Physiological and ecological implications of sequestered cardenolides in the milkweed bugs (Heteroptera: Lygaeinae)

Dissertation to obtain the doctoral degree of Natural Sciences (Dr. rer. nat.)

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“mein Laboratorium ist die Natur”
Ernst Stahl (1848-1919)

“I have no good ideas, but I have got good data to support other people's ideas.”
Thomas Eisner (1929-2011)

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1. The dissertation submitted on the topic ***"Physiological and ecological implications of sequestered cardenolides in the milkweed bugs (Heteroptera: Lygaeinae)"*** is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

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2022.07.15 _____

Place, Date

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Publications and Presentations arising from thesis

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Contribution to the chapters

For Chapter 2, the oxidative stress assays were carried out by Cecilia Heyworth in the lab of Prof. Jonathan D. Blount as a part of her master's thesis at the University of Exeter, United Kingdom.

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Zusammenfassung

Im Laufe der Evolution brachten Pflanzen ein breites Spektrum an bioaktiven, chemischen Verbindungen (Pflanzentoxine) hervor, um sich gegen Antagonisten, darunter eine Vielzahl von pflanzenfressenden Insekten, zu schützen. Dabei ist bemerkenswert, dass viele Insekten Pflanzentoxine in ihrer Nahrung nicht nur tolerieren können, sondern sogar dazu in der Lage sind, die Verbindungen zu ihrem eigenen Vorteil zur Abwehr von Fressfeinden und Parasitoiden zu speichern (Sequestrierung). Um die physiologischen sowie ökologischen Auswirkungen von Pflanzentoxinen auf die Evolution der Pflanzen-Insekten-Fressfeind-Interaktion zu verstehen, ist die Untersuchung beider Anpassungen -Resistenz und Sequestrierung- erforderlich. Die chemische Abwehr geht zudem meist mit auffälligen Farben und Mustern einher, welche als Warnsignale dienen, um Fressfeinde auf die Ungenießbarkeit der Insekten aufmerksam zu machen (Aposematismus).

Ritterwanzen (Heteroptera: Lygaeinae) sind häufig generalistische Samenräuber und weisen eine charakteristische schwarz-rote Färbung auf. Die Wanzen bevorzugen Samen toxischer Pflanzen aus der Familie der Apocynaceae (Hundsgiftgewächse). Diese enthalten Cardenolide, welche die ubiquitär vorkommende Na^+/K^+-ATPase hemmen, die essenzielle physiologische Funktionen im tierischen Organismus einnimmt. Lygaeinae besitzen aufgrund einiger Aminosäure-Substitutionen in der Na^+/K^+-ATPase eine ausgeprägte Resistenz gegenüber den toxischen Cardenoliden und sequestrieren diese zum Schutz vor Fressfeinden.

Die übergeordnete Fragestellung meiner Dissertation ist, ob die Sequestrierung von Toxinen bei aposematischen Vertretern der Lygaeinae physiologische (z. B. Wachstum, Lebensspanne, Fruchtbarkeit, Produktion von Farbpigmenten und Umgang mit oxidativem Stress) und ökologische Kosten (z. B. Universalität der Toxin vermittelten Abwehr) verursacht. Mithilfe einer artifiziellen Diät, welche Cardenolide in drei ansteigenden Konzentrationen enthielt, zog ich Nymphen von vier Lygaeinae-Arten (*Oncopeltus fasciatus*, *Caenocoris nerii*, *Arocatus longiceps* und *Spilostethus pandurus*) und eine eng verwandte Feuerwanzenart (*Pyrrhocoris apterus*) bis zum Adultstadium auf. Das Wachstum wurde bei allen Arten durch Gewichtsanalysen verfolgt, weitere Fitnessparameter wurden nur für *Oncopeltus fasciatus* ermittelt. Farbintensität von *O. fasciatus* sowie oxidativer Stress wurden mittels Bildaufnahmen und biochemischen Methoden zum Nachweis von Lipidperoxidation (Malondialdehyd oder MDA), Superoxiddismutase (SOD) und Gesamtglutathiongehalt (GSH) in allen Nymphen-Stadien bis hin zum adulten Tier gemessen. Um zu verstehen, warum ein Schutz gegen bestimmte Prädatoren nicht bei allen Wanzenarten nach dem Verzehr herzglykosidhaltiger Samen

beobachtet wird, untersuchte ich, ob das Ergebnis der Räuber-Beute-Interaktion durch die strukturelle Variation innerhalb derselben Verbindungsklasse oder durch die Insektenspezies beeinflusst wurde. Dazu zog ich zwei Arten von Ritterwanzen (*Lygaeus equestris* und *Horvathiolus superbus*) auf Samen zweier phylogenetisch nicht verwandter Wirtspflanzen (Ranunculaceae: *Adonis vernalis* und Plantaginaceae: *Digitalis purpurea*) auf, deren Cardenolide die Wanzen sequestrierten und führte Prädationsexperimente mit Florfliegenlarven durch. Die Menge an sequestrierten Toxinen habe ich mittels Hochleistungsflüssigkeitschromatographie erfasst.

Meine Untersuchungen ergaben, dass die mit der Nahrung aufgenommenen Pflanzentoxine das Wachstum der sequestrierenden Nahrungsspezialisten (*O. fasciatus* und *C. nerii*), nicht aber das der sequestrierenden Nahrungsgeneralisten, *S. pandurus*, beschleunigten, obwohl sie alle Toxin resistente Na^+/K^+-ATPasen besitzen. Unter Toxin-Exposition war das Wachstum der *A. longiceps* Nymphen (resistent und nicht sequestrierend) unbeeinflusst, während das der *P. apterus* (nicht resistent und nicht sequestrierend) beeinträchtigt war. Darüber hinaus erreichten *O. fasciatus* Nymphen unter Toxin-Exposition früher das Adultstadium und lebten zudem länger, als Individuen, die auf einer Kontroll-Diät ohne Toxine aufgezogen wurden. Allerdings produzierten sie deutlich weniger Nachkommen, wenn sie nach Erreichen des adulten Stadiums nicht auf eine Toxin freie Diät umgestellt wurden. Weitergehend konnte ich zeigen, dass jene *O. fasciatus*, welche auf Diät mit hohen und mittleren Cardenolid-Konzentrationen aufgezogen wurden, deutlich geringere GSH-Werte aufwiesen. Wanzen mit höheren GSH-Werten zeigten leuchtendere Signalfarben, die jedoch nicht mit der Sequestrierung korrelierten. Neben physiologischen Aspekten hing die Wahrscheinlichkeit, ob Ritterwanzen den Angriff eines Fressfeindes überlebten, stark von den strukturellen Unterschieden der sequestrierten Toxine ab.

Zusammenfassend konnte ich zeigen, dass Cardenolid-Konsum einen positiven Effekt auf die Gesamtfitness mancher Ritterwanzen hat, was im Widerspruch zur aktuellen Lehrmeinung steht, dass durch Sequestrierung physiologische Kosten verursacht werden. Der oxidative Zustand der Tiere weist dennoch auf potentielle physiologische Kosten der Toxin-Sequestrierung hin. Die Wirkung der Toxin-Sequestrierung auf Fressfeinde wird durch strukturelle Variationen der toxischen Verbindungen zur Abwehr beeinflusst, sie hängt daher vom ökologischen Kontext, sprich der Verwendung bestimmter Wirtspflanzen ab. Die vorliegende Dissertation gibt einen Einblick in die physiologischen und ökologischen Konsequenzen von Sequestrierung bei aposematischen Insekten und verhilft zu einem besseren Verständnis der Wechselwirkungen zwischen Pflanzen, Insekten und Fressfeinden.

Summary

Through the course of evolution, plants have evolved a broad range of bioactive chemical compounds (i.e., plant toxins) to protect themselves against antagonists including a plethora of insect herbivores. Strikingly, many insects cannot only cope with plant toxins in their diet but also store the compounds for their own benefit to ward off predators and parasitoids (aka sequestration). Thus, sequestering insects exploit their host-plants in at least two ways- as a dietary resource and as source of chemical defense. Integration of both adaptations (i.e., resistance and sequestration) is required to understand the physiological and ecological implications of plant toxins on the evolution of plant-insect-predator interactions. Regarding interactions with higher trophic levels, chemical defense is mostly associated with conspicuous colors and patterns, and these are considered warning signals, that alert predators to their unpalatability as food - aposematism.

Milkweed bugs (Heteroptera: Lygaeinae) have a predilection for toxic plants, and possess a distinctive black and red coloration. Although many milkweed bugs are generalist seed predators, they commonly feed on plants in the family Apocynaceae (milkweed) which often contain toxic cardenolides. Cardenolides inhibit the ubiquitous Na^+/K^+-ATPase, an essential animal enzyme mediating essential physiological functions. Milkweed bugs possess pronounced insensitivity towards cardenolides due to a few amino acid substitutions in the Na^+/K^+-ATPase (i.e., target site insensitivity) and sequester cardenolides for protection against their predators.

The overarching question remains whether chemical defenses, in aposematic individuals sequestering toxins, incur physiological costs, such as effects on growth or other fitness parameters like longevity and fecundity, production of color pigments, and handling oxidative stresses, and/or ecological costs, such as universality of toxin defense. Using an artificial diet, I raised larvae of four milkweed bug species (*Oncopeltus fasciatus*, *Caenocoris nerii*, *Spilostethus pandurus* and *Arocatus longiceps*) and a closely related pyrrhocorid bug species (*Pyrrhocoris apterus*) on three increasing dietary doses of cardenolides, and assessed the increase in growth by recording the mass until adult. Additionally, I investigated the life-history parameters only in *O. fasciatus*. To understand if milkweed bugs exhibit honest signaling, using same artificial diet treatment, the color intensity of *O. fasciatus* was measured by taking photographs in each larval stage until adulthood. To understand if toxin sequestration in milkweed bugs imposes oxidative stress, biomarkers of oxidative stress was measured through biochemical assays for lipid

peroxidation (malondialdehyde, or MDA), superoxide dismutase (SOD), and total glutathione content (GSH). To understand why protection against certain predators is not observed in all bug species although they feed on seeds containing cardenolides, I tested if the outcome of predator-prey interaction was mediated by the structural variation within the same class of compound or by the insect species. For this purpose, I raised two milkweed bug species (*Lygaeus equestris* and *Horvathiolus superbus*) on the seeds of two phylogenetically unrelated host plants (Ranunculaceae: *Adonis vernalis* and Plantaginaceae: *Digitalis purpurea*) from which the bugs sequestered cardenolides, and carried out predation assays with lacewing larvae. The amount of toxins sequestered by the milkweed bugs was estimated using high performance liquid chromatography.

My research revealed that dietary plant toxins increased growth in the sequestering specialists (*O. fasciatus* and *C. nerii*) but not in the sequestering generalist, *S. pandurus*, despite all possessing toxin-resistant Na^+/K^+-ATPases. Under exposure to the dietary toxins, the growth of *A. longiceps* nymphs (resistant and non-sequestering) was unaffected, while that of *P. apterus* (non-resistant and non-sequestering) was impaired. In addition, *O. fasciatus* nymphs developed to adults faster and lived longer as adults under toxin exposure when compared to individuals raised on the control diet, but produced significantly fewer offspring unless being transferred to a toxin-free diet after reaching adulthood. Furthermore, I showed that *O. fasciatus* raised on the high and medium levels of dietary cardenolides had significantly lower levels of GSH. Bugs with more GSH levels had brighter warning signals but these signals were not related to sequestration. Besides physiological aspects, the chance of milkweed bugs surviving a predator attack strongly depended on the structural differences of sequestered toxins.

Overall, I found that cardenolide consumption exerts a positive effect on overall fitness in milkweed bugs, a conclusion in disagreement with current theory predicting costs of sequestration. Oxidative state may be a fundamental aspect where costs lie in aposematic individuals sequestering toxins, and the effect of plant-toxin sequestration on predators is affected by the structural variation of defensive compounds and therefore depends on the ecological context, i.e., host-plant use. My dissertation provides insight into the implications of physiology and ecology on sequestering aposematic insects, giving us a better understanding of plant-insect-predator interactions.

General Introduction

Background

Plants and the herbivores that feed on them dominate biodiversity on land. Plants have evolved refined mechanisms to cope with herbivorous insects, and in turn, insects have developed adaptations to overcome plants' defenses. Apart from the physical defenses such as thorns, latex, trichomes, etc., plants also possess a myriad of secondary metabolites (i.e., plant toxins) that enables escape from insects. However, insects evolve and adapt to the plants producing specific toxins. This reciprocity of adaptations led to speciation, giving rise to existing biological diversity (Futuyma and Agrawal, 2009).

Although plants and insects possess their own specific and individual traits, some evolve to be intertwined, leading to the arise of coadapted strategies like the sequestration of plant toxins and aposematic coloration among insects. This thesis centers on the physiological and ecological implications of sequestered plant toxins in specialist herbivorous insects.

Sequestration Of Plant Toxins

In non-adapted insects, plant toxins deter herbivory, inhibit digestion (Fürstenberg-Hägg et al., 2013), and/or directly act on a specific toxin-receptor (Mithöfer and Boland, 2012). In adapted insects, many species accumulate plant toxins while feeding on their hosts, using them for defense against antagonists, a phenomenon called sequestration (Duffey, 1980). Classically, sequestration is defined as the process of selective uptake (Frick and Wink, 1995; Willinger and Dobler, 2001), transport (Strauss et al., 2013), modification (Heckel, 2014), storage (Zagrobelny et al., 2014) and deployment of plant toxins (Duffey et al., 1978; Bramer et al., 2017) for the insects' own defense. In brief, sequestration is the uptake of toxins from plants (i.e., the first trophic level) by insects (i.e., the second trophic level) to protect themselves against parasitoids and predators (i.e., the third trophic level) (Petschenka and Agrawal, 2016).

Lincoln Brower published the first study on anti-predator effects of sequestered cardenolides in aposematic monarch butterflies (*Danaus plexippus*). He demonstrated that toxic monarch butterflies, developing from the caterpillars that consumed cardenolide-rich milkweed

plants (*Asclepias* spp.), induced emesis in blue jays (Brower, 1969). Since then, various studies have shown that toxin sequestration is a common phenomenon found in more than 275 insect species sequestering toxins from at least 40 plant families (Opitz and Müller, 2009; Beran and Petschenka, 2022).

Aposematic Coloration

Chemical defenses are frequently associated with characteristics colors and patterns. This conspicuousness in prey are advertisements or warning signals to alert predators to their unpalatability as food, a phenomenon known as aposematism (Ruxton et al., 2019). Aposematic theory posits that predators learn to avoid toxic or distasteful prey when the prey is more conspicuous, creating a selective pressure for toxic prey to be as distinctive as possible (Sherratt, 2002).

Aposematism is observed in a diverse array of animal groups such as frogs (Summers and Clough, 2001), birds (Dumbacher et al., 2008), snakes (Kikuchi et al., 2014), and various orders of insects. For example, paper wasps (*Polistes dominula*) have black and yellow color patterns, and the wasps with brighter colors have larger venom glands (i.e., containing more defensive toxins) (Vidal-Cordero et al., 2012). Ladybird beetles such as *Harmonia axyridis* and *Coccinella septempunctata* are bright red with black spots and are defended with alkaloids (Bezzerides et al., 2007; Blount et al., 2012). The benefits of chemical defense against predator attack have most likely enabled the evolution from ancestrally cryptic appearance to more conspicuous coloration in many species (Sherratt and Beatty, 2003). Evidence for this evolutionary advance can be seen in phylogenetics; for example, the warning colors in *Papilio* spp. have evolved at least four times (Prudic et al., 2007).

Cost Of Defense

A crucial question remains: does chemical defenses incur costs in aposematic individuals? If an animal synthesizes chemical defense compounds, there are costs in expressing the necessary enzymes for toxin production, the energy required to store the toxins, and preventing autotoxicity. In sequestration, some of these costs are avoided by obtaining the toxins from host-

plants, but other associated costs are present, for example transportation of toxins across the gut and the metabolism (biotransformation) of toxins. Together with these costs, there is also a cost of toxin resistance such as target site mutation. Although of great importance, less is known about the cost-benefit outcome of different mechanisms involved in chemical defense. However, in the burnet moth (*Zygaena filipendulae*), the cost of *de novo* synthesis of cyanogenic glucosides is higher compared to the cost of sequestration (Fürstenberg-Hägg et al., 2014). Moreover, *de novo* synthesis and sequestration of cyanogenic glucosides may trade-off, as shown for *Heliconius* butterflies, indicating costs of both strategies (Engler-Chaouat and Gilbert, 2007).

The production costs of warning signals would guarantee that individuals honestly advertise their chemical defenses, i.e., level of toxicity. For example, if a predator learns to avoid an aposematic individual, the prey can afford to invest less in toxicity, since it already benefits from possessing conspicuous coloration (Pfennig et al., 2001). In this scenario, where the evolution of warning signals is unstable, a prey will honestly advertise its toxicity using its coloration only if the production of the warning signal is costly - thus, a mimic would not be able to cope with high levels of toxicity (Guilford and Dawkins, 1993). If a prey advertises defense using warning signals, naïve predators would be attracted; therefore, a non-toxic conspicuous prey would face higher predation rates than cryptic prey (Zahavi, 1977). If this is the case, the cost of conspicuous coloration in increasing potential attacks by predators can only be tolerated by the most toxic prey (Speed et al., 2010).

Furthermore, the production of color pigments is costly (Srygley, 2004). Specific pigment molecules are known to have antioxidant properties (Oettl and Reibnegger, 2002), and some of these pigments are employed by aposematic species in their signals (McGraw, 2005; Griffith et al., 2006). In the resource competition model (Blount et al., 2009), the cost of chemical defenses in aposematic individuals is assumed to be in the form of oxidative stress. Therefore, there exists a tradeoff in which an individual must allocate pigment molecules either to their coloration, or use them to combat against potential oxidative stress caused by sequestering, producing, and/or maintaining chemical defenses.

Cardenolide Sequestration In Aposematic Milkweed Bugs

Cardenolides are produced by ten different plant families with a great diversity (more than 500) of chemical structures (Malcolm, 1991; Luckner and Wichtl, 2000). Cardenolide toxicity is due to the specific inhibition of the ubiquitous enzyme Na^+/K^+-ATPase (Lingrel, 1992; Emery et al., 1998). The Na^+/K^+-ATPase is a cation carrier essential for major physiological functions such as the generation of neuronal action potentials and maintenance of an electrochemical gradient across the cell membrane (Jorgensen et al., 2003), and cardenolides bind to the extracellular domain of the Na^+/K^+-ATPase α-subunit (ATPα1) (Kaplan, 2002; Li and Langhans, 2015).

Insects in at least six orders, including milkweed bugs (Heteroptera: Lygaeinae), milkweed butterflies (Lepidoptera: Danaini), and certain leaf beetles (Coleoptera: Chrysomelidae) show common adaptations to counter cardenolide toxicity (Dobler et al., 2015). These groups possess modified forms of Na^+/K^+-ATPases that are resistant to cardenolides. A few amino acid substitutions mediate resistance in the first extracellular loop of the Na^+/K^+-ATPase α-subunit, a phenomenon referred to as target-site insensitivity. This mechanism of resistance to cardenolides resulted in a high level of molecular convergence, i.e., often identical amino acid substitutions at the same positions mediating resistance in varied taxa (Dobler et al., 2012; Zhen et al., 2012).

My thesis focuses on milkweed bugs, a diverse group of over 600 species occurring on five continents (Slater and O'Donnell, 1995). The most intriguing feature of this group is their predilection for toxic plants. They are seed feeders that primarily use the cardenolide-producing plant family, Apocynaceae and cardenolide-producing plants from unrelated families, as host plants (Petschenka et al., 2022). They sequester cardenolides to ward off predators (Evans et al., 1986); for example, species that are found consuming Apocynaceae include *Oncopeltus fasciatus* (Dallas, 1852) on *Asclepias* spp., *Caenocoris nerii* (Germar, 1847) on *Nerium oleander* and *Spilotethus pandurus* (Scopoli, 1763) on *N. oleander* and *Calotropis* spp. Species are found on unrelated cardenolide-producing plant families include *Lygaeus equestris* (Linnaeus, 1758) on

Adonis vernalis (Ranunculaceae) (Kugelberg and Solbreck, 1972; Rabitsch and Deckert, 2007) and *Horvathiolus superbus* (Pollich, 1781) on *Digitalis spp.* (Plantaginaceae) (Wachmann et al., 2004; Aukema et al., 2005). Milkweed bugs also possess morphological adaptations in a double-layered epidermis to sequester and deploy a cardenolide-rich secretion under predator attack (Scudder and Meredith, 1982; Bramer et al., 2017). Cardenolide resistance and sequestration are most likely ancestral features in the milkweed bugs, which may account for the species' evolutionary success (Bramer et al., 2015). Besides mechanisms to sequester plant toxins, milkweed bugs also exhibit conspicuous coloration with black and red color patterns, warning signals that indicate potent defenses to predators (Sillén-Tullberg, 1985; Mappes et al., 2005).

Research Questions

Under an ecological view, the costs of possessing defenses are assumed to be compensated by increased protection against predators (Bowers, 1992; Camara, 1997). In other words, the costs of chemical defenses are often outweighed by their benefits, and such costs are not always easy to detect and estimate (Lindstedt et al., 2010). The evidence for potential costs such as effects on growth or other fitness parameters including longevity and fecundity, production of color pigments, and handling oxidative stresses is very scarce (Zvereva and Kozlov, 2016). Therefore, understanding the costs of chemical defense will require comparative analyses integrating a variety of physiological and ecological parameters. This dissertation broadly examines the following questions:

i) Are there physiological costs of cardenolide sequestration in milkweed bugs?

ii) Are there costs of signaling in aposematic milkweed bugs? Do the bugs show honest quantitative signaling?

iii) Do the costs of sequestration in milkweed bugs compensate universally in terms of protection against predators?

i) Are there physiological costs of cardenolide sequestration in milkweed bugs?

I designed an experiment to disentangle the effects of the traits cardenolide resistance and sequestration on growth by comparing four milkweed bug species and the European firebug

Pyrrhocoris apterus. These species have different combinations of both traits (i) non-resistant Na^+/K^+-ATPases and no sequestration (*P. apterus*), (ii) resistant Na^+/K^+-ATPases but no sequestration (*A. longiceps*), or (iii) both resistant Na^+/K^+-ATPases and sequestration (*O. fasciatus, C. nerii* and *S. pandurus*. Moreover, I tested if dietary cardenolides also influence additional life-history parameters such as developmental time and fecundity in *O. fasciatus*. Discerning the possible costs of toxin sequestration will advance our understanding to which extent the insect species would specialize on the toxic host plant.

ii) Are there costs of signaling in aposematic milkweed bugs? Do these bugs show honest quantitative signaling?

In some aposematic species the intensity or brightness of their signal correlates either positively (Bezzerides et al., 2007; Vidal-Cordero et al., 2012) or negatively (Darst et al., 2006; Wang, 2011) with their toxicity. Milkweed bugs vary in their quantity and quality of sequestered cardenolides in nature (Isman et al., 1977). Moreover, the color intensity of the black and red patterns in the milkweed bugs also varies (Rodríguez-Clark, 2004). In *O. fasciatus*, the primary red pigments are pteridines (such as xanthopterin, isoxanthopterin, and 2-amino-4-hydroxypteridine), and pterins (such as erythropterin) (Good and Johnson, 1949; Bartel et al., 1958; Hudson et al., 1959), and pigments have been shown to have antioxidant properties (McGraw, 2005). As the resource competition model aims to explain both the positive and negative correlations between defense and signal depending on the resource state (Blount et al., 2009), *O. fasciatus* is an ideal species to test the model. If it is not costly for milkweed bugs to sequester cardenolides, their variations in defense and signal quality are unexplained. They may then be honestly signaling their level of defense, allocating antioxidants between pigments and against oxidative stress. Furthermore, Blount's model assumes a physiological association between defense and signal quality in which a shared resource is depleted in the production and maintenance of both traits. This work examines this assumption of the model.

Approach To Estimate Costs

It is challenging to estimate the potential costs that an organism bears. I selected cardenolide sequestration as a single factor manipulation to measure costs (if any) in milkweed bugs, as single factor manipulation is the most reliable and informative method (Stearns, 1992). For the work described in Chapters 1 and 2, I established an artificial diet and raised milkweed bug larvae on three increasing doses of cardenolides in the diet until adulthood (Figure 1). The advantages of artificial diet include uniform nutrition and, in my case, manipulation with a desired dose of toxins, enabling me to carry out reductionist (i.e., without a complex and potentially interfering chemical environment) assays to test ecological and evolutionary hypotheses. In Chapter 1, I assessed the increase in growth by recording the larval mass and investigated additional life-history parameters. In Chapter 2, we (in collaboration with C. Heyworth) carried out experiments to test if there is a link between the level of defense (i.e., amount of sequestered cardenolides), signal quality (i.e., color intensity), and oxidative stress levels due to toxin sequestration in the milkweed bugs. We measured the color intensity by taking photographs in each larval stage until adulthood. Additionally, we measured the oxidative stress through biochemical assays for lipid peroxidation (malondialdehyde, or MDA), superoxide dismutase (SOD), and total glutathione content (GSH).

Figure 1: *Oncopeltus fasciatus* adults feeding on the artificial diet

iii) Do the costs of sequestration in milkweed bugs compensate universally in terms of protection against predators?

The effects of structurally diverse cardenolides against different natural enemies remain largely unknown. It was recently shown that the European milkweed bugs, *H. superbus* and *L. equestris* were protected against insectivorous birds when they sequestered cardenolides from the seeds of *D. purpurea* and *A. vernalis*, respectively (Petschenka et al., 2022). However, only *H. superbus* larvae gained protection against larvae of the predatory lacewing *Chrysoperla carnea*; this outcome was unexpected because both insect species had sequestered cardenolides, although from different plant species. To investigate this further, I used a full factorial design to test whether this outcome was mediated by structural differences in plant chemistry between *D. purpurea* and *A. vernalis* or by the insect species. Specifically, I raised both species of milkweed bugs on the seeds from both species of host plants and carried out predation assays with lacewing larvae. This work investigates on the mechanism that mediates different outcomes against predators within the same class of toxins.

