How Bright and How Nasty: The Economics of Variable Aposematic Traits

Submitted by Hannah Cecilia Heyworth to the University of Exeter as a thesis for the degree of Master of Philosophy in Biological Sciences, January 2022.

Abstract

Although the theory about the evolution and maintenance of aposematism predicts distinct and uniform warning signals, variation in both signal and defence is common across many taxa, and some species also show correlations between signal and defence. One hypothesis to explain these correlations is honest signalling of defence in prey through the framework of resource competition enforcing a tradeoff. Competition for antioxidant molecules that have dual functions as pigments and in protecting against oxidative stress from toxin sequestration or production have been suggested as a specific candidate resource limitation that could explain warning signal honesty. In Chapter 2 I report an experiment using the large milkweed bug (*Oncopeltus fasciatus)* as an aposematic model prey that naturally varies in colour and toxicity. By raising milkweed bugs on diets of controlled toxicity and measuring their defence, signal expression, and oxidative stress, I test the expectations of the resource competition model. I found that milkweed bugs overall did not show a correlation between signal in terms of colouration and their level of chemical defence, but that there was a relationship between signal and glutathione amount, a measure of total antioxidant capacity, in the most toxic bugs. These results suggest a mechanistic link between oxidative stress, warning signals and chemical defences in large milkweed bugs. In Chapter 2 I briefly review the concept of dietary wariness, and in Chapter 4 I apply this in practice, using praying mantids (*Hierodula membranacea*) as a generalist invertebrate predator. I present them with *O. fasciatus* raised on one of three diets that differ in chemical defence, one species of nontoxic seeds (*Helianthus annus*) and two different species of their toxic host plants (genus *Asclepias*). I tested mantids with nontoxic milkweed bugs and measured their level of neophobia and dietary wariness, then tested them with the *Asclepias*-raised bugs to measure their avoidance learning. Mantids did not learn to avoid the milkweed bugs, even when possibly facing chronic poisoning from consuming them. My results suggest that avoidance learning of toxic prey in predators is not universal. These experiments further our understanding of variation in aposematic traits in prey by examining two key but less explored hypotheses for why signals might vary: resource competition and dietary wariness.

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Declaration

Chapter 2 of this thesis was based on a joint research project with Prayan Pokharel (PP), a PhD student at the University of Hohenheim, and this chapter will also comprise a section of his doctoral thesis. I designed the concept for the research with my supervisors. I reared the insects, collected images, and processed the samples with PP. Independently, I analysed the images, conducted the oxidative state analyses, led the statistical analysis, and led the writing of the original draft of the chapter and manuscript. PP analysed the cardenolide content of the samples, visualised the data, and contributed to writing. A further author contribution statement is located at the beginning of Chapter 2. Chapters 1, 3 and 4 were independent work.

Chapter 1: General Introduction

1. Introduction

The evolutionary and ecological relationships between predators and prey have long fascinated scientists since the times of Darwin (1859) and Wallace (1889), from the cyclical waxing and waning predator and prey populations (Abrams and Matsuda, 1997; Peterson et al., 1984) to never-ending arms races where each species develops adaptions to counter the other (Abrams, 2000). Natural selection ensures that prey species constantly evade predation pressure through new strategies, whether behavioural, physiological, or chemical, and that predators constantly overcome these defences.

Predation is a process rather than an event, which Endler (1991) defined as having distinct stages: encounter, detection, identification, approach, subjugation, and consumption. Strategies that prey use to avoid death will vary at each of these stages, and predator responses will too. Therefore, to understand how defences and evasion evolve, it is critical to examine the predator-prey relationship from both perspectives, and to identify which stage of the predation sequence the prey may be combating. Through the shifting balances of predation pressure has arisen the evolution of various prey defensive strategies (Ruxton et al., 2018), including crypsis, disruptive colouration, deimatism, and aposematism.

In this introduction, I will take a broad overview of the predator-prey relationships, describing the different defences that prey can use against predators. I will first describe strategies to reduce detectability and identification, and then on how prey avoid approach, subjugation, and consumption. I will focus in detail on aposematic prey species, and the variability in their defences. I will first examine the evidence for variability in prey signals and defences and the reasons suggested for this variability, with both theoretical frameworks, experimental case studies, and field study examples. I will then discuss how predators can also vary in their responses to aposematic prey, in terms of predator mixes, behaviour like neophobia and dietary conservatism, and the abundant evidence for predator avoidance learning. I shall then introduce the two model species used in the experiments within this thesis, the large milkweed bug (*Oncopeltus fasciatus)* and the giant Asian mantis (*Hierodula membranacea),* exploring why they are pertinent species for these experiments, and reviewing the literature of their history in research. Finally, I shall explain the broad questions the experiments in this thesis aimed to understand, and why they are important.

2. Part 1: Prey

2.1. Crypsis: background matching, disruptive colouration, and masquerade

Crypsis is one of the most widespread means of reducing the risk of predation in animals during the detection and identification stages of the predation sequence, wherein the predator is searching for prey (Endler, 1991). Crypsis encompasses various prey characteristics that decrease the prey's risk of detection (Stevens and Merilaita, 2009a). Most crypsis research has been about visual methods like background matching, in which the animal appears similar to their direct substrate. Theoretical and experimental evidence has supported this strategy as effective compared to animals not matching their background, as requires costs from predators in terms of greater search time, and discriminatory ability (Gendron and Staddon, 1983; Staddon and Gendron, 1983; Stevens and Merilaita, 2009a; Szopa-Comley et al., 2020). However, matching the background may not always be the optimal concealment mechanism, and Thayer (1918) proposed that an animal's outline may still give away its presence. Crypsis can also be achieved through disruptive coloration, where patterns break up the animal's appearance and body outline, making them more difficult to perceive. Disruptive colouration has been found experimentally to sometimes be a more effective strategy than crypsis, with predators taking a longer time to find and identify disruptively coloured prey (Stevens and Merilaita, 2009a, 2009b). Prey can also benefit from being misidentified through masquerade, where prey are detectable but resemble inanimate objects in their environment (Skelhorn, 2018; Skelhorn et al., 2010a). Masquerade has been tested experimentally and found to be an effective anti-predator mechanism, especially when predators are in environments with the model object present (Skelhorn and Ruxton, 2011; Skelhorn et al., 2010b; Valkonen et al., 2014).

