that female caterpillars may have additional protection mechanisms compared to their male counterparts.

Female caterpillars ate more diet, regardless of hederagenin levels, than males (Fig. 1). This may be because female caterpillars, compared to males, must accumulate sufficient resources at the larval stage for egg production as adults (Wheeler, 1996; Wheeler et al., 2000). It also means that females are exposed to higher levels of plant chemical defenses, in the case of our study hederagenin.

Comparing constitutive male and female gene expression, female caterpillars expressed more *doublesex-like* transcripts (Fig. 4A,C, 6A). This gene is regulated through alternative splicing but associated with sex-related differentiation. Male caterpillars exposed to low levels of hederagenin-induced expression of *CYP₄₅₀ 6B2-like* (LOC113493761)(Fig. 4B, 5B). In female caterpillars exposed to high hederagenin-spiked diet, there is a striking upregulation of a gene encoding a cytochrome P₄₅₀ 6B7-like protein (LOC113492289) (Fig. 4C, 5C).

Cytochrome P₄₅₀ enzymes are involved in phase I detoxification, catalyzing the oxygenation of xenobiotic to facilitate their excretion or provide sites for phase II reactions, such as the addition of glutathione by glutathione *S*-transferases (GSTs)(Feyereisen, 2012; Esteves et al., 2021; Amerzian et al., 2021). Lepidopterans have superfamilies of detoxification genes (i.e. cytochrome P₄₅₀s, GSTs, carboxylesterases) that may reflect their herbivorous lifestyle exposing them to a diversity of plant specialized metabolites, but this also makes them adept at insecticide detoxification leading to pesticide resistance. The *T. ni* genome contains 108 genes that encode cytochrome P₄₅₀ enzymes compared to 90 in the Brassicacea specialist *P. xylostella* and 84 in the mulberry specialist *Bombyx mori* (You et al., 2013; Fu et al., 2018). Most of the detoxification transcripts were constitutively expressed; a sex-specific upregulation of two *CYP6B* (LOC113493761 and LOC113492289) genes were observed (Fig. 4C,D, 5A-C), recognizing the importance of members of the cytochrome P₄₅₀ 6B family in plant specialized metabolite and insecticide detoxification (Table 1)(Hlavic, 2011; Lu et al., 2021; Nauen et al., 2022). However, the increase of *CYP6B7-like* (LOC113492289) was ~ 90% higher in female insects exposed to high hederagenin levels in the diet, compared to males on this diet that showed signs of stress-related gene expression (Fig. 5C). Though not definitive, bioinformatic tools identified a few select carbons as possible sites for cytochrome P₄₅₀ modification of hederagenin (Supplemental Table S4).

Table 1

Detoxification of plant specialized metabolites (PSMs) or insecticides by cytochrome P₄₅₀ 6B enzymes in the 6B class. Higher *CYP6B7* expression was observed in female caterpillars fed hederagenin (Fig. 6).

