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Rozprawa doktorska

Charakterystyka działania wybranych glikoalkaloidów z rodziny *Solanaceae* na kluczowe procesy metaboliczne u chrząszcza *Tenebrio molitor*

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I. Winkiel M.J., Chowański S., Gołębiowski M., Bufo S.A., Słocińska M., *Solanaceae* glycoalkaloids disturb lipid metabolism in the *Tenebrio molitor* beetle, Metabolites 2023; 13, 1179, DOI: 10.3390/metabo13121179

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III. Winkiel M.J., Chowański S., Walkowiak-Nowicka K., Gołębiowski M., Słocińska M., A tomato a day keeps the beetle away – the impact of *Solanaceae* glycoalkaloids on energy management in the mealworm *Tenebrio molitor*

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   IF: **5.9** (2022); punktacja MNiSW: **200**

2. Winkiel M.J., Chowański S., Słocińska M. Anticancer activity of glycoalkaloids from *Solanum* plants: A review, Frontiers in Pharmacology 2022; 13, 979451, DOI: 10.3389/fphar.2022.979451

   IF: **5.6**; punktacja MNiSW: **100**

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   IF: **3.8**; punktacja MNiSW: **70**

4. Chowański S., Walkowiak-Nowicka K., Winkiel M.J., Marciniak P., Urbański A., Pacholska-Bogalska J. Insulin-like peptides and cross-talk with other factors in the regulation of insect metabolism, Frontiers in Physiology 2021; 12, 701203, DOI: 10.3389/fphys.2021.701203

   IF: **4.8**; punktacja MNiSW: **100**

5. Komasa A., Winkiel M.J., Kwaśniewska-Sip P., Cofta G. Synthesis, spectroscopic, theoretical and antifungal activity study of gemini 3-hydroxy- and 3-hydroxymethylpyridinium dibromides, Journal of Molecular Structure 2018; 1171, 888-897, DOI: 10.1016/j.molstruc.2018.06.062

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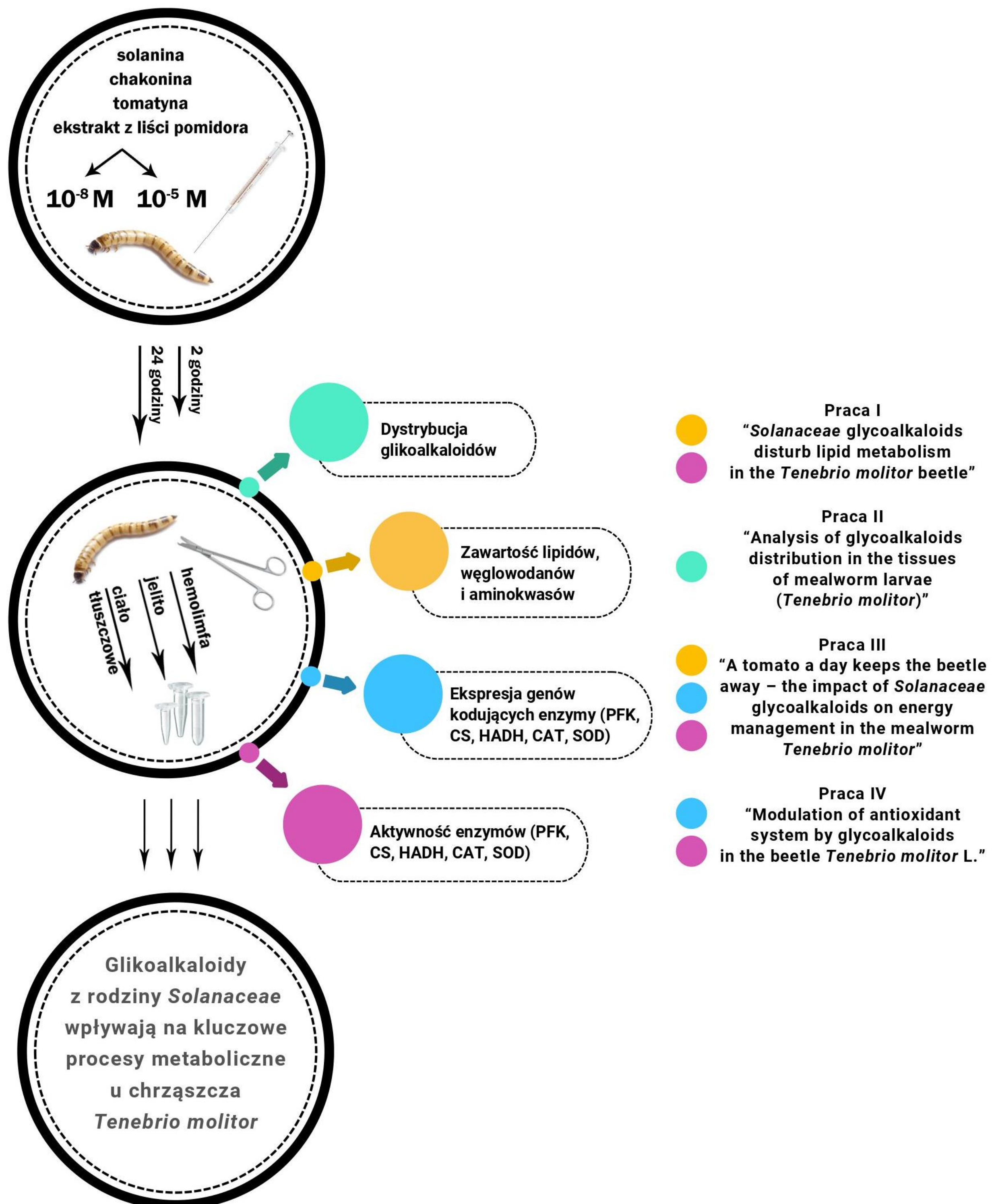
Wykaz stosowanych skrótów

CAT	Katalaza
CHA	Chakonina
CS	Syntaza cytrynianowa
EXT	Ekstrakt z liści pomidora
GA	Glikoalkaloidy
GC-MS	Chromatografia gazowa sprzężona ze spektrometrią mas
HADH	Dehydrogenaza hydroksyacylo-CoA
LC-MS	Chromatografia cieczowa sprzężona ze spektrometrią mas
PFK	Fosfofruktokinaza
ROS	Reaktywne formy tlenu
RT-qPCR	Ilościowa reakcja łańcuchowa polimerazy w czasie rzeczywistym
SOD	Dysmutaza ponadtlenkowa
SOL	Solanina
TAG	Triglicerydy
TOM	Tomatyna

Schemat badań



Streszczenie i słowa kluczowe

Środki ochrony roślin są stosowane w ogromnych ilościach do ograniczania populacji szkodników, co przyczynia się do zanieczyszczenia środowiska i stanowi zagrożenie dla innych organizmów. Związki pochodzenia roślinnego dają możliwość ograniczenia użycia pestycydów. Są to substancje biodegradowalne, bardziej bezpieczne w stosowaniu, a także stosunkowo łatwe i tanie w pozyskaniu. Glikoalkaloidy (GA) to wtórne metabolity roślinne, które wykazują znaczną aktywność biologiczną, także w organizmach owadów. Mechanizmy ich działania nie zostały jednak precyzyjnie poznane. Celem przedstawionej rozprawy doktorskiej było określenie wpływu wybranych GA na kluczowe procesy metaboliczne larw chrząszcza *Tenebrio molitor*. W tym celu przeprowadzono szereg badań, w których testowano wpływ solaniny, chakoniny i tomatyny oraz ekstraktu uzyskanego z liści pomidora. Analizowano efekty działania GA po ich aplikacji za pomocą iniekcji w dwóch stężeniach 10^{-8} i 10^{-5} M. Testowane tkanki izolowano po 2 i po 24 godzinach od aplikacji GA. Z racji funkcji troficznej, skupiono się na analizie zmian w obrębie jelita, ciała tłuszczowego i hemolimfy. Pozwoliło to również na określenie specyficzności tkankowej GA. Prace badawcze rozpoczęto od analizy zmian poziomu GA w poszczególnych tkankach w czasie, po ich wcześniejszej aplikacji na drodze iniekcji. Następnie określono wpływ testowanych związków na poziom substratów energetycznych (lipidów, węglowodanów i aminokwasów) w tkankach owada. Ponadto, dokonano pomiaru zmian poziomu ekspresji genów kodujących kluczowe enzymy szlaków metabolicznych: glikolizy (fosfofruktokinaza), cyklu Krebsa (syntaza cytrynianowa) i β-oksydacji kwasów tłuszczowych (dehydrogenaza hydroksyacylo-CoA), a także głównych enzymów antyoksydacyjnych (dysmutaza ponadtlenkowa, katalaza) i białek szoku cieplnego HSP70 po aplikacji GA. Dodatkowo, określono wpływ GA na aktywność wymienionych enzymów oraz proces peroksydacji lipidów. Na podstawie uzyskanych wyników stwierdzono, że mechanizmy działania testowanych związków w tkankach mącznika różnią się w zależności od rodzaju i stężenia GA, czasu inkubacji oraz typu badanej tkanki. Związki te wpływają na poziom ekspresji genów i aktywność białek zaangażowanych w kluczowe ścieżki metaboliczne. Poza tym, GA zmieniają zawartość składników odżywczych w tkankach owadów, prawdopodobnie w wyniku zwiększonego zapotrzebowania energetycznego podczas wzmożonego procesu detoksykacji. Badane związki znacząco wpływają na metabolizm chrząszczy, co sugeruje możliwość ich potencjalnego zastosowania jako naturalnych bioinsektycydów.

Słowa kluczowe: mącznik młynarek; owady; metabolizm; bioinsektycydy; wtórne metabolity roślinne

Abstract and keywords

Plant protection products are used in huge quantities to reduce pest populations, which contributes to environmental pollution and poses a threat to other organisms. Plant-derived compounds offer an opportunity to reduce the use of classic pesticides. They are biodegradable, safer to use, easy and inexpensive to obtain. Glycoalkaloids (GAs) are secondary plant metabolites that exhibit significant biological activity, also in insect organisms. However, their mechanisms of action have not been precisely studied. The aim of the presented PhD thesis was to determine the effects of selected GAs on key metabolic processes in larvae of the beetle *Tenebrio molitor*. For this purpose, a series of experiments were carried out, in which solanine, chaconine, tomatine, and tomato leaf extract were tested at two concentrations, 10^{-8} and 10^{-5} M. The selected compounds were administered to larvae by injection. Tissues were isolated 2 and 24 hours after GA application. Tested samples were prepared separately from the gut, fat body and hemolymph to determine the tissue specificity of the observed effects, because of trophic function of these tissues. The study began with quantitative analysis of GAs over time after their injection. Subsequently, the effects of these compounds on nutrient levels (lipids, carbohydrates and amino acids) in the insect's tissues were determined. In addition, changes in the expression of genes encoding key enzymes of metabolic pathways: glycolysis (phosphofructokinase), Krebs cycle (citrate synthase) and fatty acid β-oxidation (hydroxyacyl-CoA dehydrogenase), as well as major antioxidant proteins (superoxide dismutase, catalase) and heat shock protein HSP70 after GA application were measured. In addition, the effect of GAs on lipid peroxidation process and the catalytic activity of the mentioned enzymes was determined. Based on the results, it was found that the mechanisms of action of the tested compounds in mealworm tissues vary depending on the type and concentration of GA, incubation time and the type of tissue tested. These compounds regulate gene expression and activity of proteins involved in key metabolic pathways. Besides, GAs alter the nutrient content of insect tissues, probably as a result of increased energy requirements during detoxification. The tested compounds significantly affect beetle metabolism, suggesting their potential use as natural bioinsecticides.

Keywords: mealworm; insects; metabolism; bioinsecticides; secondary plant metabolites

Wstęp

Środki ochrony roślin, zwane potocznie pestycydami, są szeroko stosowane w produkcji rolnej. Służą do ograniczenia populacji organizmów wyrządzających szkody, między innymi w celu zwiększenia plonów upraw rolnych, zmniejszenia strat żywności i ograniczenia chorób zakaźnych. Ich działanie powinno być skierowane tylko względem określonych gatunków organizmów. Stosowanie środków ochrony roślin niesie jednak ze sobą zagrożenie zanieczyszczenia wód i gleb, uodpornienia się organizmów na szkodliwe substancje, oddziaływania na organizmy pożyteczne i niebędące celem aplikacji, a także stanowią bezpośrednie zagrożenie dla zdrowia i życia człowieka (Kowalska & Kowalski, 2019). Z danych EUROSTAT wynika, że w 2021 roku w Polsce nastąpił wzrost zużycia środków ochrony roślin w porównaniu z rokiem 2013 o 21,4%. Z tego powodu poszukuje się związków bardziej bezpiecznych, o niższym stopniu oddziaływania na środowisko.

Jednym ze sposobów ograniczenia stosowania pestycydów jest wykorzystanie związków pochodzenia roślinnego. Substancje te mogą stanowić mniejsze zagrożenie dla ludzi i środowiska, ponieważ są łatwiej biodegradowalne i produkowane w naturalny sposób przez rośliny. Poza tym, często działają bardziej selektywnie na określone grupy organizmów. Zaletą jest również aspekt finansowy, ponieważ ich pozyskanie jest stosunkowo tanie w porównaniu z chemiczną syntezą pestycydów (Souto i in., 2021). Grupą związków wytwarzanych przez rośliny są glikoalkaloidy (GA) - wtórne metabolity produkowane głównie przez gatunki z rodziny *Solanaceae*, takie jak ziemniak, *Solanum tuberosum* L., pomidor, *Solanum Lycopersicum* L. oraz psianka czarna, *Solanum nigrum* L. Są to cukrowe pochodne alkaloidów o budowie pierścieniowej. Przykładami GA są: chakonina (CHA), solanina (SOL) i tomatyna (TOM). Synteza tych związków znacznie wzrasta w warunkach stresowych. U roślin stanowią one naturalną obronę przed patogenami i roślinożercami. GA wykazują szeroki zakres aktywności biologicznej u różnych gatunków zwierząt, także u owadów (Chowański i in., 2016; Friedman, 2002, 2006; Milner i in., 2011).

Mechanizmy działania i dystrybucja GA w różnych tkankach owadów pozostają w znacznym stopniu niepoznane. Aby móc wykorzystać GA przy opracowywaniu nowych, bezpiecznych metod zwalczania owadów, będących szkodnikami, niezbędna jest wiedza o tym jak działają one na poziomie komórki. Zaburzenia metabolizmu komórki mogą prowadzić do jej śmierci, a w konsekwencji do zakłócenia funkcjonowania i śmierci całego organizmu. Dlatego celem przedstawionej pracy było określenie wpływu wybranych GA na kluczowe procesy metaboliczne chrząszcza *Tenebrio molitor* (mącznika młynarka).

Sposób dystrybucji ksenobiotyków w tkankach owadów ma istotne znaczenie w kontekście mechanizmów ich działania. Mogą być one wydalane z organizmu w różnym tempie, a także charakteryzować się zmiennym powinowactwem do poszczególnych tkanek. Ponadto ksenobiotyki, po dostaniu się do organizmu owada, mogą być transportowane pomiędzy tkankami, zanim ulegną przemianom i wydaleniu na zewnątrz ciała (Gao i in., 2022). W większości badań GA były podawane owadom z pożywieniem i/lub w postaci ekstraktu, dlatego nie wiadomo, jakie stężenia wywołują zmiany w procesach fizjologicznych u tych zwierząt. Z tego powodu jednym z zadań badawczych zaplanowanych w ramach tej pracy było przeprowadzenie analizy ilościowej GA w poszczególnych punktach czasowych po podaniu tych związków do ciała larw *T. molitor* (Praca II *Analysis of glycoalkaloids distribution in the tissues of mealworm larvae (Tenebrio molitor)*). Umożliwiło to ocenę, jak szybko są one eliminowane i/lub metabolizowane przez chrząszcza. Dodatkowo, oceniono przeżywalność larw po aplikacji GA, aby określić potencjalną toksyczność i długoterminowe skutki działania tych związków.

Wiedza dotycząca dystrybucji GA w tkankach owadów pozwoliła na zaplanowanie kolejnych badań. Wprowadzenie ksenobiotyku do organizmu owada może skutkować zaburzeniem zawartości substratów energetycznych w tkankach, koniecznych do prawidłowego metabolizmu komórek. Kluczowymi związkami chemicznymi służącymi do przetwarzania energii są lipidy. To szeroka grupa substancji, obejmująca m.in. kwasy tłuszczowe, triglicerydy (TAG), fosfolipidy i steroidy. Oprócz funkcji energetycznej, biorą udział m.in. w tworzeniu błon komórkowych czy przekazywaniu sygnałów w komórkach (Arrese & Soulages, 2010; Canavoso i in., 2001; Stanley-Samuelson i in., 1988). Aby ocenić potencjalny wpływ GA na metabolizm lipidów, przeanalizowano poziom wolnych kwasów tłuszczowych, steroli, estrów i TAG, a także aktywność kluczowego enzymu β-oksydacji kwasów tłuszczowych, HADH, który katalizuje utlenianie 3-hydroksyacylo-CoA do 3-ketoacylo-CoA (Chandel, 2021b) (Praca I *Solanaceae glycoalkaloids disturb lipid metabolism in the Tenebrio molitor beetle*). Zmiany ekspresji *HADH* opisano w Pracy III.

Istotnymi metabolitami energetycznymi są także węglowodany i aminokwasy. Owady magazynują energię w postaci glikogenu w ciele tłuszczowym. Z kolei głównym węglowodanem krążącym w hemolimfie jest trehaloza, która zbudowana jest z dwóch cząsteczek glukozy (Arrese & Soulages, 2010; Tellis i in., 2023). Glukoza jest przekształcana w procesie glikolizy do pirogronianu, a enzymem limitującym ten proces jest fosfofruktokinaza (PFK), która fosforyluje fruktozo-6-fosforan do fruktozo-1,6-bisfosforanu. Z kolei kluczowym

enzymem cyklu Krebsa jest syntaza cytrynianowa (CS), która katalizuje reakcję kondensacji szczawiooctanu i acetylo-CoA (Chandel, 2021a). Podstawową funkcję budulcową w organizmach żywych pełnią białka zbudowane z aminokwasów. Związki te mogą być wykorzystywane u owadów również jako źródło energii (Chen, 1966). Analiza poziomu węglowodanów i aminokwasów w tkankach owadów, a także pomiar ekspresji genów kodujących PFK i CS oraz ich aktywność enzymatyczna były kolejnymi zadaniami wykonanymi w ramach przygotowania tej rozprawy doktorskiej (Praca III *A tomato a day keeps the beetle away – the impact of Solanaceae glycoalkaloids on energy management in the mealworm Tenebrio molitor*). Uzyskane wyniki pozwoliły określić, czy potencjalne zmiany w aktywności metabolicznej komórek wpływają na dostępność substancji odżywczych dla innych tkanek.

Detoksyfikacja ksenobiotyków wprowadzonych do organizmu owada często związana jest z tworzeniem reaktywnych form tlenu (ROS). Związki te w niskich ilościach są wynikiem prawidłowego funkcjonowania komórki, ponieważ pełnią m.in. funkcje sygnalizacyjne. Jednak zbyt wysokie stężenie ROS w tkankach i zbyt niska efektywność działania systemu antyoksydacyjnego mogą skutkować wystąpieniem stresu oksydacyjnego. Jednym z elementów tworzących wspomniany system obronny są enzymy antyoksydacyjne, takie jak katalaza (CAT) i dysmutaza ponadtlenkowa (SOD). Enzym SOD katalizuje reakcję dysmutacji anionorodnika ponadtlenkowego, natomiast CAT przeprowadza hydrolizę nadtlenku wodoru do wody i tlenu. Stres oksydacyjny prowadzi do degradacji lipidów, białek i kwasów nukleinowych, a więc kluczowych składników energetycznych i budulcowych każdej komórki (Chaitanya i in., 2016; Felton & Summers, 1995). Dlatego celem kolejnej pracy była ocena wpływu GA na poziom ekspresji *MnSOD, CAT* i *HSP70*, a także na aktywność katalityczną SOD i CAT oraz proces peroksydacji lipidów (Praca IV *Modulation of antioxidant system by glycoalkaloids in the beetle Tenebrio molitor L.*). Umożliwiło to analizę działania systemu antyoksydacyjnego pod wpływem testowanych GA.

Zaburzenia energetyczne komórki mogą skutkować jej śmiercią, dlatego precyzyjne poznanie mechanizmów działania GA na metabolizm owadów jest niezwykle istotne, gdyż może umożliwić zaprojektowanie nowych środków ograniczania populacji szkodników. Ponadto, wyniki badań mają kluczowe znaczenie poznawcze i przyczyniły się do poszerzenia wiedzy dotyczącej fizjologii owadów w kontekście radzenia sobie ze stresem środowiskowym tj. odpowiedź organizmu na ksenobiotyki. Dodatkowo, uzyskana wiedza będzie mogła zostać

wykorzystana np. w farmakologii, ponieważ wiele alkaloidów jest stosowanych jako leki (Heinrich i in., 2021).

Cel pracy

Celem przedstawionej rozprawy doktorskiej było określenie wpływu wybranych GA na procesy metaboliczne chrząszcza *T. molitor* na poziomie genów oraz białek.

W tym celu przeprowadzono szereg badań obejmujących analizę dystrybucji aplikowanych GA w tkankach larw, a także określenie wpływu testowanych związków na poziom substratów energetycznych u owadów. Ponadto, dokonano pomiaru zmian ekspresji genów kodujących kluczowe enzymy szlaków energetycznych: glikolizy (PFK), cyklu Krebsa (CS) i β-oksydacji kwasów tłuszczowych (HADH), a także głównych białek antyoksydacyjnych (MnSOD, CAT) i opiekuńczych (HSP70) po aplikacji GA. Analizie poddano również aktywność wymienionych enzymów po iniekcji testowanych związków larwom *T. molitor*. Określono zależność zaobserwowanych efektów od stężenia testowanych GA (10^{-8} i 10^{-5} M), czasu inkubacji (2 i 24 godziny), a także analizowanej tkanki owadów (hemolimfa, jelito, ciało tłuszczowe).

Materiały i metody

Badania przeprowadzono na larwach chrząszcza *Tenebrio molitor* L. Jest to popularny gatunek modelowy, często wykorzystywany w badaniach farmakologicznych, toksykologicznych, fizjologicznych i środowiskowych (Adamski i in., 2019). Jest łatwy w hodowli ze względu na niewielkie wymagania życiowe. Ponadto *T. molitor* jest szkodnikiem, który przyczynia się do powstania strat w magazynach zbożowych (Hagstrum i in., 2013). Hodowla chrząszcza była prowadzona w Zakładzie Fizjologii i Biologii Rozwoju Zwierząt UAM.

Do badań wykorzystano syntetyczne GA: SOL, CHA i TOM oraz ekstrakt z liści pomidora (EXT) uzyskany od grupy badawczej Prof. Sabino A. Bufo z Uniwersytetu Basilicata w Potenzie, który zawierał 2.95 ± 0.25% TOM (Ventrella i in., 2016). Testowane związki iniekowano larwom owadów za pomocą mikrostrzykawki Hamilton w formie roztworów o stężeniach 10^{-8} i 10^{-5} M w płynie fizjologicznym dla *T. molitor*. Stężenia TOM w roztworze ekstraktu odpowiadały stężeniom czystych GA w aplikowanych roztworach. Kontrolę stanowiły owady iniekowane roztworem fizjologicznym.

Po iniekcji larwy inkubowano 2 lub 24 godziny. Następnie, za pomocą narzędzi mikrochirurgicznych izolowano kluczowe tkanki budujące oś troficzną owada: hemolimfę, jelito i ciało tłuszczowe. Odpowiednio przygotowane próbki wykorzystano do przeprowadzenia następujących analiz za pomocą wymienionych metod:

- dystrybucja GA (LC-MS),
- zawartość lipidów i aminokwasów (GC-MS) oraz poziom węglowodanów (spektrofotometria),
- zmiany ekspresji genów *PFK, CS, HADH, CAT*, *MnSOD* i *HSP70* (RT-qPCR),
- aktywność enzymatyczna PFK, CS, HADH, CAT i SOD oraz proces peroksydacji lipidów (spektrofotometria).

Uzyskane wyniki poddano analizie z użyciem odpowiednich testów statystycznych.

Wyniki i wnioski

Analiza dystrybucji SOL i CHA została przeprowadzona w hemolimfie, jelitach (zawierających pokarm) i w pozostałych tkankach owadów (łącznie) z dominującym udziałem ciała tłuszczowego. Pomiar zawartości GA określono po 30 minutach, po 1.5 godziny, po 8 godzinach oraz po 24 godzinach. Największy udział procentowy wprowadzonej do ciała owadów ilości testowanych związków zaobserwowano w próbkach ciała tłuszczowego. Największym stężeniem w przeliczeniu na masę tkanki charakteryzowała się z kolei hemolimfa, w której stężenie GA zmniejszało się z upływem czasu. Nie wykryto żadnego z produktów hydrolizy GA, dlatego jednym z możliwych mechanizmów ich detoksykacji może być utlenianie i/lub sekwestracja. Związki te mogą być wydalane przez cewki Malpighiego z odchodami lub z kutikulą podczas linienia. Co więcej, procesy wydalania GA zachodzą stosunkowo wolno, ponieważ po 24 godzinach nadal oznaczono znaczną ilość SOL i CHA (odpowiednio ponad 60 i 70% zaaplikowanej ilości), jednak nie zaobserwowano śmiertelności wśród owadów w ciągu 10 dni od podania GA. Szybkość eliminacji CHA w całym organizmie owada była najwyższa bezpośrednio po wstrzyknięciu (0-0.5 godziny), natomiast SOL była eliminowana najszybciej w przedziale 0.5-1.5 godziny. Zaprezentowane wyniki są istotne w kontekście interpretacji danych otrzymanych w kolejnych z przeprowadzonych badań związanych z wpływem GA na metabolizm owadów.