There is also indication of other types of non-visual crypsis (Ruxton, 2009) for example chemical crypsis, where animals deliberately alter their scent to avoid predators – this has been found in the puff adder, a species that masks its scent to predators (Miller et al., 2015).

2.2. Deimatism and eyespots

Once predators detect and identify prey, other strategies evolve to counter approach, subjugation, and consumption, for example deimatic (or startle) displays. Animals exhibiting deimatism are generally cryptic at a distance, and can be palatable (for example many mantid species perform startle displays (Vidal-García et al., 2020)), but once identified by a predator suddenly flash a colourful display (reviewed in Umbers et al., 2017). This rapid display surprises the hunter and can allow the prey to escape, a theory tested feasible in experiments (Holmes et al., 2018).

Similarly, eyespots are a form of anti-predator signal wherein the prey animal has patches of colouration that appears like eyes, a strategy especially known in Lepidoptera (Stevens, 2005). These false eyes can increase prey survival rates, and the strategy is thought to function either as imitation of a predator eyes, inciting fear in the receiver (Olofsson et al., 2013), or by deflecting predator attacks to less vulnerable areas of the prey animal's body (Lyytinen et al., 2004a; Prudic et al., 2015).

2.3. Aposematism

Aposematism, first defined in the nineteenth century, describes prey animals that possess toxic or distasteful chemical defences, also called unpalatable (Ruxton et al., 2018) and that have conspicuous colours and patterns (Poulton, 1890; Ruxton et al., 2018). In aposematism theory, these attention-grabbing colour and patterns are a form of advertisement, that alert potential predators to their unsuitability as food (Ruxton et al., 2018). Aposematic prey are therefore predicted to be conspicuous and distinctive to predators, typically visually, but also through other senses (Sherratt, 2002). Aposematism is found in a wide range of taxa, from butterflies (Prudic et al., 2019) to frogs (Summers and Clough, 2001), beetles (Lindstedt et al., 2017) to snakes (Kikuchi et al., 2014), mammals (Lartviere and Messier, 1996) to birds (Dumbacher et al., 2008), and potentially plants (Lev-Yadun, 2009), although this thesis will focus on animals. Warning colours have evolved alongside or after chemical defence many times - for example, four times alone in the genus *Papilio* (Prudic et al., 2007a) - and its widespread existence implies a strong, if not permanent, evolutionary advantage (Kikuchi et al., 2021).

Aposematism comes with the advantage of protection from predators through an advanced warning system that can trigger innate wariness in predators, and is reinforced through avoidance learning where predators learn to associate the appearance of the prey with the negative consequences of attacking them (Ruxton et al., 2018). Frequency-dependent selection by predators reinforces aposematism, with predators avoiding the commonly found conspicuous, unpalatable prey (Endler, 1988). However, this same selective pressure is also predicted to eliminate rare, novel conspicuous morphs (for example, Chouteau et al., 2016; Lindström et al., 2001), therefore posing a problem as to how aposematism originally evolved. Researchers have posited kin selection as one solution for this (Malcolm, 1986), as one aposematic individual's death from predation may not be the end of the new signal, it if gains protection for other, related individuals with the same trait. There is evidence for this, in that warning signals are more effective in groups warning signals (Riipi et al., 2001), and that many aposematic species are also gregarious (Ruxton and Sherratt, 2006). On the other hand, other studies have provided evidence for individual selection being a possible mechanism, through both innate predator biases against certain signals, and avoidance learning where the prey individual is not killed (Halpin et al., 2008; Wiklund and Järvi, 1982).

Warning signals have historically been defined as exclusively visual signals, for example colouration (Cott, 1940; Wallace, 1877) and/or patterning, and the majority of research still primarily focuses on visual signals (for example Crothers and Cummings, 2013; Hegna et al., 2013; Lindstedt et al., 2010). Aposematic animals are usually identified as such due to their bright colouration like red, yellow and black, although other colours are sometimes suggested, like white in some desert animals (Cloudsley-Thompson, 1979) and blue in the blue-ringed octopus (Whitelaw et al., 2019). There is increasing interest in measuring the visual signals of aposematic prey from multiple predators' visual perspectives. An individual organism may face predation pressure from a wide range of taxa, for example from avian predators as well as invertebrate species. These two groups have vastly different visual systems, so warning colouration may be successfully communicated to one group but not the other. Historically in aposematism research prey signals were observed and recorded from the human visual system and defined as conspicuous by what humans see, but the human visual system is not always ecologically relevant. For this reason, in recent years analysing visual signals through the visual systems of their ecologically relevant predators has become the ideal option (for example, see Boevé et al., 2013; Stuart-Fox et al., 2006; Ximenes and Gawryszewski, 2019). This may be especially important for when the visual systems differ dramatically, for example as compared to humans, avian predators can see ultraviolet (UV) wavelengths, and UV signals have been shown to be important for bird foraging (Church et al., 1998; Honkavaara et al., 2002; Lyytinen et al., 2004b).