Quantification Of Cardenolides

Across all chapters/objectives, the amount of toxins sequestered by the milkweed bugs was estimated using high performance liquid chromatography (HPLC).

Thesis Aims

To conclude, this thesis aims to investigate various aspects of cardenolide sequestration in aposematic milkweed bugs across different scales, including

i) the physiological costs of sequestration (Chapter 1),

ii) mechanistic linkages between sequestration and warning signals (Chapter 2), and

iii) non-universal benefits of sequestration against predators (Chapter 3).

Chapter 1 - Physiological Costs Of Sequestration

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RESEARCH ARTICLE

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

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Abstract

Sequestration, that is, the accumulation of plant toxins into body tissues for defense, was predicted to incur physiological costs and may require resistance traits different from those of non-sequestering insects. Alternatively, sequestering species could experience a cost in the absence of toxins due to selection on physiological homeostasis under permanent exposure of sequestered toxins in body tissues. Milkweed bugs (Heteroptera: Lygaeinae) sequester high amounts of plant-derived cardenolides. Although being potent inhibitors of the ubiquitous animal enzyme Na^+/K^+-ATPase, milkweed bugs can tolerate cardenolides by means of resistant Na^+/K^+-ATPases. Both adaptations, resistance and sequestration, are ancestral traits of the Lygaeinae. Using four milkweed bug species (Heteroptera: Lygaeidae: Lygaeinae) and the related European firebug (Heteroptera: Pyrrhocoridae: *Pyrrhocoris apterus*) showing different combinations of the traits "cardenolide resistance" and "cardenolide sequestration," we tested how the two traits affect larval growth upon exposure to dietary cardenolides in an artificial diet system. While cardenolides impaired the growth of *P. apterus* nymphs neither possessing a resistant Na^+/K^+-ATPase nor sequestering cardenolides, growth was not affected in the non-sequestering milkweed bug *Arocatus longiceps*, which possesses a resistant Na^+/K^+-ATPase. Remarkably, cardenolides increased growth in the sequestering dietary specialists *Caenocoris nerii* and *Oncopeltus fasciatus* but not in the sequestering dietary generalist *Spilostethus pandurus*, which all possess a resistant Na^+/K^+-ATPase. We furthermore assessed the effect of dietary cardenolides on additional life history parameters, including developmental speed, longevity of adults, and reproductive success in *O. fasciatus*. Unexpectedly, nymphs under cardenolide exposure developed substantially faster and lived longer as adults. However, fecundity of adults was reduced when maintained on cardenolide-containing diet for their entire lifetime but not when adults were transferred to non-toxic sunflower seeds. We speculate that the resistant Na^+/K^+-ATPase of milkweed bugs is selected for working optimally in a "toxic environment," that is, when sequestered cardenolides are stored in the body.

KEYWORDS

cardenolides, fitness costs, life history traits, milkweed bugs, Na^+/K^+-ATPase, sequestration, trade-off

1 | INTRODUCTION

Chemical defenses are widespread among animals and remarkably diverse across species. Many insect herbivores accumulate secondary metabolites from their host plants and utilize them for their defense to ward off natural enemies, a phenomenon called sequestration (Opitz & Müller, 2009; Petschenka & Agrawal, 2016). For example, caterpillars of the monarch butterfly (*Danaus plexippus*) sequester cardenolides from milkweed plants (*Asclepias* spp.). On the contrary, other insects produce their toxins via *de novo* synthesis as observed in leaf beetles (Chrysomelidae) (Pasteels et al., 1988) or butterflies producing cyanogenic glycosides (Heliconiinae) (Brown & Francini, 1990). Sequestration of toxins from plants and *de novo* synthesis of toxins may trade-off evolutionarily as was suggested for *Heliconius* butterflies (Engler-Chaouat & Gilbert, 2007) indicating a cost of both strategies. Under an ecological view, costs of possessing defenses are assumed to be compensated by gained protection against predators (Bowers, 1992; Camara, 1997).

It was speculated that the physiological costs of *de novo* synthesis of defensive compounds are higher compared to the costs of sequestration of plant toxins (Fürstenberg-Hägg et al., 2014; Zvereva & Kozlov, 2016), which may explain why sequestration is a common phenomenon currently reported for more than 250 insect species acquiring toxins from at least 40 plant families (Opitz & Müller, 2009). Nevertheless, sequestration of chemical defenses may incur physiological costs (Camara, 1997; Reudler et al., 2015) since sequestering insects are exposed to high concentrations of toxins stored within their body tissues. In accordance, physiological costs of toxin resistance (here: insecticides) such as reduced energetic resources, lifespan, and fecundity have been shown in several insects including mosquitoes (Carriere et al., 1994; Rivero et al., 2011) and bed bugs (Gordon et al., 2015). Besides physiological costs, sequestration may interfere with the insect immune system as indicated by a compromised immune response of buckeye caterpillars (*Junonia coenia*) dependent on the amount of iridoid glycosides in their diet (Smilanich et al., 2009). Contrarily, dietary toxins were also suggested to interact positively with the immune system of sequestering insects such as caterpillars of the monarch butterfly (Tan et al., 2019) and the tobacco hornworm (*Manduca sexta*) (Garvey et al., 2021) indicating system-specific differences. However, empirical evidence on the benefits of defenses is more apparent than their costs, and the costs of chemical defenses are not always easy to detect (Lindstedt et al., 2010; Ruxton, 2014). In line with this, evidence for actual physiological costs such as negative effects on growth or other fitness parameters like longevity and fecundity is very scarce (Zvereva & Kozlov, 2016) and understanding the costs of sequestration will require reductionist comparative analyses integrating a diversity of physiological and ecological parameters.

Trade-offs play a crucial role in an organism's life history and occur when a beneficial change in one trait is linked to an unfavorable change in another trait causing a cost (Stearns, 1989). Life history theory suggests that fitness determining traits such as longevity and fecundity are negatively associated with each other (Flatt, 2011; Holliday, 1994). However, results are contradictory with studies showing positive, negative, or zero correlation between these two traits among individuals within a population (Bell, 1986; Van Noordwijk & de Jong, 1986). Generally, life history trade-offs result from compromises in resource allocation across growth, survival, maintenance, and reproduction under challenges such as predation occurring in an ecosystem (Levins, 1968; Roff, 1992; Sibly & Calow, 1986; Walsh & Reznick, 2010). Regarding chemical defense, an organism's potential physiological costs may be estimated as trade-offs between investments in defense and other physiological parameters such as growth, longevity, or fecundity (Camara, 1997; Ruxton et al., 2019).

Cardenolides are produced by more than 10 different plant families (Luckner & Wichtl, 2000; Malcolm, 1991) and are toxic to animals because they specifically inhibit the ubiquitous enzyme Na^+/K^+-ATPase (Emery et al., 1998; Lingrel, 1992). Na^+/K^+-ATPase is a cation carrier responsible for essential physiological functions such as the generation of neuronal action potentials and maintenance of an electrochemical gradient across the cell membrane (Jorgensen et al., 2003). Remarkably, insects from at least five orders, including milkweed bugs (Heteroptera: Lygaeinae), milkweed butterflies (Lepidoptera: Danaini), and leaf beetles (Coleoptera: Chrysomelidae), show common adaptations to cardenolides (Dobler et al., 2015). Resistance in these groups is mediated by target site insensitivity due to a few amino acid substitutions in the first extracellular loop of the alpha subunit of Na^+/K^+-ATPase (ATPα), resulting in a high level of molecular convergence, that is, often the identical amino acid substitutions at the same positions confer resistance (Dobler et al., 2012, 2015; Zhen et al., 2012).

Milkweed bugs are seed feeders primarily found on Apocynaceae species and on unrelated cardenolide-producing plants (Petschenka et al., 2020). Besides feeding on cardenolide-containing plants, milkweed bugs also sequester cardenolides to ward off predators (Evans et al., 1986; Pokharel et al., 2020). Alteration of the Na^+/K^+-ATPase in the Lygaeinae is probably correlated with several duplications of the ATPα1 gene resulting in four ATPα1 paralogs (A, B, C, and D) found in *Oncopeltus fasciatus* and *Lygaeus kalmii* (Dalla & Dobler, 2016; Yang et al., 2019; Zhen et al., 2012). Moreover, cardenolide-resistant Na^+/K^+-ATPases and the ability to sequester cardenolides are most likely synapomorphic traits of the Lygaeinae, which may account for the milkweed bugs' evolutionary success (Bramer et al., 2015).

The goal of our study was to evaluate if cardenolide exposure and sequestration causes physiological costs or benefits, and if these effects differ across closely related milkweed bug species possessing different combinations of the traits "resistance" and "sequestration" (i.e., having resistant/sensitive Na^+/K^+-ATPases and sequestering/not sequestering). Our set of species included dietary specialist and generalist milkweed bug species as well as the European linden bug *Pyrrhocoris apterus* (Linnaeus, 1758, Pyrrhocoride) having no adaptations to cardenolides for comparison (Figure 1).

The milkweed bug species we used were *O. fasciatus* (Dallas, 1852), *Caenocoris nerii* (Germar, 1847) (both resistant and sequestering, dietary specialists), *Spilotethus pandurus* (Scopoli, 1763) (resistant and sequestering, dietary generalist), and *Arocatus longiceps* (Stal, 1872) (resistant and not sequestering, dietary specialist). As Stearns (1992) considers manipulating a single factor the most reliable and informative method to measure costs (or trade-offs, if any), we manipulated cardenolide concentration in the diet as a single factor. For this purpose, we established an artificial diet approach and raised larvae on increasing doses of cardenolides in the diet. We assessed growth over the course of development. Furthermore, we determined the amount of sequestered cardenolides using liquid chromatography (HPLC-DAD).

To test for effects on potential trade-offs between life history parameters due to dietary cardenolides, we investigated the influence of dietary cardenolides on the longevity and fecundity in *O. fasciatus*. Trade-offs have been measured in the field (Clutton-Brock, 1982; Clutton-Brock et al., 1983) and in the laboratory (Partridge & Farquhar, 1981), for example, by genotypic studies in *Drosophila melanogaster* (Rose & Charlesworth, 1981a, 1981b), and by phenotypic studies in *Daphnia pulex* and *Platyias patulus* (Bell, 1984a, 1984b). How different traits will interact depends on ecological factors (e.g. nutrition or predation) and the physiological state (e.g. developmental stage or fecundity) of an organism. Thus, trade-offs can change across different environments in different species or even within the same species. Therefore, a life history trade-off probably may only appear in a species under a particular set of conditions, such as stress (Reznick, 1985).

To explicitly investigate the influence of dietary toxins in milkweed bug species, we set out to test the following hypotheses: (i) insect species having different physiological traits (resistance and sequestration) and ecological strategies (generalist and specialist) will react differently to dietary toxins, (ii) sequestering species will experience costs, either in the presence or absence of toxins, and (iii) the fecundity-longevity trade-off will be altered by dietary toxins.

2 | MATERIALS AND METHODS

2.1 | Preparation of artificial diet

We followed Jones et al.'s method to prepare an artificial diet for *Oncopeltus fasciatus* (Jones et al., 1986) but used a modified approach to offer the diet to the bugs. Sunflower seeds (25 g), wheat germ (25 g), casein (25 g), sucrose (10 g), Wesson's salt (4 g), vitamins (Vanderzant Vitamin mix, 5 g), methyl 4-hydroxybenzoate (1 g), sorbic acid (0.5 g), olive oil (7.5 g), and toxins (only for the treatment groups, not for controls) were blended in 200 ml of water until the mixture was homogenous. Agar (7.5 g) was boiled separately in 300 ml of water in a microwave. After 5 min, when the agar had slightly cooled down, the agar and the mixture were combined and

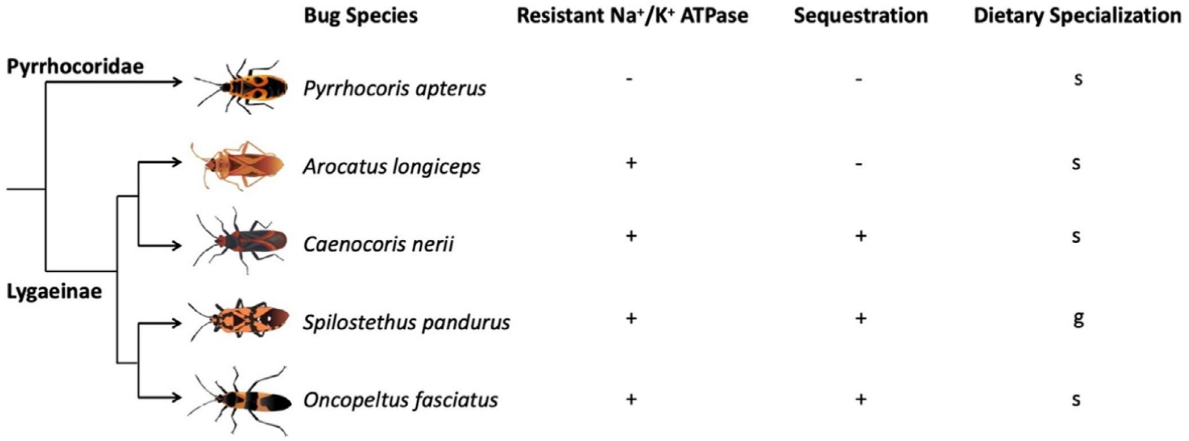

FIGURE 1 Overview of the Heteroptera species used in the experiments with their key traits. We compared four species of Lygaeinae sharing cardenolide-resistant Na^+/K^+-ATPase (+ = resistant; − = sensitive) but differing in their ability to sequester cardenolides (+ = sequestering; − = not sequestering). Within the sequestering milkweed bug species, *Oncopeltus fasciatus* and *Caenocoris nerii* may be classified as host-plant specialists (s = specialist), while *Spilostethus pandurus* uses a wide variety of host plants (g = generalist). *Arocatus longiceps* is specialized on *Platanus* and *Ulmus* which are devoid of cardenolides, and lost its ability to sequester cardenolides in the course of evolution. *Pyrrhocoris apterus* belongs to the relatively closely related family Pyrrhocoridae and is specialized on cardenolide-free Malvaceae. Furthermore, it is known to possess a sensitive Na^+/K^+-ATPase, and, based on our recent analyses, does not sequester cardenolides. Phylogenetic relationships of Heteroptera species are based on Bramer et al. (2015)

blended again. The obtained paste was poured into plastic boxes and stored at 4°C for further use. For the feeding assays, an aliquot of diet was filled into the lid of a 2 ml Eppendorf tube and sealed with a piece of stretched parafilm to be used as an "artificial seed." Using portions from the same initial bulk of the diet, we added increasing amounts of an equimolar mixture of crystalline ouabain and digitoxin (Sigma-Aldrich, Taufkirchen, Germany) to prepare diets with 2 ("low"), 6 ("medium"), or 10 ("high") mg cardenolide/g dry weight of diet. The initial diet without cardenolides added was used as a control. We used the polar ouabain and the relatively apolar digitoxin to mimic the condition that plants typically produce an array of cardenolides with a wide polarity spectrum. The concentration of cardenolides in the diet was chosen to be in the range of natural cardenolide concentrations observed in *Asclepias* seeds (Isman, 1977). For each batch of diet prepared, we verified the concentrations of cardenolides across all dietary treatments by using high-performance liquid chromatography (HPLC, Section 2.2).

2.2 | Quantification of cardenolides

To verify the amount of cardenolides in the artificial diet, cubes of diet (approx. 20–25 mg dry weight) were freeze dried, weighed, and added to a 2 ml screw-cap tube containing approximately 900 mg of zirconia/silica beads (ø 2.3 mm, BioSpec Products, Inc., Bartlesville, OK, US). One ml HPLC-grade methanol containing 0.01 mg/ml of oleandrin (PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany) as an internal standard was added to the tube, and diet samples were homogenized for two cycles of 45 s at 6.5 m/s in a Fast Prep™ homogenizer (MP Biomedicals, LLC, Solon, OH, US). After centrifugation at 16,100 g for 3 min, supernatants were transferred into fresh tubes. Original samples were extracted two more times with 1 ml of pure methanol as described above. All the supernatants of a sample were combined, evaporated to dryness under a stream of nitrogen gas, and resuspended with 100 µl methanol by agitating tubes in the Fast Prep™ homogenizer without beads. Subsequently, samples were filtered into HPLC vials using Rotilabo ® syringe filters (nylon, pore size: 0.45 µm, ø 13 mm, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Finally, 15 µl of the extract was injected into an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, US) equipped with a photodiode array detector, and compounds were separated on an EC 150/4.6NUCLEODUR® C18 Gravity column (3 µm, 150 mm × 4.6 mm, Macherey-Nagel, Düren, Germany). Cardenolides were eluted at a constant flow rate of 0.7 ml/min at 30°C using the following acetonitrile–water gradient: 0–2 min 10% acetonitrile, 13 min 95% acetonitrile, 18 min 95% acetonitrile, 23 min 10% acetonitrile, and 5 min reconditioning at 10% acetonitrile. The same HPLC method was used for quantification of sequestered cardenolides in *O. fasciatus* and *P. apterus*, respectively. For the analysis of sequestered cardenolides in *C. nerii*, *S. pandurus*, and *A. longiceps*, we used a different acetonitrile–water gradient to achieve improved separation of polar cardenolides: 0–2 min 16% acetonitrile, 25 min 70% acetonitrile, 30 min 95% acetonitrile, 35 min 95% acetonitrile, 37 min 16% acetonitrile, and 10 min reconditioning at 16% acetonitrile. We interpreted peaks with symmetrical absorption maxima between 216 and 222 nm as cardenolides (Malcolm & Zalucki, 1996) and integrated peaks at 218 nm using the Agilent ChemStation software (B.04.03). The amount of cardenolides in a sample was quantified based on the peak area of the known concentration of the internal standard oleandrin.

2.3 | Insect colonies

Oncopeltus fasciatus were obtained from a long-term laboratory colony (originally from the United States) maintained on sunflower seeds. We collected specimens of *P. apterus* in the vicinity of linden trees (*Tilia* spp., Malvaceae) and specimens of *A. longiceps* from under the bark of plane trees (*Platanus* spp., Platanaceae) in Giessen, Germany. Specimens of *S. pandurus* and *C. nerii* were collected from a *Nerium oleander* habitat close to Francavilla di Sicilia, Messina, Sicily, Italy. In the laboratory, insect colonies were reared in plastic boxes (19 × 19 × 19 cm) covered with gauze in a climate chamber (Fitotron® SGC 120, Weiss Technik, Loughborough, UK) at 27°C, 60% humidity, and a day/night cycle of 16/8 h under artificial light. We reared all insects on organic sunflower seeds (Alnatura GmbH, Darmstadt, Germany), supplied water in cotton-plugged Eppendorf tubes, and included a piece of cotton wool as a substrate for oviposition. In addition to sunflower seeds, *P. apterus* was provided with approximately 10 freshly chopped mealworms twice a week.

For the experiments described below, we used first-generation offspring from field-collected *P. apterus* and *A. longiceps* (maintained as described above), whereas *S. pandurus* and *C. nerii* offspring were obtained from colonies maintained in the laboratory for more than four generations.

2.4 | Growth assay

We carried out feeding assays to investigate the influence of increasing doses of dietary toxins on the growth of larvae of four species of milkweed bugs (*O. fasciatus*, *C. nerii*, *S. pandurus*, and *A. longiceps*) and an outgroup, *P. apterus*. These species either lack the ability to tolerate and sequester cardenolides (*P. apterus*), possess a cardenolide-resistant Na^+/K^+-ATPase, and can sequester cardenolides (*O. fasciatus*, *C. nerii*, and *S. pandurus*) or possess a cardenolide-resistant Na^+/K^+-ATPase but lost the ability to sequester (*A. longiceps*) (Figure 1). We placed three second instar (L2) larvae from the stock colonies in a Petri dish (60 mm × 15 mm, with vents, Greiner Bio-One, Frickenhausen, Germany) lined with filter paper (Rotilabo® round filters, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) that was supplied with an artificial seed (either being devoid of cardenolides, or possessing a cardenolide concentration of 2, 6, or 10 µg/mg dry weight) and a water source (a 0.5 ml Eppendorf tube plugged with cotton wool). The artificial seeds were replaced once in 2 weeks. All Petri dishes were

spatially randomized and maintained in a controlled environment (KBWF 240 climate chamber, Binder, Tuttlingen, Germany) at 21°C, 60% humidity, and a day/night cycle of 16/8 h under artificial light. The growth of larvae was assessed twice a week over a period of 3 weeks by sedating all bugs of a Petri dish with CO_2 and weighing them jointly. After reaching adulthood, at least one bug per Petri dish was transferred to a toxin-free diet for 10 days to avoid a potential bias from toxins remaining in the gut (i.e., not being sequestered) by purging. Finally, bugs were killed by freezing, freeze-dried, weighed, extracted and analyzed by HPLC to estimate the amount of sequestered cardenolides (Section 2.2). We also estimated the amount of excretion products on filter paper that may provide an indication of food intake (Appendix S1). For *O. fasciatus*, growth assays were carried out in batches. Altogether three experiments ($n = 10$ per treatment for experiments I and II; $n = 5$ per treatment for experiment III) were carried out. Additionally, we also carried out growth assays using a different *O. fasciatus* strain (Figure S3). Sequestration of cardenolides in *O. fasciatus* was only evaluated in specimens from the experiments I and II.

2.5 | Life history assays with *Oncopeltus fasciatus*

2.5.1 | Developmental time

Since the effects of dietary cardenolides on growth were most pronounced in *O. fasciatus*, we carried out a separate experiment to assess additional life history parameters including duration of larval development, adult lifespan, and body size under the influence of dietary cardenolides in this species. We chose the medium-dose cardenolide (6 μg/mg dry weight) because we observed that *O. fasciatus* showed maximal growth on this diet in our previous experiment. The experimental setup was similar to that of the growth assay. However, here only one L2 larva was placed in each Petri dish to monitor the time of larval development. Petri dishes lined with filter paper either containing medium-dose diet or control diet without toxins and a water source (Section 2.4) were spatially randomized and kept in a climate chamber (Fitotron® SGC 120, Weiss Technik, Loughborough, UK) at 27°C, 60% humidity, and a day/night cycle of 16/8 h under artificial light. We checked the Petri dishes every day for dead individuals, raised the bugs until adulthood, and observed them until they died. We also measured the body length of adult males and females raised on the two different diets using a Vernier caliper.

2.5.2 | Reproductive fitness

We conducted two additional experiments to assess the effect of dietary cardenolides on reproductive fitness. We raised L2 larvae in bulk (around 50 individuals) until adulthood in plastic boxes (19 × 19 × 19 cm) covered with gauze either on two artificial seeds of medium-dose or control diet and a water source (four Eppendorf tubes of 2 ml plugged with cotton wool). Boxes were kept in a climate chamber (Fitotron® SGC 120, Weiss Technik, Loughborough, UK) at 27°C, 60% humidity, and a day/night cycle of 16/8 h under artificial light. Artificial seeds were replaced once in 2 weeks. At least 3 (but not older than 6)-day-old males and females from the same treatment were paired in Petri dishes (9 cm × 1.5 cm, with vents, Greiner Bio-One, Frickenhausen, Germany) lined with filter paper and a water source (a 2 ml Eppendorf tube plugged with cotton wool). Additionally, we included a piece of cotton wool as a substrate for oviposition. Petri dishes were spatially randomized and kept under the same conditions as described above. In a first experiment, adult bugs were supplied with the same type of artificial diet that they were raised upon (i.e., either control or medium-dose cardenolide artificial seeds). In nature, adults of *O. fasciatus* disperse after reaching adulthood and forage for other seeds besides *Asclepias* spp. (Feir, 1974). Therefore, we carried out a second experiment under the same conditions as described above in which pairs of bugs were supplied with approx. 20 sunflower seeds instead of artificial seeds. Since a substantial portion of eggs in both experiments were unviable (possibly due to the use of an artificial diet), we counted only hatchlings and not the total number of eggs produced by each female over its entire lifespan. Especially after transfer to sunflower seeds, viable eggs were only produced by 52% of the females (13 of 25) raised on the artificial diet without toxins and by only 39% of the females (10 of 26) raised on the artificial diet with toxins. Remarkably, the proportion of females laying viable eggs was much higher in females remaining on artificial diet (>80%; 13 of 15 females on the control vs. 12 of 15 females on the toxic diet). For statistical analysis, females producing no viable eggs were excluded and their inclusion did not change the direction of the results.

2.6 | Statistical analysis

Statistical analyses were computed using JMP® Pro 15 statistical software (SAS Institute, Cary, NC, US). All data were $1og_{10}$ transformed to achieve homogeneity of variances and normality of residuals. For feeding experiments, we analyzed sequential data on larval masses with repeated measures ANOVA followed by LSMeans Tukey HSD test to assess potential differences across treatments. We compared the amounts of sequestered cardenolides across treatments and milkweed bug species by ANOVA followed by LSMeans Tukey HSD test and included bug species and treatment as model effects. Additionally, we estimated Pearson's correlation coefficients between body mass and concentration of sequestered cardenolides in *O. fasciatus*. Body length data were analyzed by ANOVA followed by LSMeans Tukey HSD test, including sex, treatment, and the interaction between sex and treatment as model effects. Lifespan and number of hatchlings were analyzed by ANOVA followed by LSMeans differences Student's *t*-test, including treatment as the model effect. For *O. fasciatus*, "experiment" was always included as a model

effect in our statistical analysis. Sample sizes for every experiment are mentioned in the figure legends. Probability values <0.05 were considered statistically significant.