Znając dystrybucję GA w tkankach chrząszczy, w kolejnym etapie badań dokonano oceny wpływu testowanych GA na metabolizm lipidów. Zaobserwowano zwiększoną ilość kwasów tłuszczowych w ciele tłuszczowym 24 godziny po iniekcji tych związków. W tym samym czasie, stężenie TAG zmniejszyło się, co może świadczyć o zwiększeniu intensywności hydrolizy tych związków. Skutkowało to uwolnieniem kwasów tłuszczowych. Jednocześnie odnotowano obniżenie aktywności HADH, co mogło spowodować zahamowanie procesu β-oksydacji i nagromadzenie kwasów tłuszczowych w badanej tkance. W hemolimfie owadów również nastąpił wzrost poziomu kwasów tłuszczowych, co może wskazywać na transport tych związków z innych tkanek i narządów. Substancje te mogą być wykorzystywane do syntezy feromonów lub/i składników kutikuli. Uzyskane wyniki wskazują na zmianę profilu lipidowego w tkankach mącznika oraz zmianę aktywności kluczowego enzymu katalizującego jeden z etapów β-oksydacji kwasów tłuszczowych. Zaobserwowane zmiany zależą jednak od stężenia SOL, CHA i TOM, a także czasu inkubacji oraz rodzaju badanej tkanki. Co więcej, działanie EXT różni się od efektów powodowanych przez TOM. Wyniki te przyczyniły się

do poszerzenia wiedzy dotyczącej wpływu GA na metabolizm kluczowych substratów energetycznych, którymi są lipidy.

Ważnymi substratami energetycznymi są także węglowodany. Wyniki wskazują, że TOM i EXT wpływają na stężenie trehalozy w hemolimfie mącznika. Co ciekawe, testowane GA nie zmieniają zawartości trehalozy, glukozy i glikogenu w ciele tłuszczowym. Zmierzono również poziom aminokwasów i stwierdzono, że TOM i EXT powodują akumulację większości oznaczonych aminokwasów w tkance tłuszczowej 24 godziny po iniekcji, jednocześnie zmniejszając ich zawartość w hemolimfie. Sugeruje to potencjalny transport aminokwasów pomiędzy tkankami. Efektu tego nie zaobserwowano po zastosowaniu SOL i CHA, co wskazuje na odmienny mechanizm działania tych związków. Zaobserwowane zmiany mogą wynikać z degradacji białek i/lub wzmożonych reakcji katabolicznych, w trakcie których powstaje ATP niezbędne jako źródło energii w procesach detoksykacji. Co ciekawe, testowane GA regulują także aktywność i ekspresję genów kodujących kluczowe enzymy ważnych szlaków metabolicznych, tj. PFK, CS i HADH. Również w przypadku tych analiz, zaobserwowany efekt zależy od stężenia GA, rodzaju badanej tkanki oraz czasu inkubacji, jaki upłynął od iniekcji. Wyniki wskazują również na istnienie możliwych mechanizmów kompensacyjnych. Zmniejszona aktywność badanych enzymów po aplikacji GA korelowała często ze wzrostem poziomu ekspresji *PFK, CS* i *HADH*, szczególnie w ciele tłuszczowym owada. Przeprowadzone badania sugerują wpływ testowanych GA na proces glikolizy, cykl Krebsa, a także szlak β-oksydacji kwasów tłuszczowych, co może przekładać się na zmieniony metabolizm energetyczny u larw mącznika.

Wprowadzenie ksenobiotyku do organizmu owada często skutkuje zwiększoną produkcją ROS, a także wzmożonym działaniem systemu antyoksydacyjnego w celu uniknięcia wystąpienia stresu oksydacyjnego. Po iniekcji GA zaobserwowano zwiększoną aktywność SOD w ciele tłuszczowym larw. Co ciekawe, aktywność tego enzymu w jelicie zmniejszyła się, a także nastąpił wzrost aktywności katalitycznej CAT w tej tkance. Wyjaśnieniem różnic może być większa odporność jelita na stres oksydacyjny spowodowana szybkim przemieszczaniem się pokarmu. Z drugiej strony, być może poziom ROS po aplikacji GA jest na tyle duży, że hamuje to aktywność SOD w jelicie. Poza tym, odnotowano brak zmian ekspresji *MnSOD* na skutek traktowania GA, przy jednoczesnym wzroście ekspresji *CAT* i *HSP70.* Wzrost ekspresji tych genów może być wynikiem zwiększonego zapotrzebowania na degradację ROS, które powstają na skutek działania GA, oraz mechanizmem przyczyniającym się do wzrostu odporności komórek na potencjalny stres oksydacyjny (King & MacRae, 2015). Stan

ten skutkuje degradacją lipidów, węglowodanów i kwasów nukleinowych, prowadząc do zaburzeń w funkcjonowaniu komórki i całego organizmu. Być może GA zwiększają produkcję ROS w komórkach owadów (Adamski i in., 2014; Büyükgüzel i in., 2013), ponieważ zaobserwowany w przedstawionych badaniach wzrost zawartości kwasów tłuszczowych (Winkiel i in., 2023) może w rezultacie prowadzić do ich wzmożonej produkcji. Dodatkowo ROS, a także produkty peroksydacji lipidów działają destrukcyjnie na białka i prowadzą do ich degradacji, a także utraty aktywności katalitycznej (Pardini, 1995). Jest to zgodne z uzyskanymi wynikami, ponieważ GA nie tylko wpływały na poziom malondialdehydu, markera peroksydacji lipidów, ale także prowadziły do zmniejszonej aktywności wielu analizowanych enzymów, takich jak PFK czy HADH. Dodatkowo, odnotowane zmiany w poziomie ekspresji genów również mogą być wynikiem zaburzeń powodowanych przez powstały stres oksydacyjny (Pardini, 1995).

Celem przedstawionej pracy było określenie wpływu wybranych GA na procesy metaboliczne u chrząszcza *T. molitor.* Na podstawie wyników uzyskanych w ramach przygotowania przedstawionej rozprawy doktorskiej stwierdzono, że mechanizmy działania testowanych związków w tkankach mącznika młynarka są złożone. Efekty powodowane przez stosowane metabolity roślinne są zróżnicowane, ponieważ zależą zarówno od rodzaju testowanego GA, jego stężenia, czasu inkubacji, jak i od typu badanej tkanki. Generalnie GA zmieniają ekspresję genów i aktywność białek zaangażowanych w kluczowe ścieżki metaboliczne komórek. Poza tym, testowane metabolity roślinne wpływają na zawartość substratów energetycznych u owadów, prawdopodobnie w wyniku zwiększonego zapotrzebowania energetycznego, spowodowanego koniecznością detoksykacji aplikowanych związków. Substancje te są metabolizowane i/lub wydalane z organizmu owada w stosunkowo wolnym tempie, co stwarza możliwość ich akumulacji w tkankach i zwiększa szanse potencjalnego zastosowania jako bioinsektycydów. Mimo że badane stężenia związków nie powodują efektów letalnych, to na podstawie wyników przedstawionych badań można stwierdzić, że znacząco zmieniają one metabolizm chrząszczy, co w konsekwencji może prowadzić do zaburzenia rozwoju i reprodukcji szkodników, a tym samym do ograniczenia ich populacji. Ponadto, potencjalne zastosowanie GA w postaci dodatku do chemicznych środków ochrony roślin pozwoli zwiększyć efektywność i bezpieczeństwo stosowania, a także zmniejszyć zużycie tradycyjnych pestycydów (Spochacz i in., 2020). Niezbędne są jednak kolejne badania, które pozwolą powiązać prostszy, w kontekście szerokiej skali zastosowania, sposób aplikacji GA (np. oprysk roślin) z rzeczywistą zawartością tych związków w organizmie

owada, a także przetestować wyższe, letalne dla mącznika młynarka stężenia GA, przy jednoczesnej kontroli bezpieczeństwa tych substancji dla innych organizmów, w tym dla człowieka.

Podsumowanie

Badania przeprowadzone w ramach przedstawionej rozprawy doktorskiej wskazują, że SOL, CHA, TOM i EXT wpływają na metabolizm energetyczny larw *T. molitor* poprzez zmianę poziomu wybranych lipidów, węglowodanów i aminokwasów. GA wpływają także na kluczowe szlaki metaboliczne: glikolizę, cykl Krebsa, jak również szlak β-oksydacji kwasów tłuszczowych. Zmiany zaobserwowano dla istotnych enzymów tych procesów, zarówno na poziomie genów, jak i białek, a uzyskany efekt jest tkankowo-specyficzny. Przyczyną tych efektów może być zaburzone funkcjonowanie systemu antyoksydacyjnego, a także zróżnicowany sposób dystrybucji i kumulacji GA w tkankach owada. Zmiany w poziomie metabolitów istotnych z punktu widzenia funkcjonowania komórki oraz modyfikacje aktywności i ekspresji enzymów wywołane przez GA mogą prowadzić do zaburzeń rozwoju, reprodukcji i metamorfozy, a w konsekwencji zmniejszyć populację szkodników. Wyniki badań przestawionych w ramach tej pracy przyczyniają się zatem do zrozumienia mechanizmów działania wtórnych metabolitów roślinnych na owady i wskazują na możliwość wykorzystania GA jako potencjalnych naturalnych bioinsektycydów.

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Kopie prac wchodzących w skład rozprawy doktorskiej

Praca I

Solanaceae glycoalkaloids disturb lipid metabolism in the *Tenebrio molitor* beetle

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który jest częścią mojej rozprawy doktorskiej, polegał na zaplanowaniu badań, pozyskaniu funduszy, zebraniu materiału do analiz i przygotowaniu próbek, przeprowadzeniu analiz (określenie poziomu triacylglicerydów oraz analiza aktywności dehydrogenazy 3-hydroksyacyl-CoA), opracowaniu i interpretacji wyników, przeprowadzeniu analiz statystycznych, napisaniu manuskryptu, opracowaniu wykresów (Fig. 1-5), wprowadzeniu korekt, przygotowaniu manuskryptu do publikacji oraz na korespondencji z redakcją czasopisma.

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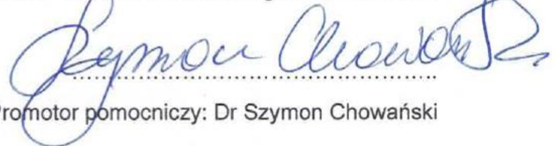
Promotor: Prof. UAM dr hab. Małgorzata Słocińska



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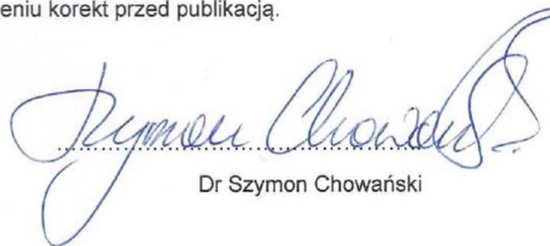
Winkiel M.J., Chowański S., Gołębiowski M., Bufo S.A., Słocińska M. Solanaceae glycoalkaloids disturb lipid metabolism in the Tenebrio molitor beetle, Metabolites 2023, 13, 1179, DOI: 10.3390/metabo13121179,

który jest częścią rozprawy doktorskiej Magdaleny Joanny Winkiel, polegał na przygotowaniu próbek do analizy aktywności dehydrogenazy 3-hydroksyacyl-CoA, nadzorowaniu przedstawienia wyników badań oraz na wprowadzeniu korekt przed publikacją.



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który jest częścią rozprawy doktorskiej Magdaleny Joanny Winkiel, polegał na oznaczeniu poziomu kwasów tłuszczowych, steroli i estrów w próbkach oraz na wprowadzeniu korekt manuskryptu przed publikacją.

KIEROWNIK
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I declare that my participation in the preparation of the article:

Winkiel M.J., Chowański S., Gołębiowski M., Bufo S.A., Słocińska M. *Solanaceae glycoalkaloids disturb lipid metabolism in the Tenebrio molitor beetle*, Metabolites 2023, 13, 1179, DOI: 10.3390/metabo13121179,

which is part of the doctoral dissertation of Magdalena Joanna Winkiel, consisted of preparing the tomato leaves extract for analyses and making corrections before publication.

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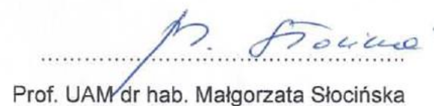
który jest częścią rozprawy doktorskiej Magdaleny Joanny Winkiel, polegał na nadzorowaniu organizacji badań oraz na wprowadzeniu korekt przed publikacją.



Prof. UAM dr hab. Małgorzata Słocińska

Praca II

Analysis of glycoalkaloids distribution in the tissues of mealworm larvae (*Tenebrio molitor*)

Magdalena Joanna Winkiel, Szymon Chowański, Maria Sulli, Gianfranco Diretto, Małgorzata Słocińska

Manuskrypt jest na etapie recenzji w czasopiśmie *Scientific Reports*

Analysis of glycoalkaloids distribution in the tissues of mealworm larvae (*Tenebrio molitor*)

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Keywords: solanine; chaconine; detoxification; plant secondary metabolites; insect; mass spectrometry

Abbreviations:

GAs - glycoalkaloids

SOL - α-solanine

CHA - α-chaconine

G - gut sample

H - haemolymph sample

FB - sample prepared with the whole larvae without gut and haemolymph

Abstract

Solanine (SOL) and (CHA) are glycoalkaloids (GAs) produced mainly by *Solanum* plants. These plant secondary metabolites affect insect metabolism; thus, they have potential to be applied as natural plant protection products. However, it is not known which GA concentration induce physiological changes in animals. Therefore, the aim of the study was a quantitative analysis of SOL and CHA in the larvae of *Tenebrio molitor* using LC-MS technique to assess how fast they are eliminated or metabolised. In the experiment, the beetles were injected with 2 μL of 10^{-5} M SOL or CHA solution, which corresponds to dosage range 0.12–0.14 ng/mg body mass. Then, 0.5, 1.5, 8 and 24 hours after GA application, haemolymph (H), gut (G), and the rest of the larva body (FB), were isolated. GA was reported in all samples tested during 24 hours with the highest percentage of the amount applied in the FB, while the highest concentration was measured in the H sample. The SOL and CHA concentration decreased in the hemolymph over time, while it did not change in other tissues. CHA has the highest elimination rate immediately after injection, while SOL slightly later. None of the GA hydrolysis products were detected in the tested samples. One possible mechanism of detoxification of GA may be oxidation and/or sequestration. They may be excreted by Malpighian tubules, with feces or with cuticles during molting. The results presented are significant because they facilitate the interpretation of studies related to the effects of GAs on insect metabolism.

42 Introduction

43 Plants produce many compounds, called primary metabolites, which are essential for their growth, development,
44 and metabolism. During primary metabolic reactions, by-products called plant secondary metabolites (PSMs) are
45 created. PSMs are frequently involved in the defence mechanisms activated as a reaction to many different stresses
46 such as changes in environmental conditions, infections, or herbivore feeding [1]. Alkaloids are one of a major group
47 of PSMs with around 10,000 derivatives reported [2]. In particular, steroidal glycoalkaloids (GAs), such as solanine
48 (SOL) and chaconine (CHA), contain glycoside residues attached to the nitrogenous aglycone part and are
49 produced mainly by *Solanaceae* crop plants, such as potato (*Solanum tuberosum* L.) and tomato (*Solanum*
50 *lycopersicum* L.). GAs can be found almost in all plant organs, including leaves, stems, roots, and tubers [3]. GAs
51 demonstrate high biological activity [4]; they are toxic to cells from various groups of organisms; thus, GAs are the
52 defence against herbivores and pathogenes. On the other hand, their cytotoxic, antioxidant, antivirial and
53 antimicrobial properties can be used in the drug industry [5,6].

54 GAs are biologically active substances, and their effects on cells were reviewed by many researchers [7,8]. For
55 example, GAs are strong inhibitors of acetylcholinesterase and butyrylcholinesterase, which catalyse the
56 hydrolysis of the acetylcholine neurotransmitter in the nervous system [8]. They can affect the cell division process
57 by inducing the ornithine decarboxylase enzyme and can modulate Ca^{2+} and Na^{+} transport across cell membranes.
58 Moreover, GAs form complexes with cholesterol what leads to cell disruption and leakage of content of the cell.
59 Some reports indicate teratogenicity effects in animals caused by GAs with CHA being more toxic than SOL [7].
60 The toxic effect of GAs depends on many factors, such as the type of carbohydrate chain in the structure, the
61 presence of a nitrogen atom in the GA ring, and the pH value [7]. In mammals, the LD_{50} values for GAs are similar
62 in different species. In rodents, the metabolism of GAs is defined by low absorption, rapid excretion, and
63 hydrolysis to alkaloids, which are less toxic. Due to poor absorption, the intraperitoneal LD_{50} values are much
64 lower than the values calculated after GA consumed orally. For example, intraperitoneal LD_{50} for SOL is 34 mg/kg
65 body weight in mice, while for GA consumed orally, the LD_{50} is more than 1000 mg/kg body weight [7].

66 GAs also have a wide range of insecticidal activity [4,9,10]. Pure GAs and leaf extracts of *S. tuberosum* and *S.*
67 *lycopersicum* added to the culture medium caused malformations and reproduction disturbances in *Drosophila*
68 *melanogaster* M. [11]. More specifically, SOL, CHA, and tomatine were found to be toxic against stored product
69 insects: the red rust flour beetle (*Tribolium castaneum* H.), and the rice weevil (*Sitophilus oryzae* L.) [12]. Similarly,
70 SOL and its extract from tomato leaves administered with food affected fertility, fecundity and survival of *Galleria*
71 *melonella* L. [13,14]. In this species, the impact of solasonine and *Solanum nigrum* L. extract was also studied. These
72 substances were found to affect the composition of hemolymph metabolites, as well as the ultrastructure of fat
73 body and midgut cells (et al., 2021). GAs and their extracts also exhibit cardioactive properties that were shown
74 in *T. molitor* and *Zophobas atratus* B. beetles [16,17]. Sublethal effects of GAs, such as disturbed development, food
75 intake, and reproduction, were observed in *T. molitor* after addition of pure GAs and *S. nigrum* fruit extract to the
76 food [18]. Injection of GAs and tomato leaf extract into *T. molitor* larvae affects their lipid metabolism [19], in turn
77 administration of *S. nigrum* fruit extract modulates insect immune system activity [20] in this species.

78 SOL and CHA are the main GAs of potato tuber, which is one of the most important agricultural products.
79 According to FAO data, a total of 376 million tons of potatoes were produced worldwide in 2021 (FAOSTATs,
80 2021). Colorado potato beetle (*Leptinotarsa decemlineata* S.), potato ladybird (*Henosepilachna*
81 *vigintioctopunctata*, F.), and potato tuber moth (*Phthorimaea operculella*, Z.) belong to the most dangerous potato
82 pests [21]. Thus, it is crucial to discover mechanisms of GAs action in insects, because it might to develop new
83 strategies against crop pests.

84 As GAs affect insect metabolism, they have potential to decrease insect survival or by disturbing fecundity reduce
85 insect population thereby they can be applied as natural plant protection products [22]. However, it is not known
86 exactly which GA concentrations induce the mentioned physiological changes in animals, because in most studies,
87 the GAs were administered with food, in the extract form or/and incubation time was chosen arbitrarily. Therefore,
88 **the aim of the study was to perform a quantitative analysis of these compounds at particular time points**
89 **after the injection of GAs into the body of the larvae of *T. molitor*** to assess how fast they are eliminated or
90 metabolised by the beetle. Additionally, the survivability of larvae after GAs injection was measured to assess the
91 potential lethality and long-term effects of GAs treatment.

92

Results

Mass spectrum

The SOL and CHA amount in the tested samples of haemolymph (H), gut (G) and the remaining part of the larvae (FB) from the insects injected with tested GAs were measured using LC-HRMS. More in detail, the sample mass spectrum is shown on the Fig. 1. For SOL and CHA, the area of the M+H ions of m/z were 868.5053 and 852.5104 m/z (Dppm<3), respectively. The calibration curves were established with appropriate analytical standards. Retention time for SOL was 10.8 min, while for CHA – 11.1 min.

Changes of SOL content over time

0.5 h after SOL application, almost all of the applicated GA amount 69.5±2.95 ng (~100%) was still present in the insect body (Fig. 2). During the next 1 hour, the total amount of SOL started to drop by around 12.2±2.50 ng (~17.5%) and it was the highest decrease in total SOL content in time. Therefore, we observed a delay in starting the elimination of SOL from the insect body. During the next 6.5 h we observed a further decrease on average by only 3.0±4.00 ng (~4.4%) and another 3.0±2.19 ng (~4.3%) drop in the quantity of SOL at 8 h of the analysed period. After 24 h, 51.3±8.76 ng (~73.9% of the administered amount of SOL) still remained in the insect body. The distribution of SOL in different tissues also changed during the time analysed. At the first time point (0.5 h after injection), the lowest amount of total GA was located in the gut (10.4±1.55 ng, ~15.0% of the applied amount of GA). Two times more SOL was detected in the haemolymph – 20.0±7.24 ng (~28.9% of applicated SOL) and 39.1±5.23 ng (~56.3% of injected GA amount) was spread across remaining parts of the larvae body, mainly in the fat body. One hour later, the compound level in the gut drops to 9.4±1.91 ng (what constitute ~13.5% of applicated amount) but increased to 15.9±2.30 ng (~22.9%) in the next time point and finally reached the level of 9.4±1.17 ng (~13.5%) after 24 h. In the samples obtained from haemolymph, the amount of SOL systematically dropped to 15.9±3.97 ng (~22.9%), 7.7±0.67 ng (~11.1%) and 5.3±0.90 ng (~7.6%) 1.5, 8 and 24 h after application, respectively. In the FB sample, we observed the lowest fluctuation in the content of SOL. At each time point, the SOL content in the samples was similar and reached the value 32.1±7.26 ng, 30.6±10.15 ng and 36.7±8.89 ng (between ~44.1 and ~52.8% of applied SOL) after 1.5, 8 and 24 h injection.

The highest total amount of SOL at 0.5 time-point was noticed in the FB when the whole mass of tissue was taken into account (Fig. 2). Knowing the total mass of each tissue used for sample preparation, we also calculated the concentration of SOL in each of them. The results (Fig. 3) showed that in FB we noticed the lowest and constant concentration (between 0.08±0.018 and 0.09±0.015 ng/mg). That indicates a low affinity of SOL for that tissue. The highest concentration 0.5 h after GA injection was observed in haemolymph (0.85±0.244 ng/mg). It was more than 9 times higher than in FB (0.09±0.015 ng/mg) and four times higher than in gut (0.21±0.031 ng/mg) in that time point. During 24 h, the concentration of SOL in haemolymph decreased over 4-times (to 0.20±0.018 ng/mg), but still, at each time point, it was higher than in FB (Fig. 3). The SOL concentration in the gut ranges between 0.16±0.035 and 0.22±0.028 ng/mg and it does not change with time. The ratio of SOL concentration in different tissues changed from 1.0:9.3:2.3 (FB:H:G) to 1.0:7.8:1.5, 1.0:3.7:2.8 and 1.0:2.4:2.0 (Table 1), respectively at each next time point. Equalization of concentrations in haemolymph (H) and gut (G) sample at 8 h and 24 h time-point can be the reason why the elimination of SOL slowed down significantly after 8 and 24 h. Whereas, the lack of significant changes in SOL concentrations in FB may result from the fact that during the whole tested period, the concentration in that tissue was lower than in the hemolymph.

Changes of CHA content over time

At the first checked time point (0.5 hour after injection), there was 59.9±1.75 ng (~87.9%) of the applicated CHA amount (68.2 ng) in the entire insect body; therefore, the elimination of CHA has started just after application (Fig. 4). During the next 1 hour, its level decreased to 58.8±4.34 ng (~86.3%), and at the next time points we measured 56.9±9.45 ng (~83.0%) and 43.0±4.16 ng (~63.1%) of applied CHA as still presented in the insect body, 8 and 24 h after application.

Regarding the distribution of GAs in tested tissues, at the beginning of the experiment (0.5 h variant), the lowest total level of GA was in the gut (9.5±1.50 ng, ~13.9% of the applicated amount). Almost two times higher level of CHA was observed in the haemolymph (16.6±2.04 ng, ~ 24.3% of applied CHA), and 33.9±4.72 ng (~49.7% of the amount of GA amount) was located in the FB sample. In the gut, CHA level increased slightly 1.5 h after injection to 10.9±1.64ng (~16.0% of the applicated amount), but decreased to 10.0±1.77ng (~14.7%) at the next

time point and reached the 11.0±0.62 ng amount (~16.2%) again after 24 h. In haemolymph samples, CHA amount decreases systematically during the whole experiment to 12.4±2.06 ng (~18.2%), 10.7±2.61 ng (~15.7%), and 6.5±0.91 ng (~9.5%) 1.5, 8 and 24 h after injection respectively. In FB samples, the amount of CHA increases during 8 hours after application, reaching 36.2±3.63ng (~53.1%), while it decreases at the 24 time point to 25.5±3.28 ng (~37.3% of injected CHA).

Similarly to SOL, when we consider the whole mass of tissue, the highest amount of CHA was detected in the FB samples (Fig. 4). However, the lowest concentration expressed as ng per mg was calculated in these samples and was between 0.06±0.010 and 0.09±0.013 ng/mg (Fig. 5). There were no significant changes in CHA concentration during the whole experiment neither in FB nor in gut samples (0.16±0.014-0.20±0.037 ng/mg). The highest GA concentration at all time points tested was observed in the haemolymph (0.28±0.058-0.77±0.129 ng/mg). It was more than 9 times higher than in FB (0.08±0.012 ng/mg) and almost 4 times higher than in the gut (0.20±0.037 ng/mg) at the 0.5 time point (Table 2). The greatest changes in CHA concentration during 24 h were also detected in that tissue (almost 3-times). The proportion of GA concentration in gut samples (G) with other tissues increased at the 24 time point. The ratio of CHA concentration in different tissues changed from 1.0:9.3:2.4 (FB:H:G) to 1.0:6.8:2.1, 1.0:4.6:2.1 and 1.0:4.4:2.5 (Table 2), respectively, at each next time point.

Changes of GAs elimination/accumulation rate

The concentration of SOL and CHA in the samples changed with a different rate (Fig. 6). SOL started to be eliminated from FB already during 0.5 and 1.5 h after injection with a rate $2.01 \cdot 10^{-4} \pm 1.319 \cdot 10^{-4}$ ng/mg/min (Fig. 6A). Furthermore, the elimination rate tends to be higher during this period than between 1.5 and the next time point of 8 h ($9.79 \cdot 10^{-6} \pm 3.233 \cdot 10^{-5}$ ng/mg/min). During the last period (8-24 h), GAs slowly accumulated slowly ($9.12 \cdot 10^{-6} \pm 7.160 \cdot 10^{-6}$ ng/mg/min). On the other hand, CHA accumulated in FB during all the tested periods; at the beginning of the experiment (0.5-1.5 h) with a rate $6.04 \cdot 10^{-5} \pm 1.941 \cdot 10^{-4}$ ng/mg/min, during the next tested period $1.14 \cdot 10^{-6} \pm 1.092 \cdot 10^{-4}$ ng/mg/min and in the last one $1.84 \cdot 10^{-5} \pm 3.264 \cdot 10^{-5}$ ng/mg/min.