Research on aposematism is also expanding beyond visual and chemical defences, and increasingly research is examining several other possible avenues for signalling, for example through olfactory signals like pyrazines, often released by prey species and known to be deterrent (Rowe and Guilford, 1996, 1999). Unpleasant tastes are also recognised as potential signals, and there have been connections drawn between toxins and bitter flavours (Nissim et al., 2017; Skelhorn and Rowe, 2006a). As well as signalling through one modality, many aposematic species have multimodal signalling, using several modalities at once to enhance their effect (Partan and Marler, 1999). It is currently thought that multimodal signalling can greatly enhance the efficacy of an aposematic strategy (Rowe and Halpin, 2013).

Aposematic prey species may synthesise secondary metabolites as defence or sequester them from their host species (Burdfield-Steel et al., 2018; Fürstenberg-Hägg et al., 2014; Nishida, 2002; Opitz and Müller, 2009). In insects, sequestration of toxins from their host plants is common, and the relationships between sequestering species and their hosts are as complex as those from prey to predators (reviewed in Opitz and Müller, 2009). These secondary defences can be challenging to define (Marples et al., 2018), but the majority of aposematism research refers to primarily toxic or distasteful chemical defences, also called unpalatable (Ruxton et al., 2018). Some authors consider wider types of defence, including mechanical defences and strength of escape, as all relevant to aposematism, and contained within the phrase ‘unprofitability’ (Mappes et al., 2005). This broader definition is useful but makes it difficult to distinguish between defence and signal, for example unprofitability can include distastefulness as a defence, but in multimodal signalling an unpleasant taste could also be a gustatory signal. To focus on the salient points that relate to my experiments, in this thesis I will use the terms unpalatable and toxic, and my definition of aposematism will focus on prey animals with toxins as chemical defence.

Much of the dynamic nature of aposematism is not yet understood. Across all species, few exhibit warning colouration, and the prevalence of conspicuous signals varies across ecosystems, but it is not fully clear why (Kikuchi et al., 2021). Even with one species, aposematism as a state is not static; this balance may change over time, and animals can evolve over time from conspicuous to cryptic, or cryptic to conspicuous (Arbuckle and Speed, 2015; Wang et al., 2021). These changes may be due to shifts in the tradeoff between the costs of conspicuousness and/or toxicity and the benefits of enhanced predator learning, making their current strategy suboptimal. This was found in amphibians, where the warningly coloured groups divided into new species more quickly, but also lost warning colouration quickly, with certain groups reverting to crypsis (Arbuckle and Speed, 2015). These shifts could be climatic or environmental (Hegna et al., 2013) and one can imagine scenarios of potential changes in this balance, for example an influx of new predators with different visual systems, a change in temperature making melanisation beneficial, or behavioural changes in female mate choice leading to sexual selection being of increasing importance (Gordon et al., 2015).

Aposematism is a constantly expanding and infinitely detailed field of study, primarily because every predator-prey relationship is different from the next. There is variation in prey, both in terms of signalling and defence (reviewed in Briolat et al., 2018a; Speed et al., 2012), as well as a wide array of potential predator classes. For decades there has been a growing appreciation for the importance of this variation in aposematic prey species (for example Crothers and Cummings, 2013; Ihalainen et al., 2007; Lindstedt et al., 2010), and the possible causes of it.

2.4. Variation in warning visual signals

The origin and evolution of warning signals and associated chemical defences are intrinsically linked to their intended receivers, predators. As Guilford and Dawkins (1991) explained, the evolution of signals is dependent upon the receivers' psychology, in terms of how easily they detect and discriminate the prey as potential food, and how easily they learn and remember them. Aposematism is an exploitation of these receiver biases in their predators, by both predator generalisation of the warning colours (Guilford and Dawkins, 1991), and by enhancing learning with the negative reinforcement from chemical defence (Skelhorn and Rowe, 2006a, 2006b, 2006c; Skelhorn et al., 2016).

In any given aposematic species signalling theory predicts uniformity in warning signals, because consistency in appearance has been shown to improve predator learning and memory (Gittleman and Harvey, 1980; Roper and Redston, 1987; Roper and Wistow, 1986; for more examples see Ruxton et al., 2018). Generalisation of warning signals is thought to be crucial to many predator-prey relationships and is indeed the basis for mimicry rings (Sherratt, 2008). In a mimicry ring, numerous species are superficially similar in appearance, sharing the same set of warning colours or patterns. The most well-known mimicry rings are the *Heliconius* butterflies of tropical South America, with four commonly used rings, or groups of similarly appearing butterfly species (Llaurens et al., 2014; Mallet and Gilberg, 1995; Sheppard et al., 1985; Turner, 1976). All of the species within the ring benefit from predator generalisation of avoidance, and those that fall outside it face predator attack (Chouteau et al., 2016; Ihalainen et al., 2012). Mimicry rings are based on many of the species inside being unpalatable, but they also allow for Batesian mimics, those that have no defence of their own but are still avoided by predators, for example hoverflies, black and yellow insects without the stinging defences of the other species in the ring (Edmunds and Reader, 2014). To be conspicuous and palatable would be a risky strategy if predator generalisation was not high.

Despite experimental evidence for predator generalisation, and the existence of mimicry rings, variability in visual signals has been identified at numerous levels, ranging from within-populations to groups of related species (Briolat et al., 2018a). Joron and Mallet (1998) discussed variation in warning signals, specifically in view of Müllerian mimicry complexes. They emphasised the importance of predator behaviour, geographic and temporal divergence of mimicry ring species, and the possibility of unpalatable models escaping the diluting effect of mimics by changing their colouration. More recently, Briolat et al. (2018a) published a comprehensive review on the subject of variation in warning signalling, reiterating and expanding on many of the earlier made points, which I summarise briefly below.