3 | RESULTS

3.1 | Influence of dietary cardenolides on growth

We examined the influence of dietary toxins on the growth of *P. apterus*, *O. fasciatus*, *C. nerii*, *S. pandurus*, and *A. longiceps* (Figure 2) using an artificial diet containing increasing doses of cardenolides (Figure S1). Growth of *P. apterus* was compromised substantially [$F(3, 36) = 8.83$, $p < .001$] upon exposure to dietary cardenolides ($p < .001$, LSMeans Tukey HSD). In contrast, *S. pandurus* [$F(3, 35.81) = 1.5$, $p = .23$] and *A. longiceps* [$F(3, 40) = 1.11$, $p = .36$] grew equally well across all diets. Remarkably, cardenolides had a positive effect on growth in *O. fasciatus* [$F(3, 88.01)$ * 5.33, $p = .002$] and *C. nerii* [$F(3, 36) = 5.69$, $p = .003$]. We observed increased growth in the presence of dietary toxins across all doses in *O. fasciatus* (low vs. control, $p = .008$; medium vs. control, $p = .005$; high vs. control, $p = .015$, LSMeans Tukey HSD). Compared to a diet without cardenolides, *C. nerii* grew better on the low- ($p = .002$) and the high-dose ($p = .02$), but not on the medium-dose diet ($p = .09$). Since our laboratory strain of *O. fasciatus* was highly inbred, we carried out the same experiment with a different laboratory strain of *O. fasciatus* and obtained similar results, but here only low-dose was statistically significant from control (Figure S3).

The amount of excretion products was not influenced by the presence of toxins across diets for the milkweed bug species, *C. nerii* [$F(3, 14) = 1.38$, $p = .29$], *S. pandurus* [$F(3, 15) = 0.53$, $p = .67$], and *A. longiceps* [$F(3, 5) = 0.96$, $p = .48$], but there was an effect for *P. apterus* (lower in the presence of cardenolides, $F(3, 15) = 4.69$, $p = .02$). *Oncopeltus fasciatus* [$F(3, 19) = 8.34$, $p < .001$] excreted similar amounts when fed on either control, low-, or medium-dose diet, but less on the high-dose diet compared to low ($p = .004$) and medium dose ($p = .002$), but similar to control ($p = .25$). This suggests that stronger growth in *O. fasciatus* and *C. nerii* is not due to increased food uptake mediated by a phagostimulatory effect of dietary cardenolides (Figure S4).

3.2 | Cardenolide sequestration

Pyrrhocoris apterus did not sequester any cardenolides (Figure 3a). We found substantial differences regarding the concentration of sequestered cardenolides across all dietary treatments for *S. pandurus* [$F(2, 12) = 12.11$, $p < .001$], *O. fasciatus* [$F(2, 27) = 15.99$, $p < .001$], and *C. nerii* [$F(2, 12) = 18.16$, $p < .001$]. Compared to the other species, *O. fasciatus* sequestered remarkably higher amounts of cardenolides ($p < .001$). Although *A. longiceps* possesses a resistant Na^+/K^+-ATPase, we observed only very small concentrations of

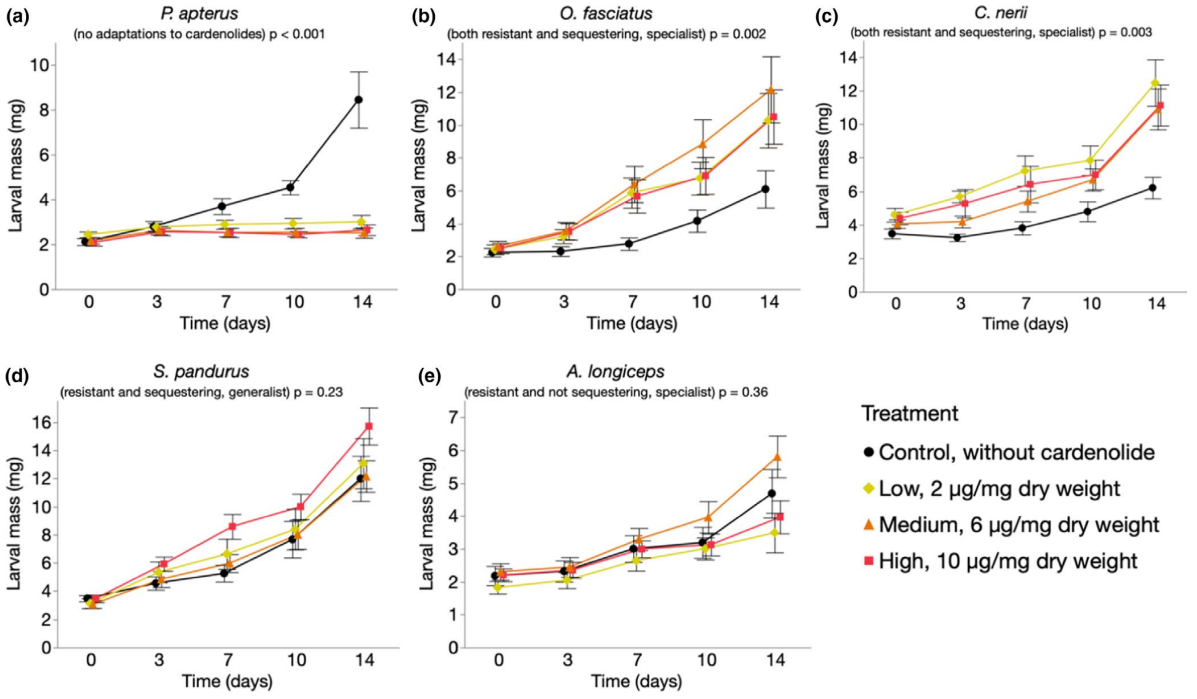

FIGURE 2 Growth of bugs on artificial diet with increasing doses of cardenolides. Each data point represents the mean (±SE) of larval mass at a given time. (a) *Pyrrhocoris apterus* ($n = 10$ per treatment), (b) *Oncopeltus fasciatus* ($n = 25$ per treatment, three experiments), (c) *Caenocoris nerii* ($n = 10$ per treatment), (d) *Spilostethus pandurus* ($n = 10$ per treatment), and (e) *Arocatus longiceps* ($n = 10$–13 per treatment)

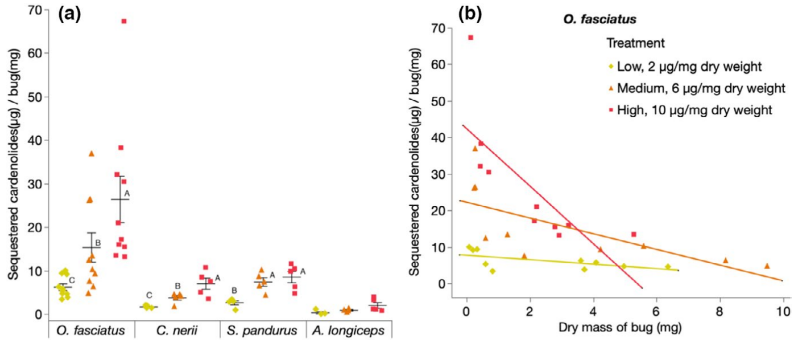

FIGURE 3 Sequestration of cardenolides by four species of milkweed bugs on artificial diet with increasing amounts of cardenolides. (a) Total amounts of cardenolides sequestered per species [$n = 5$, per treatment for all species except *Oncopeltus fasciatus* ($n = 10$)] and dietary cardenolide concentration. Horizontal bars represent the mean concentration (± SE) of sequestered cardenolides. Within the same bug species, different letters indicate significant differences across treatments and dots represent jittered raw data. (b) Correlations between dry body mass and concentration of sequestered cardenolides in *O. fasciatus* ($n = 10$ per treatment). Trend lines represent the linear least squares regression fits to data points and dots represent jittered raw data

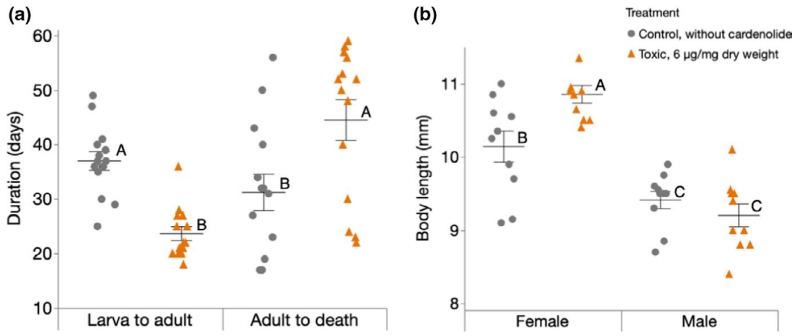

FIGURE 4 Effect of cardenolides on developmental time, lifespan, and body size of *Oncopeltus fasciatus*. (a) Each horizontal line represents the mean (± SE) number of days taken by *O. fasciatus* larvae ($n = 14$ per treatment) to turn adult (left side), and until death after reaching adulthood (right side, n = 14 per treatment); within the same category (left or right), different letters indicate significant differences between diets. (b) Each horizontal line represents the mean (± SE) body length of adult (female or male) *O. fasciatus* ($n = 10$ per treatment); different letters above bars indicate significant differences. Larvae were either raised on toxic diet or a control diet. Dots represent jittered raw data

sequestered cardenolides which is consistent with earlier findings (Bramer et al., 2015).

Spilostethus pandurus sequestered similar amounts of cardenolides from the diet with the intermediate and with the highest concentration of cardenolides ($p = .89$). In contrast, we found dose-dependent cardenolide sequestration in *O. fasciatus* (low vs. medium, $p = .011$; low vs. high, $p < .001$; medium vs. high, $p = .05$) and in *C. nerii* (low vs. medium, $p = .01$; low vs. high, $p < .001$; medium vs. high, $p = .05$). Specifically, *O. fasciatus* sequestered 6.23 ± 0.76, 15.36 ± 3.39, and 26.43 ± 5.32; *C. nerii* sequestered 1.66 ± 0.14, 3.77 ± 0.52, and 7.02 ± 1.3 µg; and *S. pandurus* sequestered 2.64 ± 0.45, 7.43 ± 0.98, and 8.55 ± 1.29 µg cardenolides per mg dry weight (mean ± SE) from the low-, medium-, and high-dose diet, respectively. Additionally, the sequestration data in *O. fasciatus* revealed an inverse relationship between body mass and concentration of sequestered cardenolides [low, $r(10) = -0.7$, $p = .03$; medium, $r(10) = -0.92$, $p < .001$; high, $r(10) = -0.98$, $p < .001$] (Figure 3b).

Besides the total amounts of cardenolides sequestered, we also compared the number of structurally different cardenolides across the sequestering species (Figure S2). Based on retention time comparison using an authentic standard, ouabain was sequestered as such. However, a peak with the retention time of digitoxin was not detected, but we found up to three compounds with a cardenolide spectrum and increased polarity probably representing digitoxin metabolites. Remarkably, we found more than one and up to three putative digitoxin metabolites in *S. pandurus* and *C. nerii*. In *O. fasciatus*, we did not find any digitoxin metabolites.

3.3 | Influence of dietary cardenolides on lifespan, longevity, and body size of *Oncopeltus fasciatus*

Dietary cardenolides showed substantial effects on the developmental speed and longevity of *O. fasciatus*. Larvae raised on

cardenolide-containing diet developed faster into adults [$F(1, 26) = 42.79$, $p < .001$, LSMeans differences Student's t-test] and adults resulting from larvae raised on cardenolide-containing diet lived longer [$F(1, 26) = 6.39$, $p = .02$, LSMeans differences Student's t test] compared to individuals raised on cardenolide-free diet (Figure 4a). Furthermore, cardenolides affected adult body size [$F(3, 36) = 22.31$, $p < .001$]. Females raised on toxic diet were the largest across all combinations (i.e., sex vs. diet; $p < .001$, when compared to males on toxic or control diets; and $p = .02$, when compared to females on the control diet, LSMeans Tukey HSD). The body length of male *O. fasciatus* was not different between the two diets ($p = .74$, LSMeans Tukey HSD) (Figure 4b).

3.4 | Influence of dietary cardenolides on reproductive fitness of *Oncopeltus fasciatus*

We observed substantial effects on the reproductive fitness of female *O. fasciatus* upon exposure to dietary cardenolides. When we continued feeding females after reaching adulthood on the same diet they were fed upon as larvae, *O. fasciatus* on cardenolide-containing diets produced less hatchlings than individuals raised on cardenolide-free diet [$F(1, 23) = 15.82$, $p = .001$, LSMeans differences Student's t-test]. In contrast, *O. fasciatus* from cardenolide-containing and cardenolide-free diets produced similar numbers of hatchlings, when both groups were fed with sunflower seeds after reaching adulthood [$F(1, 21) = 0.52$, $p = .48$, LSMeans differences Student's t-test] (Figure 5).

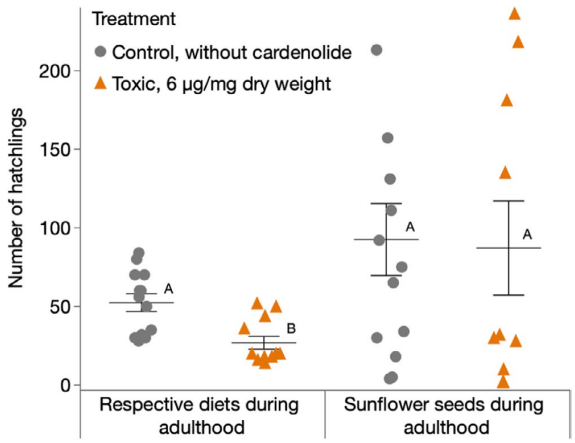

FIGURE 5 Reproductive success of *Oncopeltus fasciatus* in the presence or absence of dietary cardenolides. Each horizontal bar represents the mean (±SE) total number of hatchlings produced by *O. fasciatus* females until death. Larvae were either raised on toxic diet or control diet until adulthood. A group of bugs stayed on the respective diets after reaching adulthood (left; n for control = 13, n for toxic = 12), while the other group was transferred to sunflower seeds (right; n = 13 for control, n = 10 for toxic). Females producing no viable eggs at all were excluded from the analysis. Within the same group, different letters indicate significant differences. Dots and triangles represent jittered raw data

4 | DISCUSSION

We investigated if dietary cardenolides affected the growth of closely related species of milkweed bugs (*O. fasciatus*, *C. nerii*, *S pandurus*, and *A. longiceps*) and the outgroup species, *P. apterus* possessing different combinations of the traits "cardenolide resistance" and "cardenolide sequestration" and having different dietary strategies (generalist vs. specialist). Remarkably, dietary cardenolides increased growth in the sequestering specialists, *O. fasciatus* and *C. nerii*, but not in the sequestering generalist *S. pandurus*. *Oncopeltus fasciatus* nymphs completed their development 2 weeks earlier and lived on average 13 days longer during the adult stage under cardenolide exposure when compared to individuals raised on control diet, but produced less offspring unless being transferred to a cardenolide-free diet (sunflower seeds) after reaching adulthood.

Empirical evidence on the effect of dietary plant toxins on insect growth is ambiguous as different studies suggest contradictory effects. Nevertheless, several studies found effects that are in agreement with our study. For example, the growth of *O. fasciatus* was faster when raised on *Asclepias* species containing higher amounts of cardenolides (*A. syriaca* and *A. hirtella*) than when raised on species with lower cardenolide contents (*A. incarnata* and *A. viridiflora*) (Chaplin & Chaplin, 1981). Additionally, the African danaid butterfly *Danaus chrysippus*, having a cardenolide-resistant Na^+/K^+-ATPase (Petschenka et al., 2013), developed faster and produced larger adults when reared on *Calotropis procera* containing cardenolides compared to caterpillars raised on *Tylophora* spp. lacking cardenolides (Rothschild et al., 1975). Furthermore, *Zygaena filipendulae* larvae reared on the plant *Lotus corniculatus* containing cyanogenic glucosides developed faster, and the larvae showed decelerated development when reared on transgenic *L. corniculatus* free of cyanogenic glucosides (Zagrobelny et al., 2007).

In contrast to these studies, caterpillar growth of the milkweed butterfly species *Euploe core*, *D. plexippus*, and *D. gilippus* was unaffected by cardenolides across eight *Asclepias* species ranging from very low to very high cardenolide contents (Petschenka & Agrawal, 2015). Notably, all the studies mentioned above focused on insect feeding performance on intact plants or plant organs such as leaves or seeds that naturally represent highly complex diets (but see Bowers, 1984). This could be one reason why contradicting outcomes in response to the same class of chemical compounds were observed even within related plant species. Here, we used an artificial diet to control for variation across dietary treatments rigorously.

We showed that cardenolides had a positive effect on growth in *O. fasciatus* and *C. nerii*, both of which may be categorized as dietary specialists feeding on seeds of *Asclepias* spp. and seeds of *Nerium oleander*, respectively. We speculate that the positive impact on growth upon exposure of toxins may be due to selection on the resistant Na^+/K^+-ATPases to function optimally in a "toxic environment," that

is, in the body tissues of a milkweed bug storing large amounts of cardenolides. In other words, there could be functional trade-offs of cardenolide-adapted Na^+/K^+-ATPases in a physiological environment that is devoid of cardenolides, a phenomenon that could be called "evolutionary addiction." Alternatively, better growth could be due to increased consumption of the larvae mediated by cardenolides as phagostimulants (Pantle & Feir, 1976). Nevertheless, our excretion data hint toward equal consumption of diet regardless of dietary cardenolide concentration (Figure S4). Since endogenous and sequestered defenses may trade-off (Engler-Chaouat & Gilbert, 2007), the lack of cardenolides for sequestration could lead to a higher investment towards endogenous defenses (i.e., defensive secretions) and therefore impair growth on the cardenolide-free control diet. Although such a trade-off has been suggested to occur in the milkweed bug *Lygaeus equestris* (Havlikova et al., 2020), metathoracic scent glands were shown to be reduced in *O. fasciatus* and other milkweed bugs (Aldrich, 1988; Schaefer, 1972) making this explanation rather unlikely.

The milkweed bug species *A. longiceps* is specialized on plants producing no cardenolides such as *Platanus* spp. or *Ulmus* spp. However, due to its evolutionary history, *A. longiceps* possesses resistant Na^+/K^+-ATPases, but has lost the ability to sequester cardenolides (Bramer et al., 2015). In contrast, *S. pandurus* possesses resistant Na^+/K^+-ATPases, and feeds on a wide array of host plants (Péricart, 1998; Vivas, 2012), including cardenolide producing species such as *Nerium oleander* and species of *Calotropis* from which it sequesters cardenolides (Abushama & Ahmed, 1976; Von Euw et al., 1971). For both species, dietary cardenolides did not influence growth, that is, they grew equally well on all diets.

The lack of a positive effect of dietary cardenolides on growth in *A. longiceps* may be associated with the inability of this species to sequester cardenolides. Accordingly, its Na^+/K^+-ATPases may have undergone a different selection regime compared to the sequestering species. In other words, their putative suite of Na^+/K^+-ATPases may have adapted to the absence of cardenolides in the body tissues secondarily. Alternatively, or in addition, there could be further physiological mechanisms involved mediating between sequestration and growth such as gaining energy from metabolizing sequestered toxins. Nevertheless, the latter seems rather unlikely as an explanation for increased growth in *O. fasciatus* and *C. nerii*, since the sugar moieties in digitoxin are dideoxy sugars not known to be easily metabolized as an energy resource (Liu & Thorson, 1994) and ouabain (carrying a rhamnose moiety) was found to be sequestered as such (Scudder & Meredith, 1982).

For *O. fasciatus*, we found negative correlations between sequestered cardenolides and body dry masses within the dietary treatments, which contrasts with the observed increased growth under cardenolide exposure. This correlation, however, was weaker on the low and the medium diet compared to the diet with the highest concentration of cardenolides. At the same time, growth in *O. fasciatus* was highest on low and medium diet compared to the most toxic diet which could point to a dose dependency of the observed positive effect of sequestered cardenolides, suggesting that our findings are not necessarily in contrast with the increased growth under cardenolide exposure. In addition, we used fresh weights for the growth experiments and dry weights for the assessment of sequestration which may further complicate the comparison of these two experiments.

While it is not surprising that cardenolide-resistant Na^+/K^+-ATPases alleviate toxicity of dietary cardenolides in both species, *A. longiceps* and *S. pandurus*, it is an open question why the sequestering *S. pandurus* did not show increased growth under cardenolide exposure. Although *O. fasciatus* sequesters substantially higher amount of cardenolides in comparison to *S. pandurus*, it seems unlikely that the extent of sequestration is the underlying mechanism, given that *C. nerii* sequesters concentrations similar to *S. pandurus* not showing increased growth. Along the same lines, cardenolide metabolism may also not explain the patterns of growth since we found pronounced differences in metabolism between *O. fasciatus* and *C. nerii* both showing increased growth under cardenolide exposure. However, the putative adaptations underlying generalist feeding behavior of *S. pandurus* likely caused different selection pressures in this species and may thus interfere with the pattern of amino acid substitutions across the different Na^+/K^+-ATPases or lead to differential expression of the Na^+/K^+-ATPase genes across different tissues.

The outgroup species, *P. apterus*, belongs to a different family (Pyrrhocoridae) and is not adapted to cardenolides, that is, it has a cardenolide-sensitive Na^+/K^+-ATPase and is not able to sequester cardenolides (Bramer et al., 2015). The lack of a resistant Na^+/K^+-ATPase most likely explains why larval growth in this species was compromised substantially by dietary cardenolides. Alternatively, or in addition, reduced growth could be due to feeding deterrence mediated by dietary cardenolides as indicated by the reduced amount of excretion products observed during our feeding trial.

Although specialist insects can successfully feed on toxic host plants, it is generally expected that the underlying resistance traits incur costs via trade-offs, which are expected to depend on the ecological context and the molecular mechanisms involved (Peterson et al., 2016; Smilanich et al., 2016). As *O. fasciatus* raised on cardenolide-containing diet developed faster into adults and had a longer lifespan compared to those raised on cardenolide-free diet, cardenolide exposure clearly influences the fecundity-longevity trade-off. A faster development and an extended lifespan both indicate higher fitness. Nevertheless, individuals exposed to dietary cardenolides produced a lower number of hatchlings contradicting higher fitness. This apparent disadvantage, however, could be alleviated by maternal transfer of cardenolides to the eggs mediating protection against predators (Newcombe et al., 2013). Moreover, we found that feeding on a non-toxic diet (i.e., sunflower seeds) as adults can compensate for this effect. This likely resembles the natural situation since *O. fasciatus* larvae feed and cluster around on *Asclepias* seeds or seedpods, while adults disperse and forage on various plants (Feir, 1974). Contrary to our findings, it was shown that male *O. fasciatus* fed with *A. syriaca* seeds invested in reproduction at the expense of survival when compared to those fed with sunflower

seeds (Attisano et al., 2012). In this study, however, only the diet of male specimens was manipulated and female *O. fasciatus* were exclusively fed with sunflower seeds. Moreover, due to the use of milkweed seeds, it is not possible to directly attribute the observed effects to cardenolides. In conclusion, it seems likely that cardenolides exert a positive effect on overall fitness in *O. fasciatus*, which is in disagreement with theory predicting costs of sequestration.

Dealing with xenobiotics can require energy allocation towards metabolism or can cause pleiotropic effects due to particular molecular mutations that might confer a selective advantage in the presence of xenobiotics but incur a cost in their absence (Coustau et al., 2000; Mauro & Ghalambor, 2020). Although it is unclear how the function of Na^+/K^+-ATPase could be related to growth and longevity in milkweed bugs, the enzyme is involved in many physiological processes apart from being a cation carrier suggesting many unknown non-canonical functions of Na^+/K^+-ATPase (Liang et al., 2007), which could provide a mechanistic link. In *O. fasciatus*, the three gene copies (α1A, α1B, and α1C) encoding α- subunit of cardenolide-resistant Na^+/K^+-ATPases have diverse functions (Lohr et al., 2017) and duplicated α1 gene copies do not only vary in number and identity but also show specific expression patterns in different body tissues (Dobler et al., 2019; Yang et al., 2019; Zhen et al., 2012) allowing for complex regulation.

In conclusion, reduced growth in the absence of cardenolides in highly specialized milkweed bugs with cardenolide-resistant Na^+/K^+-ATPases suggests a novel type of physiological cost arising in the absence of plant toxins. Mechanistically, this cost could be due to negative pleiotropic effects mediated by resistant Na^+/K^+-ATPases not functioning optimally in a physiological environment lacking cardenolides. Furthermore, the observed effects of cardenolides on the fecundity-longevity trade-off probably leading to increased fitness in *O. fasciatus* may be due to optimized resource allocation under the influence of sequestered cardenolides. Our study suggests a concept that is contradictory to the general assumption that sequestered plant toxins produce physiological costs and our results indicate a further level of coevolutionary escalation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Prayan Pokharel: Conceptualization (equal); data curation (lead); formal analysis (equal); investigation (lead); methodology (equal); validation (equal); visualization (lead); writing – original draft (equal); writing – review and editing (equal). **Anke Steppuhn:** Formal analysis (equal); supervision (supporting); validation (equal); writing – review and editing (equal). **Georg Petschenka:** Conceptualization (lead); data curation (equal); formal analysis (equal); funding acquisition (lead); methodology (equal); project administration (lead); resources (lead); supervision (lead); validation (equal); writing – original draft (equal); writing – review and editing (equal).

DATA AVAILABILITY STATEMENT

All raw data reported in this manuscript were archived in Dryad: https://doi.org/10.5061/dryad.q2bvq83m3.