The elimination rate of SOL in haemolymph (Fig. 6B) tends to be highest during the first tested period 0.5-1.5 h ($3.78 \cdot 10^{-3} \pm 3.532 \cdot 10^{-3}$ ng/mg/min), and it is almost 19 times higher than in FB. In the next timespans, SOL was removed from haemolymph more slowly ($8.82 \cdot 10^{-4} \pm 8.588 \cdot 10^{-6}$ and $8.13 \cdot 10^{-5} \pm 3.081 \cdot 10^{-5}$ ng/mg/min). Changes in CHA elimination rate in the hemolymph are very similar to SOL but slightly lower ($2.97 \cdot 10^{-3} \pm 2.278 \cdot 10^{-3}$; $5.44 \cdot 10^{-4} \pm 5.035 \cdot 10^{-5}$ and $1.07 \cdot 10^{-4} \pm 8.223 \cdot 10^{-5}$ ng/mg/min in the subsequent tested periods). However, the changes are not statistically significant.

During 0.5-1.5 h, the elimination rate of SOL in gut (G) was the highest ($1.50 \cdot 10^{-3} \pm 9.079 \cdot 10^{-4}$ ng/mg/min). However, it accumulated between 1.5 and 8 h with a rate $2.46 \cdot 10^{-4} \pm 1.609 \cdot 10^{-4}$ ng/mg/min ($p<0.001$). In the next timespan, SOL tends to be eliminated again with $5.27 \cdot 10^{-5} \pm 6.186 \cdot 10^{-5}$ ng/mg/min rate. CHA was eliminated from the gut during the whole experiment with the highest rate during the 0.5-1.5 period ($2.18 \cdot 10^{-4} \pm 4.066 \cdot 10^{-4}$ ng/mg/min). SOL is eliminated from the gut during the first period tested faster than CHA ($p<0.01$).

The changes of the calculated, detected amount of GAs in the whole larvae (the sum of GAs in FB, G, and H) are shown in Fig. 6D. In total, SOL and CHA were eliminated from the larvae body throughout the entire experiment. During the first period tested, CHA is eliminated faster (0.275±0.0584 ng/min) than during the next one (0.012±0.0659 ng/min) ($p<0.0001$) and with the highest rate in the whole experiment. SOL is eliminated the fastest 0.5-1.5 h after injection with a rate 0.135±0.0278 ng/min. This value is higher than in the first tested period ($p<0.05$) and higher than in the next one ($p<0.01$). CHA is eliminated over 7 times faster (0.275±0.0584 ng/min) than SOL (0.037±0.0386 ng/min) during 0.5 h after injection ($p<0.0001$). However, in the next tested period (0.5-1.5 h), the elimination rate of SOL is more than 11 times higher (0.135±0.0278 ng/min) than CHA (0.012±0.0659 ng/min) ($p<0.01$). During the next timespans (1.5-8 h and 8-24 h) the elimination rates of GAs are much slower than before and reached values between 0.003±0.0023 and 0.014±0.0157 ng/min.

Survivability of larvae after GAs application

The survivability of the *T. molitor* larvae after 2 μL of SOL and CHA 10^{-5} M application were analyzed for 10 days (Fig. 7). In the three replicates together, there was one dead larva observed in the control with physiological saline application, four larvae for variant with SOL and two larvae after CHA injections. However, there were no significant changes in survival compared to the control. The first dead larva in the experimental variant with SOL was observed one day after injection, while for CHA treatment – after two days.

Discussion

Xenobiotics are chemical compounds that are not natural components of a living organism but are exposed to them. They undergo metabolic processes, including absorption, distribution, biotransformation, and excretion. The xenobiotic can enter the insect organism through the cuticle, eggshell or orally. Then, the detoxification processes begin. The compounds undergo modifications and degradations, including oxidation-reduction reactions that increase compounds solubility and facilitate their elimination from the organism. After that, the modified xenobiotic is excreted firstly from cells and finally from the organism by different types of transporters [23].

Many insects that are crop pests are exposed to glycoalkaloids. **The purpose of the study was to perform a quantitative analysis of GAs at particular time points in different tissues after their injection to assess how they are distributed and accumulated through the insect organism, as well as how fast they are metabolised and eliminated by the insect.**

Under natural conditions, GAs can enter the insect body with food. Most studies concerning feeding the insects with plants containing GAs or of preparation of artificial diet with the addition of GAs [13–15,18,24]. However, without knowledge about the exact concentration of these compounds in insects, it is impossible to understand the precise mechanism of GA action. Thus, in this research we applied SOL and CHA by injection to deliver the exact amount of the compound to the larvae. We have tested samples of haemolymph, gut and samples obtained from the remaining part of the insect body, mainly consisting of fat body, Malphighian tubules, and cuticle. The insect haemolymph is composed of fluid plasma containing hemocytes, and it circulates around the other tissues in the insect body. The fat body tissue fills the body cavity, surrounding the digestive tract. It is immersed in the hemolymph, which facilitates the exchange of metabolites. It is the main organ of the intermediary metabolism of insects. Therefore, it is not surprising that the applicated GAs were detected in the fat body sample and haemolymph (Fig. 2, 4). However, the results also indicate that the GAs were transported to the insect gut (Fig. 2, 4). They might be transported from haemolymph directly, or/and with Malpighian tubules. It may be one of the explanations of the GAs loss in haemolymph with time. The Malpighian tubules are long tubes which are connected to the gut between midgut and hindgut. They build up the excretory system, which is responsible for maintaining homeostasis [25].

When a xenobiotic enters the insect organism, it may undergo different detoxification reactions. The type of process depends on the chemical nature of the compound. GAs are classified as glycosides, because they are composed of carbohydrate chain and the aglycon part connected with glycosidic bond. Glycosides, in turn, belong to acetal compounds with the general formula $R_2C(OR')_2$ [10]. Acetals are obtained during the nucleophilic addition of two molecules of an alcohol to an aldehyde or ketone in the presence of an acid catalyst [26]. This condensation reaction is called acetalisation. Acetals are stable to bases, reducing agents, as well as nucleophiles; however, they break down in acid environment [26]. SOL and CHA are produced in plants *through* the cholesterol pathway, in a glycosylation reaction of carbohydrates (carbonyl compounds) with solanidine (alcohol) [7,8]. Additionally, GAs are derived from alkaloids.

The biotransformation of GAs involves the hydrolysis process, which leads to a few different products. Carbohydrate groups are susceptible to hydrolysis in acids as well as to hydrolysis catalysed by enzymes. Detaching particular sugar molecules lead, first, to β-compounds, then γ-derivatives are formed. The aglycon part called solanidine remains, when all sugar chains are cut off from the SOL or CHA molecule (Fig. 8) [7,8]. Hydrolysis of the glycosidic bond results in the loss of the GAs activity [10], thus, the biotransformation is an ability of many organisms (to avoid the toxicity), as well as of different plant species (to eliminate autotoxicity risk), although, nitrogen-containing chain often shows high resistance to transformation. Many bacteria species have the ability to metabolise GAs by detaching the carbohydrate group or oxidising the hydroxyl groups [27]. Plants and phytopathogenic fungi contain glycosidases that hydrolyse GA molecules. However, it is not known whether mammals glycosidases also have such properties [7,8]. Glycosidases were identified in insects of various orders, such as Orthoptera, Hymenoptera, and Coleoptera [28]. These enzymes were also reported in adults of *T. molitor* [29] as well as in larvae [30] were also reported. However, contrary to expectations, any of the GA hydrolysis products were detected in the study. One possible explanation is that glycosidases present in *T. molitor* larvae have high substrate specificity and do not react with GA compounds.

245 Anyway, insects developed complex protection systems for defence against different xenobiotics [23]. Some toxic
246 molecules can be metabolised into easily excreted compounds and eliminated from the body by the excretory
247 system. Other xenobiotics can be modified to safer chemicals to facilitate their accumulation in tissues [25]. The
248 tested GAs do not cause lethal toxicity during 10 days after application (Fig. 7), thus, insects can use a variety of
249 strategies to deal with the xenobiotics. One of the physiological adaptation of the organism to prevent poisoning
250 is the rapid intestinal passage, which protects against the accumulation of toxins [31]. Usually, in detoxification
251 processes, cytochrome P-450 is involved. It catalyses the oxidation of different xenobiotics, such as
252 phytochemicals and insecticides [32]. For example, nicotine (another alkaloid of *Solanaceae* plants) given with food
253 to *Manduca* larvae induces the P-450 cytochrome in the midgut epithelium. Nicotine presented in the haemolymph
254 was metabolized and the product of its oxidation was actively transported to Malpighian tubules with a nonspecific
255 alkaloid pump and excreted [25,33]. Active transport of alkaloids to urine was also reported in larvae of *Rhodnius* and
256 *Pieris* [34]. However, G-strophanthin, a cardiac glycoside, is also actively transported in *Zonocerus*, while in *Locusta*
257 it moves passively into the Malpighian tubules [35]. The detoxification enzymes act in the fat body and Malpighian
258 tubules; however, they are the most active in the insect midgut [25,36]. Some species, for example butterfly *Danaus*
259 *plexippus,* maintain the oxidising conditions in the midgut to defend against plant-derived compounds [37]. In
260 *Spodoptera litura,* many detoxification-related genes were up-regulated after tomatine treatment. In addition to
261 the P450 genes, glutathione S-transferases, ABC transport enzyme, UDP-glucosyltransferases and
262 carboxylesterases were also upregulated, mainly in the midgut and fat body [36]. The molecular mechanisms
263 involved in the action of all these enzymes in *Spodoptera* were described in the review [38], while regulation of their
264 expression in insects was described in the study [39]. Besides the oxidation system, xenobiotics which enter the insect
265 body can be sequestered and stored in the cuticle, glands or in the haemolymph [25,40]. For example, *Oncopeltus*
266 *fasciatus* (Hemiptera) is able to sequester g-strophanthin [41]. Thus, one possible mechanism of detoxification of
267 GAs can be oxidation and/or sequestration.

268 Various xenobiotics are removed from the insect organism in a different way. The elimination path depends on the
269 type of detoxification processes that take time. This is the first study to analyse changes in GA concentration in *T.*
270 *molitor* tissues over time. The change in applied SOL percentage in different time points shows Fig. 2. The
271 oxidation/excretion processes do not occur during the first 30 minutes after application because almost all the
272 amount of GA was detected in the samples. At the end of the experiment (24 hours after injection), 73.9% of the
273 applied SOL still remained in the larvae. On the other hand, the CHA content was much lower than the SOL
274 percentage after 0.5 h (87.9% of the applicated CHA) and 24 h after injection (63.1%) (Fig. 4). The results indicate
275 that CHA are eliminated immediately after injection, while there is a delay in SOL elimination. Moreover, GAs
276 excretion processes are relatively slow because 24 hours is not enough to remove all GA amounts from the larvae
277 organism.

278 According to expectations, the highest percentage of applied GA among the tissues tested was in the FB sample
279 (Fig. 2, 4), which mainly contained a fat body and Malpighian tubules, due to the function of these tissues described
280 above. Furthermore, these results are consistent with other studies, because the lipid droplets in the fat body of the
281 *T. molitor larvae,* as well as the *G. melonella* larvae treated with the extract of *S. nigrum,* solasonine, and
282 solamargine showed decreased homogeneity and lysis of the content of lipid droplets [15,18]. Thus, GAs can alter fat
283 body structure. Moreover, SOL, CHA and tomatine affect lipid metabolism [19]. Despite GA delivery by injection
284 through the cuticle, the compounds were also detected in the gut tissue. It indicates that GAs can be transferred to
285 the gut, which is involved in GA metabolism and/or elimination. This finding was also reported by (Li et al., 2023),
286 in which GA accumulation was studied in the potato tuber moth *P. operculella*. In this research, the concentration
287 of GAs applied to the insects with food were analyzed in head, foregut, midgut, hindgut, cuticula and feces of
288 larvae. In the insects fed with potato leaves, SOL was detected in feces and midgut, while CHA was excreted with
289 feces and accumulated in hindgut, head, midgut and cuticle (order of decreasing GA content). In the insects fed
290 with 0.3% GAs containing artificial diet (1 mL of CHA and 0.75 mL of SOL), SOL was found in midgut and feces,
291 while CHA was detected in midgut, hindgut, feces, head and cuticula (order of decreasing GA content). None of
292 these GAs was detected in the foregut. Unfortunately, neither haemolymph nor fat body was studied in this
293 research. The excretion of GAs with feces might be the most effective method of their detoxification. These results
294 are consistent with our suggestions that SOL and CHA are excreted by *T. molitor* mainly with feces and cuticle.

295 The concentration of GAs in insects depends on the type of tissue (Fig. 3,5), and these compounds are eliminated
296 at different rates (Fig. 6). The concentration of GAs in the FB sample is relatively low and does not change with
297 time (Fig. 3,5), thus showing a low affinity for that tissue. Moreover, their concentration change rate is almost
298 constant (Fig. 6A). In the haemolymph, SOL and CHA concentrations decreased during 8 h after application (Fig.

299 3,5), and the elimination rate tends to be the highest at the beginning of the experiment (Fig. 6B). In the gut,
300 similarly to FB sample, the GAs concentration is also quite low and there are no changes in its concentration with
301 time (Fig. 3,5). On the other hand, during the first tested period (0.5-1.5 h), SOL is eliminated at the fastest rate in
302 the whole experiment, and significantly quicker than CHA (Fig. 6C). A possible explanation of this finding might
303 be that GAs present in haemolymph are transported to the gut (directly, or/and with the Malpighian tubules),
304 maintaining the constant, maximum level. This result can also be explained by the direct transfer of these
305 compounds to the cuticle. Taking into account the whole insect, tested GAs were eliminated from the larvae body
306 throughout the entire experiment (Fig. 6D). Thus, in addition to GA transport between tested tissues, SOL and
307 CHA must be eliminated outside the body, for example, with feces. These results corroborate the findings of [24]
308 who reported the GA excretion with feces as well as with the cuticle. It is possible that the GA amount in the gut
309 as well as in the fat body samples would decrease when it would reach the saturated concentration in the
310 haemolymph. The observed changes might be attributed to the sequestration of some of these plant secondary
311 metabolites in the insect body as well.

312 In the present study, SOL and CHA were injected into the larvae of *T. molitor* and the percentage amount of GAs
313 was analysed in different tissues within 24 hours at particular time points. Tested GAs were reported in the samples
314 of gut, haemolymph and the remaining tissues together (mainly fat body and Malpighian tubules), with the highest
315 percentage in the last ones. The present study raises the possibility that SOL and CHA are not hydrolized in the
316 larvae of *T. molitor* by glycosidases because none of the hydrolysis products were detected in the tested samples.
317 One possible mechanism of detoxification of GAs can be oxidation and/or sequestration. On the other hand, the
318 GAs concentration was the highest in the haemolymph. SOL and CHA concentration decreased in the haemolymph
319 during the experiment, while it did not change in other tissues. Thus, they may be excreted by Malpighian tubules,
320 with feces or with cuticles during molting. Moreover, GAs excretion processes are relatively slow because 24
321 hours is not enough to remove all the applicated GAs amount from the larvae organism. Despite this, there are no
322 lethal effects during 10 days since GAs administration. The rate of CHA elimination in the entire insect was the
323 highest immediately after injection (0-0.5 h), while SOL was eliminated the fastest later (between 0.5-1.5 h). The
324 presented results are significant because they facilitate the interpretation of the conducted research and future
325 research related to the effects of GAs on insect metabolism. Further work is needed to explore the longer-term
326 excretion of GAs in insects, as well as to evaluate the impact of the way in which insects are exposed to GAs on
327 the detoxification processes of these compounds.

328
Methods

329
Insects

330 The larvae of *T. molitor* beetles were obtained from the colony cultured at the Department of Animal Physiology
331 and Developmental Biology at the Faculty of Biology of Adam Mickiewicz University in Poznań, Poland at
332 constant temperature (26 ± 0.5 °C), humidity (65 ± 5%) and photoperiod 12:12 h light to dark. The food consisted
333 of oat flakes and fresh carrots. Only feeding larvae from the 15th to 16th instar of approximately 120 to 140 mg
334 of weight were selected for the experiments.

335
Compounds and Treatment Procedure

336 Saline solutions of synthetic SOL (≥95.0%, Cat. No. S3757) and CHA (≥95.0%, Cat. No. PHL80075) (Sigma-
337 Aldrich, Merck, Darmstadt, Germany) were used in experiments at a concentration of 10^{-5} M. The insects were
338 injected with 2 μL of GAs solution, which corresponds to 69.45 ng of SOL or 68.17 ng of CHA per one sample
339 composed of 4 larvae (dosage range 0.12–0.14 ng/mg body mass). This concentration was selected based on the
340 literature and our previous studies and causes different metabolic and developmental disorders [15,16,18,19]. The tested
341 compounds were administered to larvae by injection using a microsyringe (Hamilton). The injection was made on
342 the abdominal side of the larva behind the last pair of legs after 8 min of CO_2 anaesthesia.

343
Tissue Isolation and Samples Preparation for MS analyses

344 Samples of selected tissues (haemolymph (H), gut (G), and the rest of the larva body (FB), which mainly consists
345 of the fat body), were isolated 0.5, 1.5, 8 and 24 hours after GA injection. Before isolation, larvae were
346 anaesthetised with CO_2. We chose those tissues because of their role in the distribution, metabolism, and
347 detoxification of xenobiotics within insect body [25,42]. Haemolymph was collected using an automatic pipette after
348 cutting the legs of the first pair. After decapitation and cutting off the last segment of the abdomen, the gut was
349 isolated. Guts were not cleaned of food residuals. The rest of the larva body was then placed in Eppendorf tubes.

350 The isolation was performed on ice to avoid sample degradation. After isolation, gut and fat body samples were
351 weighed to determine the fresh mass of tissues and the volume and weight of the haemolymph in each sample
352 were measured. In the next step, the samples were homogenized in the fresh prepared extraction buffer (methanol
353 1% acetic acid with daidzein 1 μg/mL) using a pestle homogenizer (Fisherbrand, Ottawa, ON, Canada) and mixed
354 at RT OV with a laboratory cradle (KL-942). Finally, the samples were centrifuged (10.000 RPM, 20 min, 4°C),
355 filtered with syringe filters (0.22 μm), and the supernatant was transferred to a new tube for LC-HRMS analyses.

356 LC-HRMS analyses

357 Samples of isolated tissues extracts were transferred (0.5 ml) into vials for LC/MS analysis (Mini-UniPrep®
358 syringeless filters with 0.2 μm pore size, PTFE membrane, Whatman) and analyzed with a LC system equipped
359 with a photodiode array detector (Dionex) and coupled to a Q-exactive Mass Spectrometer (Thermo Fisher
360 Scientific). LC separation was performed with (A) water (0.1% formic acid) and (B) acetonitrile:H_2O 90:10 (0.1%
361 formic acid) injecting 5 μL of sample on a C18 Luna column (Phenomenex), 2.1 × 100 mm, 2.5 μm particle size.
362 Column hoven temperature was set at 40°C. Total run time was 32 min and flow rate 0.250 ml/min, with an elution
363 system as follows: 0 to 0.5 min 95% A/5% B, 24 min 25% A/75% B, and 26 min 95% A/5%, as previously
364 described [43]. Ionization was obtained by Heated Elettrospray Source (HESI) operating in both positive and negative
365 ionization mode. Sheath and auxiliary gas 40 and 10 units, respectively. Probe heater temperature was 330 °C, the
366 capillary temperature was 250 °C, and the S-lens RF level was set at 50. The acquisition was performed in the
367 mass range 110–1600 m/z both in positive and in negative ion modes with the following parameters: resolution
368 70,000, microscan 1, AGC target 1 × 106, maximum injection time 50. SOL and CHA were quantified by LC–MS
369 in HESI positive ionization mode, integrating the area of the M+H ions of m/z 868.5053 and 852.5104 m/z
370 (Dppm<3), respectively, using calibration curves established with analytical standards SOL (≥95.0%, Cat. No.
371 S3757) and CHA (≥95.0%, Cat. No. PHL80075) , and normalizing on the on the weight of tissue used for the
372 extraction. Standard solutions were prepared in methanol 1% acetic acid at a concentration of 50 ng/ml and then
373 serially diluted to working concentrations. All the solvents used were LC-MS grade (Merck, Darmstadt, Germany).

374 Survivability

375 The survivability of *T. molitor* larvae during 10 days after GA injection. The numbers of living and dead larvae
376 were recorded every day for each experimental variant and each repetition. Each experiment was repeated three
377 times with 15 larvae per replicate.

378 Statistical analysis

379 Statistical calculations were made using Graphpad Prism 8.0.1 and two-way ANOVA test, Log-rank test (Mantel-
380 Cox). The normality was checked with the Shapiro–Wilk test.

381 Data Availability Statement

382 The data analysed during this study are included in this published article.

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471

472 ## Author Contributions

473 Conceptualization: Magdalena Joanna Winkiel, Szymon Chowański, Małgorzata Słocińska; Methodology:
474 Magdalena Joanna Winkiel, Szymon Chowański, Maria Sulli, Gianfranco Diretto, Małgorzata Słocińska; Material
475 preparation: Magdalena Joanna Winkiel; Data collection and analysis: Maria Sulli, Magdalena Joanna Winkiel;
476 Writing - original draft preparation: Magdalena Joanna Winkiel; Writing - review and editing: Magdalena Joanna
477 Winkiel, Szymon Chowański, Maria Sulli, Gianfranco Diretto, Małgorzata Słocińska; Supervision: Szymon
478 Chowański, Małgorzata Słocińska. All authors have read and agreed to the published version of the manuscript.

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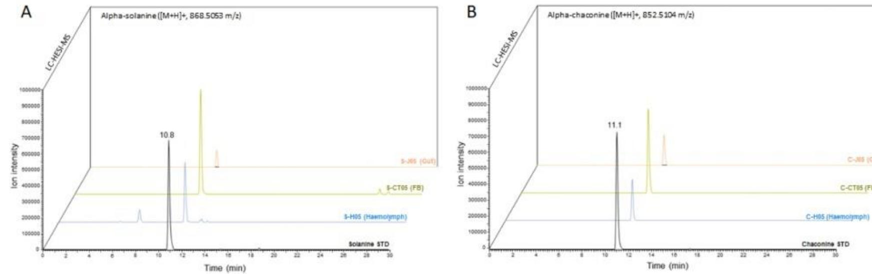
481 ## Competing interests

482 The author(s) declare no competing interests.

483 Figure legends and Tables

484 **Mass spectrum**

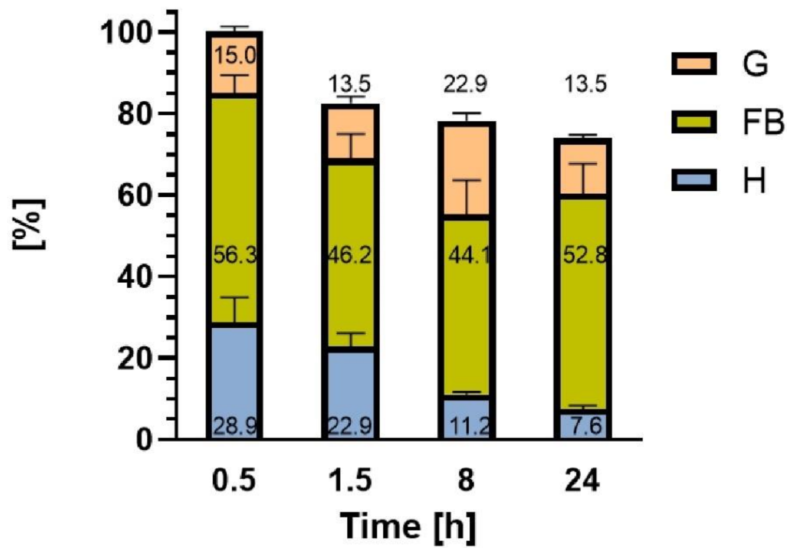


485

486 **Fig. 1** Accurate MS spectrum of SOL (A) and CHA (B) extracted from the haemolymph (H), gut (G) and the
487 remaining part of the larvae (FB) 0.5 hour after injection and analyzed by LC-HESI_MS alongside authentic
488 standards (STD).

489

490 **Changes of SOL content over time**



491

492 **Fig. 2** Percentage content of total applied SOL in each of the samples obtained from the haemolymph (H), gut (G)
493 and the remaining part of the larvae (FB) 0.5, 1.5, 8 and 24 hours after injection. Data are shown as mean with
494 SEM. The pooled samples were used with $n = 4$, and for each experimental variant, three independent replicates
495 were performed.