Caterpillar species	Compound (Plant specialized metabolite (PSM) or insecticide (I))	Type of cytochrome P ₄₅₀ 6B	Reference
Black swallowtail, <i>Papilio polyxenes</i>	Linear furanocoumarins (PSM)	CYP6B1 CYP6B3 CYP6B4	Ma et al., 1994; Wen et al., 2003; Wen et al., 2006, Hung et al., 1977
Corn earworm, <i>Helicoverpa zea</i>	Flavone, indoles, furanocoumarins, chlorogenic acid (PSM), a-cypermethrin, aldrin, diazinon (I)	CYPB8	Li et al., 2004
Two-tailed swallowtail, <i>Papilio multicaudata</i>	Linear and angular furanocoumarins (PSM)	CYPB33	Mao et al., 2007
Fall armyworm, <i>Spodoptera frugiperda</i>	Indole, xanthotoxin (PSM)	CYP6B40	Giraud et al., 2015
Fall armyworm, <i>Spodoptera frugiperda</i>	Indole, indole-3-carbinol, quercetin, 2-tridecanone, xanthotoxin (PSM)	CYP6B39	Giraud et al., 2015
Cotton bollworm, <i>Helicoverpa armigera</i>	Pyrethroid (PSM, I)	CYP6B7	Abd El-Latif, et al., 2004
Cotton bollworm, <i>Helicoverpa armigera</i>	Pyrethroid fenvalerate (I)	CYP6B7	Ranasinghe et al., 1998; Ranasinghe et al., 1999
Cotton bollworm, <i>Helicoverpa armigera</i>	Preexposure to the quercetin (PSM, flavonoid) increases tolerance to lamda-cyhalothrin (I)	CYP6B6 CYP6B8 CYP321A1	Chen et al., 2018b
Tobacco cutworm, <i>Spodoptera litura</i>	Preexposure to lead increases tolerance to a-cypermethrin (I)	CYP6B47 CYP9A39	Zhou et al., 2012
Tobacco cutworm, <i>Spodoptera litura</i>	Preexposure to copper increases tolerance to xanthotoxin (PSF)	CYP6B50	Lu et al., 2019

Of particular interest is the major facilitator superfamily-like transporter (LOC113492899); even though this gene was differentially expressed in male caterpillars exposed to low hederagenin, in both males and

females, gene expression increased with dosage suggesting that this transporter may be involved in hederagenin and/or hederagenin-derivative transport (Fig. 3D).

The host plants that herbivorous caterpillars feed upon likely affect their ability to detoxify the defensive specialized metabolites found in those plants. Caterpillars of *T. ni* feed on plants on the genus *Barbarea* and are, thus, exposed to hederagenin-type saponins. This possibly explains why hederagenin is not toxic or an antifeedant to *T. ni* caterpillars, compared to previous studies using hederagenin glucosides with other caterpillar species (Shinoda et al., 2002, Liu et al., 2019). What was striking is that female caterpillars ate 7% more diet than males, implying that, in nature, they may be exposed to more noxious plant compounds than males (Fig. 1). This was reflected in the detoxification-related gene expression where *CYP6B7-like* (LOC113492289) expression was 90% higher in female caterpillars exposed to higher hederagenin levels compared to their male counterparts (Fig. 4C, 5C). However, detoxification enzymes not only metabolize plant specialized metabolites but also other xenobiotics such as insecticides. Indeed, sex-linked resistance has been identified in a number of insect species (examples: Heckel et al 1998; Kanga et al., 2001; Reyes et al., 2008; Banazeer et al., 2022). Thus, determining male- and female-specific LD₅₀s and understanding sex-specific resistance strategies has important implications for pesticide management and sustainable agricultural practices.

Abbreviations

DEGs

Differentially expressed genes

Declarations

ACKNOWLEDGEMENTS

Our insect colony was established from *Trichoplusia ni* eggs and first instars received from the Insect Production Services (Canadian Forestry Service). We are grateful to them for the insects and also all the advice given to us on insect maintenance. The generation of RNA libraries and RNA-Seq was conducted by Genome Québec and transcriptomic analysis was enabled by support provided by the Digital Alliance of Canada (alliancecan.ca).

FUNDING

We gratefully acknowledge our funding sources for supporting this research: the Natural Science and Engineering Research Council (NSERC) Discovery Grant (2019-04516) as well as support from the Fonds de recherche du Québec – nature et technologies (FRQNT) Centre SÈVE (RQT00335) to JCB. The authors declare no financial or non-financial interests related to this research.

AUTHOR CONTRIBUTIONS

The research was primarily conducted by Yinting Chen (experimental design, experiments, data collection, analyses), under the supervision of Brian T. Driscoll and Jacqueline C. Bede. Ryan J. Smith was involved in assistance with caterpillar maintenance and experiments. Christine LaFleur was involved in molecular training and assisted in this part of the experiments. Transcriptomic data analysis was conducted by Yinting Chen under the direction of Diljot Kaur. The manuscript was written by Yinting Chen and Jacqueline C. Bede and incorporated comments from all co-authors. All authors have read and approved the final manuscript.