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Chapter 2 - Mechanistic Linkages Between Sequestration And Warning Signals

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Antioxidant availability trades off with warning signals and toxin sequestration in the large milkweed bug (*Oncopeltus fasciatus*)

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Abstract

In some aposematic species the conspicuousness of an individual's warning signal and the concentration of its chemical defence are positively correlated. Several mechanisms have been proposed to understand this phenomenon including resource allocation trade-offs where the same limiting resource is needed to produce both the warning signal and chemical defence. Here, the large milkweed bug (*Oncopeltus fasciatus*: Heteroptera, Lygaeinae) was used to test whether allocation of antioxidants, that can impart colour, trades against their availability to prevent self-damage caused by toxin sequestration. We investigated if (i) the sequestration of cardenolides is associated with costs in the form of changes in oxidative state; and (ii) that oxidative state can affect the capacity of individuals to produce warning signals. We raised milkweed bugs on artificial diets with increasing quantities of cardenolides and then examined how this affected signal quality (brightness and hue) across different life stages. We then related the expression of warning colours to the quantity of sequestered cardenolides and indicators of oxidative state – oxidative lipid damage (malondialdehyde), and two antioxidants: total superoxide dismutase, and total glutathione. Bugs raised on the high and medium dietary cardenolide treatments had significantly lower levels of the antioxidant glutathione. Glutathione also tended to decrease in all

individuals with increasing levels of sequestered cardenolides. Bugs with more total glutathione had brighter warning signals but these signals were not related to sequestration. Our results give tentative support for a physiological cost of sequestration in milkweed bugs and a mechanistic link between antioxidant availability and warning signals.

Keywords: Aposematism; Honest signalling; Cardenolides; Oxidative state; Resource competition

1. Introduction

The conspicuous colours of aposematic animals serve as important signals of chemical defences (Wallace 1889; Sherratt 2002). In some aposematic animals warning signals and chemical defences are positively correlated, both when looking within species (Bezzerides *et al.* 2007; Blount *et al.* 2012; Maan *et al.* 2012; Vidal-Cordero *et al.* 2012) and across species (Cortesi and Cheney, 2010). Several mechanisms have been proposed to understand this phenomenon (Blount *et al.* 2009; Lee, Speed & Stephens 2011; Holen & Svennungsen 2012). One of the mechanisms that predicts a positive relationship between conspicuousness and defence in prey is resource allocation trade-offs, where variable access to resources may result in differential costs of signalling (Blount *et al.* 2009; Holen & Svennungsen 2012). One possible shared resource is energy, which can be limiting for the sequestration or biosynthesis of toxins (Holloway *et al.* 1991), and the expression of warning signals (Srygley 2004). Another, is antioxidants, which are relevant if sequestration of toxins imposes metabolic costs in terms of oxidative stress (Ahmad 1992; Tollrian & Harvell 1999; Blount *et al.* 2009).

If pigments used in prey warning signals play a dual role in producing both the signal and also in preventing self- damage when storing toxins (due to their antioxidant properties), then variable access to antioxidants may result in differential costs of signalling and explain the positive correlations between warning signal intensity and toxicity. There are many critiques of this idea, in particular providing a plausible mechanisms for why warning signals should have differential costs (Guilford & Dawkins 1993). But in their resource competition model Blount et al. (2009), explored how prey should divide their resources between a signal and a defence and

found that signal conspicuousness and defence level could be positively or negatively related within a population at evolutionary equilibrium, depending on the range of resource levels present. The potential influence of antioxidant availability and oxidative stress on the development of aposematic displays has received limited empirical attention (Ojala *et al.* 2005; Sandre, Tammaru & Mand 2007). But recently, Blount et al (2021) provided evidence for differential costs of signalling in monarch butterflies (*Danaus plexippus*) explained by a link between the production of coloration and protection from autotoxicity. In their study, Blount et al (2021) enabled monarchs to sequester varying amounts of toxins by rearing them on different milkweed hostplants (*Apocynaceae*). Because different hostplants vary in many traits (not just chemical defence), the costs they detect may not only result from varied sequestration. A study that only varies chemical defence content while holding other traits constant could provide a clearer test for a mechanistic link between oxidative stress, warning colours, and sequestration of chemical defences.

Here we test Blount et al's (2009) resource competition model using the large milkweed bug, *Oncopeltus fasciatus* (Hemiptera, Lygainae), which are conspicuously patterned orange and black insects. *O. fasciatus* feed on seeds or seedpods of milkweed plants (*Asclepias* spp; Feir 1974; Burdfield-Steel & Shuker 2014) which produce cardenolides (Brower 1969; Roeske *et al.* 1976). *O. fasciatus* not only tolerate cardenolides, but also sequester these toxins for their own defence in a specialised, vacuolated layer of cells beneath their outer layer of epidermis (Duffey *et al.* 1978; Scudder & Meredith 1982; Bramer, Friedrich & Dobler 2017). *O. fasciatus* vary in the amount and structure of the cardenolides they sequester when feeding on the same host species (Isman, Duffey & Scudder 1977) and the intensity of their colouration also varies in the wild (Rodríguez-Clark 2004; Davis 2009). The pigments determining colouration in *O. fasciatus* are pteridines such as xanthopterin, isoxanthopterin, and 2-amino-4-hydroxypteridine, and pterins such as erythropterin (Good & Johnson 1949; Bartel, Hudson & Craig 1958; Hudson, Bartel & Craig 1959). These pigments have the potential to function as biological antioxidants (McGraw 2005).

Because directly controlling the level of antioxidant defence that manifests in an animal is challenging experimentally, we modulated the quantity of diet-derived toxin available to the individual to test whether (1) the quantity of sequestered cardenolides by *O. fasciatus* is associated with changes in oxidative state; and (2) whether oxidative state affects the capacity of *O. fasciatus* to produce warning signals displays. We used an artificial diet to modulate dietary toxins. We raised *O. fasciatus* on diets with an increasing amount of cardenolides and measured individual signal quality (brightness and hue) and toxicity (sequestered cardenolides) across different life stages. We also measured indicators of oxidative state - lipid peroxidation (malondialdehyde), and two components of antioxidant defence: superoxide dismutase, and total glutathione. We predicted that there would be a positive correlation between individual levels of cardenolides sequestered by *O. fasciatus* and oxidative lipid damage. We also predicted that if highly toxic prey cannot bear the oxidative cost of investing in both sequestration and pigmentation traits then individuals with the highest levels of oxidative lipid damage should have the lowest investment in signals, and this trade-off should be strongest in the treatment exposed to the highest levels of food-plant cardenolides (i.e., a treatment x oxidative damage interaction effect on signalling). By using a controlled artificial diet, and a model species that naturally varies in both signal and toxicity, in this study we can rigorously test the assumptions of the resource competition model, as well as contribute to the growing literature examining honest signalling in aposematic species.

2. Materials and Methods

2.1. Insect rearing and artificial diet

O. fasciatus were obtained from a long-term laboratory colony (originally from the United States) maintained on sunflower seeds. *O. fasciatus* develop through five instars, from their first larval stage (L1) through L2, L3, and L4, to their fifth (L5), after which they moult into adults. We started our rearing experiment using third larval stage (L3) bugs. We split our experiments into three batches. In each batch, we divided 60-100 L3 *O. fasciatus* larvae from a breeding colony into four treatment groups of 15-25 individuals each.

We raised *O. fasciatus* (N total = 192) on four diets, three with increasing amounts of added ouabain and digitoxin, and one as a control diet with no added toxins. We followed Pokharel et al. 's (Pokharel, Steppuhn & Petschenka 2021) method to prepare an artificial diet which consisted of sunflower seeds, wheat germ, casein, sucrose, Wesson's salt, vitamins, methyl 4-hydroxybenzoate, sorbic acid, olive oil, and cardenolides (only for the treatment groups, not for controls), which were combined with Agar and were provided in the lids of 2 ml Eppendorf tube sealed with a piece of stretched parafilm to create an 'artificial seed'. The control (C) diet had no cardenolides added, and Low (L), Medium (M), and High (H) diets had an added 2, 6, and 10 mg cardenolides (an equimolar mixture of digitoxin and ouabain (Sigma-Aldrich, Taufkirchen, Germany; Supplementary Fig. S1) per g dry weight of diet. These three concentrations were chosen as they fall within the range of dietary toxins naturally present in milkweed seeds (*Asclepias spp.*; (Isman et al., 1977b). The groups were reared in plastic boxes (15 x 11 x 5 cm) with water supplied in Eppendorf tubes plugged with dental cotton and two portions of the artificial diet that were replenished once per week.

2.2. Photography and colour analysis

We checked the insect boxes daily to monitor the bugs' moulting. We took photographs of *O. fasciatus* individuals at the approximate end of larval stages 4 and 5 and twice within the adult stage (recently moulted adults A1, and adults 5 to 10 days after moulting A3). For A1 adults, the photographs were taken approximately one day after the imaginal moult, so that the bright red colouration apparent in the first hours after moulting had transformed to regular adult colouration. Individuals were only photographed once for image analysis. We used a Nikon D7000 digital SLR camera (Nikon, Tokyo, Japan) and a UV-Nikkor 105mm f/4.5s. The lens was fitted with a custom-built ring illumination and filter changer that illuminated the bugs with LEDs emitting light with a wavelength between 380-780 nm (Supplementary Fig S2) and allowed switching between a Baader UV-IR blocking filter (Baader Planetarium, Mammendorf, Germany; permitting only visible spectrum light from 420 to 680 nm) and a Baader UV pass filter (permitting ultraviolet light from 320 to 380 nm). Approximately half of the individuals in each

dietary treatment group at each life stage were randomly selected for photography. We sedated individual insects using CO_2 and photographed them with elytra facing upwards on a colour palette (ColorChecker Passport Photo 2, X-rite, Pantone©, Michigan, USA), alongside an identifying label and a 40% Spectralon® grey standard (Labsphere Inc., North Sutton, NH, USA). We took three pictures with increasing exposure times (0.2, 0.33, and 0.77) with an aperture of 1.3 x for both filters, i.e., six pictures per insect.

Photographs were analysed using micaToolbox (Troscianko & Stevens 2015) in ImageJ software 1.51 (Rasband 1997-2018). Because digital cameras often show a non-linear relationship between the pixel value recorded and changes in light intensity, the images were first calibrated to linearize the RGB pixel values' relationship with light intensity (Stevens *et al.* 2007) and to convert the camera's RGB values to linearized and device-independent sRGB. Because *O. fasciatus* reflect negligible amounts of UV we used only photographs in the visible spectrum and converted the sRGB values to L*a*b* colour space (CIELAB 1976; Commission Internationale de l'Eclairage; http://cie.co.at; Luo 2014). CIELAB colour space represents colour in triplet coordinates of lightness and hue that approximates the red-green and yellow-blue opponent channels of humans (Luo 2014). Euclidean distances in this colour space approximate perceived colour differences. In each photograph we delineated consistent indicative regions for one red section on the bugs' wings (Supplementary Fig S3) and then used the micaToolbox to measure the red, green, and blue and L, A, and B values.

2.3. Homogenisation of samples

After photography, the bugs were placed into labelled Eppendorf tubes, weighed, and flash-frozen in liquid nitrogen, and stored at -80 °C. Due to their smaller size, L4 larvae were pooled into groups of two to have enough material for chemical and oxidative state assays. Each sample was homogenised in a 1:20 ratio of PBS buffer solution (pH 6.6, 50 mM, with 1 mM EDTA) to bug body mass using a FastPrep™ homogenizer (MP Biomedicals, LLC, US) at 10 m/s for 15 s. Tubes were centrifuged at 16,000 x g and 4 °C for 4 min, and the clear supernatant of the homogenate was transferred to a new 2 mL Eppendorf tube. Four aliquots were taken from each

homogenate and placed into individual Eppendorf tubes. All aliquoting was done on ice. First, for the total glutathione (GSH) assay, 150 µL metaphosphoric acid (MPA) was added to 150 µL homogenate, vortexed, and left at room temperature for 5 min. The resulting mixture was centrifuged at 956 x g and 4 °C for 2 min, and the supernatant pipetted into a new 1.5 mL Eppendorf tube. Second, for the superoxide dismutase (SOD) assay, 50 µL homogenate was added to a new 1.5 mL tube with 50 µL sugar buffer (PBS with 12.6 mM mannitol and 4.2 mM sucrose) and vortexed. Third, for HPLC analysis, 100 µL of the homogenate was transferred to a new 1.5 mL Eppendorf tube. The fourth remaining aliquot was used for malondialdehyde (MDA) analysis. All samples were then frozen at -80 °C.

2.4. Determination of oxidative stress and cardenolide concentration

We performed three oxidative state assays from the aliquoted homogenates: total glutathione (GSH), total superoxide dismutase (SOD), and malondialdehyde (MDA). These assays were chosen to obtain a broad overview of biomarkers of oxidative state. GSH is as an antioxidant molecule which serves as a nucleophilic co-substrate to glutathione transferases in the detoxification of xenobiotics and is an essential electron donor to glutathione peroxidases in the reduction of hydroperoxides (Arias & Jakoby 1976). SOD is a metalloenzyme that catalyses the dismutation of superoxide into oxygen and hydrogen peroxide, forming a crucial part of intracellular antioxidant defences (Malmström, Andréasson & Reinhammer 1975). MDA is formed by the β-scission of peroxidised polyunsaturated fatty acids, and therefore is a definitive marker of oxidative lipid damage (Lapenna *et al.* 2001).

2.4.1. Total glutathione (GSH)

Total GSH was assayed by measuring the enzymatic recycling method of glutathione reductase for the quantification of GSH (Cayman Chemical, #703002). The homogenate was diluted (1:2 v/v) to fit the absorbance values within the range of the standard curve. Samples were assayed in duplicated, as per the kit instructions. Data are reported as nmol per mg of bug.

2.4.2. Superoxide dismutase (SOD)

Total SOD was assayed by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine. We followed the instruction of the kits (Cayman Chemical #706002) except that we diluted the samples (1:50 v/v) to ensure that SOD activity fell within the range of the standard curve. Samples were assayed in duplicate and reported as units of SOD activity (U) per mg of bug.

2.4.3. Malondialdehyde (MDA)

MDA was measured using HPLC with fluorescence detection (Agilent Technologies, Santa Clara, CA, USA), using a modified version of Agarwal and Chase's method (Agarwal & Chase 2002; Nussey *et al.* 2009). All chemicals were HPLC grade, and chemical solutions were prepared with ultra-pure water (Milli-Q Synthesis; Millipore, Watford, UK). We transferred a 20 µL aliquot of each homogenised sample into 2ml capacity polypropylene screw-top microcentrifuge tubes and added 20 µL butylated hydroxytoluene (BHT; 0.05% w/v in 95 % ethanol), 40 µL 2-thiobarbituric acid (TBA; 42 m*M*), and 160 µL phosphoric acid (H_3PO_4; 0.4M). Samples were capped, vortexed for 2 s, and then heated at 100°C for 1h in a dry bath incubator to allow formation of MDA-TBA adducts. Samples were centrifuged for one min at 13,300 x g, cooled for 5 min on ice before adding 160 µL n-butanol to each tube and vortexing for 10 s. Tubes were centrifuged for 3 min at 12,000 x g at 4 ^{o}C, before the upper butanol phase wad collected and transferred to an HPLC vial for analysis. Samples (20 µL) were injected into a HPLC system fitted with a Hypersil™ ODS C18 column (5µm, 100 x 4.6 mm, HSA-212-510R, Fisher Scientific, USA). The mobile phase was methanol buffer (40:60 v/v), the buffer was an anhydrous solution of potassium monobasic phosphate (50 mM) at pH 6.8 (adjusted using 5M potassium hydroxide solution), running isocratically over 3.5 min at a flow rate of 1 mL/min at 37 °C. Data were collected using a fluorescence detector (RF2000; Dionex Corporation, USA) set at 515 nm (excitation) and 553 nm (emission). For calibration a standard curve was prepared using serial dilutions of 5 µM 1,1,3,3-tetraethoxypropane (which hydrolyses to produce MDA) in 40% ethanol. Data are presented as nmols MDA per mg of bug.

2.4.4. Cardenolide analysis

To analyse the amount of sequestered cardenolides in the sample aliquot, we freeze-dried to remove the water content, and vortexed the residue in 1 ml HPLC-grade methanol containing 0.01 mg/ml of oleandrin (PhytoLab GmbH & Co. KG, Germany) as an internal standard. To facilitate dissolution of cardenolides, we immersed the sample in an ultrasonic bath for 30 min. After centrifugation at 16,100 x g for 3 min, the supernatant was collected and the sample was extracted one more time with 1 ml of pure methanol. The supernatants were pooled and dried under a flow of nitrogen gas. We dissolved the remaining pellet in 100 µl methanol by agitating in the Fast Prep™ homogenizer and filtered into HPLC vials using Rotilabo® syringe filters (nylon, pore size: 0.45 µm, diameter: 13 mm, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). We injected 15 µl of sample into an Agilent HPLC (Agilent technologies, Santa Clara, US) equipped with an EC 150/4.6 NUCLEODUR® C18 Gravity column (3 µm, 150 mm x 4.6 mm, Macherey-Nagel, Düren, Germany) and a photodiode array detector. Cardenolides were separated and eluted at a constant flow rate of 0.7 ml/min at 30 °C using the following acetonitrile-water gradient: 0–2 min 16% acetonitrile, 25 min 70% acetonitrile, 30 min 95% acetonitrile, 35 min 95% acetonitrile, 37 min 16% acetonitrile, 10 min reconditioning at 16% acetonitrile. Peaks with symmetrical absorption maxima between 218 and 222 nm were recorded as cardenolides (Malcolm & Zalucki 1996). Finally, we estimated the amount of cardenolides per sample by comparing the sum of all cardenolide peak areas to the area of the internal standard.

2.5. Statistical analysis

All analyses were performed in R through RStudio software (RStudio 2021.09.2+382). We analysed how treatment affected the quantity of cardenolides sequestered using a linear model with robust standard errors using the package 'sandwich' (Zeileis, Köll & Graham 2020). We analysed how sequestration affected oxidative state with a linear model including all pairwise interactions between treatment, sequestered cardenolides, instar, and batch, and removed model terms using the drop1 function to find the minimal adequate model and compared models using anova and AIC. We computed contrasts between the significant fixed effects using estimated marginal means in the package *emmeans* (Lenth *et al.* 2021). We analyzed how treatment,

sequestered cardenolides and oxidative state affected signal quality (L* brightness, and hue A* and B*) using a linear model, and explored interactions between the two continuous variables using a response surface analysis in the R package rsm version 2.10.3 (Lenth 2009). Data was visualised using JMP® Pro 15 statistical software (SAS Institute Inc. 1989–2021).

3. Results

3.1. Effect of diet on sequestration

All *O. fasciatus* on experimental diets sequestered cardenolides and all on control diets had no cardenolides (Figure 1), and the amount sequestered by individuals increased significantly with increasing cardenolide concentration in the diet (Control vs Low: estimate = 0.56 ± 0.06, $t = 8.91$, $p < 0.0001$; Control vs Medium: estimate = 1.69 ± 0.08, $t = 20.82$, $p < 0.0001$; Control vs High: estimate = 2.28 ± 0.12, $t = 19.12$, $p < 0.0001$). The concentration of sequestered cardenolides significantly increased with age, with each instar storing higher concentrations than the previous instar (L4 vs L5: estimate = 0.23 ± 0.10, $t = 2.17$, $p = 0.03$; L4 vs A1: estimate = 0.43 ± 0.10, $t = 4.08$, $p < 0.0001$; L4 vs A3: estimate = 0.59 ± 0.11, $t = 5.37$, $p < 0.0001$). Batch 1 sequestered significantly more cardenolides than batch 2 (estimate = -0.16 ± 0.07, $t = -2.15$, $p = 0.03$), and batch 3 sequestered significantly more than batch 1 (estimate = 0.31 ± 0.10, $t = 3.13$, $p = 0.002$).

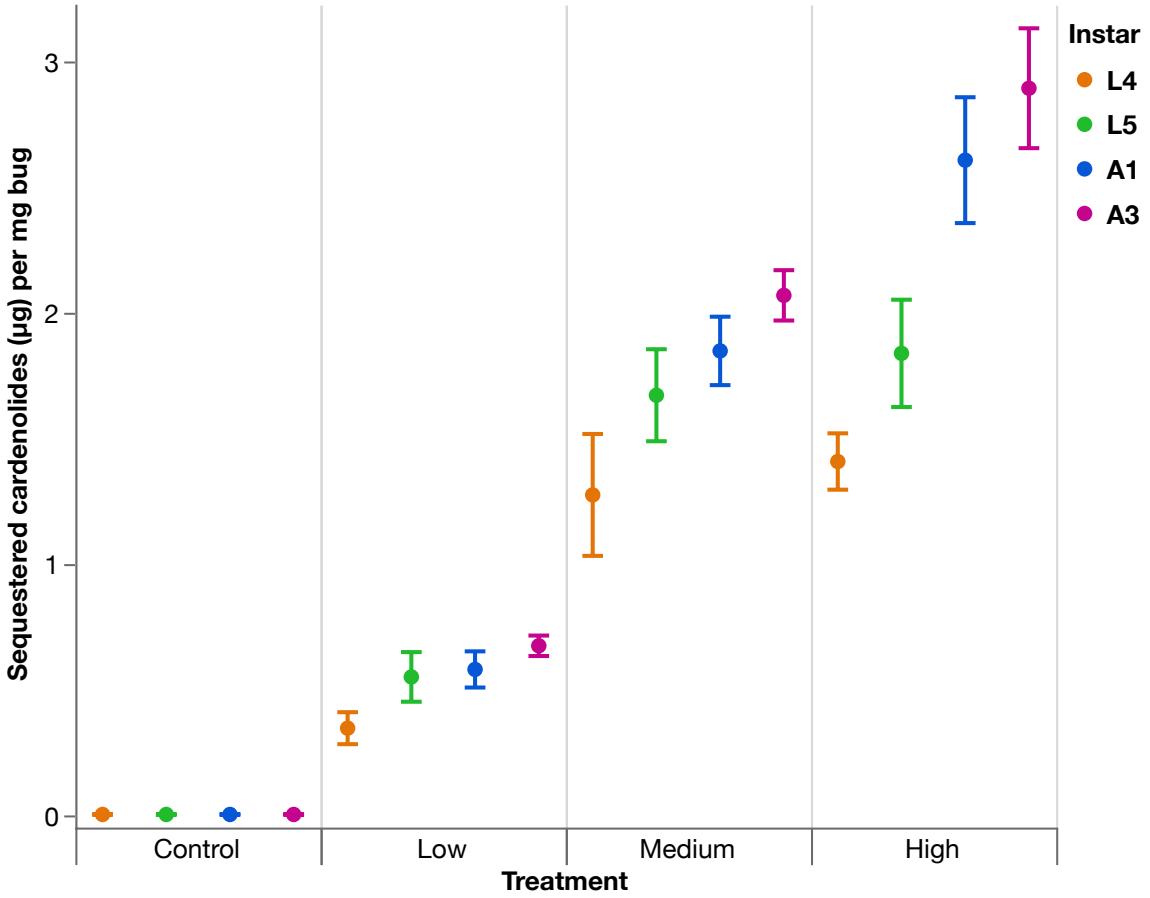

Figure 1. Mean concentration (±SE) of cardenolides sequestered (µg/mg) by instars L4 and L5, and by adults A and A3 of *Oncopeltus fasciatus* individuals when raised Control (no cardenolides), Low (2 mg/g), Medium (6 mg/g), and High (10 mg/g) cardenolide diets of roughly equimolar ouabain and digitoxin.

3.2. Cardenolide sequestration and oxidative stress

We predicted that an increase in sequestered cardenolides would have a positive association with oxidative damage measured through malondialdehyde (MDA), and a negative association with the antioxidant defences - superoxide dismutase (SOD), and total glutathione content (GSH).

MDA was not significantly affected by treatment (Control – Low: estimate = - 0.0009 ± 0.0009, t = -1.01, p = 0.32; Low – Medium: estimate = 0.0003 ± 0.0009, t = -0.31, p = 0.76; Medium – High: estimate = -0.000006 ± 0.0008, t = -0.007, p = 0.99) and did not correlate with cardenolide concentration across individuals (estimate = -0.0001 ± 0.0003, t = -0.35, p = 0.72). The difference

between MDA concentrations between larval stages L4 and L5 was not significant (estimate = 0.0007 ± 0.0009, t = -0.74, p = 0.88), but MDA levels increased when *O. fasciatus* reached adulthood, and again when they became older adults (L5-A1: estimate = -0.004 ± 0.0008, t = -4.91, p < 0.0001; A1-A3: estimate = -0.004 ± 0.0009, t = -5.16, p < 0.0001).

We found a significant interaction between treatment and instar on SOD activity (F = 3.31, p = 0.0009). We explored this by separating our analyses by instar. Interestingly, larval instar L4 in the high cardenolide treatment had significantly lower levels of SOD activity than those in the control treatment (L4 estimate = -0.07 ± 0.03, t = -2.53, p = 0.02), whereas we observed a reverse effect in larval instar L5, where those in the high cardenolide treatment had significantly higher levels of SOD activity than those in the control treatment (L5 estimate = 0.06 ± 0.02, t = 2.57, p = 0.01). There was no significant effect of treatment on SOD activity in the adult bug A1 (F (3, 48) = 1.51; p = 0.22). However, older adult bug A3 in the high cardenolide treatment had significantly lower levels of SOD activity than those in the control treatment (A3 estimate = -0.09 ± 0.04, t = -2.56, p = 0.01). Batch 1 bugs had significantly higher levels of SOD than batch 2 (estimate = -0.06 ± 0.01, t = -4.25, p <0.0001), and bugs in batch 3 did not differ in SOD activity to batch 1 (estimate = 0.01 ± 0.01, t = 0.78, p = 0.44).

We found a significant interaction between instar and individual level of sequestration (F = 4.93, p = 0.003), and also between batch and individual level of sequestration (F = 3.81, p = 0.02) on SOD activity. We explored these interactions by separating our analyses by batch and by instar. SOD activity decreased with increased sequestration in batch 1 but this was not significant at the alpha 0.05 level (estimate = -0.02 ± 0.01, t = -1.82, p = 0.07), but not in batch 2 (estimate = -0.008 ± 0.01, t = -0.73, p = 0.47), or batch 3 (estimate = 0.008 ± 0.007, t = 1.14, p = 0.26). SOD activity significantly increased with increasing sequestration in L5 nymphs (estimate = 0.03 ± 0.009, t = 3.884, p = 0.0003), but was not affected by sequestration in L4 nymphs (estimate = -0.01 ± 0.01, t = -0.53, p = 0.60), A1 adults (estimate = 0.007 ± 0.006, t = 1.11, p = 0.27) or A3 adults (estimate = -0.02 ± 0.01, t = -1.47, p = 0.15).