496

497

11

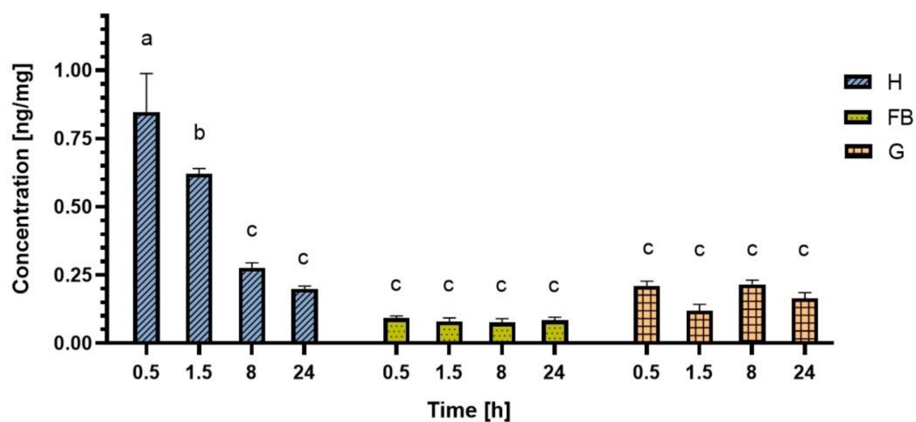
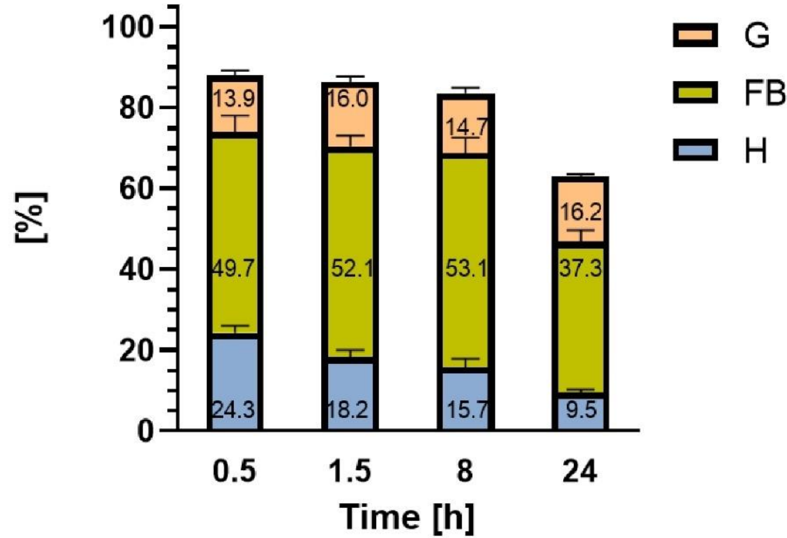


Fig. 3 Concentration of SOL in analyzed tissues as ng/mg in haemolymph (H), gut (G) and the remaining part of the larvae (FB) 0.5, 1.5, 8 and 24 hours after injection. Data are shown as mean with SEM. The pooled samples were used with *n* = 4, and for each experimental variant, three independent replicates, a two-way ANOVA test, were performed.

Table 1 SOL concentration ratio in haemolymph (H), gut (G) to the concentration in the remaining part of the larvae (FB) 0.5, 1.5, 8 and 24 hours after GA injection. For better clarity, the concentration in FB was considered as 1.

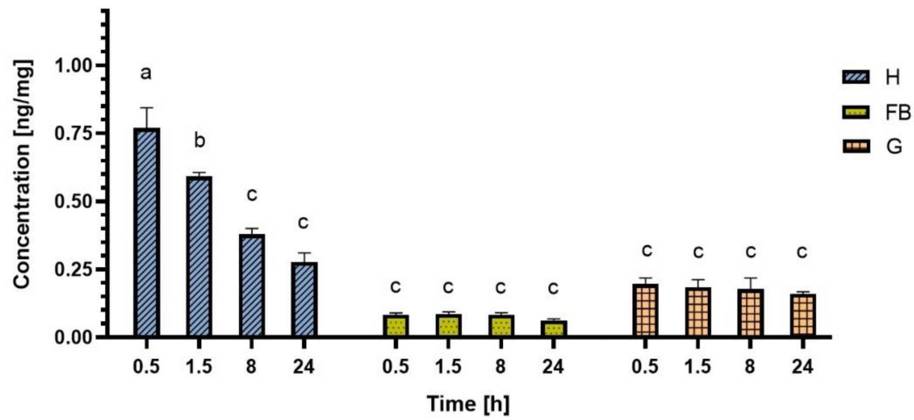
Time after GA injection [h]	Sample		
	FB	H	G
0.5	1.0	9.3	2.3
1.5	1.0	7.8	1.5
8	1.0	3.7	2.8
24	1.0	2.4	2.0

508 **Changes of CHA content over time**



509

510 **Fig. 4** Percentage content of total applied CHA in each of samples obtained from haemolymph (H), gut (G) and
511 the remaining part of the larvae (FB) 0.5, 1.5, 8 and 24 hours after injection. Data are shown as mean with SEM.
512 The pooled samples were used with $n = 4$, and for each experimental variant, three independent replicates were
513 performed.



514

515 **Fig. 5** Concentration of CHA in analyzed tissues as ng/mg in haemolymph (H), gut (G) and the remaining part of
516 the larvae (FB) 0.5, 1.5, 8 and 24 hours after injection. Data are shown as mean with SEM. The pooled samples
517 were used with $n = 4$, and for each experimental variant, three independent replicates were performed, the two-
518 way ANOVA test.

519

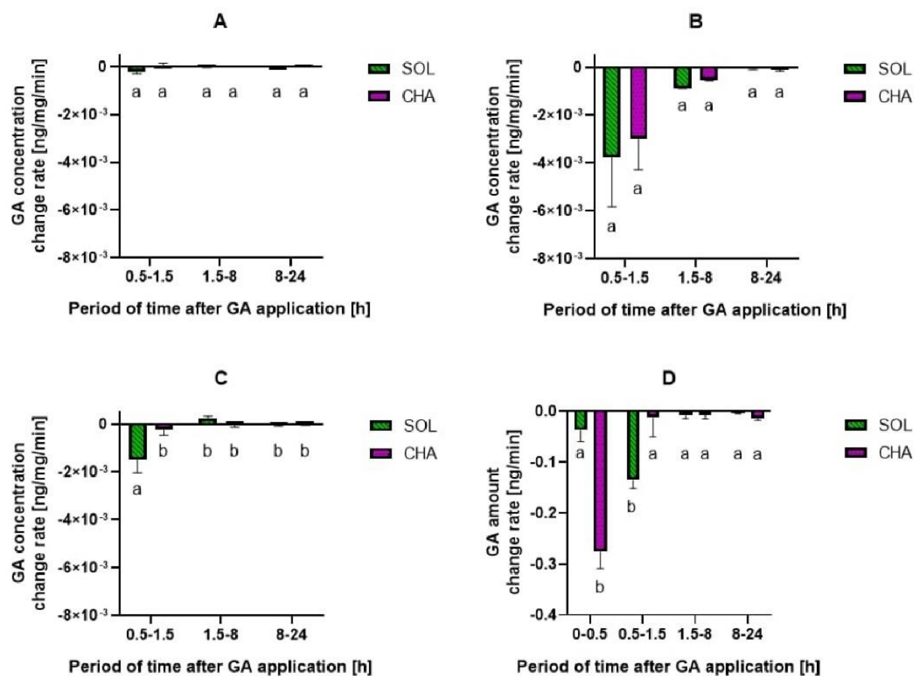
520 **Table 2** CHA concentration ratio in haemolymph (H), gut (G) to the concentration in the remaining part of the
521 larvae (FB) 0.5, 1.5, 8 and 24 hours after GA injection. For better clarity, the concentration in FB was considered
522 as 1.

Time after GA injection [h]	Sample		
	FB	H	G
0.5	1.0	9.3	2.4
1.5	1.0	6.8	2.1
8	1.0	4.6	2.1
24	1.0	4.4	2.5

523

524 **Changes of GAs elimination/accumulation rate**

525



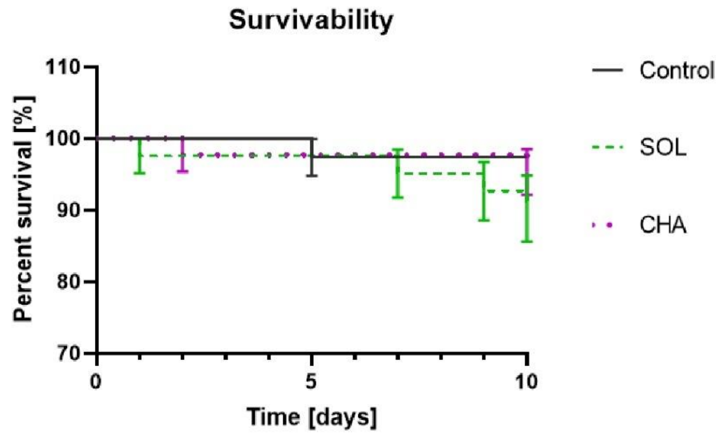
526

527 **Fig. 6** Rate of changes in SOL and CHA concentration in each tissues (A), H (B), G (C) samples as ng/mg/min
528 and in the whole larva (D) as ng/min 24 hours after GA injection. Values above zero mean accumulation, while
529 negative values mean elimination rate compared with the previous tested time period. The lower the negative
530 values, the higher the elimination rate. Higher positive values mean higher accumulation rate. The pooled samples
531 were used with n = 4, and for each experimental variant, three independent replicates were performed.

532

14

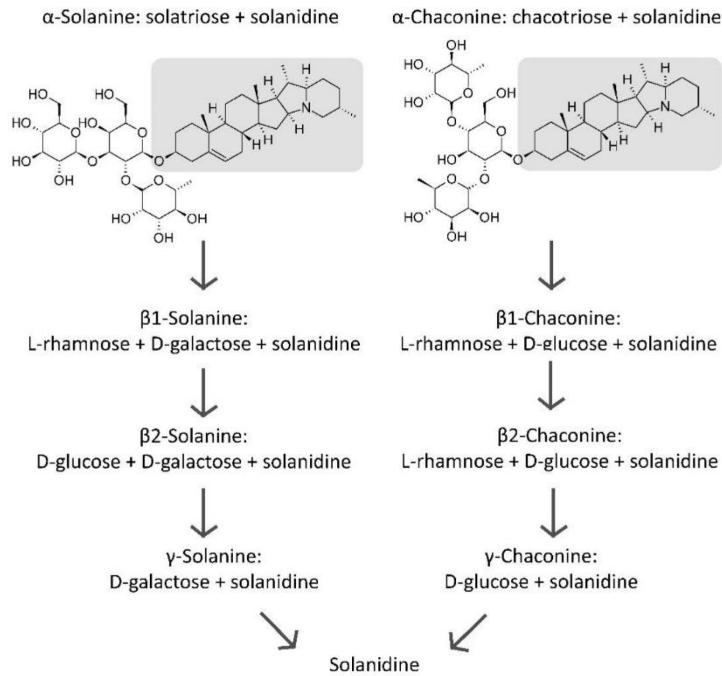
533 **Survivability of larvae after GAs application**



534

535 **Fig. 7** The survivability of larvae after GA injections. The error bars are shown as mean with SE, n=15, for each
536 experimental variant, three independent replicates were performed, log-rank test (Mantel-Cox).

537



538

539 **Fig. 8** Hydrolysis products of GAs.

540

15

Oświadczenia autora i współautorów

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Winkiel M.J., Chowański S., Sulli M., Diretto G., Słocińska M. Analysis of glycoalkaloids distribution in the tissues of mealworm larvae (Tenebrio molitor),

który jest częścią mojej rozprawy doktorskiej, polegał na zaplanowaniu doświadczeń, zebraniu materiału do analiz i przygotowaniu próbek, opracowaniu i interpretacji wyników, przeprowadzeniu analiz statystycznych, napisaniu manuskryptu, opracowaniu wykresów (Fig. 2-8) oraz tabel (Tab. 1-2), wprowadzeniu korekt i przygotowaniu manuskryptu do publikacji.

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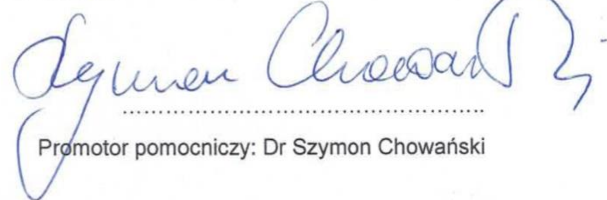

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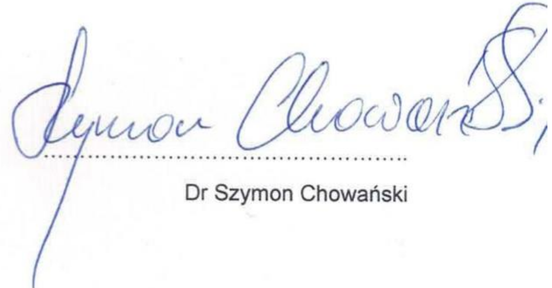

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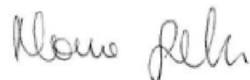
Winkiel M.J., Chowański S., Sulli M., Diretto G., Słocińska M. *Analysis of glycoalkaloids distribution in the tissues of mealworm larvae (Tenebrio molitor)*,

which is part of the doctoral dissertation of Magdalena Joanna Winkiel, consisted of supervising the methodology of preparing samples, performing MS analyses, preparing Figure 1, and making corrections before publication.



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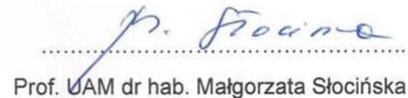

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Prof. UAM dr hab. Małgorzata Słocińska

Praca III

A tomato a day keeps the beetle away – the impact of *Solanaceae* glycoalkaloids on energy management in the mealworm *Tenebrio molitor*

Magdalena Joanna Winkiel, Szymon Chowański, Karolina Walkowiak-Nowicka, Marek Gołębiowski, Małgorzata Słocińska

Manuskrypt jest na etapie recenzji w czasopiśmie *Insect Science*

1 # A tomato a day keeps the beetle away – the impact of *Solanaceae* glycoalkaloids on energy management in the mealworm *Tenebrio molitor*

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20

21 Abbreviations:

22 CHA - α-Chaconine

23 CS - Citrate synthase

24 EXT - Tomato leaf extract

25 GAs - Glycoalkaloids

26 GC-MS - Gas chromatography – mass spectrometry

27 HADH - β-Hydroxyacyl-CoA dehydrogenase

28 PFK - Phosphofructokinase-1

29 SOL - α-Solanine

30 TAGs - Triglycerides

31 TCA - Tricarboxylic acid

32 TOM - α-Tomatine

33

34 ## Abstract

35 Solanine (SOL), chaconine (CHA), and tomatine (TOM) are plant secondary metabolites, produced
36 mainly by the species of *Solanaceae* family, such as tomato *Solanum lycopersicum* L. These
37 glycoalkaloids (GAs) have a wide range of biological activity, also in insects. However, their
38 mechanisms of action are not precisely understood. The purpose of the study was to investigate how
39 pure GAs and tomato leaf extract (EXT) affect glycolysis, Krebs cycle and β-oxidation of fatty acid
40 pathways in *Tenebrio molitor* L. beetle. For this purpose, the larvae were injected with SOL, CHA,
41 TOM, and EXT at two concentrations (10^{-8} and 10^{-5} M). For experiments, fat body, gut, and
42 heamolymph samples were collected 2 and 24 hours after injection. Then, the changes in the
43 expression level of phosphofructokinase, citrate synthase, and β-hydroxyacyl-CoA dehydrogenase
44 were measured using the RT-qPCR technique. The catalytic activity of these enzymes and the
45 carbohydrate level in insects after GA treatment were determined by spectrophotometric method.
46 Furthermore, the analysis of the amount of amino acids in tissues was performed with a GC-MS
47 technique. The results obtained show that the GAs changed the activity and expression of the genes
48 encoding key enzymes of crucial metabolic pathways. The effect depends on the type of GA
49 compound, the tissue tested, and the incubation time after treatment. Furthermore, TOM and EXT
50 affected trehalose concentration in the insect hemolymph and led to accumulation of amino acids in
51 the fat body. The observed changes may indicate a protein degradation and/or enhanced catabolism
52 reactions for the production of ATP used in detoxification processes. These results suggest that GAs
53 alter energy metabolism in the mealworm *T. molitor*. The study contributes to our understanding of
54 the mechanisms of action of secondary metabolites of plants in insects. This knowledge may allow
55 the design of new natural biopesticides against insect pests because proper energy metabolism is
56 necessary for the survival of the organism.

57

58 Keywords: citrate synthase; phosphofructokinase; metabolic pathway; nutrients; plant secondary
59 metabolite; glycoalkaloid

60

61 ## 1. Introduction

62 Glycoalkaloids (GAs) are plant secondary metabolites produced primarily by many *Solanaceae*
63 plants, such as tomato *Solanum lycopersicum* L., potato *Solanum tuberosum* L., and eggplant
64 *Solanum melongena* L. These compounds are composed of a steroidal carbon skeleton connected to
65 1-4 carbohydrates. For example, solanine (SOL) and chaconine (CHA) contain solanidine as an
66 aglycon part, while tomatine (TOM) is built from a tomatidine skeleton. Carbohydrate chains in SOL,
67 CHA and TOM are called solatriose, chacotriose, and lycotetraose, respectively (Zhao et al., 2021).
68 GAs play a defensive role against various pathogens and herbivore species. These plant secondary
69 metabolites exhibit a wide range of biological activities, such as anti-inflammatory, cytotoxic, and
70 antimicrobial activity (Zhao et al., 2021). GAs disrupt cell membranes through binding to cholesterol
71 molecules and inhibit acetylcholinesterase and butyrylcholinesterase enzymes. Moreover, these
72 compounds impact the process of cell division, as well as the ion transport (Ca^{2+}, Na^{+}) across cell
73 membranes (Friedman, 2006; Milner et al., 2011). GAs may inhibit the growth of cancer cells, for
74 example, by inhibiting angiogenesis, as well as apoptosis induction, because of their antiproliferative
75 and pro-apoptotic activity. They affect many signalling pathways in tumour cells acting through
76 different molecular mechanisms (Winkiel et al., 2022). Recent evidence suggests that SOL regulates
77 glycolytic pathway *in vitro* in non-small cell lung cancer, decreasing the expression level of the genes

78 encoding glycolysis-related proteins, such as glucose-6-phosphate isomerase, aldolase A and lactate
79 dehydrogenase A (Zou et al., 2022), and in human renal cancer, reducing the expression of HIF-1α
80 protein (Wang et al., 2021).

81 Insects store energy reserves in the form of glycogen and triglycerides (TAGs) in adipocytes, the fat
82 body cells. Moreover, this tissue synthesizes most of the metabolites. Glycogen is a polymeric form
83 of glucose, which is used as a glycolytic substrate and, for example, for chitin production. This
84 polysaccharide is synthesized from dietary carbohydrates and amino acids. Glycogen is utilized
85 mostly in the form of trehalose which is the main circulating sugar in the haemolymph. It is secreted
86 into that tissue by adipocytes with cellular membrane transporters (Arrese and Soulages, 2010).
87 Glucose may be used for the synthesis of trehalose, glycogen and lipids. Fatty acids, which serve for
88 ATP production during β-oxidation, are stored in the fat body in the form of TAGs, which are
89 constituted of glycerol and three fatty acid molecules. By conversion into diglyceride, trehalose, or
90 proline can be used in some insects as a key energetic substrates in flight muscle (Arrese and
91 Soulages, 2010). The proline amino acid is produced in the fat body from acetyl-CoA and alanine and
92 is released to the haemolymph. This amino acid synthesis is often connected to the fatty acid β-
93 oxidation because inhibition of β-oxidation blocks the release of trehalose induced by adipokinetic
94 hormone (Arrese and Soulages, 2010; Bursell, 1981). In general, insects were found to contain higher
95 amino acid amounts compared to the other animal species. Amino acids are utilized for proteins
96 production, therefore, they fulfill structural and developmental functions (Chen, 1966). The
97 following amino acids: arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine,
98 leucine, isoleucine, and valine considered essential for mammals are also necessary for the growth
99 of *Tenebrio molitor* L. larvae (Chen, 1966; Davis, 1975).

100 The main substrate for glycolysis is glucose, which is converted into pyruvate during the glycolysis
101 that occurs in the cytosol. In this pathway, two ATP moieties are generated. One of the three key
102 regulatory glycolysis reactions is the process catalyzed by phosphofructokinase-1 (PFK). This enzyme
103 is necessary for irreversible phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate.
104 The reaction is regulated by a feedforward activation mechanism, as well as by citrate, the
105 intermediary metabolite of the Krebs (tricarboxylic acid –TCA) cycle. Some glycolytic intermediates
106 can enter other biosynthetic pathways. For example, the product of the reaction catalyzed by PFK
107 can be converted to dihydroacetone phosphate, which, in turn, in the next step is transformed into
108 glycerol 3-phosphate, a substrate for TAGs production (Chandel, 2021a). In the presence of oxygen,
109 pyruvate is usually oxidized to acetyl-CoA during oxidative decarboxylation, and then converted to
110 two CO_2 moieties in the Krebs cycle which occurs in mitochondria. The reaction of citrate synthesis is
111 catalyzed by citrate synthase (CS) which is a marker enzyme of the TCA cycle, at the gateway into the
112 cycle from pyruvate *via* acetyl-CoA. Besides the pyruvate generated in the glycolysis process, fatty
113 acids can be used as the substrate for the TCA cycle. These compounds are the main source of ATP
114 during the low glucose level in the cell. One of the steps of β-oxidation of fatty acids is the
115 conversion of L-β-hydroxyacyl-CoA to β-ketoacyl-CoA, catalyzed by β-hydroxyacyl-CoA
116 dehydrogenase (HADH) (Chandel, 2021b).

117 As glycolysis, Krebs cycle, and β-oxidation of fatty acids are important processes of ATP production in
118 cells, the question is, if GAs can affect these reactions. The first study on the effect of GAs on blood
119 sugar levels was reported already in 1967 year (Satoh, 1967). At that time, it was predicted that SOL
120 may act as a hyperglycemic agent in rats. However, later this issue did not attract interest among
121 scientists, who focused on other effects caused by GAs. In insects, GAs were found to alter the
122 functioning of numerous processes from feeding through reproduction to behaviour (Chowański et
123 al., 2016). Only recently have some relationships between GAs and lipid metabolism been

124 established. We have previously reported that the application of GAs and tomato leaf extract into *T.*
125 *molitor* larvae affects the content and composition of lipid compounds in the insects' haemolymph
126 and fat body. Furthermore, HADH activity decreased after GA application, especially in the fat body,
127 which may affect ATP production (Winkiel et al., 2023). After treatment with solamargine,
128 solasonine and *Solanum nigrum* L. extract, a loss of homogeneity of lipid droplets and their regularity
129 of shape were observed in the *T. molitor* beetle and *Galleria mellonella* L. moth (Spochacz et al.,
130 2021, 2018). These compounds also affected the ultrastructure of midgut cells, as well as
131 carbohydrate, lipid, and amino acid content in the fat body and haemolymph of insects (Spochacz et
132 al., 2021, 2018). However, it is still unknown which mechanisms underlie these metabolic
133 fluctuations in insects. We do not know if the observed changes are a result of the impact of GAs on
134 the level of genes encoding crucial enzymes of metabolic pathways or their influence on the protein
135 level.

136 ATP production is necessary for the survival of cells and the entire organism. Thus, **the study aimed**
137 **to verify if SOL, CHA, TOM, and EXT may alter key steps of glycolysis, Krebs cycle and β-oxidation**
138 **of fatty acids at the gene and protein levels, and the content of energy substrates in tissues of *T.***
139 ***molitor* larvae**. Exploring the effects of GAs on energy-producing processes in insects, especially in
140 pests that cause significant losses in crops, as well as grain stores, seems especially important.
141 Umbalancing of energetic homeostasis by GAs can impact the condition of individual insects and, in
142 consequence, reduce the population of harmful insect species (Manosathiyadevan et al., 2017). The
143 obtained data extends the knowledge about GAs mechanisms of action what is necessary to
144 consider these compounds as potential promising biopesticides.

145

146 # 2. Materials and methods

147 ## 2.1 Insects

148 The larvae of *T. molitor* beetles were obtained from the colony cultured at the Department of
149 Animal Physiology and Developmental Biology at the Faculty of Biology of Adam Mickiewicz
150 University in Poznań, Poland at constant temperature (26 ± 0.5 °C), humidity (65 ± 5%) and
151 photoperiod 8:16 h light to dark. The food consisted of oat flakes and fresh carrots. Only feeding
152 larvae from the 15th to 16th instar of approximately 120 to 140 mg of weight were selected for the
153 experiments.

154 ## 2.2 Compounds and treatment procedure

155 Saline solutions of synthetic GAs: SOL (≥95.0%), CHA (≥95.0%), and TOM (≥95.0%) (Merck Sigma-
156 Aldrich) were used in experiments at concentrations of 10^{-8} M (dosage range for SOL and CHA 0.12–
157 0.14 pg/mg body mass, for TOM 0.15–0.17 pg/mg body mass) and 10^{-5} M (dosage range for SOL and
158 CHA 0.12–0.14 ng/mg body mass, for TOM 0.15–0.17 ng/mg body mass). The concentrations of GAs
159 were selected based on the literature and our previous studies in which we observed different
160 metabolic and developmental disorders (Spochacz et al., 2021, 2018; Winkiel et al., 2023). The GA
161 extract from tomato leaves (EXT) was obtained from the research group of Prof. Sabino A. Bufo from
162 Basilicata University in Potenza, Italy, and tested by our group (Marciniak et al., 2019; Ventrella et
163 al., 2016, 2015). EXT displayed the presence of the major GAs (2.95 ± 0.25%), tomatine, and two
164 other minor GAs lycotetraose, namely, dehydrotomatine and filotomatine (Ventrella et al., 2016).
165 EXT contained the same concentration of tomatine as the 10^{-8} and 10^{-5} M solutions of this GA, which
166 allowed comparing the effects of the extract and the pure GA. The physiological solution, isosmotic

167 for *T. molitor*, was used as a control (NaCl 16 mg/mL, KCl 1.4 mg/mL, $CaCl_2$ 1 mg/mL). The tested
168 compounds were administered to larvae by injection using a microsyringe (Hamilton) in a volume of
169 2 µL, and the final concentration in the haemolymph was 10^{-9} and 10^{-6} M. The injection was made
170 on the abdominal side of the larva behind the last pair of legs after 8 min of CO_2 anaesthesia.

171 2.3 Tissue isolation

172 Depending on the experimental variant, tissue isolation was performed 2 or 24 h after GA injection.
173 The tissues were isolated after 8 min of anaesthesia with CO_2. The trophic tissues (haemolymph, gut,
174 and fat body) that play a key role in maintaining metabolic balance, as well as detoxification, were
175 used for analysis. Haemolymph also distributes lipids and applied substances through the entire
176 organism of insects, while the fat body is involved in GA hydrolysis/metabolism, and the gut is
177 responsible for the removal of waste metabolites. The hemolymph was collected after cutting the
178 legs of the first pair using an automatic pipette. After decapitation and cutting off the last segment
179 of the abdomen, the larvae were cut along the dorsal side and then spread on the Petri dish with
180 pins. Afterward, the fat body and gut were washed with saline, isolated with microsurgical tweezers,
181 and placed in Eppendorf tubes. Additionally, guts were cleaned of food residues. The isolation was
182 performed on ice to avoid sample degradation. Before further preparation, the samples were stored
183 at −80 °C. In the experiments, samples pooled from several individuals were used.