2.5. Forms of, and explanations for, warning signal variation

Variation in warning signalling can come in many forms; on continuous scale, for example of brightness or colour saturation, as distinct morphs within a group (Losey et al., 1997; Thompson, 1984) or as different morphs between groups (Dugas et al., 2020; Thurman et al., 2018; Willink et al., 2014a). These different types of variation can also occur at the same time in one individual and can change within the span of an organism's life, for example some grasshoppers shift from cryptic when solitary to aposematic in aggregation (Simpson and Sword, 2009; Sword, 1999). The reasons behind the variability of warning colouration within the same species are manyfold and can depend on both abiotic and biotic factors; abiotic factors include resource availability and temperature, which can affect pigments such as melanism (Goulson, 1994; Hegna et al., 2013). Visual signals can depend on local resources if the organism obtains pigment from their environment, and if those resources decline or change, their signalling could be affected. This is relevant for organisms like *Arctia plantaginis*, a species of Arctiid moth that use dietary-derived flavonoids in their warning colouration (Lindstedt et al., 2010). Pigments like melanin are also used in thermoregulation in many species, including some insects (Goulson, 1994; Sugumaran, 2002; Watt, 1968) and birds (Margalida et al., 2008), and selection pressure due to climate can affect the most evolutionary advantageous colouration. Where the importance of melanin in thermoregulation is an opposing force to the efficacy of the warning signal, variation along climatic or ecological gradients could form, as has been found in adult male wood tiger moths (Hegna et al., 2013), partially explaining their continuous variation in signal.

Biotic factors affecting variation in signalling come from a variety of physiological constraints and ecological tradeoffs in which more conspicuous signals may either not be advantageous or even possible. For example, if an individual is facing disease or high parasite loads, it may not be possible for them to synthesise or obtain the pigments required for their colouration; although not found directly in an aposematic species, arctic char fish with higher parasite loads had duller carotenoid-based colouration, used for sexual signalling (Johansen et al., 2019). It is also possible for sexual selection to work alongside, or counter to, pressures of natural selection against predation. This has been found in male wood tiger moths, where the more successful morph for mating was less successful against predators (Gordon et al., 2015), and in some species of poison frogs, where although all individuals show warning colouration, males vary on a continuous spectrum in their brightness, and female mate choice is evident (Crothers and Cummings, 2013; Maan and Cummings, 2009). In *Heliconius* and *Melinaea* butterflies, Llaurens et al. (2014) found colouration differences in Müllerian mimics too small for birds to discriminate between, and hypothesised that these difference were instead involved in mate choice.

Interactions with other individuals in the same species could also theoretically affect colouration, through warning signals being used for deterring competition. For example, in a swallowtail butterfly, caterpillar patterning considered aposematic has also been found to deter conspecifics from ovipositing on those plants (Daniel R. Papaj and Ginny M. Newsom, 2005). Within individuals, ageing could also affect colouration. Senescence causes costs in phenotypic quality due to physiological changes in the body (Kirkwood et al., 1991), and this could lead to a decrease in signal quality.

2.6. Variation in chemical defence

Along with the numerous reasons behind variation in warning signals, there is also plenty of evidence for variation in chemical defence within species, both across and within groups (reviewed in Speed et al., 2012). There has been longstanding interest in the existence of this variation, and especially the persistence of less toxic aposematic individuals in a population or species, first discussed by Marshall et al. (1908) and Dixey (1919) in relation to mimicry. For example, in monarch butterflies (*Danaus plexippus*), a well-studied insect species that sequester cardiac glycosides from their host plant milkweed (*Asclepias* spp.) Brower et al. (1968) found that individuals vary greatly in their level and diversity of sequestered toxins. These studies led those authors to name a new type of mimicry, automimicry. Automimicry describes the occurrence of palatable ‘cheaters’ in a chemically defended population, which persist due to predator generalisation of the species as a whole (Brower et al., 1970). Predators avoiding the whole species or population after negative experiences with defended individuals, regardless of the chemical defence level of the next individuals, has been shown experimentally in both avian (Gamberale-Stille and Guilford, 2004) and invertebrate (Berenbaum and Miliczky, 1984) predators. However, experiments also indicate this is a frequency-dependent process, and an abundance of automimics weakens the population or species’ protection as a whole (Gamberale-Stille and Guilford, 2004; Skelhorn and Rowe, 2007a).

One possible contributing factor to the success of automimicry, in monarch butterflies at least, could be behavioural. Gregariousness has long been correlated to aposematism in many species (reviewed in Ruxton and Sherratt, 2006), as signals can be amplified in groups. These aggregations could aid the evolution or survival of automimics, as predators may reject the group as a whole once consuming very few individuals. Monarch butterflies are known to aggregate in huge colonies when migrating (Dingle et al., 2005), and although there are likely multiple reasons for it, joint protection against predators is likely to be one. Individuals with little or no chemical defences presumably benefit from their more defended conspecifics, as predators learn to avoid the whole species based on encounters with more toxic individuals.

Similar to the possible reasons behind variation in visual signal, there are many biotic and abiotic factors that could affect an individual’s chemical defences.

Biotic factors can be ecological, encompassing relationships with other species, for example in terms of the relative frequency of the relevant species in mimicry rings. In the mimicry ring of queen and viceroy butterflies both are unpalatable, but viceroy butterflies (usually considered the Batesian mimic) have increased chemical defences when queen butterflies (the model) is less abundant (Prudic et al., 2019). Other biotic factors include alternative, conflicting uses for the chemical defence, including defence against parasites, against conspecifics, or for other purposes. For example in newts, those that contained more tetrodotoxin (TTX) had a lower parasite load than the less toxic newts, indicating a dual function for their neurotoxins (Johnson et al., 2018). Similarly in burying beetles, individuals vary widely in their chemical defence, and Lindstedt et al. (2017) proposed that this is likely due to multiple functionality; the beetles use anal fluid as both a defence against avian predators, as well as for its antimicrobial properties in defending their breeding resource. The multiple functions possible for sequestered or synthesised toxins allow for different selection pressures than only predation to influence their evolution and development.