Bugs in the high and medium dietary cardenolide treatment had significantly lower levels of GSH than bugs in the control cardenolide dietary treatment (Control – High: estimate = -0.14 ± 0.04, t = -3.21, p = 0.002; Control – Medium: estimate = -0.11 ± 0.05, t = -2.34, p = 0.02). GSH levels did not differ between the control and low diet (estimate = -0.07 ± 0.05, t = -1.54, p = 0.13). Individual levels of GSH decreased with increasing levels of sequestered cardenolides but this was not significant at the alph0.05 level (estimate = -0.03 ± 0.02, t = -1.92, p = 0.056). GSH was significantly higher in L5 instar compared to L4 instar (estimate = -0.15 ± 0.05, t = -3.19, p = 0.002), and increased significantly from L4-A1 (estimate = 0.29 ± 0.05, t = 5.95, p < 0.0001), and did not differ between L4 to A3 (estimate 0.04 ± 0.05, t = 0.86, p = 0.39).

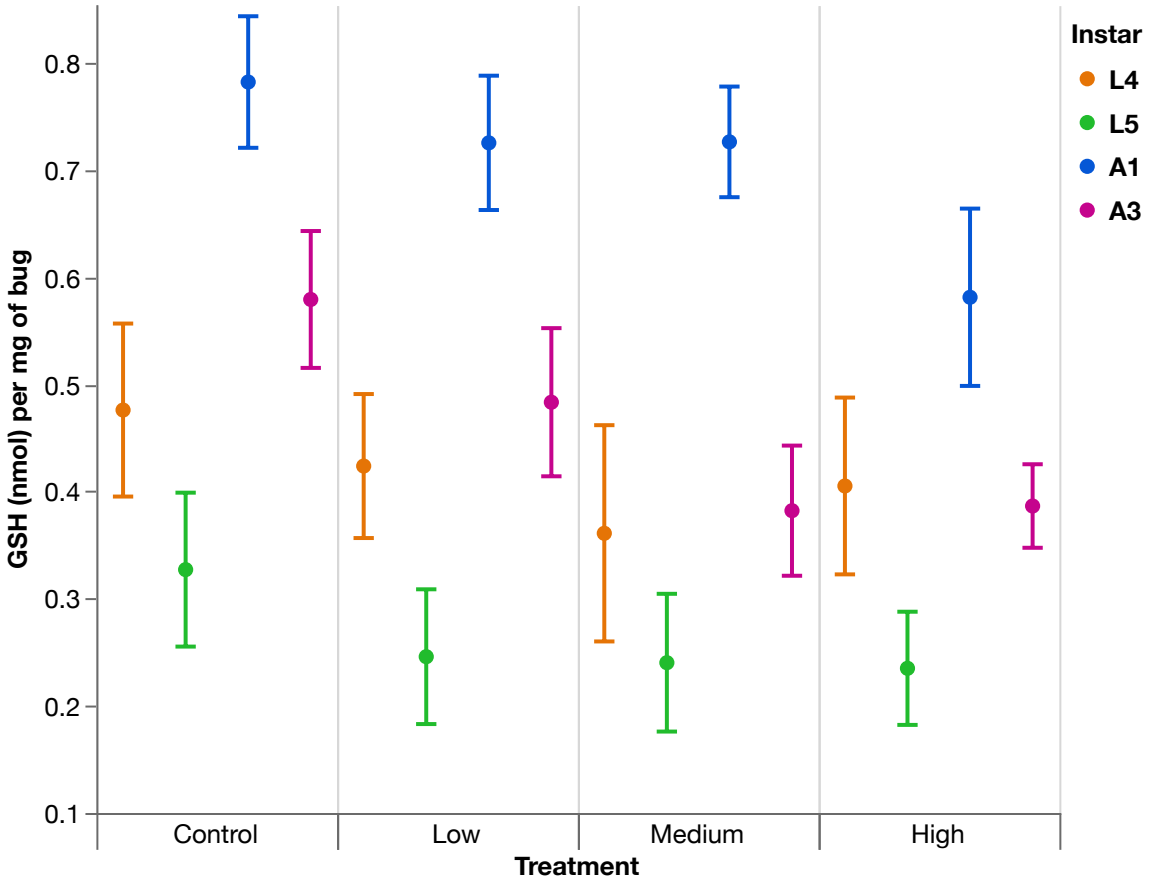

Figure 2. Total glutathione (GSH) level (µmol/mg) in *Oncopeltus fasciatus* individuals when raised on different dietary treatments. Control, Low, Medium, and High diets had 0 mg/g, 2 mg/g, 6 mg/g, 10 mg/g equimolar ouabain and digitoxin added respectively. L4, L5 represent the larval

stages Level 4 and Level 5. Adult 1 were recently moulted adults, and A3 were adult individuals one week older than this.

3.3. Sequestration, oxidative stress and warning signals

Because GSH was the only marker associated with dietary cardenolides, we proceeded to analyze its association with warning signals, but did not conduct analyses on superoxide dismutase activity or MDA effects on warning signals. We found no significant interaction between treatment and GSH on luminance so removed it from the model ($F = 1.10$, $p = 0.35$). We also found no effect of treatment on luminance and removed it from the model as well ($F = 0.21$, $p = 0.89$). Bugs with higher levels of GSH were significantly brighter than those with lower levels of GSH (Figure 3; estimate = 2.27 ± 0.71, $t = 3.22$, $p = 0.002$). There was no difference between larval instars L4 and L5 (estimate = -0.26 ± 0.54, $t = -0.48$, $p = 0.63$) or L4 and A1 (estimate = -0.64 ± 0.56, $t = -1.15$, $p = 0.25$), but adult A3 were significantly less bright than L4 larvae (estimate = -3.06 ± 0.53, $t = -5.81$, $p < 0.0001$). Batch 3 bugs were significantly brighter than batch 1 (estimate = 1.69 ± 0.43, $t = 3.91$, $p = 0.0001$) and there was no difference between batch 1 and 2 (estimate = 0.74 ± 0.47, $t = 1.58$, $p = 0.12$). We found no significant effect of individual sequestration on luminance (estimate = 0.06 ± 0.15, $t = 0.41$, $p = 0.68$).

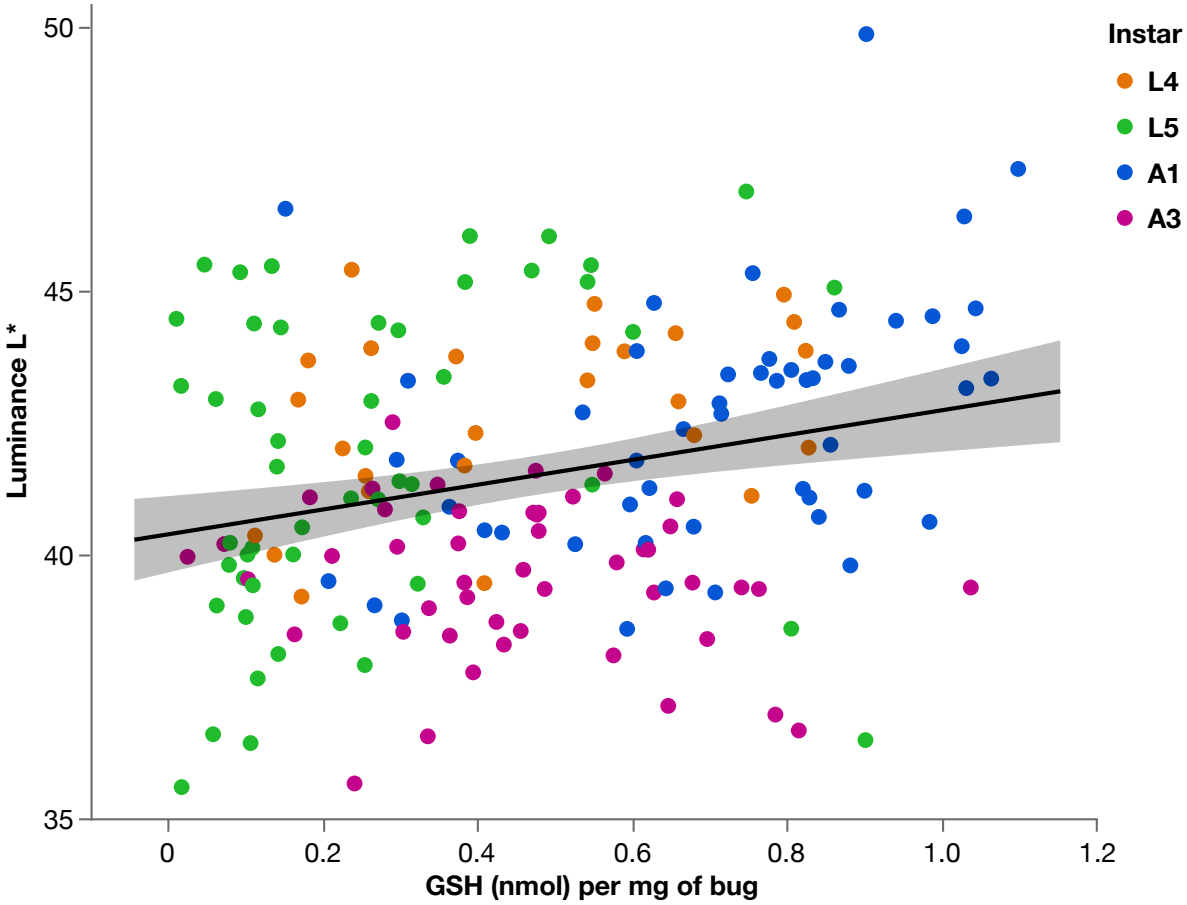

Figure 3. The correlation between total glutathione content luminance of the *O. fasciatus* wings. Larval stages are L4 and L5, and A1 and A3 are adults. Line is the smoothed conditional mean with 95% confidence intervals.

We found no significant interaction between treatment and GSH ($F = 1.09$, $p = 0.35$) and no batch effect ($F = 1.86$, $p = 0.16$) on redness so removed them from the model. Milkweed bugs in the low dietary treatment tended to be less red than those in the control treatment, but this was not significant at the alpha 0.05 levels (estimate = -0.69 ± 0.37, $t = -1.86$, $p = 0.06$). There was no difference in redness between bugs in the medium or high treatment compared to control (Control vs Medium estimate = 0.20 ± 0.36, $t = 0.56$, $p = 0.58$; Control vs High estimate = -0.43 ± 0.35, $t = -1.21$, $p = 0.22$). We found no interaction between GSH and individual cardenolide concentration on redness, so removed it from the model ($F = 1.97$, $p = 0.16$). We found no significant effect of GSH on redness (estimate = -0.17 ± 0.56, $t = -0.29$, $p = 0.77$). Instars decreased

in redness with age (L4 vs L5 estimate = -8.68 ± 0.42, t = -20.49, p < 0.0001; L4 vs A1 estimate = -11.70 ± 0.43, t = -27.05, p < 0.0001; L4 vs A3 estimate = -13.73 ± 0.41, t = -33.14, p <0.0001).

We found no significant interaction between GSH and treatment on yellowness (F = 1.98, p = 0.12) and no effect of treatment on yellowness (F = 0.80, p = 0.49). We found no significant effect of GSH on yellowness (estimate = 1.08 ± 0.87, t = 1.24, p = 0.22). Milkweed bugs became less yellow with age (L4 vs L5 estimate = -7.54 ± 0.67, t = -11.13, p < 0.0001; L4 vs A1 estimate = -7.44 ± 0.69, t = -10.80, p < 0.0001; L4 vs A3 estimate = -7.25 ± 0.65 t = -11.13, p < 0.0001). Batch 2 and 3 were significantly yellower than batch 1 (Batch 1 vs Batch 2 estimate = 1.31 ± 0.58, t = 2.27, p = 0.02; Batch 1 vs Batch 3 estimate = 2.10 ± 0.53, t = 3.95, p = 0.0001). We found no significant interaction between GSH and sequestered cardenolides on yellowness (F = 1.31, p = 0.25) so removed it from the model. We found no significant effect of sequestered cardenolides on yellowness (estimate = 0.009 ± 0.19, t = 0.03, p = 0.97).

4. Discussion

We reared large milkweed bugs *O. fasciatus* on an artificial diet with increasing concentrations of cardenolides and found intra-individual variation in sequestration, oxidative state, and colouration. By modulating the quantity of diet-derived cardenolides sequestered we found that bugs reared with medium to high levels of cardenolides had significantly lower levels of the antioxidant glutathione (GSH) than bugs that were reared without cardenolides, and that glutathione also tended to be lower in individuals with higher levels of sequestered cardenolides, independent of treatment. Bugs with higher concentrations of total glutathione had brighter warning signals, which supports the idea that variation in larval food resources is reflected in the degree of pigmentation (Davis 2009). Our results add to the recent evidence that cardenolides can have a negative effect on milkweed bug fecundity (Pokharel, Steppuhn & Petschenka 2021), and provides further support of a cost of sequestration via the depletion of antioxidants.

If pigments used in prey warning signals play a dual role in producing both the signal and also in preventing self-damage when storing toxins (due to their antioxidant properties), we predicted an interaction between treatment and oxidative state on signalling. We did not find an

interaction, instead we found that when *O. fasciatus* were raised on higher concentrations of cardenolides they had lower levels of total glutathione, which suggests that sequestration depletes this antioxidant. Glutathione is involved in detoxification processes (Enayati, Ranson & Hemingway 2005), and is well-known for detoxifying phytochemicals such as aristolochic acid (Gao et al. 2021) ansulforaphane (Villa-Cruz et al. 2009). Glutathione is also involved in the melanin synthesis pathway when pheomelanin dopaminquinone undergoes a non-enzymatic conjugation of a thiol, usually glutathione or cysteine to produce thiolated catecholamines (Ito & Prota 1977). Although the orange pigments in large milkweed bugs have been identified as pterins (Bartel, Hudson & Craig 1958), pteridines also commonly act as cofactors of enzymes involved in the melanin synthesis pathway which hydroxylate phenylalanine to tyrosine and oxidize tyrosine to DOPA (Shamim et al. 2014). Given that glutathione was depleted with increasing concentrations of sequestered cardenolides, and individuals that had low levels of GSH produced less bright warning signals, we can speculate that glutathione availability has a role in the biochemistry underlying the variation in colouration. Our results could reflect differences in how we quantified the visual signals of the bugs. In this study, we calculated hue and luminance according to trichromatic L*a*b* colour space rather than ΔS conspicuous to a specific background of a tetrachromatic visual system which is what we did for monarch butterflies in Blount et al (2021). When we analyse sRGB luminance and redness we find that bugs with higher levels of GSH are also significantly brighter (supplementary material section 9). Future research on milkweed bug colour could model appearance for a range of visual systems and natural backgrounds, but this is beyond the scope of this present study. Pigmentation and warning colours are regulated by more than one mechanism, and our results show that the relationship is likely more complex than our study quantifies, and that this warrants further psychophysical and biochemical study.

We predicted a positive correlation between individual levels of cardenolides sequestered by *O. fasciatus* and oxidative lipid damage, we did not find this, instead we found stable levels of oxidative damage (MDA) during sequestration. This suggests that *O. fasciatus* can

sustain redox state during acquisition of cardenolides. In a comparator system, the monarch butterfly (*Danaus plexippus*), increasing concentrations of sequestered cardenolides resulted increased oxidative damage (Blount et al 2021). *O. fasciatus* have significantly higher resistance to cardenolides than monarchs (Braer et al. 2015), which could be one explanation for the difference in our results. Another possibility is that we held the nutritional background constant, only varying the quantity of additional cardenolides, whereas in Blount et al (2021) monarchs were reared on whole food plants which differ not only in phytochemical profile but also in other metabolic and physical parameters which could have contributed further to changes in redox state. We also found that *O. fasciatus* only sequestered ouabain, and there were no digitoxin metabolites (as was also reported in Pokharel, Steppuhn & Petschenka 2021), so the costs of sequestration may differ when milkweed bugs are feeding on plants with more complex mixtures of cardenolides. *O. fasciatus* do experience oxidative damage in other contexts (López-Muñoz et al. 2019), so the stable levels of damage we measured here could be related to their higher cardenolide resistance rather than a general resistance to oxidative stress. A comparative analysis of related milkweed bug responses to sequestration would be a worthwhile future investigation to test this idea, and aid in our understanding of host shifts that are known to occur in the Lygaeinae (Petschenka et al. 2022).

We found significant batch effects in our results. This could be due to variation in the length of exposure to toxins during the larval development, however, we checked all batches and insect boxes daily to monitor their moulting and sampled them according to the same criteria across batches. Another reason could be genetic variance among batches which has been described in studies on flight (Palmer and Dingle 1986, 1989) and heritability of morphological traits (Rodríguez-Clark, 2004). However, our bugs came from the same long-term laboratory colony. It could also be argued that our experimental design caused these batch effects because we have a single factor treatment with four levels (diet) randomly assigned to an experimental box in each batch. This means that we potentially introduced a unique set of factors to each box and batch that contribute to the error variance of the measures of the response in that group and,

as a consequence, the error (residuals) within a batch are more similar to each other than they are to the residuals among batches. However, our batch effects were mainly at the level of sequestration rather across all measures of oxidative state and colouration and this could reflect intra-individual variation in sequestering efficiency which has been recorded in wild caught populations (Isman, Duffey & Scudder 1977). Irrespective of these limitations, our results clearly demonstrate that the amount of cardenolides sequestered can influence the redox state of large milkweed bugs, and that antioxidant availability affects warning signal brightness. Whether the variability of colour and sequestration we have measured in this experiment results in differential predation is an open question, but there is evidence in other experiments to suggest this is likely (Pokharel et al. 2020; Petschenka et al. 2022)

In conclusion, our results add to the evidence that sequestration impacts life-history traits (Pokharel, Steppuhn & Petschenka 2021), and has molecular costs in *O. fasciatus* (Dalla & Dobler 2016; Dalla, Baum & Dobler 2017). Studies documenting the costs associated with using signals and secondary defences in natural systems, and the the fitness benefits/costs of defences against enemies is important for our understanding of the ecology and evolution of aposematism (Zvereva & Kozlov 2016).

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Chapter 3 - Non-universal Benefits Of Sequestration Against Predators

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Article

Defense of Milkweed Bugs (Heteroptera: Lygaeinae) against Predatory Lacewing Larvae Depends on Structural Differences of Sequestered Cardenolides

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Abstract: Predators and parasitoids regulate insect populations and select defense mechanisms such as the sequestration of plant toxins. Sequestration is common among herbivorous insects, yet how the structural variation of plant toxins affects defenses against predators remains largely unknown. The palearctic milkweed bug *Lygaeus equestris* (Heteroptera: Lygaeinae) was recently shown to sequester cardenolides from *Adonis vernalis* (Ranunculaceae), while its relative *Horvathiolus superbus* also obtains cardenolides but from *Digitalis purpurea* (Plantaginaceae). Remarkably, toxin sequestration protects both species against insectivorous birds, but only *H. superbus* gains protection against predatory lacewing larvae. Here, we used a full factorial design to test whether this difference was mediated by the differences in plant chemistry or by the insect species. We raised both species of milkweed bugs on seeds from both species of host plants and carried out predation assays using the larvae of the lacewing *Chrysoperla carnea*. In addition, we analyzed the toxins sequestered by the bugs via liquid chromatography (HPLC). We found that both insect species gained protection by sequestering cardenolides from *D. purpurea* but not from *A. vernalis*. Since the total amount of toxins stored was not different between the plant species in *H. superbus* and even lower in *L. equestris* from *D. purpurea* compared to *A. vernalis*, the effect is most likely mediated by structural differences of the sequestered toxins. Our findings indicate that predator–prey interactions are highly context-specific and that the host plant choice can affect the levels of protection to various predator types based on structural differences within the same class of chemical compounds.

Keywords: predatory–prey interactions; multi-trophic interactions; cardiac glycosides; cardenolides; Lygaeinae; plant toxins; milkweed bugs

1. Introduction

Top-down regulation by predators is a major force controlling the dynamics of prey populations [1,2]. While many insect species defend themselves against predators with chemical compounds synthesized de novo [3], insects from at least six orders employ secondary metabolites sequestered from their host plants as a defense [4]. Plant secondary metabolites are often toxic or deterrent in order to repel herbivores [5,6] and it has been repeatedly shown that many insect species use sequestered plant toxins for protection against predators. For example, triodine swallowtail butterflies sequester aristolochic acids that are distasteful to birds [7,8], pyrrolizidine alkaloids sequestered by arctiid moths act as defensive agents against *Nephila* spider [9,10], geometrid moths sequestering grayanotoxins are protected against house-lizards [11], and lygaeid bugs and danaine butterflies gain protection against avian predators by sequestering cardenolides [12,13].

Milkweed plants (*Asclepias* spp.) produce cardenolides, and the interactions among milkweed, its specialist herbivorous insects, and predators, represent an important model system in chemical ecology and insect–plant coevolution [14–16]. Cardenolides are a group of secondary plant metabolites that are distributed across approximately 62 genera in more than 10 plant families [17,18] occurring in a wide range of habitats [19]. These compounds are an important class of natural drugs, which show cardiotonic and neurological activity in vertebrates and are also toxic for some insects [17,20,21]. The pharmacological effects of cardenolides are mediated by the specific inhibition of the ubiquitous animal enzyme Na^+/K^+-ATPase, a cation carrier that is involved in many essential physiological functions such as the generation of neuronal action potentials and the maintenance of an electrochemical gradient across the cell membrane [22,23].

Insects in at least five orders (Diptera: Agromyzidae, Lepidoptera: Danaidae, Coleoptera: Chrysomelidae, Heteroptera: Lygaeidae, Sternorrhyncha: Aphididae, and Caelifera: Pyrgomorphidae) sequester and show adaptations to cope with cardenolides. These species possess a modified form of Na^+/K^+-ATPase that is resistant to cardenolides, due to a few amino acid substitutions, a phenomenon referred to as target site insensitivity [15,16,24–27]. In some cases, the evolution of this trait seems to be associated with the ability to sequester cardenolides for defense [28]. Besides resistance, these insects also show morphological adaptations related to chemical defense mechanisms based on cardenolides. For example, the large milkweed bug *Oncopeltus fasciatus* has evolved a double-layered epidermis to store and deploy cardenolides when attacked by a predator [29,30].

Several studies provide evidence for the distastefulness of plant-derived cardenolide defenses against both vertebrate and invertebrate predators. The most prominent example is the feeding trials involving *Asclepias*-reared monarch butterflies *Danaus plexippus* and blue jays *Cyanocitta cristata*, showing pictures of rejection and disgust behaviors from the birds [31]. Further examples include the mice species *Peromyscus aztecus* and *Reithrodontomys sumichrasti* that taste-rejected both field-caught and laboratory-reared monarchs, as well as diets containing digitoxin, a pharmaceutically important cardenolide from foxglove (*Digitalis* spp.) [32,33]. Similarly, the oleander seedbug *Caenocoris nerii* (Heteroptera: Lygaeinae), reared on cardenolide-containing *Nerium oleander*, was protected against common quails *Coturnix coturnix* [34].

Besides the observations based on vertebrates, effects have also been found for invertebrate predators. Praying mantids (*Tenodera ardifolia*) vomited and showed signs of poisoning after feeding on *O. fasciatus* [35]. The orb-weaving spider *Zygiella x-notata* consumed fewer toxic oleander aphids (*Aphis nerii*) compared to non-toxic aphids. Moreover, spiders built disrupted webs when feeding on toxic aphids [36]. Similarly, *Asclepias* seed-fed adults and nymphs of *O. fasciatus* were significantly less likely to be preyed upon by *Nephila senegalensis* spiders than control bugs raised on sunflower seeds [37]. Even the eggs of milkweed-raised females of *O. fasciatus*, that are known to contain cardenolides via maternal transfer [38], were found to be protected against the larvae of the lacewing *Chrysoperla carnea* [39].

Milkweed bugs (Heteroptera: Lygaeinae) are a diverse group of over 600 species [40] that are typically aposematic, with a distinctive red and black pattern. Across their global distribution range, milkweed bug species are commonly associated with host plants belonging to the family Apocynaceae (e.g., *Asclepias* spp., *Nerium oleander*), which often contain cardenolides [19,26,41]. Several species of milkweed bugs are also associated with plants [42–44] that are phylogenetically disparate from Apocynaceae but convergently produce cardenolides [45]. For example, *Lygaeus equestris* (Linnaeus 1758) is associated with the cardenolide containing *Adonis vernalis* (Ranunculaceae) [46,47]. Similarly, *Horvathiolus superbus* (Pollich 1781) specializes on *Digitalis purpurea* (Plantaginaceae) [48,49], which also contains cardenolides [18].

We have demonstrated previously that both, *H. superbus* and *L.equestris*, were protected against avian predation when they had sequestered cardenolides from their respective host plants [45]. However, while the early instar larvae of *H. superbus* raised on *Digitalis* seeds also gained protection against lacewing larvae, *L. equestris* nymphs were not protected when raised on *Adonis* seeds [45].

Since both insect species sequester cardenolides from their respective host plants, it remains unclear which factors mediate these differences. More specifically, predator aversion could be either due to the quantitative or qualitative differences of the cardenolides sequestered. Alternatively, the differential exposure of sequestered defenses to the attacking predator by the two milkweed bug species could explain the observed differences (i.e., the differences could depend on the insect species).

We designed a full factorial experiment to determine how two insect species sequestering the same class of toxic compounds could have such different outcomes with predators. We raised the two milkweed bug species on both types of cardenolide-containing seeds (either *Digitalis* or *Adonis*), and exposed them to lacewing larvae. In addition, we compared the cardenolide profiles of the toxins sequestered by both species, and tested if the amount and identity of the sequestered toxins differed across the two diets and insect species. Specifically, we tested if the different predator tolerance was due to (i) different amounts of cardenolides sequestered from *Digitalis* compared to *Adonis* (i.e., quantitative differences), (ii) the structural differences between the cardenolides sequestered from *Digitalis* and *Adonis* (i.e., qualitative differences), and/or (iii) the differences mediated by the milkweed bug species (e.g., deployment of toxins).