184 2.4 Concentration of carbohydrates

185 The analysis of glucose, trehalose, and glycogen levels was performed using samples prepared as
186 previously described for triacylglyceride (TAG) determination (Winkiel et al., 2023). Fat body tissue
187 from 2 larvae and 16 µL of haemolymph were homogenized on ice using a pestle homogenizer in 300
188 µL and 150 µL of PBS-Tween 0.05%, respectively. Next, after incubation at 70 °C for 10 min, the
189 samples were centrifuged (10,000 RPM, 5 min, 4 °C), and the supernatant was transferred to new
190 tubes. For each experimental variant, four independent replicates were performed. The samples
191 were frozen in liquid nitrogen and stored at −80 °C until the measurements were made.

192 The tested carbohydrates were determined spectrophotometrically in undiluted haemolymph
193 samples. The fat body samples were used undiluted for glucose analyses, while diluted 10-fold with
194 PBS-Tween 0.05% for trehalose and glycogen level determination. For glucose level analyses, the
195 Glucose Assay Kit (Merck Sigma-Aldrich; GAGO20) was used according to the manufacturer's
196 protocol. Each sample (15 µL) and 50 µL of the Assay Reagent were placed on a clear-bottom 96-well
197 plate and incubated at 37 °C for 60 min. Then 50 µL of sulfuric acid (Merck Sigma-Aldrich; 339741)
198 was added as a reaction inhibitor. After 10 min, the absorbance was measured at wavelength λ =
199 540 nm at RT with a Synergy H1 Hybrid MultiMode Microplate Reader (BioTek). The glucose level in
200 each sample was calculated using the standard curve. However, it was not detected in haemolymph,
201 and it was very low glucose concentration in fat body samples. Therefore, it was neglected and the
202 concentration of trehalose was determined after the addition of trehalase (Merck Sigma-Aldrich;
203 T8778-1UN) to the Assay Reagent (1:1000), and for glycogen analyses, aminoglucosidase (Merck
204 Sigma-Aldrich; A1602) was added to the Assay Reagent (3:1000). The standards of trehalose (Merck
205 Sigma-Aldrich; T9449) and glycogen (Merck Sigma-Aldrich; G8751) were used to prepare the
206 standard curves. The carbohydrate level is expressed in µg per 1 mg of fresh tissue.

207 2.5 Concentration of amino acids

208 Gas chromatography–mass spectrometry (GC–MS) was used to measure the level of amino acids in
209 the haemolymph and fat body. For analysis, pooled samples were used with $n \geq 15$ (haemolymph) or
210 $n \geq 10$ (fat body), and the analyses were performed in triplicate. Each sample contained a minimum

of 120 µL of haemolymph or 160 mg of the fat body. After isolation, tissues were transferred into 1.5 mL glass bottles with chloroform and methanol 2:1 (v/v). The prepared samples were stored at 4 °C until measurements were taken.

Amino acids were determined with the GC–MS technique according to the method described previously (Szymczak-Cendlak et al., 2022; Winkiel et al., 2023). Briefly, amino acids were extracted in 30 mL of dichloromethane. The solvent was removed from the samples under a gentle stream of nitrogen. Components of extracts were silylated with 100 µL of a mixture of 99% bis(trimethylsilyl)acetamide and 1% chlorotrimethylsilane at 100 °C for 1 h on the day of analysis. The samples were analyzed using GC–MS on a GC/MS QP2010 SE (Shimadzu, Kyoto, Japan) equipped with a fused silica capillary column Zebron–5, 30 m × 0.25 mm i.d. and with a 0.25 µm thick film. Helium was used as the carrier gas. The ion source was maintained at 220 °C. The injector and transfer line temperatures were kept at 310 °C. Electron-impact ionization (electron energy 70 eV) was used. The column temperature was programmed at 4 °C × min−1 from 80 (held for 10 min) to 310 °C, which was held for 10 min. The amino acid level is expressed in µg per 1 mg of fresh tissue.

2.6 Quantitative analysis of gene expression

The samples for gene expression measurements were pooled from 5 individuals. Tissues (fat body and gut) were placed into 300 µL of RNA Lysis Buffer (Zymo Research; R1060-1), homogenized for 3 min using a pestle homogenizer (Fisherbrand), and the total RNA isolation was conducted using Quick-RNA™ MiniPrep Kit (Zymo Research; R1055), according to the manufacturer's protocols. The residual DNA was then removed with a Turbo DNase kit (Thermo Scientific; AM1907), and the RNA concentration was measured spectrophotometrically (DeNovix DS-11 FX+). After that, the RNA samples were frozen in liquid nitrogen and stored at −80 °C until the next steps.

The synthesis of cDNA was conducted using LunaScript® RT SuperMix Kit (Biolabs; E3010) and T100™ Thermal Cycler (BIO-RAD). The prepared cDNA samples were stored at −20 °C. Quantitative real-time PCR (RT-qPCR) analyses were performed with a SYBR Green Master mix (Thermo-Fisher Scientific; 4309155) on a C1000™ Thermal Cycler with the CFX96™ Real-Time System (BIO-RAD). The primers were designed based on sequences available in public databases (NCBI) and synthesized by the Institute of Biochemistry and Biophysics, Warsaw (Supp. Mat.). The suitability of the primers for the qPCR was tested by analyzing the melting curves. The PCR conditions for the amplified gene and the reference gene (ribosomal protein L13a (Rpl13a)), were determined and optimized before amplification. The stability of *Rpl13a* expression was validated prior to the experiment. The experiment was prepared in three biological replicates and three independent replicates for each experimental variant. Negative controls were prepared to check for possible contamination of the samples. Relative expression was calculated using the $2^{\Delta\Delta}$Ct method (Livak and Schmittgen, 2001). To confirm the results, the amplicons were sequenced by the Molecular Biology Techniques Laboratory (Faculty of Biology, Adam Mickiewicz University in Poznań) and compared with the data available in a public database (NCBI).

2.7 Enzyme activity

The activity of PFK and CS in the gut and fat body was measured in samples pooled from a minimum of 10 individuals. The tissues were placed in 250 µL (gut) or 500 µL (fat body) of physiological saline, homogenized for 3 min using a pestle homogenizer (Fisherbrand) and centrifuged (10.000 RPM, 10 min, 4 °C). The supernatant was then transferred to new tubes, and the protein concentration was measured using a Direct Detect spectrometer (Merck) (Szymczak-Cendlak et al., 2022). Total soluble proteins concentration in the gut samples ranged between 9.7 and 18.9 mg/mL, and in fat body

255 samples, they ranged between 13.2 and 29.5 mg/mL. Afterward, the samples were frozen in liquid
256 nitrogen and stored at −80 °C until the measurements were made.

257 The PFK and CS catalytic activity was measured using commercially available kits (Merck Sigma-
258 Aldrich; MAK093 and MAK193, respectively). The experiment was carried out according to the
259 manufacturer's instructions. The gut samples for the experiment were diluted 16x (PFK) or 10x (CS).
260 On the contrary, fat body samples were diluted to a total protein concentration of 3.0–4.2 μg/μL
261 with 4 mM kojic acid in PBS buffer. Then, the samples were put on the plate (PFK) or diluted again
262 with the kit buffer 50x (CS). Kojic acid was used as a polyphenol oxidase inhibitor to reduce the
263 interference of the polyphenol oxidase reaction product with the product of the reaction catalyzed
264 by the tested enzymes. The experiments were based on the spectrophotometric technique using
265 Spark Microplate Reader (Tecan, Switzerland). The absorbance was measured at wavelength $\lambda = 450$
266 nm (PFK) and $\lambda = 412$ nm (CS) at RT for 50 min (5 min intervals). Enzyme activity is expressed as mU
267 per μg of total soluble protein in the sample. The assays were prepared in three independent
268 replicates for each experimental variant.

269 2.8 Statistical analysis

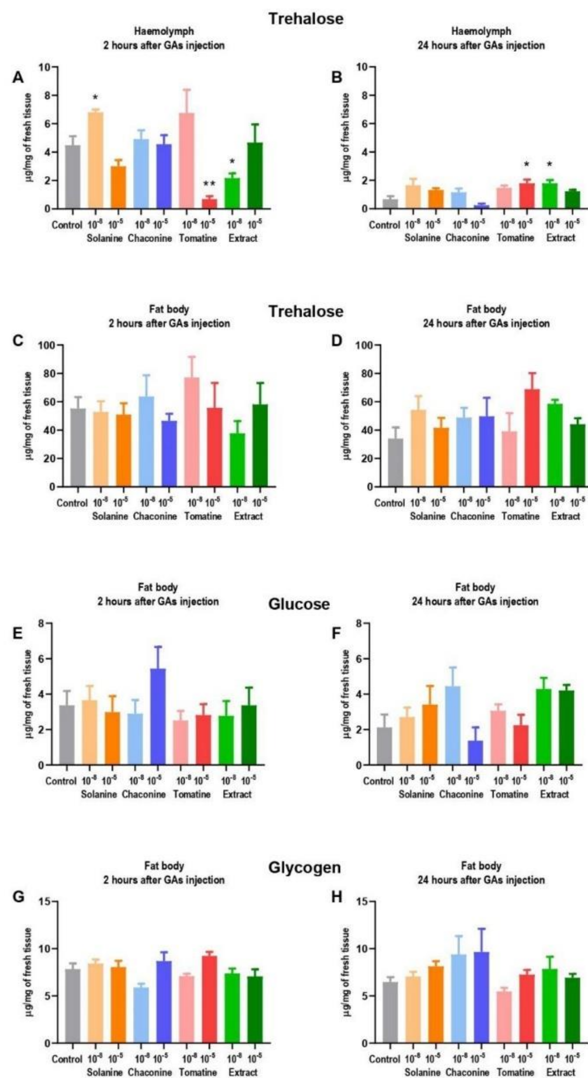
270 The results were analyzed using Graphpad Prism 8.0.1. (Department of Animal Physiology and
271 Developmental Biology AMU license). The normality of the distribution was determined using the
272 Shapiro-Wilk test. Normally distributed data were analyzed with ordinary one-way ANOVA or
273 Brown–Forsythe and Welch ANOVA with Dunnett's multiple comparison tests. Data with a non-
274 normal distribution were analyzed using Kruskal-Wallis with Dunn's multiple comparison tests.

275

276 3. Results

277 3.1 Level of carbohydrates

278



279

280 Fig. 1 Concentration of trehalose (A-D), glucose (E,F) and glycogen (G,H) in the fat body and
281 haemolymph of *T. molitor* larvae 2 (A,C,E,G) and 24 h (B,D,F,H) after injection with solanine,
282 chaconine, tomatine, extract from tomato leaves and physiological saline as a control.
283 Concentrations of the compounds 10^{-8} M (10^{-8}) and 10^{-5} M (10^{-5}) are shown on the graphs. Data are
284 expressed in µg per 1 mg of fresh fat body tissue and shown as the mean with SEM. Pooled samples
285 were used, four independent replicates were performed. The tested groups were compared with the
286 control (insects injected with physiological saline) using Brown–Forsythe and Welch ANOVA with
287 Dunnett's multiple comparison tests, ** $p \leq 0.01$, * $p \leq 0.05$.

288 The concentration of glucose, trehalose, and glycogen was analyzed in fresh tissue of fat body and
289 haemolymph after GAs injections at two concentrations 10^{-8} and 10^{-5} M. The glucose level in the fat
290 body tissue did not change compared to the control, neither 2 (Fig. 1E) nor 24 h (Fig. 1F) after GA
291 injection. The calculated glucose concentration in the samples was between 1.4 ± 2.12 and 5.4 ±
292 3.45 µg per 1 mg of fresh fat body tissue. There was a slight increase in glucose content 2 h after
293 CHA 10^{-5} M treatment, however, the change was not significant. On the other hand, 24 h after the
294 GAs application, in this experimental variant, the monosaccharide concentration tended to decrease.
295 In haemolymph, the amount of glucose was below the detection limit.

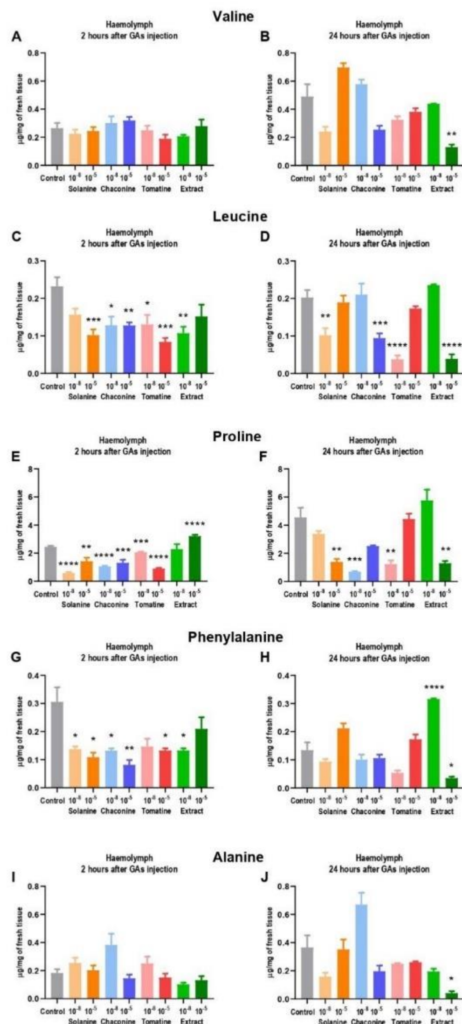
296 The trehalose concentration in the haemolymph 2 h after GAs treatment ranged from 0.7 ± 0.60 to
297 6.8 ± 0.58 µg per 1 mg of tissue (Fig. 1A), while in 24 h variant, there was between 0.2 ± 0.33 and 1.8
298 ± 0.66 µg of trehalose per 1 mg (Fig. 1B). SOL at lower concentration (10^{-8} M) significantly increased
299 the disaccharide level in haemolymph after 2 h. On the contrary, a decrease in trehalose content
300 was observed 2 h after TOM 10^{-5} M, as well as after the application of EXT 10^{-8}. Interestingly, in these
301 experimental variants, an increase in disaccharide concentration was observed 24 h after GA
302 treatment. In the fat body, the trehalose concentration in the 2 h variant was between 37.7 ± 24.68
303 and 77.2 ± 41.01 µg per 1 mg (Fig. 1C), and 24 h after GAs application it ranged from 34.1 ± 22.48 to
304 68.9 ± 32.14 µg per 1 mg of fresh tissue (Fig. 1D). No significant changes in the content of this
305 carbohydrate were reported neither 2, nor 24 hours after GA treatment.

306 The glycogen concentration in the fat body did not change as a result of GAs injection, compared to
307 the control (Fig. G,H). It ranged between 5.9 ± 1.12 – 9.2 ± 1.18 µg/mg in the case of 2 h variant, and
308 between 5.5 ± 1.10 and 9.7 ± 6.96 µg per 1 mg of fresh tissue 24 h after treatment. No amount of
309 glycogen was detected in the haemolymph samples.

310

311 3.2 Level of amino acids

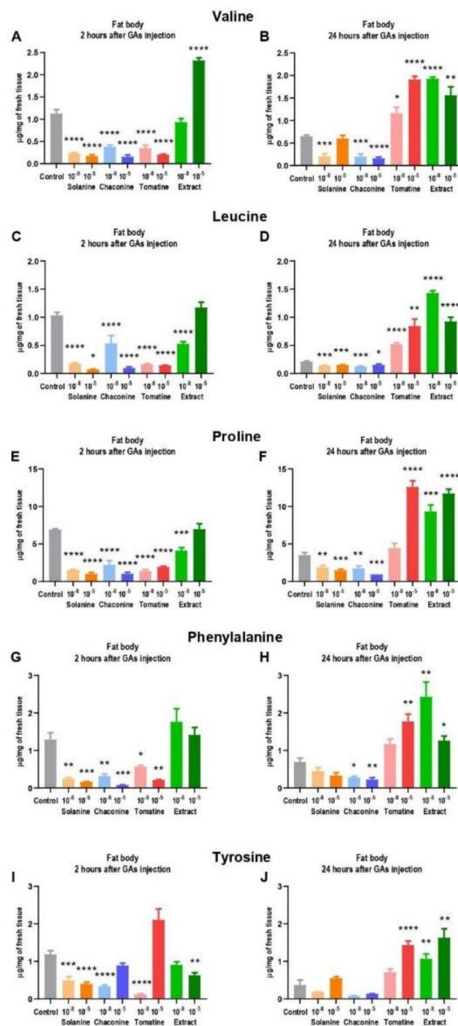
312



313

314 Fig. 2 The concentration of amino acids in the haemolymph of *T. molitor* larvae 2 and 24 h after
315 injection with solanine, chaconine, tomatine, extract from tomato leaves, and physiological saline as
316 a control. Concentrations of the compounds 10^{-8} M (10^{-8}) and 10^{-5} M (10^{-5}) are shown on the graphs.
317 Data are expressed in µg per 1 mg of fresh haemolymph tissue and shown as the mean with SEM.
318 Pooled samples were used with $n \geq 10$, and the analysis was performed in triplicate. The tested
319 groups were compared with the control (insects injected with physiological saline) using Brown–
320 Forsythe and Welch ANOVA with Dunnett's multiple comparison tests, **** $p \leq 0.0001$, *** $p \leq$
321 0.001, ** $p \leq 0.01$, * $p \leq 0.05$.

322



323

324 Fig. 3 The concentration of amino acids in the fat body of *T. molitor* larvae 2 and 24 h after injection
325 with solanine, chaconine, tomatine, extract from tomato leaves, and physiological saline as a control.
326 Concentrations of the compounds 10^{-8} M (10^{-8}) and 10^{-5} M (10^{-5}) are shown on the graphs. Data are
327 expressed in μg per 1 mg of fresh fat body tissue and shown as the mean with SEM. Pooled samples
328 were used with $n \geq 10$, and the analysis was performed in triplicate. The tested groups were
329 compared with the control (insects injected with physiological saline) using Brown–Forsythe and
330 Welch ANOVA with Dunnett's multiple comparison tests, **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$,
331 * $p \leq 0.05$.

332 The following amino acids were detected in the tested sample of haemolymph and fat body: valine,
333 leucine, proline, phenylalanine, alanine (only in haemolymph), and tyrosine (only in the fat body).
334 The valine concentration in haemolymph 2 h after GAs injection did not change (Fig. 2A). It ranged

335 between 0.2 ± 0.11 µg/mg and 0.3 ± 0.10 µg/mg of tissue. 24 h from the GAs application (Fig. 2B),
336 the valine content was more differentiated (0.1 ± 0.07 – 0.7 ± 0.12 µg/mg). In this experimental
337 variant, only a higher EXT concentration significantly decreased valine content in that tissue. In the
338 fat body, all pure GAs decreased amino acid concentration during 2 h (Fig. 3A) with the greatest
339 change after CHA 10^{-5} M treatment (more than 7-fold). On the contrary, EXT 10^{-5} M increased the
340 valine concentration 2-times (2.3 ± 0.19 µg/mg) compared to the control (1.1 ± 0.28 µg/mg). 24 h
341 after treatment with SOL 10^{-8} M, CHA 10^{-8} M, and CHA 10^{-5} M, the amino acid content in the fat body
342 was still decreased compared to the control (Fig. 3B). However, TOM and EXT increased the valine
343 concentration in the fat body. It indicates the possibility of amino acid transfer from haemolymph to
344 that tissue.

345 Leucine concentration was decreased in the haemolymph in most of the 2 h experimental variants
346 compared to the control (Fig. 2C). The greatest change was reported after TOM 10^{-5} M application
347 (almost a 3-fold decrease). 24 h after GAs injections the lower amino acid concentration was
348 observed after 10^{-8} M SOL and TOM, as well as after 10^{-5} M CHA and EXT treatments (Fig. 2D). The
349 application of all of the tested GAs after 2 h resulted in decreased leucine concentration in the fat
350 body, except for EXT 10^{-5} M, which did not affect amino acid content compared to the control (Fig.
351 3C). Leucine concentration was decreased also 24 h after SOL and CHA application (Fig. 3D).
352 However, similarly to valine, TOM as well as EXT after 24 h significantly increased amino acid content
353 in the fat body compared to the control (even almost 7-fold).

354 All of the tested GAs, except for EXT 10^{-5} M, decreased proline concentration during 2 h in the
355 haemolymph (Fig. 2E). The lowest amino acid content was reported after SOL 10^{-8} treatment (change
356 from 2.5 ± 0.27 µg/mg in the control to 0.6 ± 0.30 µg/mg after GA injection). On the contrary,
357 treatment with EXT 10^{-5} M after 2 h resulted in increased proline concentration in the haemolymph.
358 24 after injection, 10^{-8} M CHA, and TOM, as well as 10^{-5} M SOL and EXT, maintained a decrease in
359 proline concentration compared to the control (Fig. 2F). In the fat body, all pure GAs as well as EXT
360 10^{-8} M after 2 h caused a decrease in amino acid content (Fig. 3E). Similarly to the other amino acids,
361 proline concentration in the fat body was lower 24 h after treatment with SOL and (even almost 4
362 times), while higher compared to the control (also almost 4 times) after TOM and EXT injections (Fig.
363 3F).

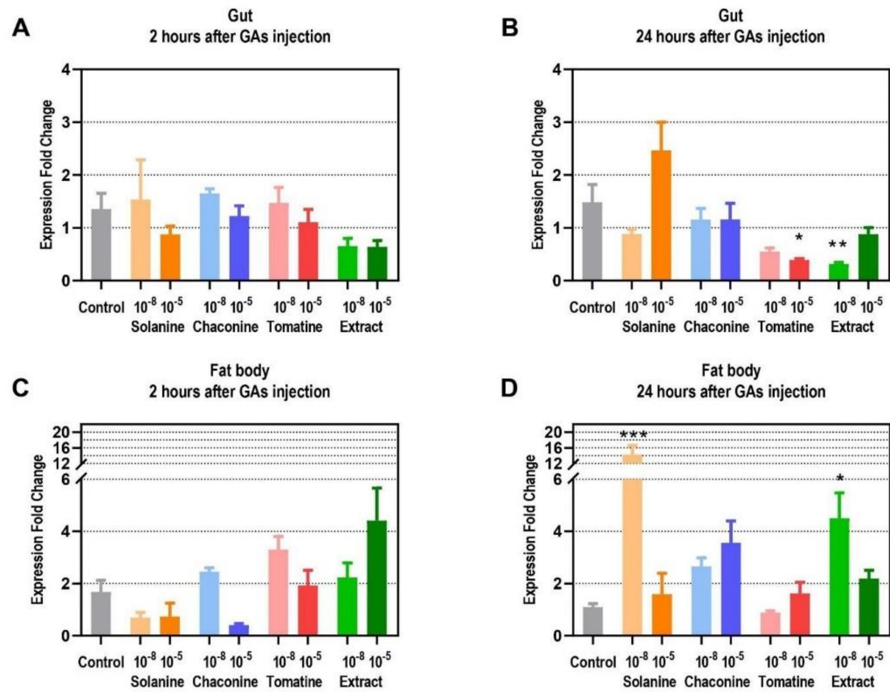
364 The phenylalanine concentration in the haemolymph 2 h after the application of pure GAs and EXT
365 10^{-8} M was reduced compared to the control (Fig. 2G). The lowest amino acid value was noted after
366 CHA 10^{-5} M injection (0.1 ± 0.07 µg/mg compared to the 0.3 ± 0.20 µg/mg in the control).
367 Surprisingly, 24 h after application, EXT 10^{-8} M caused an increase in phenylalanine concentration in
368 the haemolymph (more than 2-times), while the treatment with EXT 10^{-5} M resulted in a decrease in
369 amino acid content (more than 4-times) compared to the control (Fig. 2H). Phenylalanine
370 concentration in the fat body was decreased 2 h after pure GAs injections, while no change was
371 observed after EXT application (Fig. 3G). 24 h after CHA injection, the amino acid content in the fat
372 body remained decreased compared to the control, while it increased after TOM and EXT treatment
373 (Fig. 3H). For example, the phenylalanine concentration was 0.7±0.32 µg/mg in the control, and
374 1.8±0.64 µg/mg after TOM 10^{-8} M injection (2.6-fold change).

375 Alanine was detected only in the haemolymph. However, any of the tested GAs affected its
376 concentration which ranged between 0.1 ± 0.05 µg/mg and 0.4 ± 0.31 µg/mg (Fig. 2I). Alanine
377 concentration was significantly lower compared to the control only 24 h after the injection of EXT 10^{-}
378 ^{5} M (Fig. 2J). In the other experimental variants, no changes were reported.

379 Tyrosine was reported only in the fat body. Most of the tested GAs decreased its concentration after
380 2 h (Fig. 3I). The biggest change was calculated after TOM 10^{-8} M treatment (10-fold decrease). 24 h
381 after TOM 10^{-5} M and EXT injections, an increase in tyrosine concentration in the fat body was
382 reported (Fig. 3J), similarly to the other amino acids.