CONFLICT-OF-INTEREST

The authors declare that there were no conflicts-of-interest, financial or otherwise, with the contents of this article.

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Figures

Fig. 1

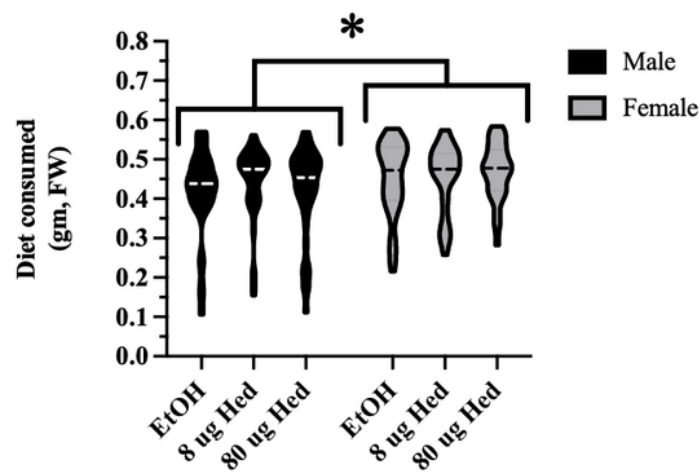


Figure 1

Differential gene expression of 4th instar *Trichoplusia ni* caterpillars fed artificial diet spiked with the saponin aglycone hederagenin. Caterpillars were fed on diet spiked with ethanol (control) or hederagenin (8 or 80 mg). Total RNA was extracted from dissected gut and Malpighian tubule samples for transcriptomics (RNA-Seq). Volcano plots illustrate the differentially expressed genes. **A)** Female caterpillars, ethanol vs 8 mg hederagenin, **B)** Female caterpillars, ethanol vs 80 mg hederagenin. No differentially expressed genes were identified, **C)** Male caterpillars, ethanol vs 8 mg hederagenin and **D)** Male caterpillars, ethanol vs 80 mg hederagenin. The inset includes a downregulated gene (*fibroin heavy chain-like*) that was removed from “D” for better presentation. Data is represented by a volcano plot ($\text{Log}_2\text{FoldChange} \geq 2$ or ≤ -2 ; $\text{padj} \leq 0.05$).

Fig. 3A

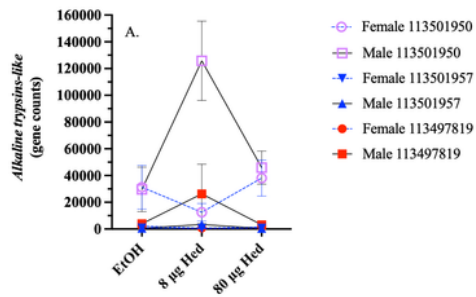


Fig. 3B

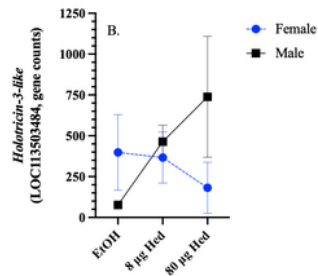


Fig. 3C

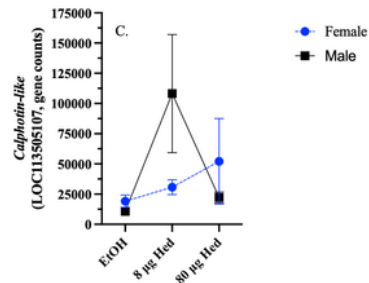


Fig. 3D

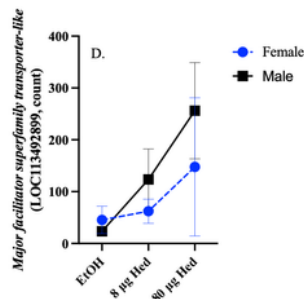


Figure 3

Sex-specific gene expression; higher expression in male *Trichoplusia ni* caterpillars fed artificial diet spiked with the saponin aglycone hederagenin. Male and female *T. ni* caterpillars were fed on diet spiked with ethanol (EtOH; control) or hederagenin (Hed; 8 or 80 mg). Total RNA was extracted from dissected gut and Malpighian tubule samples for transcriptomics (RNA-Seq). These genes have higher expression in male (black solid lines, squared) compared to female (blue, dashed lines, circle) caterpillars. **A. Three**