2. Materials and Methods

2.1. Insect Culture

We collected *Lygaeus equestris* specimens from an *A. vernalis* habitat ("Oderhänge Mallnow"), north of Lebus, Brandenburg, Germany, and the specimens of *Horvathiolus superbus* from a *D. purpurea* habitat close to Eberbach, Baden-Württemberg, Germany. In the laboratory, insect colonies were housed in plastic boxes (19 × 19 × 19 cm) covered with gauze in a controlled environment (Binder KBWF 240) at 28 °C, 60% humidity and a day/night cycle of 16/8 h under artificial light. We reared stock colonies of both species on organic sunflower seeds (Alnatura GmbH, Darmstadt, Germany) and supplied water in Eppendorf tubes plugged with cotton. We also included a piece of cotton as a substrate for oviposition. *H. superbus* used for the video-recording of aversive predator behavior were collected from a *D. purpurea* habitat close to Lollar, Hesse, Germany.

2.2. Predation Assay

We obtained *L. equestris* and *H. superbus* eggs from the stock colonies and placed them in Petri dishes (60 × 15 mm, with vents) lined with filter paper. The larvae were either raised on field-collected *Digitalis purpurea* seeds (Eberbach, Germany), commercial *Adonis vernalis* seeds (Jelitto Staudensamen GmbH, Schwarmstedt, Germany), or sunflower seeds as a control, until reaching the second instar (older larvae of *L. equestris* are too big as a prey for lacewing larvae). Water was supplied in Eppendorf tubes as described above. Lacewing larvae (*Chrysoperla carnea*) were obtained commercially (Sautter & Stepper GmbH, Ammerbuch, Germany), transferred individually into the wells of a 48-well plate, supplied with the eggs of *Sitotroga cerealella* (Katz Biotech AG, Baruth, Germany) as a diet and covered with a breathable membrane (Breathe-Easy sealing membrane, Diversified Biotech, Dedham, MA, USA). To increase the body size, lacewing larvae were allowed to feed for two days at 21 °C, 60% humidity and a day/night cycle of 16/8 h under artificial light (Binder KBWF 240 climate chamber, Tuttlingen, Germany). Before the predation experiment, each final instar lacewing larva was transferred into an empty well of a 48-well plate and starved for two days under the same conditions as described above.

Predation assays were carried out under ambient conditions in the laboratory. We exposed the second instar larvae of *L. equestris* and *H. superbus* individually to one lacewing larva in a Petri dish (60 mm diameter) and observed the behavior of the lacewing larva. If the first attack was unsuccessful, i.e., if the lacewing released the milkweed bug instantly after probing, we removed the milkweed bug. These milkweed bug larvae were individually transferred to empty Petri dishes, supplied with sunflower seeds and water, and the survival was scored on the following day. If the attack was successful, we recorded how long the lacewing larvae spent feeding on the milkweed bug larva until the lacewing

larva left its prey. We also counted the frequency of aversive behavior (mandible wiping) shown by the lacewing larvae when their attack was unsuccessful. For the illustration of aversive behavior, we recorded the mandible cleaning of lacewing larvae using a camera (Nikon D90, Nikon Corporation, Tokyo, Japan) equipped with a Sigma 105 mm 1:2.8 DG Macro lens (Sigma Corporation, Kanagawa, Japan) in a separate setup.

2.3. Chemical Analysis

To analyze the amount and the differences between the sequestered cardenolides in *L. equestris* and *H. superbus*, additional milkweed bug larvae were stored at −80 °C, and subsequently freeze-dried and weighed. The samples were homogenized with zirconia/silica beads (ø 2.3 mm, BioSpec Products, Inc., Bartlesville, OK, USA) in 1 mL HPLC-grade methanol containing 0.01 mg/mL of oleandrin (PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany) as an internal standard in a Fast Prep™ homogenizer (MP Biomedicals, LLC, Solon, OH, USA) for two cycles of 45 s at 6.5 m/s. After centrifugation at 16,100 g for 3 min, the supernatant was collected and the sample was extracted two more times with 1 mL of pure methanol. All the supernatants of a sample were pooled and evaporated to dryness under a flow of nitrogen gas. Finally, we dissolved the residues in 100 µL methanol by agitating the samples in the Fast Prep™ homogenizer without the inclusion of beads and filtered samples into HPLC vials using Rotilabo® syringe filters (nylon, pore size: 0.45 µm, diameter: 13 mm, Carl Roth GmbH & Co. KG, Karlsruhe, Germany).

We injected 15 µL of extract into an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a photodiode array detector and separated the compounds on an EC 150/4.6 NUCLEODUR® C18 Gravity column (3 µm, 150 mm × 4.6 mm, Macherey-Nagel, Düren, Germany). Cardenolides were eluted at a constant flow rate of 0.7 mL/min at 30 °C using the following acetonitrile–water gradient: 0–2 min 16% acetonitrile, 25 min 70% acetonitrile, 30 min 95% acetonitrile, 35 min 95% acetonitrile, 37 min 16% acetonitrile, 10 min reconditioning at 16% acetonitrile. Peaks with symmetrical absorption maxima between 218 and 222 nm were recorded as cardenolides [50] using the Agilent ChemStation software (B.04.03). Finally, we estimated the amount of cardenolides per sample by comparing the sum of all cardenolide peak areas to the area of the internal standard [51,52].

2.4. Statistical Analysis

We tested the hypothesis that the diet of the bugs affected their survival upon attack by a lacewing larva using the 2×3 Freeman–Halton extension of Fisher's exact test [53]. The probability values for the binomial data from the predation experiment (*survival of milkweed bugs and mandible cleaning behavior by lacewing larvae*) were computed using an online statistical tool (http://www.danielsoper.com/statcalc) [54]. All the other data were analyzed using the JMP® 14.3.0 statistical software (SAS Institute, Cary, NC, USA). Data were assessed for normal distribution by visual inspection of the q-q plots and by the Shapiro–Wilk W test. Homogeneity of variances was evaluated by visual inspection of residual plots. The duration data from the predation experiment were $\log_{10}$ transformed to achieve normal distribution and analyzed using Welch's ANOVA due to the heteroscedasticity of this dataset. We excluded one outlier (determined by the outlier box-plot in JMP) from the dataset of *L. equestris* raised on *Adonis* but the exclusion of this outlier did not change the direction of results. To assess the differences between treatments, we used the Games–Howell HSD post-hoc test. The concentrations and diversity of sequestered toxins were analyzed by ANOVA followed by LSMeans Difference Tukey HSD. We included bug species, treatment (*Digitalis* or *Adonis*), and the interaction between bug species and treatment in our model. Probability values < 0.05 were considered statistically significant.

3. Results

3.1. Predation Assay

We conducted predation trials with *H. superbus* and *L. equestris* larvae raised on either sunflower, *A. vernalis*, or *D. purpurea* seeds and the larvae of the predatory lacewing *C. carnea*. We found that a diet of *Digitalis* seeds increased the survival of both *H. superbus* and *L. equestris* ($p < 0.001$, for both insect species, Fisher's exact test) (Figure 1). In addition, the lacewing larvae showed mandible-cleaning behavior (Video S1) only after attacking *Digitalis*-raised *H. superbus* and *L. equestris* ($p < 0.001$, for both insect species, Fisher's exact test) (Figure 2). In contrast, the bugs raised on both *Adonis* (although containing cardenolides) and sunflower seeds were neither protected, nor did the lacewing larvae show mandible-cleaning behavior after attacking them (Video S2). Moreover, lacewing larvae spent significantly less time feeding on both *H. superbus* and *L. equestris* raised on *Digitalis* seeds as compared to *Adonis* and sunflower-raised bugs ($p < 0.001$, for both insect species, Games–Howell HSD) (Figure 3).

3.2. Chemical Analysis

We assessed the quantity and compared the retention times of the sequestered cardenolides in both species of bugs, raised on *Digitalis* or *Adonis* seeds (Figures 4 and 5). We found an effect of diet on sequestration ($F_{3,33} = 3.939$; $p = 0.025$, LSMeans Differences Tukey HSD, Figure 4). *Digitalis*-raised *L. equestris* sequestered lower concentrations of cardenolides than the *Adonis*-raised *L. equestris* ($p = 0.021$, LSMeans Differences Tukey HSD), whereas *H. superbus* sequestered similar concentrations of cardenolides from both types of seeds ($p = 0.998$, LSMeans Differences Tukey HSD). Regarding the diversity of sequestered cardenolides, the bugs sequestered fewer structurally different cardenolides (based on retention times) from the seeds of *Digitalis* than from the seeds of *Adonis* ($F_{3,33} = 27.623$; $p < 0.001$, LSMeans Differences Tukey HSD, Figure 4). Specifically, *L. equestris* sequestered three times more different cardenolides from *Adonis* compared to *Digitalis*. We found the same pattern for *H. superbus* although the difference was less pronounced ($p < 0.001$, LSMeans Differences Tukey HSD).

3.3. Figures, Tables and Schemes

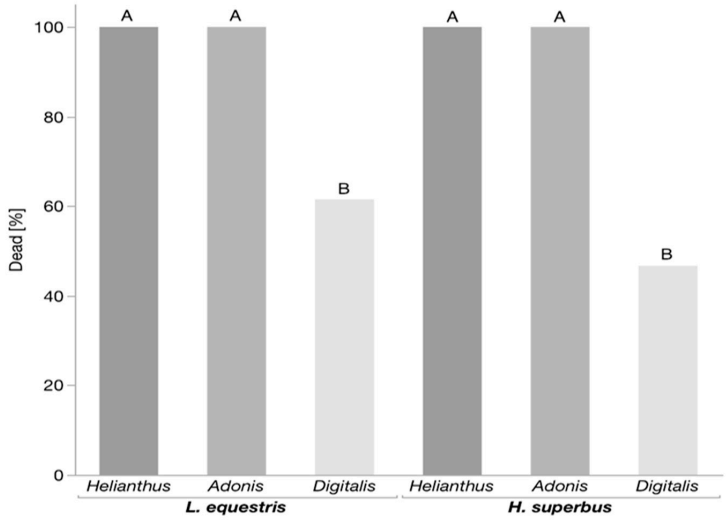

Figure 1. Survival of the milkweed bug larvae preyed upon by *C. carnea*. Bars represent the proportion of dead larvae after *C. carnea* attacks. The milkweed bugs *L. equestris* (n = 22–26 per treatment) and *H. superbus* (n = 15–16 per treatment), were raised on the seeds of either *Helianthus annus* (sunflower), *Adonis vernalis*, or *Digitalis purpurea*. Within the same insect species, levels not connected by the same letter are significantly different.

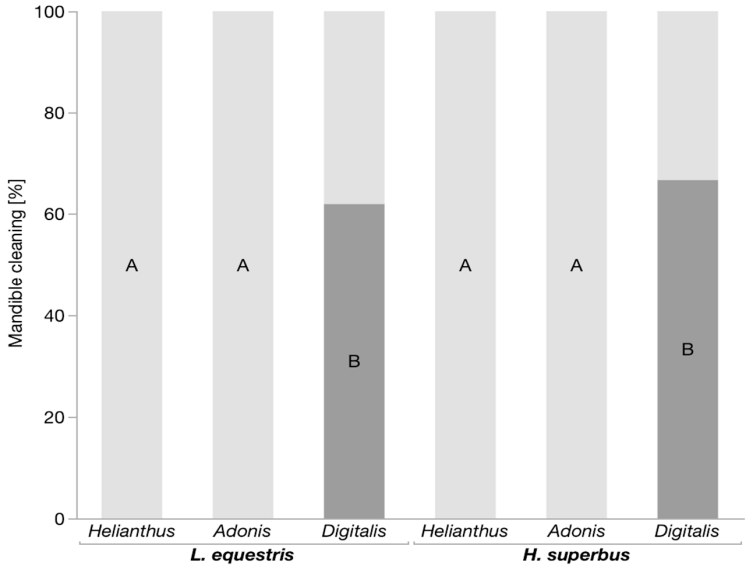

Figure 2. Mandible cleaning behavior shown by *C. carnea* after attacking milkweed bug larvae. Bars represent the proportion of lacewing larvae that cleaned their mandibles (dark grey) and that did not clean their mandibles (light grey) after attacking *L. equestris* (n = 22–26 per treatment) and *H. superbus* (n = 15–16 per treatment) larvae raised on the seeds of either *Helianthus annus* (sunflower), *Adonis vernalis* or *Digitalis purpurea*. Within the same insect species, levels not connected by the same letter are significantly different.

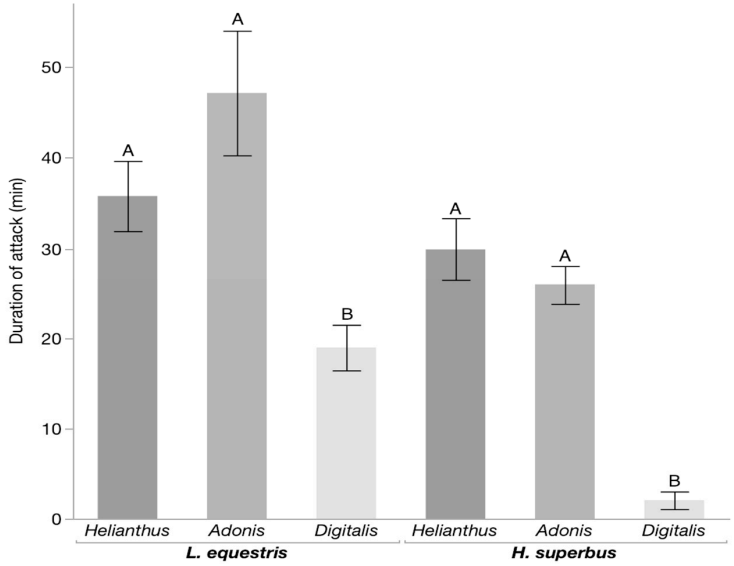

Figure 3. Feeding duration of *C. carnea* on milkweed bug larvae. Bars represent the means ± SE of the time taken by *C. carnea* to feed upon *L. equestris* (n = 22–26 per treatment) and *H. superbus* (n = 15–16 per treatment) larvae raised on the seeds of either *Helianthus annus* (sunflower), *Adonis vernalis*, or *Digitalis purpurea*. Within the same insect species, different letters above bars indicate significant differences.

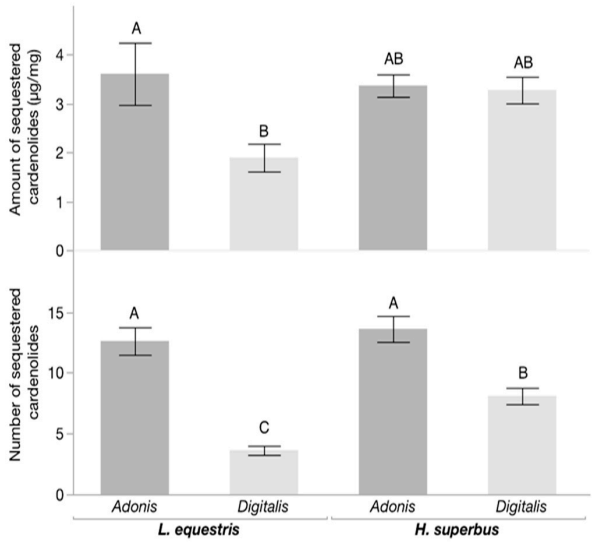

Figure 4. Quantity and number of different sequestered cardenolides by milkweed bug larvae. Bars represent the means ± SE of the concentration (µg/mg) (**top**) and the number of structurally different cardenolides sequestered (**bottom**) by *L. equestris* (n = 8 per treatment) and *H. superbus* (n = 8–10 per treatment) raised on the seeds of either *Adonis vernalis* or *Digitalis purpurea*. Different letters above bars indicate significant differences.

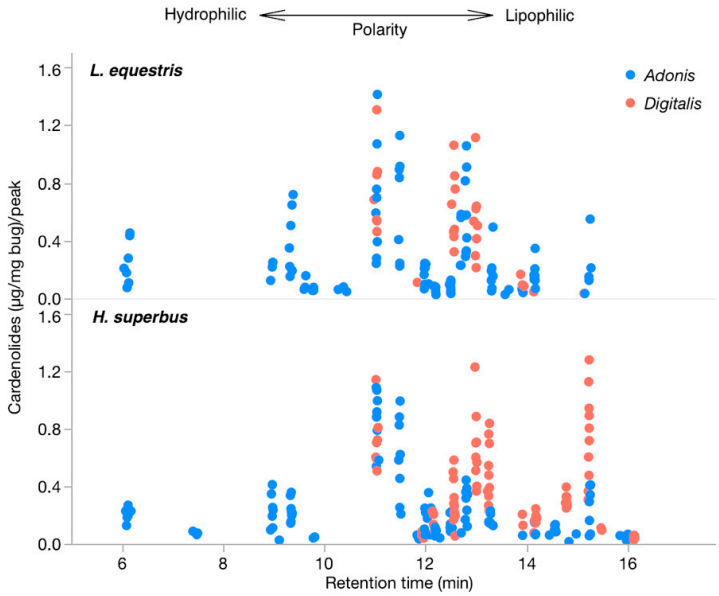

Figure 5. Concentrations of the sequestered individual cardenolides and retention times based on HPLC analysis. Each datapoint represents an individual cardenolide sequestered by a specimen of *L. equestris* (n = 8 per treatment) (**top**) and *H. superbus* (n = 8–10 per treatment) (**bottom**) raised on the seeds of either *Adonis vernalis* (blue) or *Digitalis purpurea* (red). Polar cardenolides (hydrophilic) have shorter retention times and apolar cardenolides (lipophilic) have longer retention times.

4. Discussion

It is widely accepted that sequestered phytochemicals protect herbivorous insects against their natural enemies [12]. However, our understanding of how the structural differences of sequestered

plant compounds, either within the same or across different classes of substances, can affect the outcome of predator–prey interactions, is very limited. We showed that the chance of a milkweed bug to survive a lacewing attack strongly depends on the original source of the sequestered cardenolides. Although the milkweed bugs sequestered cardenolides from both the toxic plant species (*A. vernalis* and *D. purpurea*) tested, only the bugs feeding on *Digitalis* seeds gained protection against lacewing larvae. Furthermore, our observations indicate that rejection is based on taste, as we observed aversive behavior (i.e., mandible cleaning) in the lacewing larvae after attacking bugs raised on *Digitalis* seeds. Accordingly, the lacewing larvae spent less time feeding on bugs raised on *Digitalis* seeds than on those raised on *Adonis* or sunflower seeds, indicating that the *Digitalis*-derived cardenolides show deterrent activity, but that the *Adonis*-derived cardenolides did not. Since the bugs sequestered similar or lower amounts of toxins from *Digitalis* compared to *Adonis*, it is very likely that structural features specific to one or more *Digitalis* cardenolides, rather than quantitative differences, increased the survival of milkweed bug larvae. While only cardenolides sequestered from *D. purpurea* increased survival in milkweed bugs, cardenolides from *A. vernalis* also exert some deterrent activity and decrease consumption by lacewing larvae [45]. The pattern observed here was identical for both species of milkweed bugs, rejecting the hypothesis that our initial observation was mediated by features specific to the insect species such as deployment or the discharge of toxins.

Lacewings have been used as natural predators for biological control for more than 250 years [55,56], and *C. carnea* has been employed commercially against various insect pests including lepidopterans [57,58], Colorado potato beetle [59,60], and others [61]. Lacewing prey consumption behavior was reviewed by Principi & Canard [62] and Canard & Duelli [63], and the sequence of attack was described in detail by McEwen et al. [64]. Lacewing larvae recognize their prey by contacting them with their palpi and/or antennae, followed by probing them with their mandibles for chemosensory recognition. Finally, they capture their prey and inject salivary secretions from venom glands at the base of their maxillae [65] via the mandibles, causing prey tissue to liquify, and subsequently draw it up. In our experiments, we found that lacewings rejected apparently distasteful prey, followed by mandible cleaning behavior. We hypothesized that this grooming behavior, to our knowledge reported here for the first time, removes prey toxins by rubbing mouthparts together and wiping them on the substrate.

Lacewing larvae have been reported to acquire resistance against several different pesticides, such as flonicamid (pyridines) [66] and lambda-cyhalothrin (pyrethroids) [67]. However, only a few experimental studies investigated the effects of plant toxins in herbivore diets on lacewings. One such study found that *C. carnea* larvae did not experience increased mortality when they attacked diamondback moths *Plutella xylostella* feeding on plants with toxic glucosinolates or without glucosinolates [68]. However, as mentioned above, cardenolides conferred protection to the eggs of *Asclepias*-raised *O. fasciatus* against *C. carnea* [39]. Surprisingly, cardenolides were found to be present in lacewing pupae when the larvae preyed upon *Aphis nerii* feeding on *Nerium oleander* [69] indicating an uptake of sequestered compounds from the prey by the predator. While the aforementioned examples suggest that lacewing larvae can tolerate insecticides or sequestered plant toxins to some extent, our knowledge on the aversive properties of sequestered plant toxins inducing responses such as the mandible cleaning that we described here seems to be quite limited.

Plants produce a great diversity of secondary metabolites across but also within compound classes and even on the level of individual plants. One potential hypothesis to explain the ecological significance of the diversity of observed chemical defenses is the screening hypothesis [70]. This hypothesis posits that the diversity of plant toxins is sustained to enhance the probability of a plant to possess an effective compound or a precursor of it against multiple predators or combinations of compounds working synergistically [71], thus together generating a selective advantage against a wide range of antagonists. Substantial variation of gross cardenolide content has been reported in natural populations of monarch butterflies [72] and the milkweed bugs *O. fasciatus* and *Lygaeus kalmii* [73]. In monarch butterflies, palatability to blue jays was found to vary depending on the species of milkweed used as a host plant

by the caterpillar [14]. Different species of *Asclepias* plants produce structurally diverse cardenolides that differ in their emetic potency against predators [74]. For example, monarch butterflies raised on *A. eriocarpa* had greater emetic potency than monarchs reared on *A. speciosa* [75]. Despite the fact that sequestration in milkweed bugs is comparatively well studied, the potential effects of the quantitative and structural variation of dietary cardenolides in milkweed bugs against their predators have not yet been tested empirically.

Our experiment sought to examine which factors mediated the different outcome of predator–prey interactions in closely related insect species sequestering the same class of compounds from their respective host plants. While *H. superbus* sequestered similar amounts of cardenolides from both plant species, *L. equestris* accumulated lower concentrations of cardenolides from *Digitalis* than from *Adonis*. This suggests that the observed defensive activity of cardenolides obtained from *Digitalis* was not mediated by dose, but rather by structural differences between *Digitalis*- and *Adonis*-derived cardenolides. In line with this, we observed noticeable differences in the structural diversity and polarity of sequestered cardenolides from these two plant species in both species of insects. For both insect species, we found that *Digitalis*-raised bugs sequestered fewer structurally different cardenolides than *Adonis*-raised bugs. Cardenolides sequestered from *Adonis* covered a wider polarity range than the cardenolides sequestered from *Digitalis*. Furthermore, the *Digitalis*-raised individuals of either milkweed bug species sequestered a higher proportion of apolar cardenolide compounds, potentially mediating the observed effect [76]. Here, we did not determine the identities of the individual cardenolides observed. In a previous study [45], the comparison of nine authentic cardenolides from *D. purpurea* with the cardenolides sequestered by *H. superbus* from the same plant revealed no matches based on retention times. For *L. equestris* raised on *A. vernalis* seeds, we tentatively identified cymarin, strophanthin, and k-strophantoside [45]. Although the structural identity of most cardenolides remains unknown, our findings support the hypothesis that individual plant compounds within the same chemical class can act against antagonists selectively.

Predator diversity is probably an important evolutionary driver for the observed wide variation in anti-predator defenses, as different predator species have varying tolerances to toxins, sensory abilities, and attack strategies [77]. Predators as taxonomically diverse as birds and invertebrates may exert differential selection pressures on the same prey [78]. Autogenous production as well as sequestering chemical defenses can incur physiological costs, as the organisms must tolerate active phytochemicals and sometimes modify them, whereas autogenous chemical defenses burden the species' limited resources at the expense of other functions, such as growth and survival [79–82]. These costly defenses are effective, but may only evolve to be deterrent against a wide array of natural enemies if required by predation pressure [83]. Unfortunately, our knowledge about predators attacking milkweed bugs in the field is very limited, but maybe lacewing larvae are not preying upon *L. equestris* in *A. vernalis* habitats, and therefore no selection on defenses against lacewings occurred in this species. Notably, *L. equestris* occasionally also uses *D. purpurea* as a host, suggesting that the individuals of this species show variation with regard to the predators they are protected from. Although we did not study predation on milkweed bugs in the field, this suggests that our findings also possess relevance in the field.

Although earlier literature suggested that the cardiotonic activity of *Adonis* and *Digitalis* extracts were equally potent on isolated frog hearts [84], we found that cardenolides from both plant species were perceived differently by predators. This agrees with previous work outlining how various predators reacted differently to the same prey. For example, *Neacoryphus bicrucis*, sequestering pyrrolizidine alkaloids from *Senecio*, were distasteful to green-anole lizards, however, the bugs were palatable to Fowler's toad [85]. Recently, it was also shown that two different defensive fluids from the thoracic glands and abdomen of the wood tiger moth *Arctia plantaginis* are predator specific. The moth thoracic fluids were deterrent to birds but not ants, and in contrast, the abdominal fluids deterred ants, but birds did not show any aversive response [86]. Besides the differences that may be mediated by host plant quality, the different outcomes of predator–prey interactions can also be mediated by traits of the

insects. Although *L. equestris* and *Tropidothorax leucopterus* were both feeding on cardenolide-free *Vincetoxicum hirundinaria*, only *L. equestris* was shown to be defended against domestic chicks [87]. This suggests that only *L. equestris*, but not the closely related *T. leucopterus*, was able to derive a defensive principle from this host plant. Regarding the huge structural diversity of sequestered plant secondary metabolites, future work should focus on the structure–activity relationships in the framework of predator–prey interactions.