Abiotic factors in variation in sequestration can include resource availability, or the ability of organisms to become defended. As with pigments, chemicals used for defence can be either synthesised *de novo* or sequestered from the environment or a host species. In sequestering species, resource availability is essential to obtaining chemical defences, and their toxicity is entirely dependent upon the presence of suitable host species. This limitation can also extend to species that synthesise *de novo* chemical defences, wherein the resource being limited is not the toxin itself, but rather the energy or food required to produce the toxins. For example, when an aposematic moth was reared on a limited diet, they produced less toxins in their defensive fluid, even though the amount of fluid produced was similar (Burdfield-Steel et al., 2018); similar constraints were also found in ladybirds that synthesise their defensive alkaloids (Blount et al., 2012).

There are also likely physiological and/or life history tradeoffs involved in sequestering or synthesising chemical defences that could lead to a range of strategies even within the same population. The costs of defence have been examined using theoretical models (Longson and Joss, 2006), and could be expressed in several ways. These costs could be in terms of physiology, for example immunological function; caterpillars that sequester more iridoid glycosides have compromised immune responses (Smilanich et al., 2009). Costs could also be present in terms of life history traits, for example fecundity, shown in milkweed aphids where high sequestration led to slower population growth rates (Zust et al., 2018), or general fitness, for example fat content in a swallowtail butterfly was lower in those that sequestered more toxins (Fordyce and Nice, 2008). Despite such examples, the exact cost of developing or maintaining chemical defence is not known for many aposematic species. It can be difficult to untangle where the costs lie, or if there are any at all, especially in specialist species wholly adapted to consume a toxic host species.

2.7. Theories to explain variation in aposematism

There are many underlying reasons from the prey perspective for variations in both signalling and defence, but how these variations are maintained in an ecological space is a subject of much debate. Gradations in colour and toxicity in aposematic organisms could have significant effects on predator responses, especially when they are linked within the same species. In this case, individuals in a population will show variation in both defence and signalling at the same time, leading to a combination of 'honest' and 'dishonest' individuals. In theory, qualitative honest signalling is a prerequisite of aposematism, in that a signal accurately, or honestly, represents a secondary defence (Ruxton et al., 2018; Sherratt, 2002). However, when individuals in an aposematic species have much lower amounts of chemical defence, their colouration would no longer accurately represent their defence, and they can be classed as dishonest signallers.

Dishonest signallers, or automimics, appear somewhat paradoxical. If there are costs to sequestering, producing, or maintaining a defence, usually assumed to be true, then dishonest signallers benefit by avoiding this cost. If predators generalise based on the warning colouration, and all individuals in the species are avoided, then the benefit of toxicity would be totally lost in terms of predation pressure. Automimicry would be the best strategy, and this would then destabilise the aposematic system. To answer this paradox, scientists developed a hypothesis from the framework of conventional signalling theory

(Dawkins and Guilford, 1991). This framework predicts that naïve predators would attack all conspecifics at the same rate, and the benefits of aposematism would be lost due to the dishonest signallers. Instead, it is likely that warning colouration may signal to predators not that the individual is definitively unpalatable, but that the predation should be cautious when attacking. This idea is called the ‘Go-slow hypothesis (Guilford, 1994), which has also been modelled (Holen and Svennungsen, 2012) as well as tested experimentally (Gamberale-Stille and Guilford, 2004). ‘Go-slow’ allows for a benefit of toxicity in an individual, as under this theory a small subset of a population could be dishonest signallers without the whole species losing the benefits of aposematism.

More recently, researchers have also considered whether this common variation in both defence and signalling may not be another form, or rather a more specific form, of honest signalling; this theory is called the resource competition model.

2.8. Resource competition models

Quantitative honest signalling is a hypothesis that individuals may signal not only their unpalatability, but the degree of their unpalatability, with their variations in signals. The evidence for and against quantitative honest signalling has been reviewed recently by Summers et al. (2015), as well as by White and Umbers (2021), and is summarised below.

Quantitative honest signalling is usually defined as a positive correlation between some measure of warning signal in an individual, for example luminance, saturation, or pigment quantity, with a measure of chemical defence, either through direct chemical analysis, or bioassays (María Arenas et al., 2015). Evidence has been found for such correlations across a wide range of taxa, including vertebrate and invertebrate aposematic species, and from a variety of scales, from within population analyses to across related groups of species. In insects, Bezzerides et al. (2007) found a positive correlation between elytra colour and toxicity in Asian ladybird beetles, and Vidal-Cordero et al. (2012) found that brighter paper wasps have larger toxin glands. Arenas et al. (2015) found signal honesty in four species of ladybirds, and also tested experimentally that this led to predators attacking the less conspicuous morphs more frequently. In ladybird eggs as well, positive relationships between toxicity and colour have been found; in seven-spot ladybird eggs, egg colour saturation correlated positively to egg toxin concentration (Winters et al., 2014). In sea slugs (opisthobranchs), the more toxic species were also the most conspicuous (Cortesi and Cheney, 2010). There has also been significant research with poison frogs, both within and across species. Across ten populations of the strawberry poison frog (*Dendrobates pumilio*), toxicity has been found to positively associate with colouration (Maan and Cummings, 2012), and conspicuous colour has been phylogenetically correlated with toxicity in other dendrobatids, the whole group of poison frog species (Santos and Cannatella, 2011; Summers and Clough, 2001).