383 3.3 Quantitative analysis of gene expression

384



385

386 Fig. 4 Expression fold change of PFK in a gut (A, B) and fat body (C, D) of *T. molitor* larvae 2 and 24
387 hours after application of 10^{-8} and 10^{-5} M solutions of solanine, chaconine, tomatine, the extract of
388 tomato leaves (with corresponding tomatine level), and in the control (insects injected with
389 physiological saline) compared to the L ribosomal proteins (RPL) expression. Data are shown as
390 mean with SEM. The pooled samples were used with $n = 5$. For each experimental variant, three
391 independent replicates were performed. The tested groups were compared with the control using
392 Kruskal-Wallis with Dunn's multiple comparison test, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

393

CS

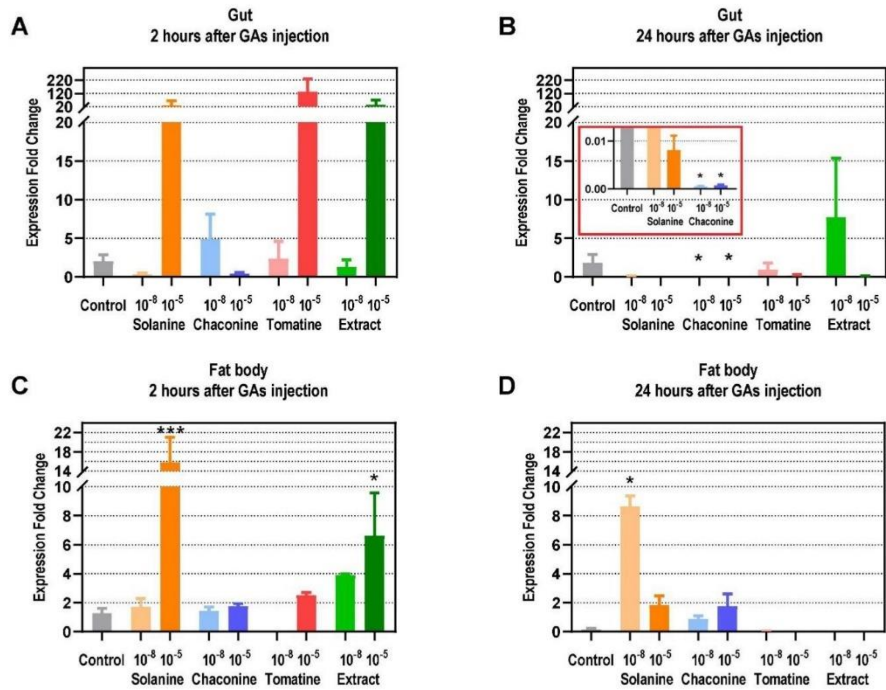
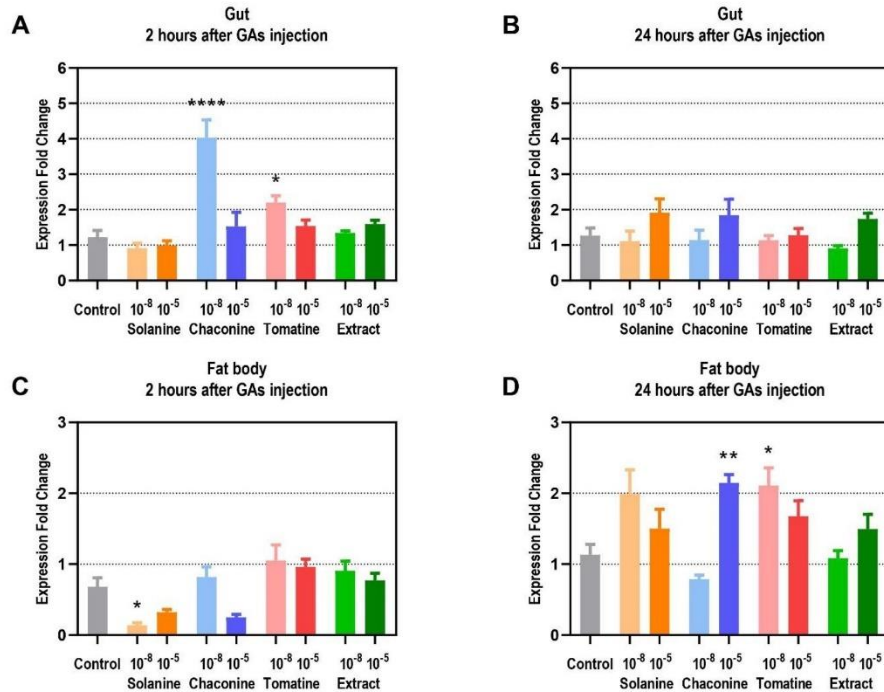


Fig. 5 Expression fold change of CS in a gut (A, B) and fat body (C, D) of *T. molitor* larvae 2 and 24 hours after application of 10^{-8} and 10^{-5} M solutions of solanine, chaconine, tomatine, the extract of tomato leaves (with corresponding tomatine level), and in the control (insects injected with physiological saline) compared to the L ribosomal proteins (RPL) expression. Data are shown as mean with SEM. The pooled samples were used with $n = 5$. For each experimental variant, three independent replicates were performed. The tested groups were compared with the control using Kruskal-Wallis with Dunn's multiple comparison test, *** $p \leq 0.001$, * $p \leq 0.05$.



403

404 Fig. 6 Expression fold change of HADH in a gut (A, B) and fat body (C, D) of *T. molitor* larvae 2 and 24
405 hours after application of 10^{-8} and 10^{-5} M solutions of solanine, chaconine, tomatine, the extract of
406 tomato leaves (with corresponding tomatine level), and in the control (insects injected with
407 physiological saline) compared to the ribosomal proteins L13a expression. Data are shown as mean
408 with SEM. The pooled samples were used with n = 5. For each experimental variant, three
409 independent replicates were performed. The tested groups were compared with the control using
410 Kruskal-Wallis with Dunn's multiple comparison test, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

411

412 The expression fold change of genes encoding PFK, CS, and HADH was calculated after GAs and
413 physiological saline (control) injections in the gut and the fat body of insects. The expression of *PFK*
414 did not change in the gut 2 h after the injection of all GAs tested (Fig. 4A). Only after 24 hours after
415 GAs application, TOM 10^{-5} M and EXT 10^{-8} M decreased the expression of the PFK genes in the gut
416 almost 4– and almost 5-folded, respectively, compared to the control (Fig. 4B). 2 h after GAs
417 treatment, similar to the gut, also in the fat body there were no changes of *PFK* expression (Fig. 4C).
418 However, 24 h after treatment, an increase in *PFK* expression was observed in this tissue after
419 injection of SOL 10^{-8} M (13 times), as well as after EXT 10^{-8} M (over 4-times compared to the control)
420 application (Fig. 4D).

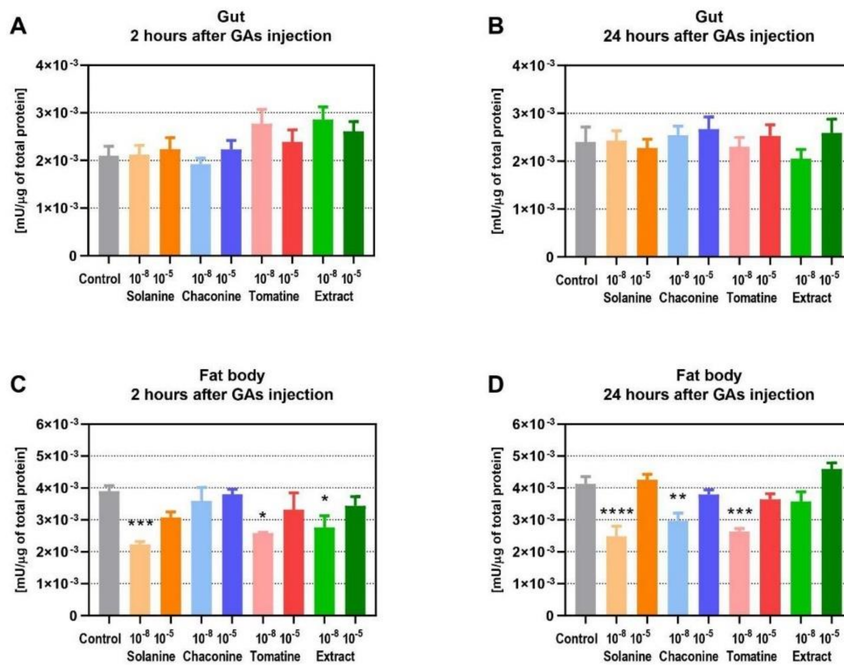
421 There was a tendency for the expression of the CS encoding gene to increase in the gut 2 h after
422 higher concentrations of SOL, TOM, and EXT injection, but the changes were not significant (Fig. 5A).
423 On the contrary, CHA treatment resulted in a considerable decrease in *CS* expression after 24 h in
424 this tissue (Fig. 5B). In the fat body, there was also an increase in *CS* expression 2 h after 10^{-5} M SOL
425 and EXT treatment reported (Fig. 5C). SOL at lower concentration increased the CS gene expression
426 24 h after injection in this tissue 53-folded (Fig. 5D). Other GAs did not affect the gene expression
427 compared to the control.

428 The expression of the genes encoding HADH increased in the gut 2 h after a lower concentration of
429 CHA and TOM injections more than 3 times and almost 2 times, respectively (Fig. 6A). After 24 h no
430 significant changes in protein gene expression were observed in this tissue with the expression fold
431 change ranging between 0.9 ± 0.22 and 1.9 ± 1.18 (Fig. 6B). In the fat body, *HADH* expression
432 decreased almost 5 times 2 h only after SOL 10^{-8} M treatment (Fig. 6C). On the contrary, an almost 2-
433 fold increase in HADH gene expression was reported 24 h after 10^{-5} M CHA as well as after 10^{-8} M
434 TOM application (Fig. 6D).

435

436 3.4 Enzyme activity

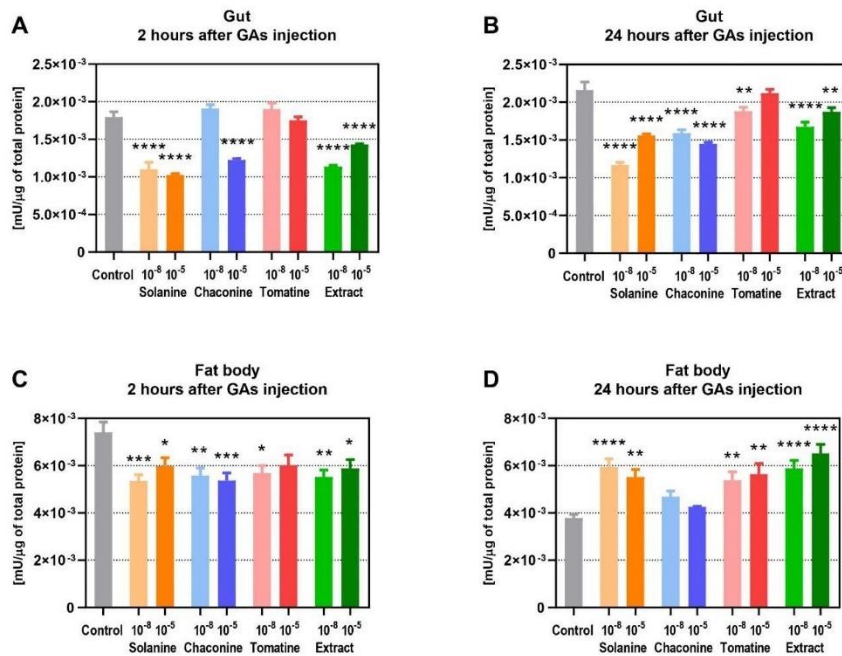
437



438

439 Fig. 7 The activity of PFK in the gut (A,B) and fat body (C,D) of *T. molitor* larvae 2 and 24 h after
440 injection with solanine, chaconine, tomatine, extract from tomato leaves, and physiological saline as
441 a control. The concentrations of the compounds 10^{-8} M (10^{-8}) and 10^{-5} M (10^{-5}) are shown on the
442 graphs. The activity is expressed as mU per µg of total soluble protein in the sample. Data are shown
443 as the mean with SEM. Samples were pooled with a minimum of 10 individuals. The assays were
444 prepared in three independent replicates for each experimental variant. The tested groups were
445 compared with the control with ordinary one-way ANOVA with Dunnett's multiple comparison test,
446 **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

447



448

449 Fig. 8 The activity of CS in the gut (A,B) and fat body (C,D) of *T. molitor* larvae 2 and 24 h after
450 injection with solanine, chaconine, tomatine, extract from tomato leaves, and physiological saline as
451 a control. The concentrations of the compounds 10^{-8} M (10^{-8}) and 10^{-5} M (10^{-5}) are shown on the
452 graphs. The activity is expressed as mU per µg of total soluble protein in the sample. Data are shown
453 as the mean with SEM. Samples were pooled with a minimum of 10 individuals. The assays were
454 prepared in three independent replicates for each experimental variant. The tested groups were
455 compared with the control with ordinary one-way ANOVA with Dunnett's multiple comparison test,
456 **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

457

458 The activity of PFK and CS was also analyzed in the gut and fat body after GAs injections. The PFK
459 activity was not affected by the tested compounds neither 2 nor 24 h after the GAs application (Fig.
460 7A,B). The PFK activity 2 h after GAs injection ranged between $1.9 \times 10^{-3} \pm 0.69 \times 10^{-3}$ and $2.9 \times 10^{-3} \pm$
461 1.47×10^{-3} mU/µg, while 24 after GAs treatment the values between $2.1 \times 10^{-3} \pm 1.05 \times 10^{-3}$ and 2.7×10^{-3}
462 $\pm 1.40 \times 10^{-3}$ mU/µg of total soluble protein in the samples were determined. On the contrary, PFK
463 activity decreased in the fat body 2 h after injection with the lower concentration of SOL, TOM, and
464 EXT (Fig. 7C). The lowest enzyme activity was observed after 10^{-8} M SOL treatment (almost a 2-fold
465 decrease compared to the control). A decrease in PFK activity was also reported 24 h after the
466 application of most GAs tested in lower concentrations: SOL, CHA, and TOM (Fig. 7D). Therefore, GAs
467 injection caused a decrease in PFK activity in the fat body, while it did not change protein activity in
468 the insect gut.

469 Treatment with SOL, EXT and 10^{-5} M CHA resulted in decreased CS activity in the gut after 2 h (Fig.
470 8A). The observed changes were also maintained 24 h after injection in that tissue (Fig. 8B).
471 Additionally, CHA 10^{-8} M and TOM 10^{-8} M also caused similar changes. Almost in all experimental
472 variants in the fat body, the CS activity was lower compared to the control (Fig. 8C). Surprisingly, the
473 SOL, TOM and EXT treatment resulted in increased enzyme activity 24 h after injection (Fig. 8D).
474 Therefore, the results indicate that the CS activity after GAs injections decreased in the gut at both
475 the incubation time tested and in the fat body 2 h after treatment. Interestingly, the CS activity was
476 increased 24 h after GAs application in the fat body, except for CHA.

477

478 4. Discussion

479 In the study, the effects of SOL, CHA, TOM and EXT on the energy metabolism of the larvae of *T.*
480 *molitor* beetle were studied. For this purpose, the level of energy metabolites in insect tissues, as
481 well as the level of gene expression and the activity of the key enzymes of glycolysis, the TCA cycle
482 and fatty acids β-oxidation were determined 2 and 24 h after GAs injection.

483 The main reserve of the energy substrates in insects are glycogen and TAGs. The effect of GAs on the
484 TAGs content in *T. molitor* larvae has previously been reported (Winkiel et al., 2023). The glycogen is
485 composed of glucose moieties, and it is stored in the fat body adipocytes. The tested GAs did not
486 alter neither glycogen nor glucose concentration in that tissue (Fig. 1E-H). In the haemolymph, these
487 carbohydrates were not detected in our study. In contrast, the increase in glycogen concentration in
488 the fat body of *T. molitor* after solamargine application, and the reduction of its content after *S.*
489 *nigrum* extract treatment were evidenced (Spochacz et al., 2018). The trehalose content did not
490 change in the fat body, as a result of GAs injection (Fig. 1C,D). However, the concentration of this
491 main circulating carbohydrate was reduced in the haemolymph 2 h after TOM treatment (in the
492 form of pure GA, and as the extract), while it increased after 24 h in these experimental variants (Fig.
493 1A,B). The decrease in trehalose content in haemolymph may be the result of the energy
494 requirement for GAs detoxification, which leads to increased nutrient catabolism. Additionally, GAs
495 increase oxidative stress in insects (Adamski et al., 2014). As trehalose has the ability to scavenge the
496 hydroxyl radical and decrease the ROS content (Felton and Summers, 1995), the later rise of this
497 disaccharide concentration may help alleviate the effect of increased ROS production. Moreover,
498 trehalose may be synthesized in insects from free fatty acids (McDougall and Steele, 1988), and, in
499 fact, it was previously reported, that GAs, especially after 24 h, increase the level of these
500 compounds in the *T. molitor* beetle (Winkiel et al., 2023). On the other hand, in previous studies on
501 *G. mellonella* larvae (Spochacz et al., 2021) it was observed that solasonine and *S. nigrum* extract did
502 not affect trehalose concentration in the insect haemolymph, which may indicate on the different

503 mechanisms of action of the tested GAs depending on the insect species, or the structure and
504 concentration of the GA compound. The application of GAs is especially important because the
505 insects in other mentioned studies were not injected but fed with the addition of GAs, which
506 certainly translates into distribution and metabolism of GAs (Spochacz et al., 2021, 2018).

507 The synthesis of amino acids takes place mainly in the fat body. They may be derived from glucose or
508 acetate, which can be incorporated to the intermediates of the glycolysis or TCA cycle. Then, the
509 amino group is added in the transamination reaction, or by adding ammonia (Chapman, 2012).
510 Besides the role of amino acids in synthesis of proteins, which are involved in transport, signalling,
511 gene expression, membrane activities, as well as acting as enzymes, these compounds have
512 additional functions related to neurotransmitters synthesis, detoxification, and ATP production
513 (Castagna et al., 1997; Manière et al., 2020). However, some amino acids, which are considered as
514 essential for larval growth (Chen, 1966; Davis, 1975), have not been detected in this research
515 (arginine, histidine, lysine, tryptophan, methionine, threonine, isoleucine). This intriguing finding
516 may be related to their trace amounts in analyzed tissues. It might also be explained by the fact that
517 these amino acids were present at a higher level in the tissues that were not analyzed in that
518 research. The following amino acids were detected in the tested tissues: valine, leucine, proline,
519 phenylalanine, and tyrosine in the fat body (Fig. 3), while valine, leucine, proline, phenylalanine, and
520 alanine in the haemolymph (Fig. 2). The obtained results indicate to the possible transport of most of
521 the identified amino acids from haemolymph to the fat body, especially 24 h after pure TOM and
522 EXT injection (Fig. 3). Their accumulation in that tissue may be the result of the protein degradation
523 after TOM treatment. However, it seems more reasonable that increased synthesis of non-essential
524 amino acids (proline, tyrosine) serves as an energy source for the severe detoxification processes
525 after 24 h since GAs application. On the other hand, tyrosine is the amino acid necessary for the
526 sclerotization of the cuticle (Andersen, 2010), usually synthesized from phenylalanine (Vavricka et
527 al., 2014). Therefore, the increased content of both amino acids after GAs injection may impact the
528 sclerotization process. The most important amino acid used for ATP production, for example, during
529 flight, is proline. It is also the key component of antimicrobial peptides (Yi et al., 2014) and plays an
530 important role in the tolerance of cold in insects (Lubawy et al., 2022; Misener et al., 2001). In the
531 haemolymph, the decrease in proline concentration was reported and these results are consistent
532 with the study that describes the decrease in proline level observed after *S. nigrum* extract and
533 solasonine in larvae of *G. mellonella* (Spochacz et al., 2021). Alanine is the amino acid used for
534 proline synthesis (Arrese and Soulages, 2010). Therefore, alanine concentration was also decreased
535 in the haemolymph 24 h after EXT injection. The other detected amino acids may constitute an
536 additional energy source with a high potential amount of ATP, which could be generated during the
537 oxidation (Bursell, 1981). Furthermore, the obtained results showed that the mechanisms of SOL and
538 CHA action are different from TOM, because after their injection no amino acid accumulation was
539 observed in the fat body tissue. The explanation may be the structures of GAs. SOL and CHA are
540 composed of solanidine and three carbohydrate molecules, while TOM contains tomatidine skeleton
541 and four carbohydrate moieties (Nepal and J. Stine, 2019), which may affect their properties and
542 mechanisms of action in insect tissues.

543 The energy substrates are utilized in the processes that produce ATP. Changes in the glycolysis
544 process have an impact on cell survival and growth, although it does not produce much energy
545 compared to oxidative phosphorylation (Xu et al., 2022). Therefore, this pathway might be the target
546 of anticancer therapy. For instance, SOL exhibited anticancer effects *via* the regulation of glycolysis
547 pathway in non-small cell lung cancer (Zou et al., 2022) as well as in human renal cancer (Wang et
548 al., 2021). It was reported that some plant secondary metabolites inhibit glycolysis enzymes activity
549 which may lead to the cell apoptosis. For example, sesquiterpenes might decrease PFK activity, the

550 key regulatory enzyme of glycolysis, in animal cells (Morrissey, 2009). Furthermore, coumarin, the
551 secondary plant metabolite, inhibited glycolysis in the *Spodoptera litura* F. moth (Xia et al., 2023). On
552 the other hand, the GA extract of *S. tuberosum* activated the glycolytic pathway in *Fusarium solani*
553 (Mart.) Sacc (Zhang et al., 2023). It reduced the amount of glucose in fungus cells and the activity of
554 hexokinase, while increasing PFK and pyruvate kinase activity. The presented study showed that GAs
555 did not affect PFK activity in the insect gut (Fig. 7A,B). However, they decreased enzyme activity in
556 the fat body, which may indicate inhibition of glycolysis (Fig. 7C,D). The changes after GAs injections
557 were also found at the gene expression level, but only 24 h after treatment. TOM and EXT reduced
558 the expression of PFK-encoding genes in the gut (Fig. 4B). On the other hand, SOL and EXT increased
559 this parameter in the insect fat body (Fig. 4D), which may be the compensatory effect of decreased
560 enzyme activity. Increased *PFK* expression in the fat body may also indicate increased intensity of
561 glycolysis in order to intensify ATP production. Similar effects of increased *PFK* expression level were
562 also observed in *Hyphantria cunea* D. moth after coumarin treatment (Yuan et al., 2024). Therefore,
563 the *PFK* expression as well as the enzyme activity were affected by GAs treatment, but the analyzed
564 parameters may differ depending on the compound and the tested tissue. The pyruvate, the product
565 of glycolysis, usually enters the mitochondria where it is oxidized to acetyl-CoA in the TCA cycle.
566 Unfortunately, there is no literature that describes the effects of GAs on the expression level of the
567 genes that encode CS in insects, as well as the activity of this protein, which is the crucial enzyme of
568 the TCA cycle. Hovewer, coumarin was recently reported to inhibit TCA cycle pathways in *S. litura*
569 moth at the gene expression level (Xia et al., 2023). Furthermore, this plant secondary metabolite
570 affects energy metabolism in *H. cunea* moth, decreasing the larval nutrient content and the
571 expression of genes involved in the mentioned process (Yuan et al., 2024). This finding is consistent
572 with the results obtained in this work, because GAs already after 2 h decrease CS activity in the
573 insect gut (Fig. 8A,B), but significant changes at the gene level were visible only 24 h after CHA
574 treatment (Fig. 5B). Also in the fat body 2 h after GAs application a decrease in CS activity was
575 reported (Fig. 8C). However, later this parameter increased compared to the control in that tissue
576 (Fig. 8D) and these results correspond to the increased expression of genes encoding CS after SOL
577 and EXT application (Fig. 5C,D). It may indicate an increase in the number of mitochondria and an
578 increase in the oxidative capacity of cells. The explanation could also be the increased energy
579 demand for stress response and GAs detoxification pathways (Rand et al., 2015).

580 Next to pyruvate, other important energy substrates are fatty acids, which may be converted during
581 β-oxidation to acetyl-CoA, the substrate of the Krebs cycle. Camptothecin in the fat body of
582 *Spodoptera frugiperda* S. was recently observed to affect the expression of important genes involved
583 in the synthesis of fatty acids. Furthermore, changes in the expression of genes related to the lipid
584 biosynthesis pathway and lipid metabolites after SOL treatment were also found in the *Curvularia*
585 *trifolii* K. fungus (Xu et al., 2023). One of the enzymes involved in fatty acid oxidation is HADH. It was
586 found to play a crucial role in lipid mobilization in insects (Arêdes et al., 2022). Our results showed
587 that the level of expression of the genes encoding HADH is higher 2 h after CHA and TOM treatment
588 in the gut (Fig. 6A). However, in the fat body this parameter is reduced (Fig. 6C), and this accords
589 with our previous observations, which showed decreased HADH activity 2 h after SOL and TOM
590 application (Winkiel et al., 2023). Interestingly, after 24 h, HADH activity in the fat body is reduced,
591 while the expression level increases (Fig. 6D). Therefore, the results indicated a possible reversed
592 correlation between protein expression and enzyme activity. Increased gene expression could be a
593 compensatory mechanism for reduced enzyme activity. Thus, GAs, as other plant secondary
594 metabolites, may alter the lipid metabolism also at the gene level.

595 5. Conclusions

596 The present study was designed to determine the effect of GAs on the concentration of energy
597 substrates and on the energy metabolism processes in the tissues of *T. molitor*. The research has
598 shown that TOM and EXT affect the trehalose concentration in the insect haemolymph. They also
599 lead to the accumulation of most of the amino acids detected after 24 h in fat body tissue, reducing
600 their content in the haemolymph, suggesting possible transport of amino acids between tissues. This
601 effect was not observed after SOL and CHA treatment, which indicates the different mechanisms of
602 action. The observed changes may be the result of protein degradation and/or enhanced catabolism
603 reactions for ATP production as an energy source for detoxification processes. The tested GAs also
604 affect glycolysis, TCA cycle, as well as fatty acids β-oxidation pathways, regulating the activity and
605 gene expression of key enzymes of these processes, but the effect depends on the type of GA
606 compound, the type of the tested tissue, and the incubation time after treatment. Furthermore, the
607 study revealed possible compensatory mechanisms related to the reduced activity of the enzymes
608 tested after application of GAs, which resulted in an increased level of expression of *PFK* and *HADH*.
609 On the other hand, the inhibited TCA pathway was reported in the insect gut, while an enhanced
610 process was noted in the fat body. Taken together, these results suggest that GAs affect the energy
611 metabolism of *T. molitor*. The study contributes to our understanding of the mechanisms of the
612 activity of plant secondary metabolites in insects. As ATP production is necessary for the survival of
613 the organism, this knowledge may contribute to the design of new natural biopesticides against
614 insect pests. However, further research should be undertaken in this topic.

615

616 Declarations of interest

617 None

618 Author contributions

619 Conceptualization: M.J.W., S.C., M.S.; Data curation: M.J.W, M.G..; Formal analysis: M.J.W, K.W.N..;
620 Funding acquisition: M.J.W., S.C.; Investigation: M.J.W., S.C., K.W.N., M.G.; Methodology: M.J.W.,
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622 M.J.W.; Writing - original draft: M.J.W.; Writing - review & editing: M.J.W., S.C., K.W.N., M.G., M.S.