5. Conclusions

Our results provide evidence that structural differences within the same class of sequestered host-plant toxins can direct the outcome of predator–prey interactions. Our findings indicate that predator–prey interactions are highly context-specific, and that investigating the effects of the diversity of chemical defenses on different predators in a community is vital for understanding tri-trophic interactions within an ecosystem.

Supplementary Materials: The following are available online at http://www.mdpi.com/2075-4450/11/8/485/s1, Video S1: Lacewing larva preying upon a milkweed bug nymph raised on sunflower seeds (control); Video S2: Lacewing larva cleaning its mandibles after attacking a milkweed bug nymph raised on *Digitalis* seeds containing cardenolides.

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General Discussion And Outlook

Deciphering the mechanisms underlying insect-plant interaction require consideration of antagonists of insects that drive multiple trophic levels. The trade-off between performance of insects on their host plants and their exposure to predators is an endless ecological theme. A deeper investigation into the costs and benefits of host plant usage enhances our understanding of the evolution of defensive strategies such as toxin sequestration and aposematism. One of the major goals was to understand how dietary plant toxins influence the physiological performance of the milkweed bugs. My dissertation revealed that toxin consumption increased growth in the sequestering specialists but not in the sequestering generalists, despite all species possessing toxin-resistant enzymes. In addition, sequestering specialist nymphs developed to adulthood faster and lived longer as adults under toxin exposure when compared to individuals raised on the control diet. However, specialists produced significantly less offspring unless being transferred to a toxin-free diet after reaching adulthood (Chapter 1). Besides a negative effect of toxins on the fecundity of *O. fasciatus*, my work also revealed a cost of toxin sequestration via the depletion of antioxidants. I showed that milkweed bugs raised on higher dietary toxins had significantly lower levels of antioxidants. The antioxidant level also tended to decrease in all individuals with increasing levels of sequestered toxins. Bugs with higher amounts of antioxidants had brighter warning signals, but these signals were not related to sequestration (Chapter 2). Finally, an important question remains in whether the gained benefits from sequestered toxins universally compensate the cost of sequestration. I found that the rates of survival against predator attack in the prey species strongly depended on the original source from which the toxins were sequestered. In other words, structural differences in the sequestered toxins directed the outcome of predator-prey interactions (Chapter 3).

Ecological Implications Of Cardenolide Sequestration

Structural Diversity Of Cardenolides In Predator-Prey Interactions

My data from Chapters 1 and 2 showed that *O. fasciatus* individuals sequestered cardenolides proportionally to the amount of cardenolides in their diets, supporting literature

suggesting sequestration is a dose-dependent process (Duffey et al., 1978). Depending upon the chemistry of the host plant cardenolides, milkweed bugs can sequester the toxins in their original forms or also can modify or detoxify them, thus covering in a wide polarity range. Generally during sequestration, cardenolides are metabolized into more polar forms (Seiber et al., 1980; Brower et al., 1984), and polar cardenolides are preferentially sequestered over nonpolar cardenolides (Frick and Wink, 1995). In accordance with this, I observed that milkweed bugs converted digitoxin (a nonpolar cardenolide) to its polar metabolites after sequestration (Chapter 1). Metabolic detoxification of cardenolides *in O. fasciatus* was often considered to be less important in terms of physiological burden in the literature (Seiber et al., 1980; Malcolm, 1994). However, recent evidence has shown that the process of sequestration could impose a burden in terms of detoxification and modification of cardenolides as seen in cardenolide-sequestering specialist monarch caterpillars (Agrawal et al., 2021). Nevertheless, the costs of adaptation, cardenolide metabolism, and sequestration in *O. fasciatus* requires more research as different cardenolides have diverse physiochemical properties (such as polarity) that directly impact on the gut uptake, distribution in the body, and excretion (Duffey, 1980).

The data presented in Chapter 3 showed that *Digitalis*-raised milkweed bugs sequestered a higher proportion of nonpolar cardenolides when compared to cardenolides sequestered by *Adonis*-raised bugs. Furthermore, individuals sequestering nonpolar cardenolides were protected more against the predator attack than individuals sequestering polar cardenolides. In future work, investigating the deterrence potential of differing cardenolides from varied plant species with a range of differing polarities against predators (both invertebrates and vertebrates) will prove important in improving our understanding of the ecological implications of sequestered compounds.

Facets of chemical defense in plants can affect predator-prey interactions by mediating effects on the insect herbivores (i.e., prey). For example, *L. equestris* and *H. superbus* feed upon the cardenolide-producing plant species *A. vernalis* and *D. purpurea*, respectively, from where both species sequester (Petschenka et al., 2022). Generally, the evolution of chemical defenses is

considered in association with the trade-offs between acquired benefits via protection against predators, and the possible costs for the acquiring and maintenance (production and/or sequestration) of these defenses. Since chemical defenses directly increase the survival of well-defended prey against the predators, the benefits of chemical defenses are more apparent than costs.

The effectiveness of sequestered plant toxins in protecting insects against their vertebrate (e.g., birds) and invertebrate (e.g., spiders) predators is widely accepted (Nishida, 2002). In my experiments, I showed that although both *L. equestris* and *H. superbus* insects sequestered cardenolides from their host plants, only those individuals feeding on *Digitalis* seeds gained protection against lacewing larvae (Chapter 3). My findings imply that the effectiveness of sequestered toxins is not universal, and rather strongly depend on the host plant chemistry. In other words, the chance of a prey in surviving a predator attack does not depend either on the prey's nature of deployment of toxins or the total quantity of toxins, but the quality of toxins (i.e., structurally diverse chemicals) sequestered by the prey.

Anti-Predator Behavioral Strategies

Milkweed bugs feed on seeds or seedpods in the open, exposing themselves to predators (Aldrich, 1988). Larvae aggregate around seedpods, communicating via pheromones (Aller and Caldwell, 1979), whereas adults disperse and forage independently (Sauer and Feir, 1973). Larvae even aggregate when feeding on an artificial diet in the laboratory (personal observation). From my dataset on *O. fasciatus* (Chapter 2), I showed that larvae were less toxic, but brighter and redder than adults. For future experiments it would be interesting to test if larvae aggregate to enhance their total aposematic signaling since aggregation has been correlated to aposematism across species (Ruxton et al., 2019), or whether aggregation is primarily to utilize their food source more effectively via communal feeding (Ralph, 1976), or alternatively simply to maintain the microclimate (Lockwood and Story, 1985).

Milkweed bug larvae have different methods of deploying chemical defense than adults. Although larvae sequester cardenolides, they release other defensive secretions such as

aldehydes from two dorsal glands (Games and Staddon, 1973), whereas adults possess a specialized double-layered epidermis wherein they store cardenolides, deploying them from the dorsolateral sides under predator attack (Scudder and Meredith, 1982; Bramer et al., 2017). In adults, the dorsal glands might have different functions, such as for pheromone production (Aldrich, 1988). I speculate that due to these differing tactics in feeding behavior and release of chemical defense, the anti-predator strategies of larvae and adult milkweed bugs may target different predator groups. I did not investigate avoidance learning behavior from the predator perspective with regards to the warning coloration of the aposematic larvae (Chapter 3). However, I reported for the first time a grooming behavior in lacewing larvae where the prey toxin is removed by rubbing and wiping the mouthparts, which I called 'mandible cleaning behavior'. The results from Chapter 2 and 3 suggest further investigations on the potential effects of diversity in chemical defenses and differing anti-predator strategies of prey on different predators in an ecosystem, to better understand multitrophic interactions.

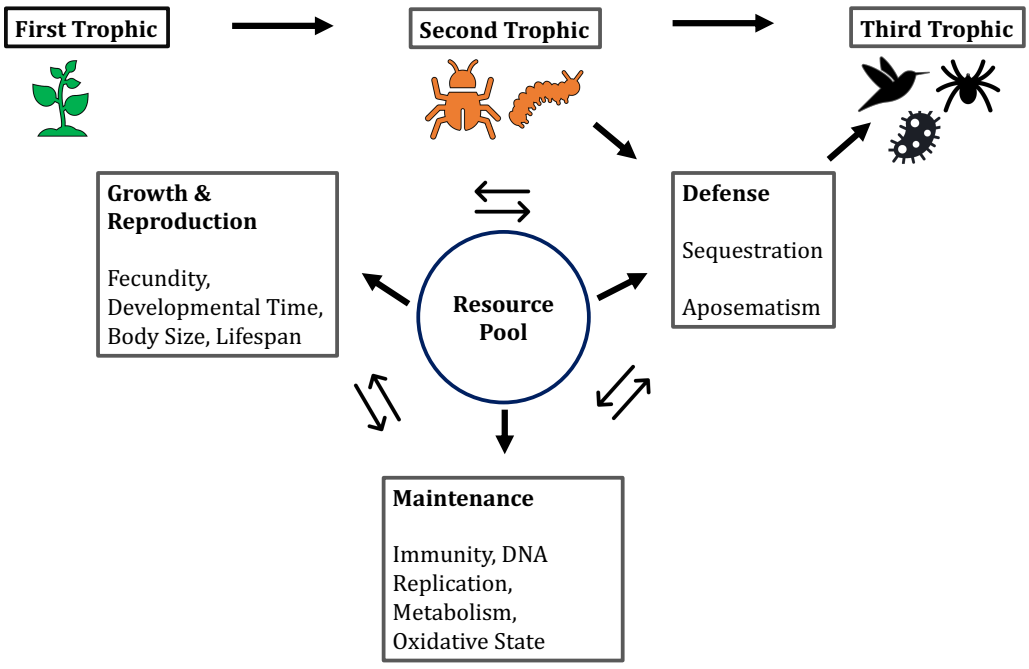

Figure 2: Toxin sequestration in an ecophysiological framework. Organisms have a finite pool of resources that must be allocated to diverse functions: growth and reproduction, maintenance,

and defense (Stearns, 1992). Sequestration is the phenomenon of uptake of toxins from host-plants (i.e., first trophic level) by insects (i.e., second trophic level) to protect themselves against predators and parasites (i.e., third trophic level). Sequestration is often associated with warning colors and patterns (aposematism), and sequestration and aposematism are the prominent suites of defenses in many species. Notably, the resource pool of a toxin-sequestering aposematic individual is finite but dynamic. Costs of defenses can be expected to be seen in many facets, including oxidative stress, reduced immune defense, impairment of growth, and reduction in fecundity. Two-headed arrows signify trade-offs arising due to allocation patterns, and an individual can change the patterns according to its varying needs of defense, growth and reproduction, and maintenance.

Physiological Implications Of Cardenolide Sequestration

Cardenolide Sequestration And Associated Costs

Empirical evidence on the costs of chemical defense on growth and reproduction is equivocal, as different studies show contrasting outcomes (see discussion, Chapter 1). However, immunosuppressive effects of iridoid glycosides have been demonstrated in specialist caterpillars of *Junoia coenia* (Smilanich et al., 2009a, 2009b; Richards et al., 2012; Lampert et al., 2014). In all mentioned studies, only high concentrations of toxins caused immune-suppression in specialists; if both generalists and specialists feed on toxins, specialists suffer more as they sequester higher amounts of toxins than generalists (Lampert et al., 2014; Lampert and Bowers, 2015). In an ecophysiological framework (Figure 2), I speculate that toxin sequestration may lead to reallocation of the resource pool mitigating the effect of toxins, rather than maintaining the immune functions. Future experiments quantifying the deterrence against natural antagonists, as well as fitness of the host, will enhance our understanding of how immune response links to multitrophic levels.

The life-history dataset (Chapter 1) showed that cardenolide-raised *O. fasciatus* lived longer as adults and were larger in size compared to individuals raised on cardenolide-free diet. However, adult fecundity was reduced when maintained on a cardenolide-containing diet for

their entire lifetime, but not when individuals were transferred to non-toxic sunflower seeds as adults. This interesting effect on fecundity maybe because the high-quality diet (here, sunflower seeds) might have rescued female fecundity from the effects of the low-quality diet (here, artificial diet) (Cirino et al., 2022).

Specialist species could experience a cost in the absence of dietary toxins due to selection on physiological homeostasis under permanent exposure to toxins. I argue that the phenomenon of positive impact on growth upon exposure of toxins could be labelled as an 'evolutionary addiction' (see Wink, 2018). Better growth in response to toxin consumption could also be due to a hormetic effect (Sebastiano et al., 2022). Growth hormesis theory predicts that hormesis is an outcome of homeostasis overcompensation (Calabrese and Baldwin, 1999; Stebbing, 2000), although this hypothesis lacks a general supporting mechanism (Thayer et al., 2005; Mushak, 2007; Jager et al., 2013). In empirical support, several studies have shown positive effects on individual fitness under insecticide stress (Celestino et al., 2014; Piiroinen et al., 2014). For example, the Colorado potato beetle (*Leptinotarsa decemlineata*) had higher adult survival rates and greater adult body mass when exposed to sublethal insecticide stress, compared to those not exposed (Margus et al., 2019). Insecticide-induced hormesis in arthropods remains a perplexing subject in terms of its functional basis and potential fitness costs.

Cardenolide Sequestration, Oxidative Stress And Warning Signal

Generally, it is expected that toxin sequestration will incur costs in an individual, suggesting a competition for resources (Figure 2) with other functions including expression of warning signals (Roitberg and Isman, 1992; Srygley, 2004) and mitigating oxidative stress (Eichenseer et al., 2002). The data presented in Chapter 2 provided a tentative support for a physiological cost of sequestration in *O. fasciatus* in terms of oxidative stress, as measured by total glutathione content (GSH). Milkweed bugs with higher concentrations of GSH had brighter warning signals, but this was not paralleled by the sequestration of cardenolides. Interestingly, male adults exhibited higher levels of superoxide dismutase (SOD) and malondialdehyde (MDA), but lower GSH than females (Annex, Chapter 2, Figure S4). The observed increase in overall

oxidative stress in males could also be due to intraspecific competition, as males often physically fight over females (personal observation). Irrespective of sex, *O. fasciatus* individuals raised on higher concentrations of cardenolides had lower levels of GSH, suggesting depletion of this antioxidant molecule by sequestration. Moreover, individuals with lower levels of GSH produced less bright warning signals. Therefore, my data suggest that GSH availability has a role in imparting coloration in *O. fasciatus*. Similar results were reported in *D. plexippus* with a mechanistic link found between MDA, redness and sequestration (Blount et al., 2021). I speculate that the availability of antioxidant molecules has a role in the biochemistry, mediating the variation in coloration and toxicity in aposematic insects. However, this realm of research requires further biochemical studies, as regulation of warning coloration is usually very complex (Wellenreuther et al., 2014; Orteu and Jiggins, 2020).

Conclusion

There is limited empirical evidence for the costs and benefits of chemical defense, and the role and effects of the diversity of chemical defenses on different predators in natural systems have received even less attention. In this thesis, I have taken initial steps into these realms of research.

As an outlook of my research for future experiments, it is important to consider that chemically defended milkweed bugs possess resistant Na^+/K^+-ATPases and these enzymes have many unknown non-canonical physiological functions (Liang et al., 2007) beyond the role of cation carrier. Moreover, several rounds of gene duplication in cardenolide-resistant Na^+/K^+-ATPases in *O. fasciatus* may have major impacts on fitness (Kondrashov, 2012). Gene copies encoding the α-subunit of cardenolide-resistant Na^+/K^+-ATPases have different functional patterns, as gene copies show tissue-specific expression patterns (Lohr et al., 2017). For example, subunits αA and αB, which exhibit higher cardenolide resistance, have reduced ion pumping activity, but subunit α1C, which exhibits lower cardenolide resistance, has higher ion pumping activity (Dalla et al., 2017; Herbertz et al., 2022). In this era of genome engineering, one could use techniques like CRISPR/Cas9 to examine the effects of individual copies of cardenolide-resistant

Na^+/K^+-ATPase genes both *in vitro* and *in vivo*. It would be enlightening to observe the pleiotropic effects of individual copies of these genes both at the molecular and organismal level, as it could help to disentangle complexities in the adaptation of physiological systems with cardenolide-resistant Na^+/K^+-ATPases in milkweed bugs.

In conclusion, it seems likely that cardenolide consumption exerts a positive effect on overall fitness in *O. fasciatus*, in contrast with much current theory. However, I did find sequestration costs in terms of fecundity, sex differences in oxidative stress, and overall oxidative stress, implying that oxidative state may be a fundamental area where these costs of sequestration lie in *O. fasciatus*. These costs are not always compensated by the benefits gained from protection against predators, as this protection is dependent on the structural differences of sequestered chemicals. Overall, my thesis provides evidence for the important role of plant-toxin sequestration against predator attack, and the effect of toxin sequestration on the physiology of insect herbivores. Both aspects of toxin sequestration, the context-dependent positive effect against predators (i.e., host-plant use) and the multifaceted effects on insect physiology, are important for our better understanding of the ecology and evolution of plant-insect-predator interaction.

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Annex

Chapter 1 Supplementary Materials

Supplementary Methods

Quantification of excretion products

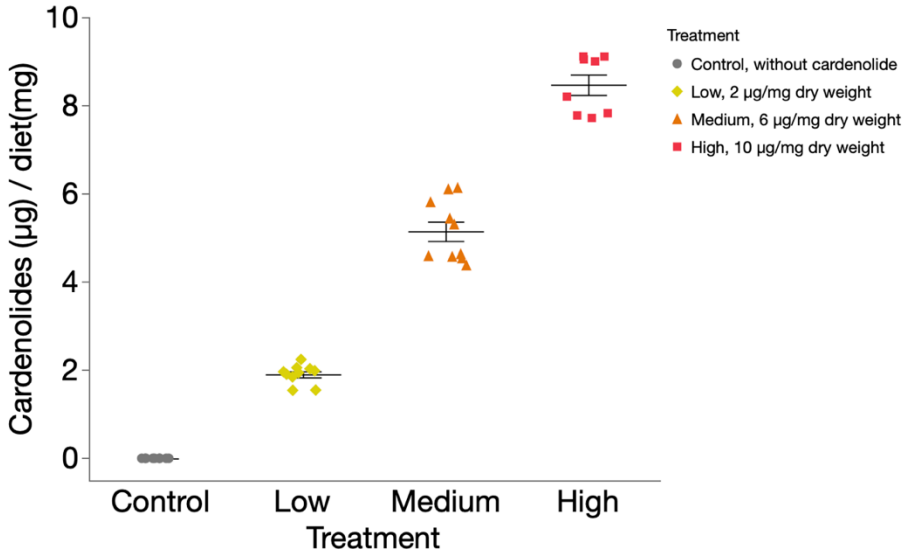
We estimated the amount of food uptake by quantifying the amount of excretion products during our feeding assay. Specifically, the area of faecal stains on filter papers lining the Petri-dishes was analysed. We only analysed filter papers from Petri-dishes in which all three bugs survived until the end of the experiment (i.e., for three weeks). Filter papers were scanned and the stained area was quantified by following the instruction of image analysis (Reinking 2007) using ImageJ 1.52k (National Institutes of Health, US). Excretion data were $\log_{10}$-transformed to achieve homogeneity of variances and normality of residuals. To test for differences in excretion across treatments data were analysed by ANOVA followed by the LSMeans Tukey HSD test in JMP.

Supplementary Results

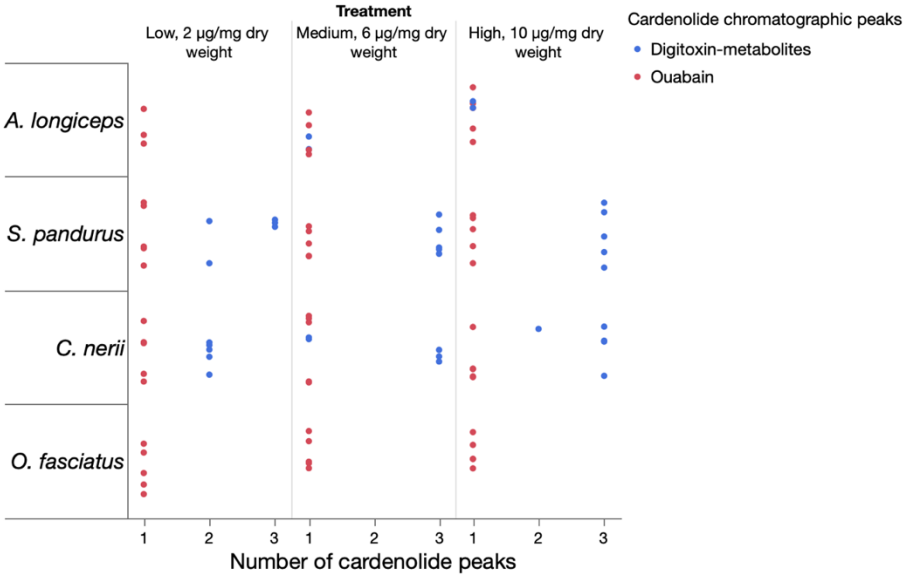
Estimation of excretion area

We estimated the area of excretion products on filter paper to assess bug feeding activity during our feeding trials. In line with increased growth of *P. apterus* on the control diet [$F(3, 15) = 4.69$, $P = 0.02$], we observed statistical differences of excretion products. Specifically, bugs excreted less waste products when fed on medium ($P = 0.01$) and high-dose ($P = 0.04$) but not on low dose diet ($P = 0.22$) compared to the control. In contrast, we found no statistical differences of excreted waste products across treatments in *C. nerii* [$F(3, 14) = 1.38$, $P = 0.29$], *S. pandurus* [$F(3, 15) = 0.53$, $P = 0.67$], and *A. longiceps* [$F(3, 5) = 0.96$, $P = 0.48$] (Supplementary Figure 4). *O. fasciatus* [$F(3, 19) = 8.34$, $P < 0.001$] excreted similar amounts when fed on either control, low or medium-dose diet. Interestingly, *O. fasciatus* excreted less in the high-dose (but similar to control) than compared to low and medium-dose. Moreover, there was a difference between excretion on medium ($P = 0.002$) and low dose ($P = 0.004$) diet as compared to the high dose diet.

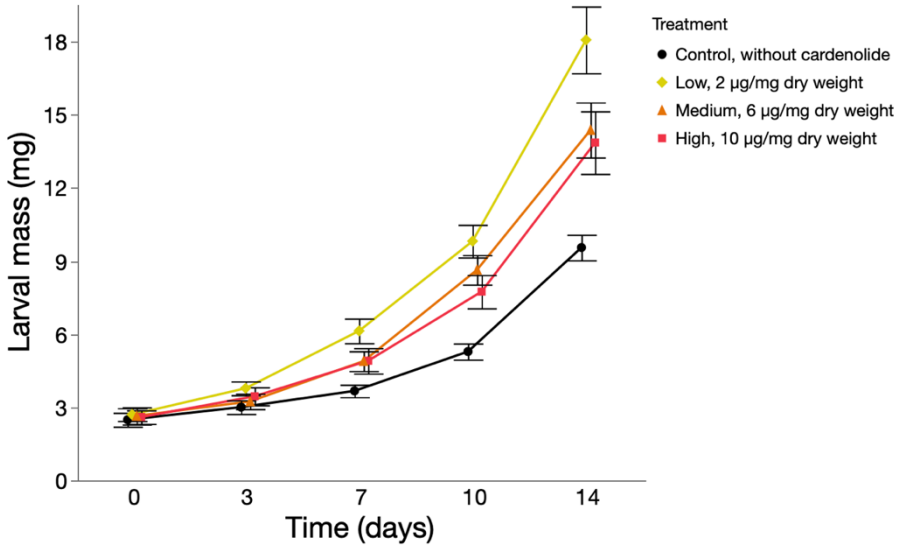
Supplementary Figures And Legends



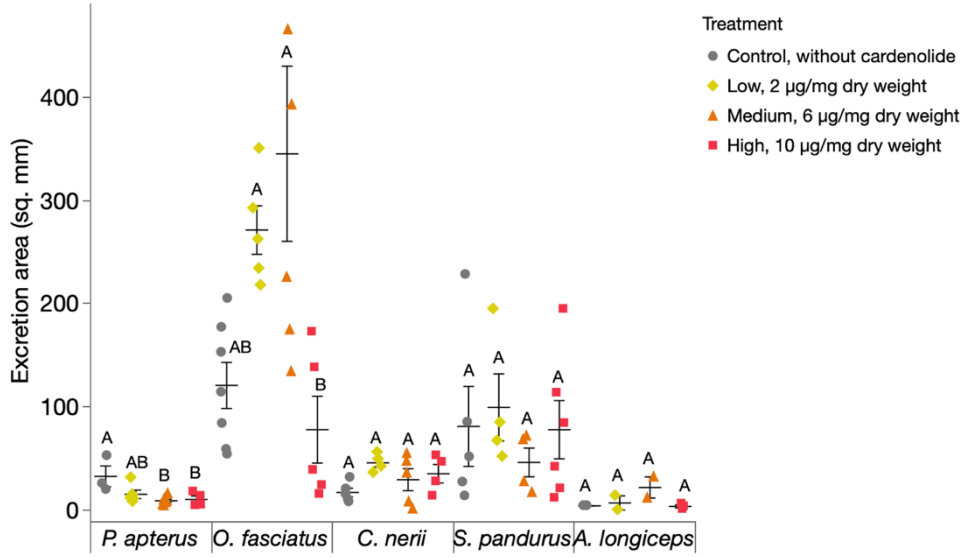
Supplementary Figure 1 Concentration of cardenolides in the artificial diet. Each horizontal bar represents the mean concentration of cardenolides (± SE) in the diet (n = 10 per treatment). Symbols represent jittered raw data.



Supplementary Figure 2 Structural diversity of sequestered cardenolide peaks by milkweed bugs. Each data point represents a bug specimen and the number of cardenolide peaks found in specimens of *O. fasciatus* (n = 10 per treatment), *C. nerii* (n = 5 per treatment), *S. pandurus* (n = 5 per treatment), and *A. longiceps* (n = 5 per treatment) when raised on artificial diet containing an equimolar mixture of ouabain and digitoxin. Chromatographic peaks with a cardenolide spectrum and a similar retention time like digitoxin were classified as digitoxin metabolites. Although we used a different HPLC method for *O. fasciatus*, the outcome is probably the same as if we had used the HPLC method used for *C. nerii*, *S. pandurus*, and *A. longiceps*. This assumption was validated by comparisons with *O. fasciatus* samples analyzed during a different set of experiments (Heyworth et al., manuscript in preparation), hence it is valid to compare *O. fasciatus* to other species.