representative *alkaline trypsin-like* genes (LOC113501950, LOC501957, LOC113497819) that have higher expression in males fed artificial diet with 8 mg hederagenin. The proteins encoded by these genes have high activity in the highly alkaline environment of the caterpillar gut (Dow, 1992). **B. *Holotricin-3-like*** (likely the homolog *gloverin*-type antimicrobial protein)(LOC113503384), **C. *Calphotin-like*** (LOC113505107), **D. Major facilitor superfamily (MFS) transporter-like** (LOC113492289).

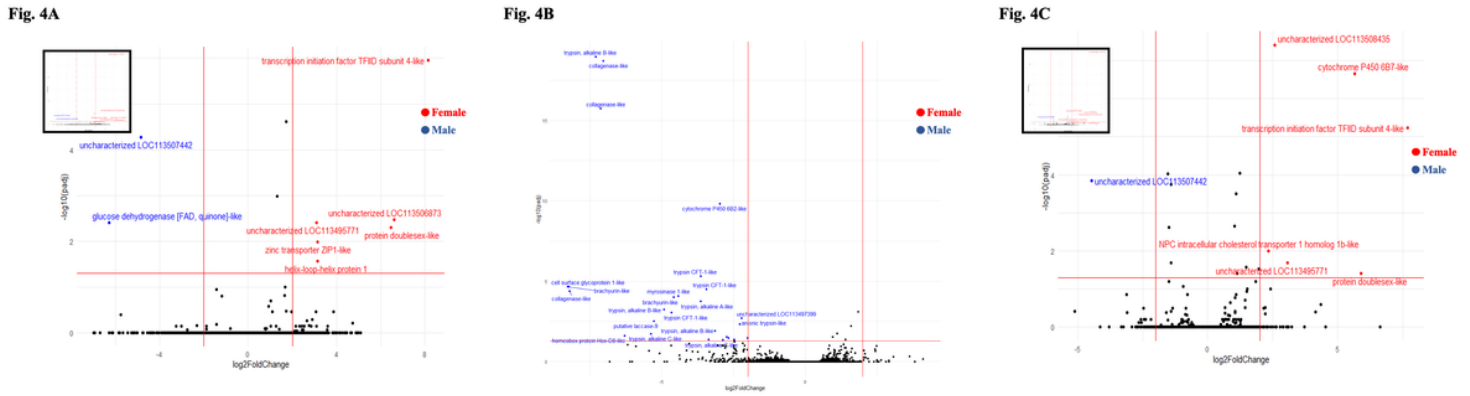


Figure 4

Differential gene expression of 4th instar *Trichoplusia ni* caterpillars fed artificial diet spiked with the saponin aglycone hederagenin; sex-specific differences. Male and female caterpillars were fed on diet spiked with ethanol (control) or hederagenin (8 or 80 mg). Total RNA was extracted from dissected gut and Malpighian tubule samples for transcriptomics (RNA-Seq). Volcano plots illustrate sex-specific differentially expressed genes. **A) Male (left, blue) vs female (right, red) caterpillars, ethanol.** The inset includes LOC113506116 which was removed from “A” for better presentation. **B) Male (left, blue) vs female (right, red) caterpillars, 8 mg hederagenin,** and **C) Male (left, blue) vs female (right, red) caterpillars, 80 mg hederagenin.** The inset includes LOC113506116 which was removed from “C” for a better presentation. Data is represented by a volcano plot ($\text{Log}_2\text{FoldChange} \geq 2$ or ≤ -2 ; $\text{padj} \leq 0.05$).