There has also been contrary evidence, with either no correlation found between toxicity and colouration, or a negative correlation, with the most toxic individuals or species being the least conspicuous. In insects, Briolat (2018b) found no convincing correlation between toxin and conspicuousness in six-spot burnet moths. With regard to poison frogs, in the granular poison frog, Wang (2011) found that the most toxic frogs are the least conspicuous morphs, showing a negative correlation. Similarly, the brighter males had less aggregate toxin in one population of strawberry poison frogs (Crothers et al., 2016), and Stuckert et al. (Stuckert et al., 2018) found no evidence for quantitative honest signalling in the mimic poison frog *Ranitomeya imitator*. Mochida et al. (2013) showed that in toxic newts (*Cynops pyrrhogaster*) there was no correlation between colour and tetrodotoxin (TTX) amount, but the authors noted as toxin amounts changed in wild newts slowly, over a decade, this time scale indicates environmental fluctuations in TTX to be the main driver. There can also be mixed or more complex results. For example, Blount et al. (2012) showed that female seven-spot ladybirds reared on limited resources showed a positive correlation between toxin and pigment levels, but males showed the opposite.

The presence of quantitative honest signalling in at least some groups and species has led to hypotheses on the underlying mechanisms maintaining it, usually involving tradeoffs. For example, Zahavi's handicap principle (Zahavi, 1975, 1977), which is usually discussed with regards to sexual selection, can also be applied to explain the maintenance of quantitative honest signalling. Applying the handicap principle, the cost of the signal to the individual enforces honesty, with only the most fit individuals able to maintain the most conspicuous warning signals.

An extension of this theory is Blount's resource competition model (Blount et al., 2009). Zahavi's principle relies on resource trade-offs but does not specify a resource that enforces the honesty gradient. It also does not account for the negative correlations between toxicity and colouration that have been found in several species. In the resource competition model, Blount et al. (2009) proposed mediator for this tradeoff: antioxidant molecules. Many classes of pigments used by organisms in signalling, for example carotenoids, pterins, and melanin, have antioxidant properties (McGraw, 2005; Sugumaran, 2002). In the resource competition model, the cost of sequestering, producing, and/or maintaining toxicity in individuals is assumed to be in the form of oxidative stress. The individual must therefore allocate pigment molecules either to its colouration, or signal, or use it for its antioxidant properties against the reactive oxygen species produced during sequestration.

The resource competition model has two potential states: high and low resource availability. In high resource environments, a negative correlation is expected between toxicity and strength of warning signal, as predicted by previous theory (Leimar et al., 1986; Ruxton and Speed, 2006), in which case it pays for a prey to divert their resources increasingly into toxins and not into warning signals, because less conspicuous but highly toxic prey encounter predators less often and have high chances of surviving attacks. In limited resource environments, if antioxidants are required to enable high levels of toxicity, signal reliability can be explained if the brightest and most toxic species gain access to more of the limiting resource than those that are less bright and less toxic – resulting in a positive correlation. This model could explain many of the examples of positive and negative correlations listed above and has the advantage of specifying a particular limiting resource.

As well as the many correlational and some experimental studies already published on the connections between toxicity and signal, further research specifically examining the three-way relationship between oxidative stress, toxicity, and colouration would add to the support for or against the resource competition model. For example, to my knowledge there are very few papers examining the link between oxidative stress and warning signals, although Flores et al. (2013) found both positive and correlations between luminance and stress in frogs, and Sandre et al. (2007) found no evidence of a link in the moth species *Orgyia antiqua*.

3. Part 2: Predators

With increasing understanding on the variability in aposematic prey, there is less focus on predator variability. This other side to the predator-prey relationship in aposematism is just as critical to understand as to look only from the prey perspective is to see only half the picture and to miss important steps in the evolutionary process. Endler (1991) made this case, and decades later it remains true that predator responses (and mitigation strategies) to prey defence is less studied, but the subject is still critical to the larger picture, as it is directly relevant to the evolution and maintenance of aposematism. It is likely that most prey species will face attack from a range of different predators, and each predator species can have different sensory modalities, tolerance of toxins, and behaviour (Endler and Mappes, 2004). What is toxic or unpalatable to one predator species may be either unnoticeable or even palatable to another (see Endler and Mappes, 2004), depending on if predators are resistant to certain toxin classes (Endler, 1988). To thoroughly research aposematism, it is important to take these ecological variations into account. Ideally, aposematic species would each be quantified in terms of their varied signals and defences, including chemical analyses of their sequestered or synthesised secondary metabolites relevant to defence. Predators too would be studied. How do predators detect, interact, and learn about aposematic prey influences the evolution of both signals and toxicity (Speed, 2001), so understanding their resistance to toxins via underlying mutations or other methods they use to evade poisoning, in their resistance to incorporating new prey types, and in their learning of distasteful prey are important for understanding what selects for and maintains variability in warning signals. The possibilities are endless for examining the predation sequence between aposematic prey and predator species, including variations in predation rates, prey mortality, behavioural responses from both prey and predator, learning and avoidance behaviour in predators, and memory and retention of avoidance.

In this section I will discuss the effects that predators can have on maintaining diversity in prey visual signals and toxicity, through predator mixes, differences in predator sensory ecology, behaviour like ‘go-slow’, associative learning, and neophobia and dietary conservatism.

3.1. Predator mix effects on maintaining diversity in aposematism

Predator variation can occur on geographic scales, with community makeup differing over distances, as well as temporally, with predator populations waxing and waning throughout the year, due to the arrival and departure of migratory species (Chesson, 1978). Prey also may face distinct predatory threats that vary according to the prey’s life stage and size (Schmitz, 2017), and which can vary according to the predator’s previous experience with toxic prey (discussed further in Section 3.6), or toxin load – for example, great tits have been found to be more wary when encountering aposematic prey when they have already consumed toxins (Hämäläinen et al., 2019). Predation can also vary with the environmental conditions (Chatelain et al., 2013). For example, a migrating monarch butterfly in California in winter, exhausted and resting, will likely face a different set of threats than in spring, in a northern city.