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629 Data statement

630 The data analysed during this study are included in this published article.

631 Supplementary material: Table 1. Primers sequences used in qPCR

632

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763

Click here to access/download;Table (Editable version);Supp. Mat. Table 1.docx

Table 1. Primers sequences used in qPCR

Name	Sequence	Amplicon length
RPL13a-F	TCGTCGTGAGATGCGAACAA	191 bp
RPL13a-R	CTGCTTCCCACGTTCTGTCT	
PFK-F	TCTCATTCAAAGCGGTGTCA	167 bp
PFK-R	GTTAATCATTGGCGGTTTCG	
CS-F	ATATCGAAACTTCCCGTTGC	176 bp
CS-R	TGGTCAGCGTGGATCACTAA	
HADH-F	CTCCCGGATTCATTGTCAAC	161 bp
HADH-R	GACCGACGTAATCGGACAAT	

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Oświadczam, że mój udział w przygotowaniu manuskryptu:

Winkiel M.J., Chowański S., Walkowiak-Nowicka K., Gołębiowski M., Słocińska M. *A tomato a day keeps the beetle away – the impact of Solanaceae glycoalkaloids on energy management in the mealworm Tenebrio molitor,*

który jest częścią mojej rozprawy doktorskiej, polegał na zaplanowaniu i przeprowadzeniu doświadczeń (oznaczenie poziomu węglowodanów, pomiar ekspresji genów oraz aktywności enzymów), zebraniu materiału do analiz i przygotowaniu próbek, opracowaniu i interpretacji wyników, przeprowadzeniu analiz statystycznych, napisaniu manuskryptu, opracowaniu wykresów, wprowadzeniu korekt i przygotowaniu manuskryptu do publikacji.

....

Mgr Magdalena Joanna Winkiel

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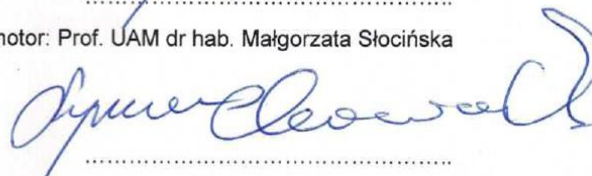

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który jest częścią rozprawy doktorskiej Magdaleny Joanny Winkiel, polegał na nadzorowaniu organizacji badań oraz na wprowadzeniu korekt w manuskrypcie.

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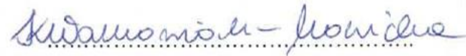
który jest częścią rozprawy doktorskiej Magdaleny Joanny Winkiel, polegał na nadzorowaniu obliczeń zmian ekspresji genów oraz na wprowadzeniu korekt w manuskrypcie.



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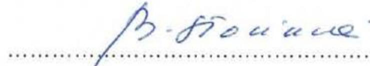
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Prof. UAM dr hab. Małgorzata Słocińska

Praca IV

Modulation of antioxidant system by glycoalkaloids in the beetle *Tenebrio molitor* L.

Magdalena Joanna Winkiel, Szymon Chowański, Karolina Walkowiak-Nowicka, Jan Lubawy, Małgorzata Słocińska

Manuskrypt jest na etapie recenzji w czasopiśmie *Ecotoxicology and Environmental Safety*

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1 # Modulation of the antioxidant system by glycoalkaloids in
2 # the beetle *Tenebrio molitor* L.

3

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19 Abbreviations:

20 CAT - Catalase

21 CHA - α-Chaconine

22 EXT - Tomato leaf extract

23 GAs - Glycoalkaloids

24 HSP - Heat shock proteins

25 MDA - Malondialdehyde

26 PSM - Plant secondary metabolites

27 ROS – Reactive oxygen species

28 SOD - Superoxide dismutase

29 SOL - α-Solanine

30 TOM - α-Tomatine

31

32 ## Abstract

Various factors may affect the antioxidative system in insects, including xenobiotics. Glycoalkaloids (GAs) are plant secondary metabolites produced mainly by the *Solanaceae* family (nightshades), such as the food crop tomato *Solanum lycopersicum* L. These compounds exhibit a wide range of biological activities and have attracted increasing interest in the context of potential insecticide properties. Therefore, the aim of the present study was to analyse the effects of GAs (solanine, chaconine, tomatine, and extracts of tomato leaves) on lipid peroxidation; the expression levels of genes encoding manganese superoxide dismutase (MnSOD), catalase (CAT), and heat shock protein 70 (HSP70); and the enzymatic activity of SOD and CAT in *Tenebrio molitor* larvae. This species is a popular model organism for toxicological and ecophysiological studies and is also a pest of grain storage. The reported changes depend on the GA concentration, incubation time, and type of insect tissue. We observed that the tested GAs affected *MnSOD* expression levels, increased SOD activity in the fat body, and reduced enzyme activity in the gut. The results showed that *CAT* expression was upregulated in the fat body and that the enzymatic activity of CAT in the gut was greater in the treated group than in the control group. Moreover, GAs affected *HSP70* expression and malondialdehyde levels in both tested tissues. This research contributes to our knowledge about the effects of GAs on the antioxidative system of *T. molitor* beetles. As efficient antioxidative system functioning is necessary for survival, the tested components may be targets of potential bioinsecticides.

Keywords: superoxide dismutase; catalase; oxidative stress; insect; mealworm; insecticide

1. Introduction

Oxygen is necessary for the life of all aerobic organisms. However, it also constitutes a danger because oxygen molecules can be reduced and form reactive oxygen species (ROS). Although ROS are produced during normal cell metabolism, at high concentrations, they may lead to protein oxidation, lipid peroxidation, and oxidative damage to DNA (Felton & Summers, 1995). Examples of ROS compounds include superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$). The superoxide anion radical is produced predominantly in mitochondria during respiratory chain reactions and as a result of phagocytic activity, while hydrogen peroxide is created by various oxidases in peroxisomes. The product of the breakdown of hydrogen peroxide in the Fenton reaction is the hydroxyl radical (Kodrík et al., 2015). The production of ROS in insects increases, for instance, during flight and bioluminescence processes (Felton & Summers, 1995) or as a result of stress conditions, such as exposure to ozone, heavy metal ions, pesticides, and ionizing radiation, as well as during the immune response (Kodrík et al., 2015). A basal amount of ROS is necessary for cell survival because it allows proper redox processes; moreover, ROS function as important signalling molecules to maintain cellular homeostasis (Mittler, 2017). However, with increasing ROS content, the risk of oxidative stress increases. This is a state that occurs when there is an imbalance between ROS concentration and antioxidant system functional efficiency (Felton & Summers, 1995).

It is believed that insects are particularly vulnerable to oxidative stress because of the structure of the respiratory system, high oxygen requirements during flight, and a diet rich in prooxidant compounds. Thus, ROS significantly influence insect development, growth, fecundity, fertility, and survival (Felton & Summers, 1995). Organisms have developed various

77 defence mechanisms against cytotoxic ROS, including enzymes and nonenzymatic
78 antioxidants. Examples of nonenzymatic antioxidants include α-tocopherol, tocotrienols, β-
79 carotene, lycopene, bilirubin, glutathione, ascorbic acid, and metal-binding proteins (ferritin,
80 transferrins) (Felton & Summers, 1995; Pardini, 1995). In turn, the main antioxidant enzymes
81 in insects are catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPOX),
82 and glutathione *S*-transferase (GSTPX) (Felton & Summers, 1995; Pardini, 1995).

83 The enzyme SOD catalyzes the dismutation of superoxide radicals to oxygen molecules and
84 hydrogen peroxide. Depending on the protein fold and metal cofactor (Mn/Fe, Cu/Zn or Ni),
85 three main families of SODs can be distinguished. In eukaryotes, MnSOD occurs
86 predominantly in mitochondria, as well as in the cytosol, and Cu/ZnSOD occurs in the outer
87 mitochondrial space. Additionally, some species have an extracellular Cu/ZnSOD (Landis &
88 Tower, 2005). MnSOD activity is essential because 90% of ROS in cells may be created in
89 mitochondria. Moreover, these organelles are vulnerable to oxidative damage due to
90 intensive oxygen metabolism and a lack of histones (Perry et al., 2010). Furthermore, it was
91 reported that the expression of *SOD* depends on the level of oxidative stress (Landis & Tower,
92 2005). Catalase catalyzes the decomposition of hydrogen peroxide to water and oxygen. This
93 enzyme is present mainly in peroxisomes (Pardini, 1995), and the main site of *CAT* expression
94 in insects is the fat body tissue (Zhang et al., 2016), midgut and hemocytes (Yamamoto et al.,
95 2005). A recent *in silico* study identified three isoforms of this enzyme in the *Tenebrio molitor*
96 beetle (Jang et al., 2024).

97 Some proteins, such as heat shock proteins (HSPs), may interact with SOD in insects (Nojima,
98 2021). The function of HSPs is to maintain cell homeostasis *via* the regulation of protein
99 folding, localization, and degradation. HSP synthesis in insects is affected by various stress
100 factors, such as extreme low and high temperatures or anoxia (King & MacRae, 2015; Lubawy
101 et al., 2022). There are four major HSP families reported in these organisms: small HSP, HSP60,
102 HSP70, and HSP90. It was previously determined that the overexpression of *HSP70* increases
103 resistance to oxidative stress in insects by reducing of ROS level. (King & MacRae, 2015).

104 *T. molitor* beetle, a yellow mealworm, is a popular model organism in various biomedical,
105 physiological, and environmental studies (Adamski et al., 2019). The breeding of the beetle is
106 undemanding and inexpensive. Moreover, the genome of this species has recently been
107 sequenced (Oppert et al., 2023), which will contribute to increasing research opportunities.
108 Additionally, the yellow mealworm is a cosmopolitan pest of grain warehouses, thus, it may
109 also serve as a model organism for toxicological studies.

110 Various xenobiotics may affect the antioxidant system in insects (Gao et al., 2022).
111 Glycoalkaloids (GAs) are compounds produced by many *Solanum* plants, such as tomato,
112 *Solanum lycopersicum* L., potato, *Solanum tuberosum* L., and the eggplant *Solanum*
113 *melongena* L. GAs include solanine (SOL), chaconine (CHA), and tomatine (TOM). These plant
114 secondary metabolites (PSMs) exhibit a wide range of biological activities in insect tissues
115 (Chowański et al., 2016). There is evidence that SOL leads to oxidative stress in these
116 organisms (Adamski et al., 2014; Büyükgüzel et al., 2013; Hasanain et al., 2015), and lipid
117 peroxidation is one of the most significant oxidative stress biomarkers. Thus, **the aim of our**
118 **study was to determine the effect of SOL, CHA, TOM and tomato leaf extract on**
119 **malondialdehyde (MDA) as a final product of lipid peroxidation, to measure the level of**
120 **oxidative stress generated by these GAs and analyse which antioxidative enzymes are**
121 **mainly involved in the antioxidative activity. Therefore, we checked the enzymatic activity**

122 **of SOD, CAT and the expression levels of *MnSOD, CAT* and *HSP70***. The obtained results
123 allowed us to answer the question of whether the oxidative stress generated by GAs is the
124 result of antioxidant system disturbances at the gene and protein levels or its inefficient
125 activity. As many insect species are crop pests, this research will broaden our knowledge
126 about the antioxidant systems in these organisms, which might be useful for the design of
127 novel biopesticides.

128

129 2. Materials and methods

130 *2.1 Insects*

131 The larvae of *T. molitor* beetles were obtained from colonies cultured at the Department of
132 Animal Physiology and Developmental Biology at the Faculty of Biology of Adam Mickiewicz
133 University, Poznań, Poland, at constant temperature (26 ± 0.5 °C), humidity (65 ± 5%) and
134 photoperiod of 8:16 h light/dark. The food consisted of oat flakes and fresh carrots. Only
135 feeding larvae with a weight between 120 and 140 mg were selected for the experiments.

136 *2.2 Compounds and Treatment Procedure*

137 In the experiments, synthetic GAs SOL (≥95.0%), CHA (≥95.0%), and TOM (≥95.0%) (Sigma–
138 Aldrich, Merck, Darmstadt, Germany) in physiological saline (PS) were used. They were
139 administered to larvae by injection using a microsyringe (Hamilton) at a volume of 2 μL and
140 at two concentrations, 10^{-8} and 10^{-5} M, which corresponds to dosages of 0.12-0.14 pg/mg
141 body mass for SOL and CHA, and 0.15-0.17 pg/mg body mass for TOM. The concentrations of
142 GAs were chosen based on the literature and our previous research in which we observed
143 different developmental and metabolic disorders (Spochacz et al., 2018, 2021; Winkiel et al.,
144 2023). The injection was performed after 8 min of CO_2 anaesthesia on the abdominal side of
145 the larva behind the last pair of legs. The GA extract from tomato leaves (EXT) was obtained
146 from the research group of Prof. Sabino A. Bufo from Basilicata University in Potenza, Italy. It
147 has been tested previously by our group (Marciniak et al., 2019; Ventrella et al., 2015, 2016),
148 showing the presence of the major GAs (2.95 ± 0.25%), TOM, and two other minor GAs,
149 namely, lycotetraose, dehydrotomatine and filotomatine (Ventrella et al., 2016). The EXT
150 contained the same concentration of TOM as the 10^{-8} and 10^{-5} M solutions of this GA, which
151 made it possible to compare the effects of the extract and pure GA treatment. As a control,
152 PS, which is isoosmotic for *T. molitor*, was used (NaCl 16 mg/mL, KCl 1.4 mg/mL, $CaCl_2$ 1
153 mg/mL).

154 *2.3 Tissue isolation*

155 Tissue isolation was performed 2 or 24 h after GA injection, depending on the experimental
156 variant. For analyses, the trophic tissues (gut and fat body) that play a key role in maintaining
157 metabolic balance as well as in detoxification were used. After anaesthesia, the larvae were
158 decapitated, and the last segment of the abdomen was removed. Next, the larvae were cut
159 along the dorsal side and spread on a Petri dish with pins. Afterwards, the fat body and gut
160 were washed with PS, isolated with microsurgical tweezers, and placed in 1.5 mL Eppendorf
161 tubes. Additionally, the guts were cleaned of food residues. The samples were pooled from
162 several individuals, depending on the experiment, which is described in detail in the following

163 sections. To avoid sample degradation, the isolation was performed on ice. The samples were
164 stored at −80 °C before further preparation.

165 *2.4 Lipid peroxidation*

166 The level of lipid peroxidation was determined in the gut and fat body samples pooled from
167 a minimum of 10 individuals. The tissues were placed in 250 µL (gut) or 500 µL (fat body) of
168 PS, homogenized for 3 min using a handheld pestle homogenizer (Fisherbrand) and
169 centrifuged (10,000 RPM, 10 min, 4 °C). The supernatant was then transferred to new tubes,
170 and the protein concentration was measured using a Direct Detect spectrometer (Merck,
171 Darmstadt, Germany) (Szymczak-Cendlak et al., 2022). Afterwards, the samples were frozen
172 in liquid nitrogen and stored at −80 °C until the measurements were conducted.

173 Lipid peroxidation was analysed by quantifying thiobarbituric acid reactive substances
174 (TBARS) using a TBARS assay kit (700870, Cayman Chemical, Tallinn, Estonia) following the
175 instructions provided by the manufacturer. Briefly, 50 µL of the sample was combined with
176 50 µL of 10% trichloroacetic acid (TCA) and 400 µL of a colour reagent (comprising
177 thiobarbituric acid (TBA), acetic acid, and sodium hydroxide) in a 2 mL vial. The vials were
178 then placed in boiling water for one hour. After that time, the vials were cooled on ice for 10
179 minutes to halt the reaction. After centrifugation at 1,600 RCF at 4 °C for 10 min, the
180 absorbance of the supernatant was measured at 530 nm using a Synergy H1 Hybrid Multi-
181 Mode Microplate Reader (BioTek, USA). The final results, derived from the standard curve of
182 MDA, are expressed as µM of MDA per µg of protein. The assay was performed in three
183 independent replicates for each experimental variant.

184 *2.5 Enzyme activity*

185 SOD and CAT catalytic activities were measured using commercially available assay kits
186 (Superoxide Dismutase Assay Kit 706002, Cayman Chemical and Catalase Activity Assay Kit
187 ab83464, Abcam, respectively). The experiments were performed according to the
188 manufacturer's instructions using the same samples used for determination of lipid
189 peroxidation levels. The gut and fat body samples for the SOD experiment were diluted 100x
190 with PBS. The experiments were based on spectrophotometric techniques using a Synergy H1
191 Hybrid MultiMode Microplate Reader (BioTek, USA). The absorbance was measured at
192 wavelengths of λ = 450 nm (SOD) and λ = 570 nm (CAT) at RT for 30 min (5 min intervals).
193 Enzyme activity is expressed as U per mg of total soluble protein in the sample. The assays
194 were prepared in three independent replicates for each experimental variant.

195 *2.6 Quantitative analysis of gene expression*

196 The samples for gene expression measurements were pooled from 5 individuals. Fat body and
197 gut tissues were placed in 300 µL of RNA lysis buffer (R1060-1, Zymo Research). Next,
198 homogenization with a pestle homogenizer (Fisherbrand) was conducted, followed by total
199 RNA isolation using a Quick-RNA™ MiniPrep Kit (R1055, Zymo Research) according to the
200 manufacturer's instructions. Then, the residual DNA was removed with a Turbo DNase kit
201 (AM1907, Thermo Scientific). The RNA concentration was measured, and the samples were
202 frozen in liquid nitrogen and stored at −80 °C until the next steps.

203 cDNA was synthesized with a LunaScript® RT SuperMix Kit (E3010, Biolabs) and a T100™
204 Thermal Cycler (Bio-Rad). The prepared cDNA samples were stored at −20 °C. Quantitative
205 real-time PCR (RT–qPCR) analyses were performed using SYBR Green Master mix (4309155,
206 Thermo Fisher Scientific) and a C1000™ Thermal Cycler with a CFX96™ Real-Time System (Bio-
207 Rad). The primers were designed based on sequences available in public databases (NCBI) and
208 synthesized by the Institute of Biochemistry and Biophysics, Warsaw (Supp. Mat. 1). Melting
209 curve analyses were performed to assess the suitability of the primers for qPCR. The PCR
210 conditions for the amplified gene and reference gene (*ribosomal protein L13a* (*RPL13A*)) were
211 determined and optimized before amplification. The stability of *RPL13A* expression was
212 validated before the experiment. Three biological and three independent replicates for each
213 experimental variant were performed. To check for potential contamination of the samples,
214 negative controls were prepared. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$
215 method (Livak & Schmittgen, 2001). To confirm the results, the amplicons were sequenced by
216 the Molecular Biology Techniques Laboratory (Faculty of Biology, Adam Mickiewicz University
217 in Poznań) and compared with data available in a public database (NCBI).

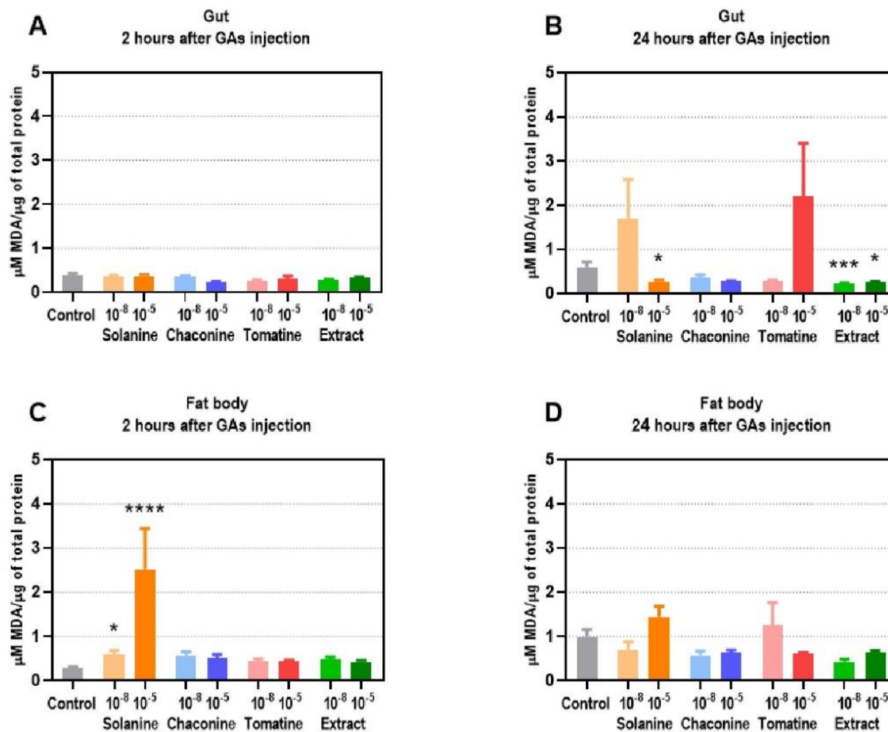
218 *2.7 Statistical analysis*

219 The results were analysed using GraphPad Prism 8.0.1. (Department of Animal Physiology and
220 Developmental Biology AMU licence). The normality of the distribution was determined using
221 the Shapiro–Wilk test. Normally distributed data were analysed with Brown–Forsythe and
222 Welch ANOVA with Dunnett's multiple comparisons test. Nonnormally distributed data were
223 analysed using the Kruskal–Wallis test with Dunn's multiple comparisons test.

224 3. Results

225 *3.1 Lipid peroxidation*

226



227 Fig. 1 The levels of MDA in the gut (A, B) and fat body (C, D) of *T. molitor* larvae 2 and 24 h after
228 injection of solanine, chaconine, tomatine, or extract from tomato leaves at concentrations of 10^{-8} M
229 (10^{-8}) or 10^{-5} M (10^{-5}), and physiological saline was used as a control. The MDA level is expressed as
230 µM per µg of total soluble protein in the sample. The data are shown as the means ± SEM. Samples
231 were pooled with a minimum of 10 individuals. The assays were prepared in three independent
232 replicates for each experimental variant. The tested groups were compared to the control group using
233 the Kruskal–Wallis test with Dunn's multiple comparisons test; **** $p \leq 0.0001$, *** $p \leq 0.001$, * $p \leq$
234 0.05.

235

236 Total soluble protein concentrations in the gut samples ranged between 9.7 and 18.9 mg/mL,
237 and in fat body samples, they ranged between 13.2 and 29.5 mg/mL. The tested GAs affected
238 the MDA levels in the gut (after 2 h: Kruskal–Wallis test, F = 17.49, $p \leq 0.05$; after 24 h:
239 Kruskal–Wallis test, F = 25.81, $p \leq 0.01$) and fat body (after 2 h: Kruskal–Wallis test, F = 25.84,
240 $p \leq 0.01$; after 24 h: Kruskal–Wallis test, F = 18.17, $p \leq 0.05$). However, compared to the
241 control, none of the tested GAs changed the MDA level in the insect gut 2 h after injection
242 (Fig. 1A). Surprisingly, 24 h after GA application, the level of MDA was significantly lower than
243 that in the control (Fig. 1B). A decrease in the MDA concentration was reported after both
244 the EXT concentration and after the SOL 10^{-5} M treatment. In contrast, SOL at both tested
245 concentrations increased the MDA level in the fat body after 2 h (Fig. 1C). The change was

246 dependent on the GA concentration. After 10^{-5} M SOL, an increase of almost 9-fold was
247 reported (2.5±2.27 after GA injection compared to 0.3±0.07 μM MDA per μg of total soluble
248 protein in the control). On the other hand, GAs did not affect the MDA level 24 h after
249 application in that tissue compared to that in the control (Fig. 1D).

250

251 *3.2 Enzyme activity*

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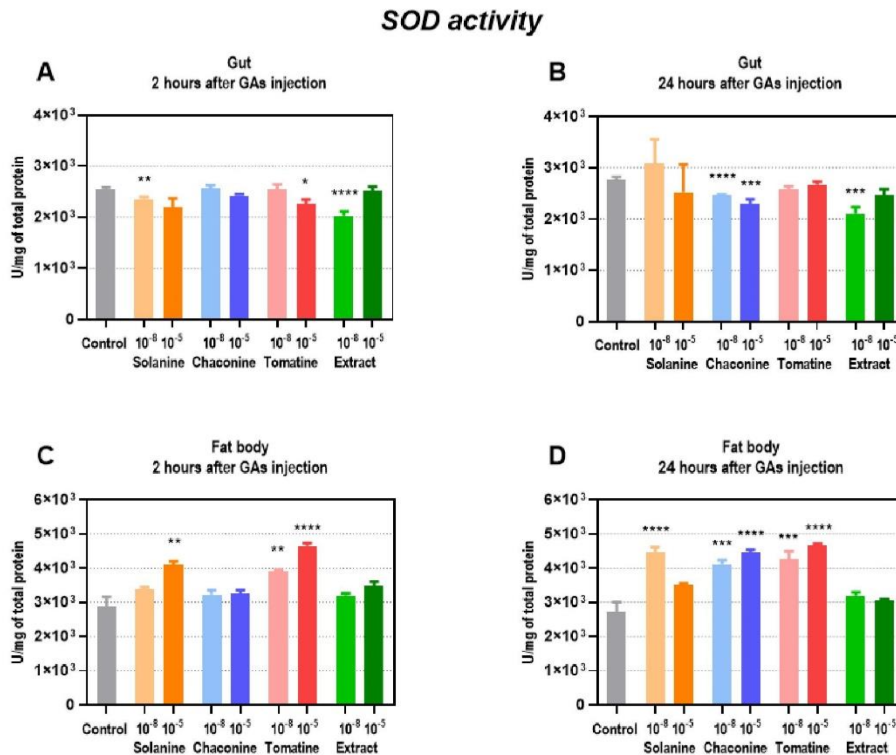

253

254 Fig. 2 The activity of SOD in the gut (A, B) and fat body (C, D) of *T. molitor* larvae 2 h and 24 h after
255 injection of solanine, chaconine, tomatine, or extract from tomato leaves at concentrations of 10^{-8} M
256 (10^{-8}) and 10^{-5} M (10^{-5}) and physiological saline as a control. The activity is expressed as U per mg of
257 total soluble protein in the sample. The data are shown as the means ± SEM. Samples were pooled
258 with a minimum of 10 individuals. The assays were prepared in three independent replicates for each
259 experimental variant. The tested groups were compared to the control group by Brown–Forsythe and
260 Welch ANOVA with Dunnett's multiple comparisons test; **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$,
261 * $p \leq 0.05$.