Supplementary Figure 3 Growth of *O. fasciatus* on artificial diet with increasing doses of cardenolides using a genetically distinct *O. fasciatus* lab strain. Each data point represents the mean mass (± SE) of larvae raised on an equimolar mixture of ouabain and digitoxin (n = 10-12 per treatment). We commercially obtained *O. fasciatus* eggs from Carolina Biological Supply Company (Burlington, NC, US). Larvae were maintained on sunflower seeds as described above and used only in this feeding assay. Overall, cardenolides had a positive effect on growth [F (3, 39) = 5.53, P = 0.003, Repeated Measures ANOVA], but only low-dose (P = 0.002) was statistically significant from control, and not the medium (P = 0.07) and high-dose (P = 0.13, LSMeans Tukey HSD).



Supplementary Figure 4 Amount of excretion products by bug species raised on increasing doses of cardenolides. Each horizontal bar represents the mean (± SE) of faecal stains on filter paper produced by *P. apterus* (n = 4-7 per treatment), *O. fasciatus* (n = 5-7 per treatment), *C. nerii* (n = 4-5 per treatment), *S. pandurus* (n = 5-6 per treatment), and *A. longiceps* (n = 2-3 per treatment) when raised on an equimolar mixture of ouabain and digitoxin. Within the same bug species, different letters indicate significant differences. Symbols represent jittered raw data.

Supplementary Reference

Reinking, L. (2007). Examples of image analysis using ImageJ. Department of Biology, Millersville University. Retrieved from https://imagej.nih.gov/ij/docs/pdfs/examples.pdf

Chapter 2 Supplementary Materials

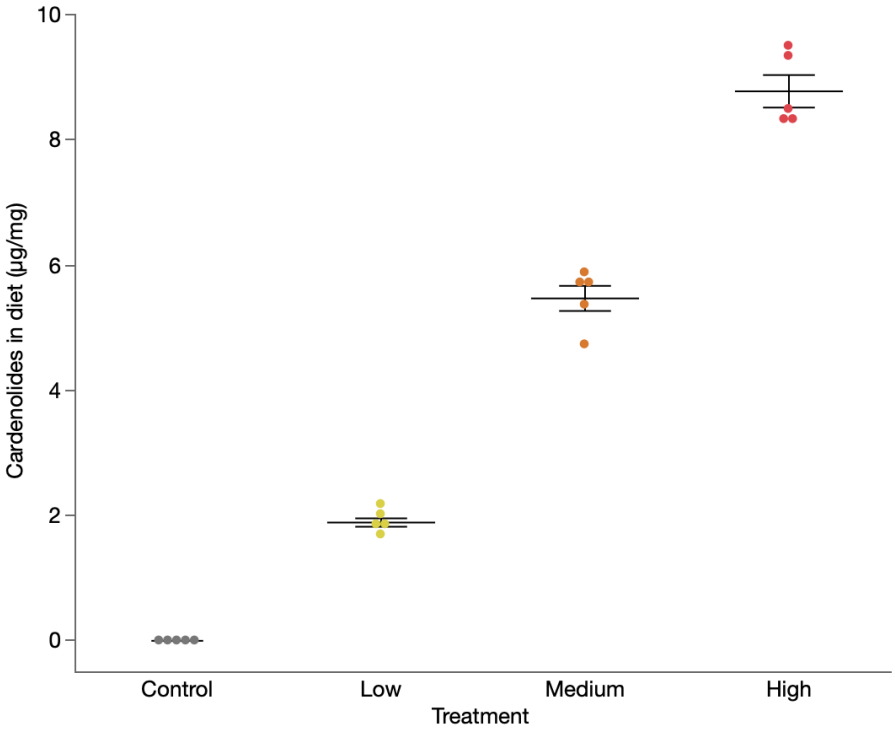


Figure S1. Amount of cardenolides in the artificial diet. Control, Low, Medium, and High diets had 0 mg/g, 2 mg/g, 6 mg/g, 10 mg/g equimolar ouabain and digitoxin added respectively.

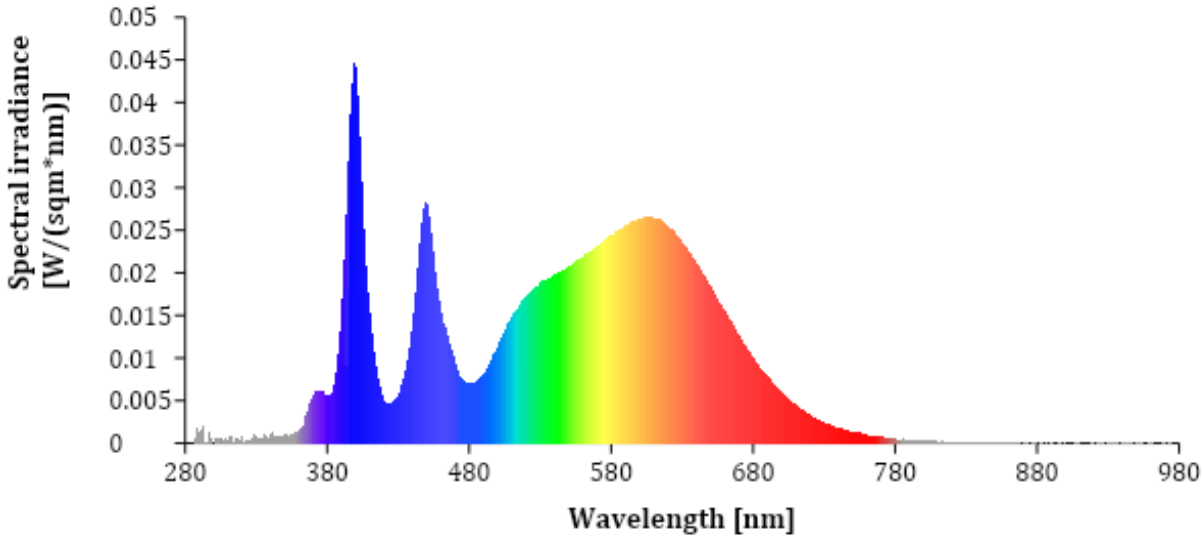


Figure S2. Reflectance spectra generated from the filter apparatus LEDs.

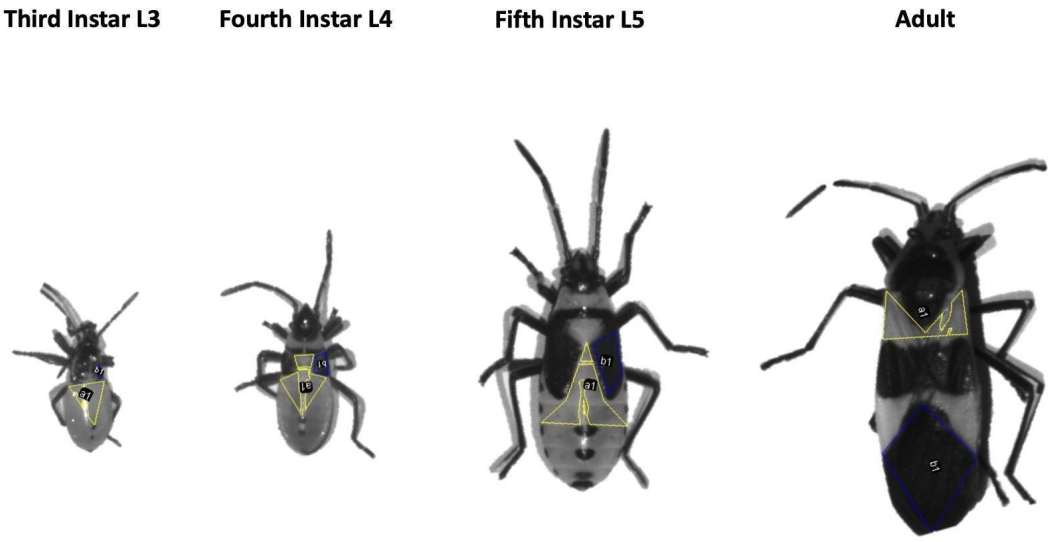


Figure S3. Regions of Interest selected in the different larval stages of *Oncopeltus fasciatus*. Yellow areas were the red selections, and blue were the black, with areas of glare removed.

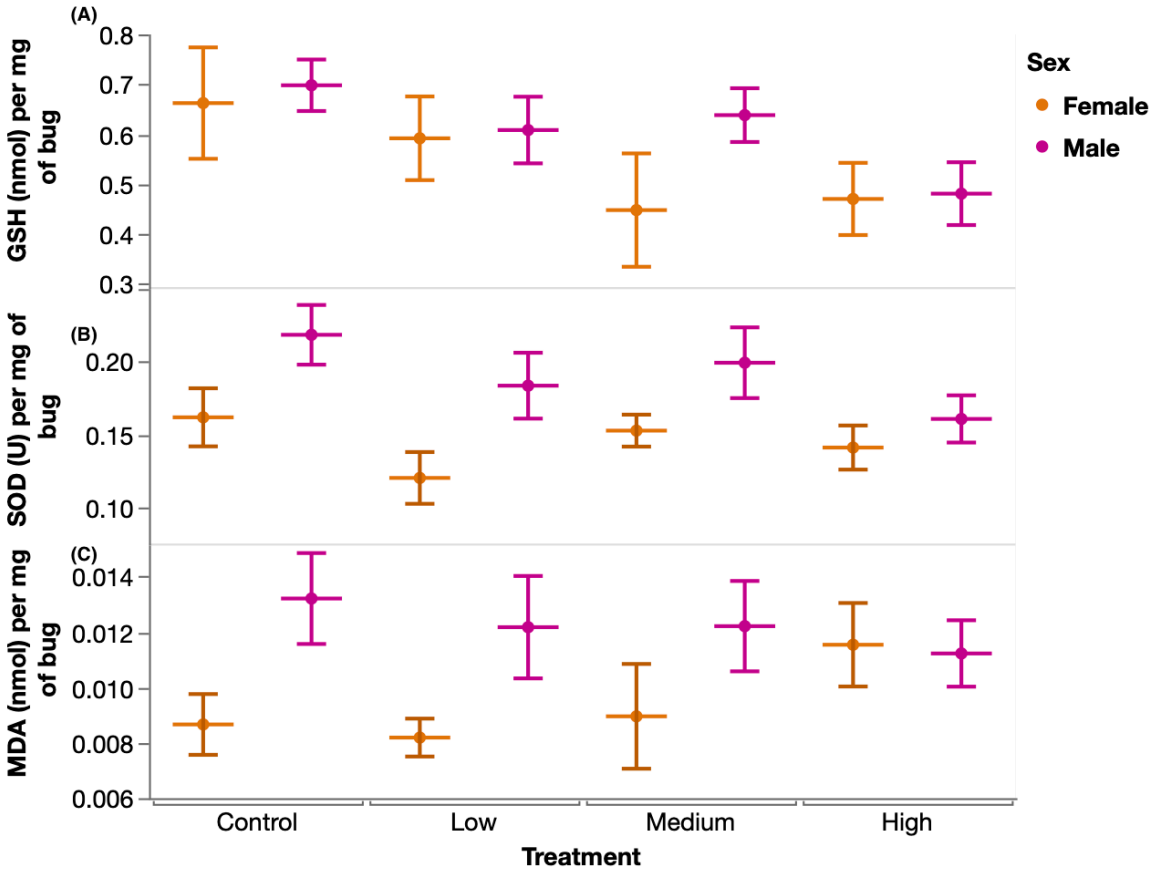


Figure S4. Oxidative stress levels by sex in *Oncopeltus fasciatus* individuals when raised on different dietary treatments. Each bar shows mean concentration (±SE) of (A) total glutathione, GSH (µmol/mg), (B) malondialdehyde, MDA (µmol/mg), and (C) superoxide dismutase, SOD (U/mg) levels in the bugs. Control, Low, Medium, and High diets had 0 mg/g, 2 mg/g, 6 mg/g, 10 mg/g equimolar ouabain and digitoxin added respectively. Male adults faced higher levels of SOD (estimate = -0.04 ± 0.02, t = -2.59, p = 0.01) and MDA (estimate = -0.003 ± 0.001, t = -1.98, p = 0.05), but similar level of GSH (estimate = -0.06 ± 0.05, t = -1.12, p = 0.27) than female adults.

Table S1 Model selection for linear model explaining the variation in MDA among *O. fasciatus* individuals. We started model selection with a model that included all pairwise interactions, and removed the terms based on the drop 1 function. P value is the comparison of models by anova.

ID	Terms in the model	AIC	df	P value
1	MDA_nmol_mg~ Treatment + Instar + Batch + Treatment:Instar + Treatment:Batch	-1530.188	168	
2	MDA_nmol_mg~ Treatment + Instar + Batch	-1544.106	183	0.48
3	MDA_nmol_mg~ Instar + Batch	-1548.782	186	0.60

Table S2 Model selection for linear model explaining the variation in MDA among *O. fasciatus* individuals. We started model selection with a model that included all pairwise interactions, and removed the terms based on the drop1 function. P value is the comparison of models by anova.

ID	Terms in the model	AIC	df	P value
1	MDA_nmol_mg~ CG_per_mg + Instar + Batch + CG_per_mg:Instar + CG_per_mg:Batch	-1461.952	171	
2	MDA_nmol_mg~ CG_per_mg + Instar + Batch	-1467.421	176	0.51
3	MDA_nmol_mg ~ Instar + Batch	-1548.782	176	

Table S3 Model selection for linear model explaining the variation in SOD among *O. fasciatus* individuals. We started model selection with a model that included all pairwise interactions, and removed the terms based on the drop1 function. P value is the comparison of models by anova.

ID	Terms in the model	AIC	df	P value
1	SOD_U_mg_bug ~ Treatment + Instar + Batch + Treatment: Instar + Treatment:Batch	-480.0196	168	
2	SOD_U_mg_bug ~ Treatment + Instar + Batch + Treatment: Instar	-481.8708	174	0.17

Table S4 Model selection for linear model explaining the variation in GSH among *O. fasciatus* individuals. We started model selection with a model that included all pairwise

interactions, and removed the terms based on the drop1 function. P value is the comparison of models by anova.

ID	Terms in the model	AIC	df	P value
1	GSH_nmol_mg~ Treatment + Instar + Batch + Treatment:Instar + Treatment:Batch	3.67	166	
2	GSH_nmol_mg~ Treatment + Instar + Batch	-14.45	181	0.76
3	GSH_nmol_mg~ Treatment + Instar	-15.34	183	0.69

Table S5 Model selection for linear model explaining the variation in GSH among *O. fasciatus* individuals. We started model selection with a model that included all pairwise interactions, and removed the terms based on the drop1 function. P value is the comparison of models by anova.

ID	Terms in the model	AIC	df	P value
1	GSH_nmol_mg ~ CG_mg_bug + Instar + Batch+CG_mg_bug:Instar + CG_mg_bug:Batch	-9.42278	169	
2	GSH_nmol_mg ~ CG_mg_bug + Instar + Batch	-12.93	174	0.30
3	GSH_nmol_mg ~ CG_mg_bug + Instar	-14.30	176	0.28

Sequestration, oxidative stress and warning signals measured by sRGB

We found no significant interaction between GSH and Treatment on redness ($F = 0.42$, $p = 0.74$) and removed this from the model. We also found no effect of batch ($F = 0.23$, $p = 0.80$) or treatment ($F = 2.27$, $p = 0.08$) or GSH ($F = 2.64$, $p = 0.11$) on redness. The bugs became significantly less red with age (L4 vs L5 estimate -0.07 ± 0.004, $t = -18.01$, $p < 0.0001$; L4 vs A1 estimate $= -0.09 \pm 0.004$, $t = -24.81$, $p < 0.0001$; L4 vs A3 estimate $= -0.10 \pm 0.004$, $t = -26.79$, $p < 0.0001$). We did not find a significant interaction between individual cardenolide content and GSH on redness and removed it from the model ($F = 1.08$, $p = 0.30$), and we also removed batch ($F = 0.19$, $p = 0.83$). We found no effect of GSH on redness (estimate $= -0.009 \pm 0.005$, $t = -1.62$, $p = 0.11$), and no effect of cardenolide concentration on redness (estimate $= -0.0009 \pm 0.001$, $t = -0.79$, $p = 0.428$). Larval instar L5, and adult stage A1 and A3 were significantly less red than stage L4 (estimate $= -0.07 \pm 0.004$, $t = -17.29$, $p < 0.0001$; estimate $= -0.09 \pm 0.004$, $t = -22.05$, $p < 0.0001$; estimate $= -0.10 \pm 0.004$, $t = -25.48$, $p < 0.0001$).

We found a no significant interaction between GSH and Treatment on luminance and removed this from the model ($F = 1.24$, $p = 0.30$). We found no significant effect of treatment on luminance ($F = 0.09$, $p = 0.96$) but bugs with higher levels of GSH were significantly brighter (estimate = 1.58 ± 0.47, $t = 3.37$, $p = 0.0009$). Bugs were significantly less bright with increasing age (L4 vs L5 estimate = -1.07 ± 0.36, $t = -3.00$, $p = 0.003$; L4 vs A1 estimate = -1.91 ± 0.37, $t = -5.15$, $p < 0.0001$; L4 vs A3 estimate = -3.79 ± 0.35, $t = -10.82$, $p = 0.0001$). Batch 3 were significantly brighter than batch 1 (estimate = 1.13 ± 0.29, $t = 3.96$, $p = 0.0001$), while there was no significant difference between batch 1 and 2 (estimate = 0.51 ± 0.31, $t = 1.63$, $p = 0.$).

Chapter 3 Supplementary Materials

Videos can be accessed from here.

https://www.mdpi.com/2075-4450/11/8/485#supplementary

Curriculum Vitae

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[Sep 2009 – Aug 2013]	*Pharmacy (BSc),* Rajiv Gandhi University of Health Sciences, Bangalore, India
[Aug 2007 – Jul 2009]	*General Science,* Higher Secondary Education Board, Caspian Valley H S School, Kathmandu, Nepal
[Jan 1997 – June 2007]	*Primary & Secondary School*, School Leaving Certificate, Bhanu Memorial English Boarding School, Biratnagar, Nepal

Work Experience

[23.08.2021- 22.01.2022]	*Visiting Scientist,* Gandaki Province Academy of Science and Technology (GPAST), Pokhara, Nepal
[02.10.2021- 30.11.2021]	*Tutor,* Center for Biotechnology, Agriculture and Forestry University (AFU), Chitwan, Nepal
[01.04.2017- 15.01.2018]	*Scientific Employee,* Department of Applied Entomology, Justus Liebig University Giessen, Germany
[01.08.2016- 31.03.2017]	*Research Assistant,* Department of Applied Entomology, Justus Liebig University Giessen, Germany
[16.05.2016- 29.07.2016]	*Research Intern,* LOEWE Center for Insect Biotechnology, Fraunhofer IME, Giessen, Germany Topic: Detection of *Wolbachia* in *Varroa* mites on honey bee colonies Supervisor: Prof. Dr. Marc F. Schetelig
[08.09.2013- 09.10.2013]	*Intern,* Alive Pharmaceutical (P) Ltd, Duhabi, Nepal
[25.02.2013- 23.03.2013]	*Trainee,* Dingla Pharmaceuticals Pvt. Ltd, Biratnagar, Nepal

Scholarships & Grants

1. "Diaspora expert for Nepal" funded by the Centre for International Migration and Development (CIM) which is jointly run by German Corporation for International Cooperation (GIZ) GmbH and the German Federal Employment Agency, Germany (August, 2021-December, 2021)

2. "Program for the new transition for prosperity", Nepal Academy of Science and Technology (NAST), Kathmandu, Nepal. *Industrial production of indigenous insects for food and feed in Nepal (2020-2021)*

3. "Graduation scholarship for Foreign Students in the Closing Phase of Their Degree", Awarded by Presidential Commission at Justus Liebig University Giessen. *Agrobiotechnology (MSc) (2015-2016)*

Conferences & Workshops

[05.09.2021 – 10.09.2021] *Virtual Oral Presentation,* 36th Annual Meeting of the International Society of Chemical Ecology, Inc. (ISCE), Stellenbosch, South Africa

[25.06.2021 - 27.06.2021] *Attendee,* Sustainable Development through effective knowledge Sharing: Concepts, Methods and Processes, Centre for International Migration and Development (CIM), German Corporation for International Cooperation (GIZ) GmbH, Germany

[24.10.2019 - 25.10.2019] *Attendee,* Science as Storytelling: From Facts to Fictions, 20th EMBL Science and Society Conference, Heidelberg, Germany

[03.03.2019 - 05.03.2019] *Oral Presentation,* Physiology Workshop, German Zoological Society (DZG), Rauischholzhausen, Germany

[05.09.2018 - 07.09.2018] *Attendee,* 4th INSECTA, International Symposium on Insects, Giessen, Germany

[13.08.2018 - 17.08.2018] *Poster Presentation,* 34th Annual Meeting of the International Society of Chemical Ecology, Inc. (ISCE), Budapest, Hungary

[14.02.2018 - 15.02.2018] *Oral Presentation,* 1st International Symposium on Himalayan Biodiversity and Bioresources, Pokhara, Nepal

[09.10.2017 - 10.10.2017] *Poster Presentation,* 1st Giessen Symposium for Insect Biotechnology, Giessen, Germany

[12.09.2017 - 15.09.2017] *Attendee,* 110th Annual Meeting of the German Zoological Society (DZG), Bielefeld, Germany

Students Supervised

1. *Bachelor Thesis 12 ECTS-Credit*, Summer Sem 2022 Jonathan Dalhäuser. Agrobiology (BSc), University of Hohenheim, Stuttgart, Germany

2. *Agrobiological Project 30 ECTS-Credit*, Summer Sem 2021 Magdalena Baumann. Agrobiology (BSc), University of Hohenheim, Stuttgart, Germany

3. *Bachelor Thesis 12 ECTS-Credit*, Winter Sem 2020/21 Christian Posch. Agrobiology (BSc), University of Hohenheim, Stuttgart, Germany

4. *Agrobiological Project 30 ECTS-Credit*, Summer Sem 2020 Fabienne Rakanovic. Agrobiology (BSc), University of Hohenheim, Stuttgart, Germany

5. *Bachelor Thesis 12 ECTS-Credit*, Winter Sem 2018/19 Marlon Sippel. Agricultural Sciences (BSc), Justus Liebig University Giessen, Germany

Scientific Publications

1. Heyworth, C*; **Pokharel, P***; Blount, JD; Mitchell, C; Petschenka, G; Rowland, H. "Antioxidant availability trades off with warning signals and toxin sequestration in the large milkweed bugs (*Oncopeltus fasciatus*) (In Preparation) [*Authors contributed equally]

2. **Pokharel, P**; Steppuhn, A; Petschenka, G. "Dietary cardenolides enhance growth and change the direction of fecundity-longevity trade-off in milkweed bugs (Heteroptera: Lygaeinae)" *Ecology and Evolution 2021, 11, 18042–18054*. https://doi.org/10.1002/ece3.8402

3. **Pokharel, P**; Sippel, M; Vilcinkas, A; Petschenka, G. "Defence of milkweed bugs (Heteroptera: Lygaeinae) against predatory lacewing larvae depends on structural differences of sequestered cardenolides" *Insects 2020, 11, 485*. https://doi.org/10.3390/insects11080485

Popular Science Articles

1. Pokharel, P. "Conservation of insects." *The Kathmandu Post*, https://kathmandupost.com/columns/2022/07/09/conservation-of-insects Published 9 July 2022

2. Pokharel, P. "Female scorpions pay a steep cost when they shed their tails for survival." *Massive Science*, https://massivesci.com/notes/scorpion-limb-loss-tail-mating/ Published 23 February 2021

3. Pokharel, P. "Should society trust science?" *Crastina*, https://crastina.se/the-crastina-microessay-competition-2020/winners/ Published 09 December 2020

4. Pokharel, P. "Whiteflies evolved to disarm plants' defenses with sugar." *Massive Science*, https://massivesci.com/notes/whitefly-insects-pest-agriculture-sugar/ Published 31 October 2020

5. Pokharel, P. "Though smelling sweet, linden trees are bad for bumblebees." *Massive Science*, https://massivesci.com/notes/linden-trees-are-bad-for-bumblebees-toxic-starvation/ Published 14 August 2019

6. Pokharel, P. "Insects are nature's little helpers." *The Kathmandu Post*, https://kathmandupost.com/columns/2019/08/06/insects-are-nature-s-little-helpers Published 06 August 2019

7. Pokharel, P. "Fall armyworms have come to Nepal from America, and they can trouble maize farmers here." *Onlinekhabar*, https://english.onlinekhabar.com/fall-armyworms-have-come-to-nepal-from-america-and-they-can-trouble-maize-farmers-here.html Published 25 June 2019

8. Pokharel, P. “Caterpillars: The Masters of Mimicry.” *Euro Scientist*, https://www.euroscientist.com/caterpillars-the-masters-of-mimicry/ Published 4 October 2018

9. Pokharel, P. “Why Some Fireflies Become Femme Fatales in Their Race for Survival.” *The Conversation*, http://theconversation.com/why-some-fireflies-become-femme-fatales-in-their-race-for-survival-91252 Published 7 February 2018

10. Pokharel, P. “The Hidden Secrets of Insect Poop.” *The Conversation*, http://theconversation.com/the-hidden-secrets-of-insect-poop-81908 Published 8 August 2017

11. Pokharel, P. “‘Himalayan Viagra’ Is Threatened by Fervent Chinese Demand and Climate Change.” *The Conversation*, http://theconversation.com/himalayan-viagra-is-threatened-by-fervent-chinese-demand-and-climate-change-78558 Published 5 June 2017

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