Fig. 5A

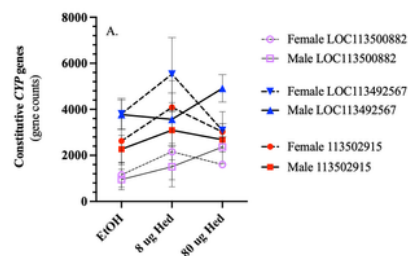


Fig. 5B

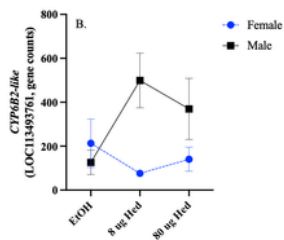


Fig. 5C

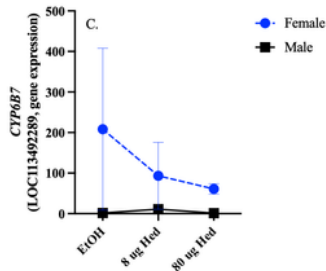


Figure 5

Sex-specific *CYP450* gene expression in *Trichoplusia ni* caterpillars fed artificial diet spiked with the saponin aglycone hederagenin. Male and female *T. ni* caterpillars were fed on diet spiked with ethanol (EtOH; control) or hederagenin (Hed; 8 or 80 mg). Total RNA was extracted from dissected gut and Malpighian tubule samples for transcriptomics (RNA-Seq). These genes have higher expression in male (black solid lines, squared) compared to female (blue, dashed lines, circle) caterpillars. **A. Three**

representative *CYP*genes (LOC113500882, LOC113492567, LOC113502915) that do not have a diet- or sex-specific difference. **B. *CYP6B2-like*** (LOC113493761) has higher expression in male caterpillars fed a hederagenin diet and C. ***CYP6B7***(LOC11349289) has higher expression in female caterpillars.

Fig. 6

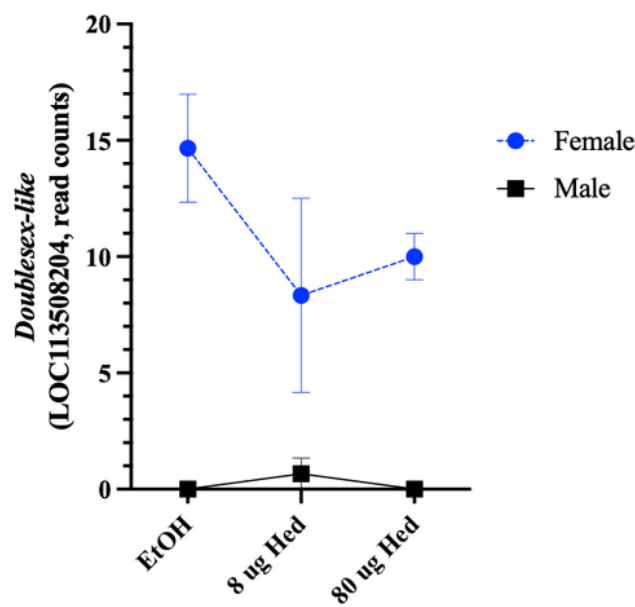


Figure 6

Sex-specific gene expression; higher expression in female *Trichoplusia ni* caterpillars. Male and female *T. ni* caterpillars were fed on diet spiked with ethanol (EtOH; control) or hederagenin (Hed; 8 or 80 mg). Total RNA was extracted from dissected gut and Malpighian tubule samples for transcriptomics (RNA-Seq). **A. Doublesex-like** (LOC113508204) has higher expression in female (blue, dashed lines, circle) compared to male (black solid lines, square) caterpillars.

Supplementary Files

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- [YCSupplemental.xlsx](#)