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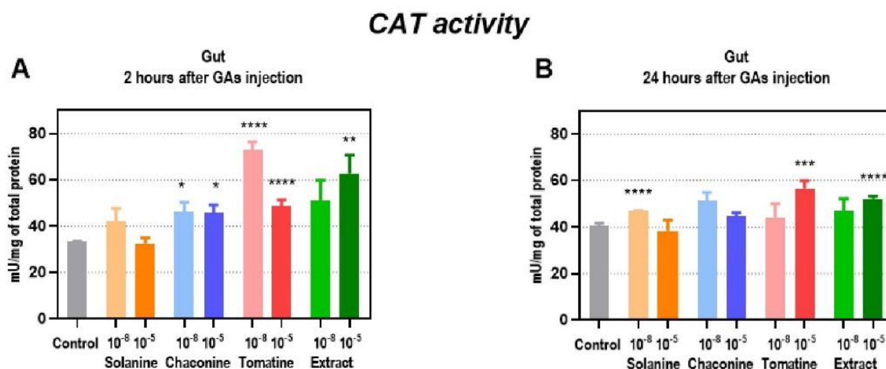

Fig. 3 The activity of CAT in the gut of *T. molitor* larvae 2 (A) and 24 h after injection (B) with solanine, chaconine, tomatine, or extract from tomato leaves at concentrations of 10^{-8} M (10^{-8}) or 10^{-5} M (10^{-5}), and physiological saline was used as a control. The activity is expressed as mU per mg of total soluble protein in the sample. The data are shown as the means ± SEM. Samples were pooled with a minimum of 10 individuals. The assays were prepared in three independent replicates for each experimental variant. The tested groups were compared to the control group by Brown–Forsythe and Welch ANOVA with Dunnett's multiple comparisons test; **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

Surprisingly, the SOD activity in the gut decreased 2 h after injection of 10^{-8} M SOL, 10^{-5} M TOM or 10^{-8} M EXT (Fig. 2A), with the greatest change reported after EXT treatment (from 2550.4±199.05 U/mg of total soluble protein in the control to 2027.4±369.60 U/mg of protein after EXT injection). This effect was also present in that tissue 24 h after the application (Fig. 2B). Additionally, a decrease in SOD activity was observed in the gut 24 h after injection of CHA at both concentrations. However, in fat body tissue, GAs had opposite effects on enzyme activity. SOD activity increased 2 h after treatment with 10^{-5} M SOL or 10^{-5} and 10^{-8} M TOM (Fig. 2C), with the highest activity occurring after the injection of 10^{-5} M TOM (4644.0±473.46 U/mg of protein). After 24 h, most of the tested GAs exhibited increased enzyme activity (Fig. 2D). The greatest value was also noted after the 10^{-5} M TOM treatment (4667.2±267.07 U/mg of protein).

The activity of CAT in the gut 2 h after GA injection was greater in almost all the experimental groups than in the control group (Fig. 3A). CHA and TOM at both concentrations, as well as 10^{-5} M EXT, significantly increased the enzyme activity, from 33.1±1.87 mU/mg of total soluble protein in the control to 72.8±15.80 mU/mg of protein (a more than 2-fold change). The increase in enzyme activity was also maintained for 24 h after TOM and EXT treatment (Fig. 3B). Additionally, the CAT activity in the insect gut 24 h after the application of 10^{-8} M SOL (46.8±0.70 mU/mg of protein) was greater than that in the control (40.7±4.46 mU/mg of protein).

3.3 Quantitative analysis of gene expression

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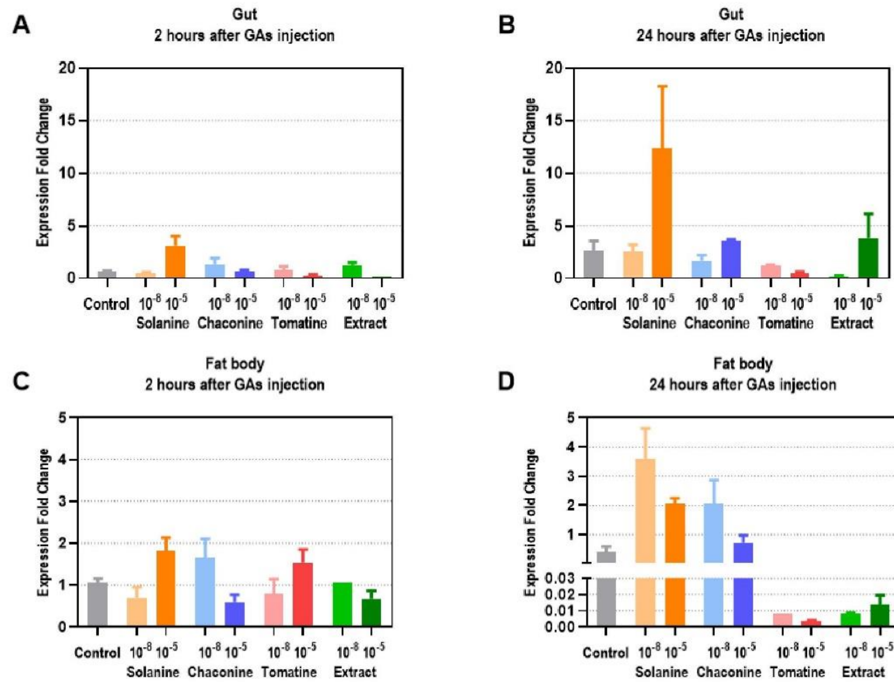

295

296 Fig. 4 The relative expression levels of *MnSOD* in the gut (A, B) and fat body (C, D) of *T. molitor* larvae
297 2 and 24 hours after the application of solanine, chaconine, tomatine, and tomato leaf extracts at
298 concentrations of 10^{-8} and 10^{-5} M; saline was used as a control. The *RPL13A* gene was used as a
299 reference gene. The data are shown as the mean and SEM. The pooled samples were used with n = 5.
300 For each experimental variant, three independent replicates were performed. The tested groups were
301 compared to the control group using the Kruskal–Wallis test with Dunn's multiple comparisons test.

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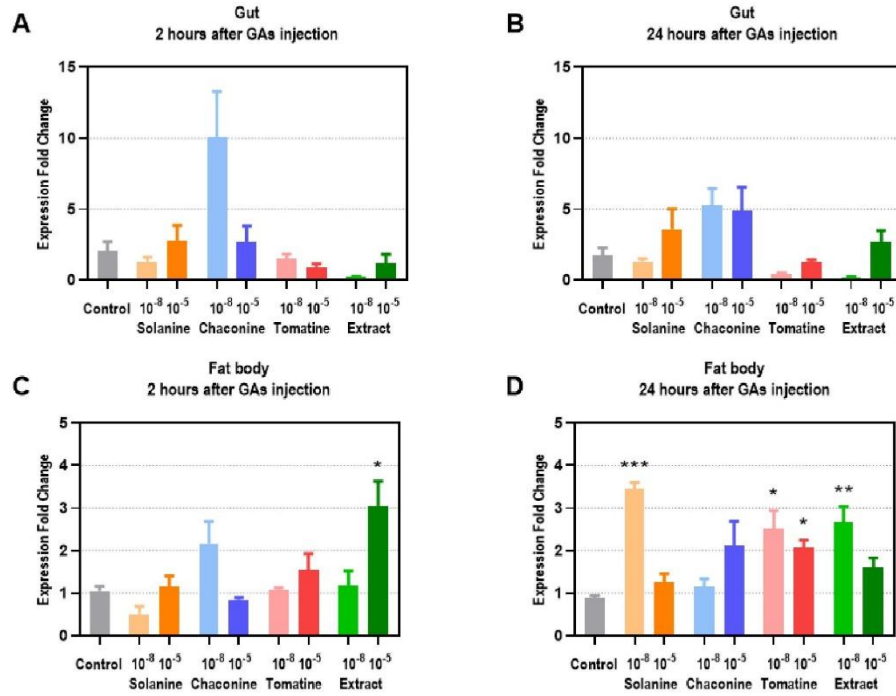

304

305 Fig. 5 The relative expression levels of *CAT* in the gut (A, B) and fat body (C, D) of *T. molitor* larvae 2
306 and 24 hours after the application of solanine, chaconine, TOM, and tomato leaf extracts at
307 concentrations of 10^{-8} and 10^{-5} M; saline was used as a control. The *RPL13A* gene was used as a
308 reference gene. The data are shown as the mean and SEM. The pooled samples were used with $n = 5$.
309 For each experimental variant, three independent replicates were performed. The tested groups were
310 compared to the control group using the Kruskal–Wallis test with Dunn's multiple comparisons test;
311 *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

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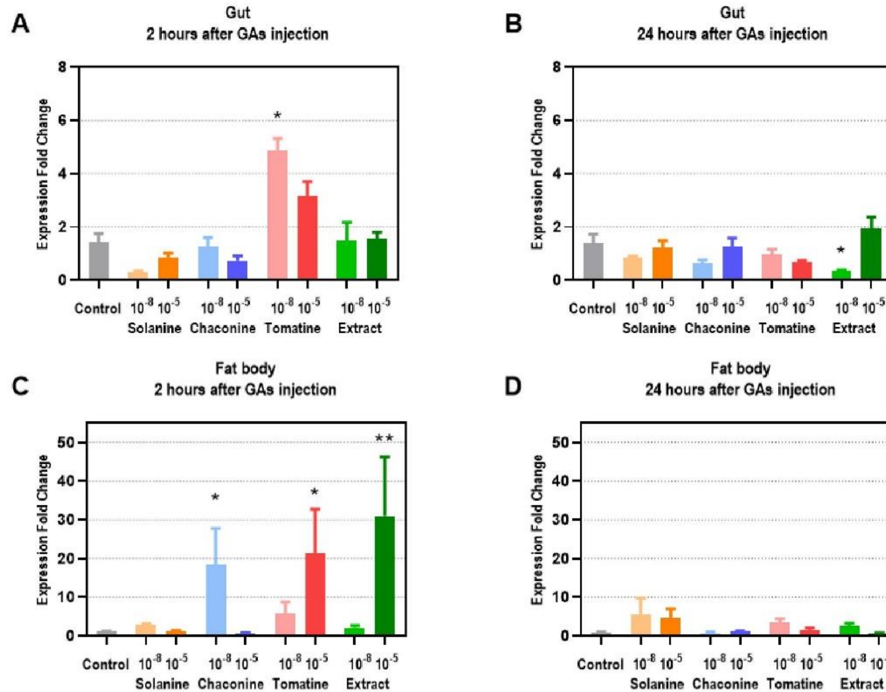

Fig. 6 The relative expression levels of *HSP70* in the gut (A, B) and fat body (C, D) of *T. molitor* larvae 2 and 24 hours after the application of solanine, chaconine, tomatine, and tomato leaf extracts at concentrations of 10^{-8} and 10^{-5} M; saline was used as a control. The *RPL13A* gene was used as a reference gene. The data are shown as the mean and SEM. The pooled samples were used with $n = 5$. For each experimental variant, three independent replicates were performed. The tested groups were compared to the control group using the Kruskal–Wallis test with Dunn's multiple comparisons test, ** $p \leq 0.01$, * $p \leq 0.05$.

The results showed that the tested compounds affected *MnSOD* expression after 2 h in the gut (Kruskal–Wallis, F = 17.06, $p \leq 0.05$) and after 24 h in the fat body (Kruskal–Wallis, F = 32.76, $p \leq 0.0001$). Depending on the time and concentration, we noticed an increase or decrease in the level of *MnSOD* expression (Fig. 4). The greatest changes were observed after the application of SOL. *MnSOD* expression in the gut 2 h after the injection of SOL at a concentration of 10^{-5} M was almost 5 times greater than that in the control (Fig. 4A) but was more than 9 times greater 24 h after the injection of 10^{-8} M SOL in the fat body than in the control (Fig. 4D). However, the observed changes were not statistically significant. No changes in *MnSOD* expression were detected in the fat body of the insects 2 h after GA application (Fig. 4C). SOL and CHA tended to increase the expression level only 24 h after GA injection (Fig. 4D). On the other hand, after 24 h, the expression of the genes encoding TOM and EXT tended to decrease.

336 The expression of *CAT* varied after the application of the tested compounds in the gut (after
337 2 h: Kruskal–Wallis test, F = 25.93, $p \leq 0.01$; after 24 h: Kruskal–Wallis test, F = 28.74,
338 $p \leq 0.001$), as well as in the fat body (after 2 h: Kruskal–Wallis test, F = 20.20, $p \leq 0.01$; after
339 24 h: Kruskal–Wallis test, F = 29.72, $p \leq 0.001$). Nevertheless, we did not observe significant
340 changes in the gut after GA injection compared to the control (Fig. 5A, B). Only a slight
341 tendency for the expression to increase was reported after SOL and CHA application, and a
342 decreasing trend after TOM and EXT treatment was noted. For example, for the 24 h variant,
343 the following ranges of expression fold changes were calculated: 1.3-5.3 (for SOL and CHA)
344 and 0.2-2.7 (for TOM and EXT), compared to the 1.7 change in the control. However, the *CAT*
345 expression level in the fat body increased 3-fold 2 h after the 10^{-5} M EXT injection (3.0 change,
346 Fig. 5C). The expression level also increased by 3.5-, 2.5-, 2.1-, and 2.7-fold after treatment
347 with 10^{-8} M SOL, 10^{-8} M TOM and 10^{-5} M TOM, respectively, and after treatment with 10^{-8} M
348 EXT (Fig. 5D). Thus, GAs at lower concentrations changed gene expression compared to that
349 of the control.

350 The results showed that the tested compounds affected the expression of *HSP70* in the gut
351 (after 2 h: Kruskal–Wallis test, F = 30.55, $p \leq 0.001$; after 24 h: Kruskal–Wallis test, F = 26.59,
352 $p \leq 0.001$) and fat body (after 2 h: Kruskal–Wallis test, F = 28.75, $p \leq 0.001$; after 24 h: Kruskal–
353 Wallis test, F = 18.32, $p \leq 0.05$). The expression level of the genes encoding HSP70 increased
354 in the gut almost 5-fold 2 h after TOM 10^{-8} M injection (4.9 change), while in the control, the
355 change was 1.4 (Fig. 6A). However, after 24 h, 10^{-8} M EXT reduced the expression level 0.3-
356 fold compared to the 1.4-fold change in the control (Fig. 6B). On the other hand, there was a
357 very large increase in gene expression in the fat body 2 h after the application of 10^{-8} M CHA,
358 10^{-5} M TOM, or 10^{-5} M EXT (an increase of almost 20 to 31-fold, Fig. 6C). During the longer
359 incubation time, no significant changes compared to those in the control were observed in
360 that tissue after GA injection (Fig. 6D).

361

362 4. Discussion

363 Various biotic and abiotic factors that exert negative impacts on insect fitness can lead to
364 oxidative stress, which is characterized by increased reactive oxygen species (ROS) levels and
365 low-efficiency antioxidative system functioning. An example may be xenobiotics, such as
366 alkaloids (Chowański et al., 2016). For instance, SOL, an alkaloid produced by *Solanum* plants,
367 elevates the level of MDA and induces protein carbonylation, the biomarkers of oxidative
368 damage, and affects glutathione *S*-transferase activity in *Galleria mellonella* larvae (Adamski
369 et al., 2014; Büyükgüzel et al., 2013). Recently, it was reported that SOL changes the
370 expression levels of detoxification enzymes, such as cytochrome P450 monooxygenases and
371 glutathione *S*-transferases (Yan et al., 2023). However, the impact of particular GAs on the
372 expression levels of *MnSOD, CAT,* and *HSP70*, as well as the enzymatic activity of SOD and CAT
373 in insects, has not been fully explored. Therefore, these parameters were analysed in our
374 research after SOL, CHA, TOM and EXT injection into the larvae of *T. molitor*. The following
375 variable conditions were used: GA concentration (10^{-8} M and 10^{-5} M), incubation time (2 and
376 24 h), and type of insect tissue (gut and fat body).

377 Many PSMs change the activity of antioxidant enzymes in insects. Cui et al. reported that
378 quercetin, a plant flavonoid, increased SOD and CAT activity in the grasshopper *Oedaleus*
379 *asiaticus* (Cui et al., 2019). Another PSM, juglone, a quinone-based compound of walnut

380 plants, changed these parameters in *G. mellonella* larvae (Altuntaş et al., 2020). This accords
381 with our observations because increased SOD activity in the fat body after pure GAs was
382 observed 2 h after the treatment. Moreover, a higher GA concentration is usually connected
383 to a greater activity change. The changes in SOD activity may be explained by the elevated
384 level of the oxidative damage biomarker MDA in that tissue. An increase in the MDA
385 concentration after SOL application was also observed in studies on *G. mellonella* (Adamski
386 et al., 2014). Moreover, more injected GAs were detected in the fat body than in the gut and
387 hemolymph, which may indicate their accumulation in that tissue (data unpublished). Thus,
388 increased ROS levels result in enhanced oxidative stress, and an inefficient response of the
389 antioxidant system may be reached by upregulating the activity of antioxidant enzymes.

390 In contrast, in the insect gut, a lower MDA concentration was observed 24 h after GA
391 application than in the control. This result is difficult to explain, but it may be the effect of
392 enhanced antioxidant system activity. The concentrations of the tested compounds in the gut
393 may have been too low for lipid peroxidation to be induced, but at the same time, they may
394 have been high enough to activate cellular mechanisms combating ROS. Interestingly, an
395 increase in CAT but a reduction in SOD activity in the insect gut after GA injection compared
396 to those in the control group were reported. The product of the dismutation of superoxide
397 radicals catalyzed by SOD, which is hydrogen peroxide, is the substrate for the reaction
398 catalyzed by CAT. Thus, this intriguing finding may be related to the excessive superoxide
399 radical production in this tissue compared to the efficacy of SOD activity in the gut. This results
400 in a lower amount of hydrogen peroxide, which enables the consecutive conversion of this
401 ROS by CAT. Moreover, hydrogen peroxide reduction is also catalyzed by glutathione
402 peroxidase. Therefore, the ability of the gut to eliminate these ROS may be greater than the
403 efficacy of superoxide radical reduction. Additionally, SODs are oxidatively inactivated
404 enzymes (Pardini, 1995); thus, ROS levels might exceed the limit of physiological adaptability
405 and lead to damage to SOD proteins. This observation is in accordance with the results of
406 Altuntas (2015), who observed previously that high xenobiotic doses lead to inhibition or a
407 decrease in SOD activity, while CAT activity increases linearly with increasing doses of
408 gibberellic acid in *G. mellonella* (Altuntaş, 2015). There are only a few studies on alkaloids,
409 but they indicate that in the gut of *Spodoptera littoralis* fed with potato, the activity of both
410 enzymes, SOD and CAT, increased (Krishnan & Kodrík, 2006). This finding may be explained
411 by lower GA doses compared to the amount of GAs injected during our study. In contrast,
412 another possible explanation for decreased SOD activity in the gut after GA treatment might
413 be greater gut resistance to oxidative stress due to faster food passage and lower GA
414 accumulation in the gut than in the fat body tissue (data unpublished).

415 There are also some reports connected to the effects of PSM on the gene expression of
416 antioxidant enzymes in insects. For example, the expression of different types of *SOD* changed
417 in the fat body of *S. frugiperda* after they were fed camptothecin alkaloids (Shu et al., 2021).
418 However, in our research, the expression of *MnSOD* did not significantly change after GA
419 treatment compared to that of the control in either the gut or the fat body. This discrepancy
420 could be attributed to many factors, such as different insect species, application methods,
421 compound concentration, and structure, which may be due to different properties and
422 mechanisms of action. On the other hand, our results showed that *CAT* expression was
423 increased in the insect fat body 2 h after EXT injection. Later, changes were observed for even
424 more of the tested compounds. These results corroborate the findings of Yuan et al., who
425 reported increased *CAT1* and *CAT2* expression in the larvae of *Hyphantria cunea* moth fed

426 coumarin (Yuan et al., 2024). According to these data, we can infer that GAs increased *CAT*
427 expression in the fat body, which might be the result of enhanced ROS reduction. Moreover,
428 because of the important role of CAT in reducing oxidative stress, antioxidant enzymes have
429 become a target of insecticides (Zhao et al., 2013).

430 The expression of *HSP70* increases in response to many stress factors, such as cold, heat,
431 dehydration, diapause, and insecticides, in many insect species (Tufail & Takeda, 2012). The
432 results revealed increased *HSP70* expression after GA injection in both tested tissues;
433 however, this effect was noted only after 2 h, which suggests that the HSP is the first-choice
434 response to protect cells from GA-induced stress. The upregulation of *HSP* expression
435 increases resistance to oxidative stress (King & MacRae, 2015), which might also be an
436 effective mechanism of antioxidative system function after GA treatment. Interestingly,
437 consistent expression patterns of *MnSOD* and *HSP60* were previously reported in Bombyx
438 mori (Nojima, 2021). However, in this study, no correlation was detected between *MnSOD*
439 and *HSP70* expression. This might be due to the slightly different functions of HSP60 and
440 HSP70 in insects (King & MacRae, 2015).

441 According to the data above, we can infer that the changes in the expression levels of the
442 genes encoding the antioxidant enzymes as well as their activity reflect the increased demand
443 for efficient antioxidative system functioning after GA treatment to avoid oxidative stress in
444 insects. In these organisms, the response to oxidative stress is regulated mainly by
445 adipokinetic hormone (AKH), which is secreted by the neuroendocrine gland *corpora*
446 *cardiaca*. AKH mobilizes energy stores of carbohydrates, lipids, and amino acids to stimulate
447 the anti-stress response. It was noted that the exposure of the fat body to the oxidative
448 stressor increases the AKH level in the haemolymph (Kodrík et al., 2015). Therefore, as GAs
449 may increase oxidative damage in insects, AKH may be correlated with changes in metabolite
450 content after GA injection. Indeed, we have observed changes in energy substrate
451 metabolism after GA injection (Winkiel et al., 2023).

452 Considering all the described results obtained in this work, we can formulate the following
453 conclusions. If the efficiency of the antioxidative system is too low, lipid peroxidation may be
454 observed as a result of ROS production, which might explain the increased level of MDA that
455 we observed. Moreover, polyunsaturated fatty acids (PUFAs) are especially vulnerable to this
456 chain reaction (Pardini, 1995). It is even believed that for this reason, the concentration of
457 PUFAs in insect tissues is very low, and this is another evolutionary mechanism by which
458 insects avoid oxidative stress (Stanley & Kim, 2020). An example of a PUFA is linoleic acid. We
459 have previously reported elevated level of this compound (9,12-octadecadienoic acid) in the
460 insect fat body 24 h after GA injection (Winkiel et al., 2023) what in consequence may lead to
461 increased production of ROS. Reactive oxygen species as well as the products of lipid
462 peroxidation, may impact activity and structure of proteins (Pardini, 1995) what is consistent
463 with our results showing decreased activity either of the key enzymes of β-oxidation of fatty
464 acids (Winkiel et al., 2023) or enzymes engagaed in glycolysis and the TCA cycle (data
465 unpublished). . Finally, nucleic acids are susceptible to oxidative damage, which might result
466 in disrupted DNA replication, transcription and translation, leading to mutations, senescence
467 and cell death (Pardini, 1995). Indeed, we have noted the altered expression of the key genes
468 involved in energy metabolism in insects (data unpublished).

469 In summary, in this study, we found that GAs affect the antioxidative system in *T. molitor*
470 beetles. The observed effects depend on the GA compound, its concentration, the incubation

time, and the tested tissue. This study contributes to our understanding of the mechanisms of GA activity in insect tissues. As an efficient antioxidative system is necessary for survival during stress conditions (Felton & Summers, 1995), its disruption may lead to a reduction in the populations of insect pests. Therefore, the implication of the obtained results is the possibility of using GAs as potential natural bioinsecticides, which is in agreement with Integrated Pest Management principles. However, further research is required to check how disturbed by GAs oxidative system affect processes of insect development and reproduction, or determine different ways of GAs application.

Conflicts of interest

None

Author contributions

Conceptualization: M.J.W., S.C., M.S.; Data curation: M.J.W.; Formal analysis: M.J.W., K.W.N.; Funding acquisition: M.J.W., S.C.; Investigation: M.J.W., S.C., K.W.N., J.L.; Methodology: M.J.W., S.C., K.W.N., J.L.; Project administration: M.J.W.; Resources: M.J.W.; Supervision: S.C., M.S.; Visualization M.J.W.; Writing - original draft: M.J.W.; Writing - review & editing: M.J.W., S.C., K.W.N., J.L., M.S.

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Data statement

The data analysed during this study are included in this published article.

Supplementary material: Table 1. sequences of Primers used for qPCR

Table 1. sequences of Primers used for qPCR

Name	Sequence	Amplicon length
RPL13A-F	TCGTCGTGAGATGCGAACAA	191 bp
RPL13A-R	CTGCTTCCCACGTTCTGTCT	
CAT-F	TGTTGGGATGGTAGTTGGGC	162 bp
CAT-R	TCCAAGGGCGACTCTTCAAC	
SOD-F	TCAAGCCGACCGTAGCAAGG	224 bp
SOD-R	AGCGCCAAAGTCTCGAGGTG	
HSP-F	CGCTTCGGCGATTTCTTTCA	174 bp
HSP-R	CGCAAGTACGACGATCCCAA	

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Oświadczam, że mój udział w przygotowaniu manuskryptu:

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który jest częścią mojej rozprawy doktorskiej, polegał na zaplanowaniu i przeprowadzeniu doświadczeń (pomiar ekspresji genów i aktywności enzymów), zebraniu materiału do analiz i przygotowaniu próbek, opracowaniu i interpretacji wyników, przeprowadzeniu analiz statystycznych, napisaniu manuskryptu, opracowaniu wykresów, wprowadzeniu korekt i przygotowaniu manuskryptu do publikacji.

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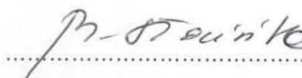

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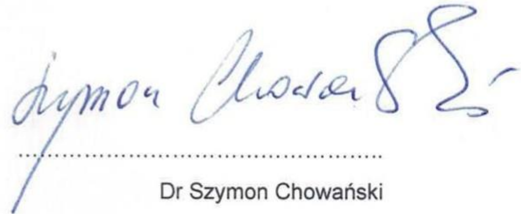

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który jest częścią rozprawy doktorskiej Magdaleny Joanny Winkiel, polegał na nadzorowaniu obliczeń zmian ekspresji genów oraz na wprowadzeniu korekt w manuskrypcie.

Dr Karolina Walkowiak-Nowicka

Poznań, 22.03.2024 r.

Dr Jan Lubawy
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ul. Uniwersytetu Poznańskiego 6
61-614 Poznań

Oświadczenie współautora manuskryptu

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....
Dr Jan Lubawy

Poznań, 22.03.2024 r.

Prof. UAM dr hab. Małgorzata Słocińska
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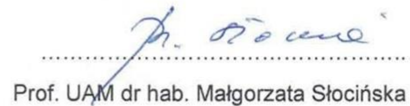

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