Theoretical work on predator mixes suggests that differences in predation selection pressure can lead to the evolution and maintenance of variation in prey colour morphs (Endler, 1988), i.e. polymorphism, and can explain the persistence of less conspicuous (or ‘weak’) warning signals (Endler and Mappes, 2004). These theories are based on variation in predator hunting, i.e., what sensory modalities they use, as well as the intensity of the predation pressure. The overall diversity of predators is expected to change selection pressure on warning colours (Sherratt, 2006). Empirical support for these theories comes from studies showing that predators vary in their responses to the signals of aposematic prey; for example, birds, crabs, and lizards all selected for different levels of conspicuousness in clay models of poison frogs (Willink et al., 2014b). Variation in prey signalling can also correlate to variation in predator communities, as has been found in several different systems. In tiger moths, those moths that coincide temporally with bat predation advertise their chemical defence with ultrasonic clicks, and those coinciding with birds instead have bright colouration (Ratcliffe and Nydam, 2008). Two morphs of the pea aphid *Acyrthosiphon pisum* are protected from parasitoid attack by the red morph, and from ladybird predation by the green morph, with morph frequencies varying depending on predator frequency (Losey et al., 1997). In the harlequin hibiscus bug, Fabricant et al. (2015; 2014) found that individuals varied in their colouration between orange and iridescent blue, and this variation correlated to areas of either greater bird or mantid predation, as iridescence is a more effective signal against avian predators. In this case, the different mix of predators affected selection on visual signals because the predators varied in their toxin acceptance (see Section 3.2). In amphibians, Mochida (2009) found that aposematic newts had more conspicuous signals when on islands, locations where they faced more avian predation, than on the mainland, where the predator community is dominated by mammals. Finally, dwarf chameleons were found to change colour differently when facing different predators, and became more cryptic to birds than to snakes (Stuart-Fox et al., 2006).

Once polymorphisms have evolved from evading selection pressure from local predator mixes, predation can then also exert disruptive selection, selecting for and maintaining polymorphisms in a species; this has been found experimentally in poison frogs, where avian predators attack ‘exotic’ morphs much more than local ones (Chouteau and Angers, 2011). By predators preferentially selecting for the non-local morph, the morph most effective as prey defence persists in that locality. However this may not always be the case; in another group of poison frogs, the local morph was not the most protected, and the persistence of a less effective signal was likely due to low gene flow between populations (Lawrence et al., 2019).

Which predator mix a prey species faces in its life will affect the selection pressure from predation (Mappes et al., 2014); this in turn determines which chemical defences are maintained and advertised. This balance may vary geographically and temporally, and it may be a more stable strategy to vary chemical defences depending on the dominant predation pressure.

3.2. How predators can vary in their defence mitigation – behavioural and physiological adaptations

As with all arms races in biology, the contest between predator and prey is both never-ending and continuously escalating. As prey evolve stronger toxins, or a wider profile of defences, predators coevolve to evade these defences (reviewed in Arbuckle et al., 2017); there are examples of this in modern history, with Australian snakes rapidly evolving resistance to toxic cane toads, a very recent invasive prey species (Phillips and Shine, 2006). Although there is less research on predator mitigation of prey defences (Endler, 1991), theoretically the variation in predator response to toxins can allow for the evolution and maintenance of variation in prey defence.

Predator toxin evasion can come in different ways, both behavioural and physiologically. Some predator species specialise to consume certain types of prey and evolve specific behavioural adaptations to avoid poisoning effects; for example, the black-eared mouse *Peromyscus melanotis* is one of the few predators of overwintering monarch butterflies. Experiments showed that they rejected the cuticles of the more toxin-laden butterflies, allowing them to consume the less toxic body parts and reducing their need to tolerate the cardenolides (Glendinning, 1990).

There are also physiological adaptations, through molecular target site insensitivity mutations, physiological barriers, or rapid excretion/detoxification of the toxin. There is little specific theoretical modelling on these hypotheses, but as Arbuckle et al. (2017) discuss, it is logical that specialist predators feeding on toxic prey would have higher resistance to those toxins. For generalist predator species, there is less selection pressure to evolve resistance to toxins. Resistance can be costly in terms of negative pleiotropic effects of molecular adaptations; dissecting behaviour and slower prey handling may translate into an overall cost in fitness in some species. Instead of evolving resistance to toxic prey, generalists can bypass these costs by simply switching their foraging to other prey types.

Of predators that do consume toxic prey, wide-ranging taxa may convergently evolve the same physiological mechanisms, even using the same mutations employed by prey species to sequester or produce the poisons in the first place.

This phenomenon is seen in tetrodotoxin resistance in pufferfish, softshell clams, and garter snakes (Soong and Venkatesh, 2006) as well as in cardiac glycoside resistance through mutated sodium-potassium pumps in amphibians, reptiles, and mammals (Ujvari et al., 2015).

These different ways in which predators mitigate toxins can vary, meaning that different predator mixes could influence variations in toxicity in prey. For example, praying mantises consume harlequin bugs with no noted negative effects, whereas birds rejected the bugs as unpalatable (Fabricant and Smith, 2014). Although this is not direct evidence for variation in toxins from different predator responses, this example shows how it could occur, as investing in toxins would be less evolutionarily beneficial in areas with more invertebrate predators. This varied predation pressure could select for different defensive strategies in different locations, maintaining a diversity of toxins in a prey species.

3.3. Sensory ecology

Variation in predator species' hunting strategies can allow for a diversity of visual signals in a prey species, and differing abilities of predators to evade toxins can influence differences in toxicity in aposematic prey (Endler and Mappes, 2004). Both these effects are underpinned by predator perception of aposematic prey, both in terms of observing the warning signals, for example through vision, auditory, and olfactory systems, as well as perceiving the chemical defence, through olfaction and taste. The increased study of multi-modal defences and signals is also revealing a complex balancing act between prey and their many predators, in which variations in multimodal signals are more effective against different predator groups (for examples see Krivoruchko et al., 2021; Ratcliffe and Nydam, 2008; Rojas et al., 2019).

Across taxa, animals sense their environment through a variety of modalities. Predators can use vision, chemosensing, echolocation, hearing, or other less-studied modalities like sensing electricity and magnetic fields, and many will use a combination of these to different degrees (Stevens 2013). With relation to predator-prey relations, the sensory ecology of predators when foraging is the most important, and this depends on the taxa. For example, many arthropod predator groups like parasitoids and marine invertebrates hunt using chemical cues (Aartsma et al., 2019; Kamio and D. Derby, 2017); volatile signals being used in hunting has also been shown in more specific taxa, like ladybirds (Pervez and Yadav, 2018). In mammals, bats use echolocation through ultrasound to detect flying insect prey in the dark of night (Jones and Holderied, 2007), and avian predators are known to primarily use visual cutes to hunt (Stevens, 2013). Different species also see different ranges of the light spectrum; snakes use infrared to detect prey (Gracheva et al., 2010), and birds use ultraviolet wavelengths in foraging decisions (Church et al., 1998; Cuthill et al., 2000). Animals also vary in their visual systems, for example from monochromatic to tetrachromatic (Mascalzoni and Regolin, 2011); in aquatic environments these differences are compounded with the addition of polarised light, which some species can detect and others cannot (Marshall et al., 2019). These different visual systems affect predator perception of conspicuousness for different colours (Stevens, 2013).

Much research focuses on warning colouration, so most examples given so far have been visual. Although warning signals can be communicated through any sensory modality, they are seen by most research as primarily visual (Ruxton et al., 2018). Due to this focus on vision, visually hunting predators have often been primarily used in behavioural experiments, usually avian predators (for example Chouteau et al., 2019; Gamberale-Stille and Tullberg, 1999; Macdougall and Stamp dawkins, 1998; Rowe and Guilford, 1996). Birds are useful as model predators against conspicuous prey, as they are adept visual hunters and can see the human visible spectrum as well as ultraviolet wavelengths. They can also distinguish prey based on brightness, as shown by great tits learning avoidance at the same rate regardless of colour in painted mealworms, but avoiding more luminant worms initially (Sandre et al., 2010). Similarly, more conspicuous models of rock lizards faced more predation attempts from birds (Stuart-Fox et al., 2003).

As they are often used as predator models, avian visual systems are usually the basis for visual modelling of prey signals (Arenas and Stevens, 2017; Blount et al., 2012; Briolat et al., 2018b; María Arenas et al., 2015). However, in other taxa the same visual signals may be perceived very differently. Some invertebrate predators cannot distinguish red as a colour (Briscoe and Chittka, 2001); as red is a ‘classic’ aposematic colour (Cott, 1940; Ruxton et al., 2018), and green a very common background colour for an animal, from leaves or moss, some prey species considered aposematic to human eyes and bird predators are cryptic to invertebrates. There are fewer experiments using visually-hunting invertebrate predators, and predators used tend to be jumping spiders (Hill, 2006; Vickers and Taylor, 2018; Vickers et al., 2021), lacewing larvae (Pokharel et al., 2020), or praying mantises (Berenbaum and Miliczky, 1984; Gelperin, 1968; Prudic et al., 2007b). This balance is changing, however, and increasingly researchers are both using more invertebrate predators in their experiments, as well as modelling vision from other predator groups than birds. As discussed previously, this was true in the case study of the hibiscus harlequin bug, which was both modelled and tested experimentally against both invertebrate and avian predators (Fabricant and Herberstein, 2015).

As well as expanding our view on visual modelling, research is also increasingly including other modalities of predator perception and embracing the other side of multimodality: the predator’s perception of multimodal signals and defences (Ratcliffe and Nydam, 2008; Rowe and Halpin, 2013). The classic yellow, red, and black colouration is typical of a visual signal (Cott, 1940), often effective against avian predators, but aposematic audio signals have been found (Olofsson et al., 2012). These target primarily audio-hunting predators, for example when tiger moths make ultrasonic clicks to effectively warn bats of their unpalatability (Rojas et al., 2017). Predators can also be deterred by tastes, with unpleasant flavours signalling unpalatability. For example birds can reject prey based on bitterness (Skelhorn and Rowe, 2006a), a trait often associated with internal toxins (Nissim et al., 2017). It can also be argued that behavioural attributes could be signals; moving slowly, or sluggishness, is a behaviour known to be associated with aposematism in various species, and could be a signal to predators that the prey is defended (Chai and Srygley, 1990; Hatle et al., 2001, 2002). Similarly, aggregations and gregariousness are associated with aposematism (Riipi et al., 2001; Ruxton and Sherratt, 2006). All these signals in any modality rely upon predator perception to be effective, and the sensory ecology in predatory species can vary widely.

The local ecosystem and immediate conditions are critical for which sensory modalities are more effective (Endler, 1993); for example, Lunt and Smee (2015) showed that a chemosensory hunter (the blue crab) was more successful in disturbed, turbid water than a visual hunter (pinfish). For conspicuous prey, environments that are less clear to see in, like turbid water, or complex light environments, could be a disadvantageous locale (Matchette et al., 2020). Visual hunters may not see them, and chemosensory hunters may not take note of their warning colouration.

3.4. Dietary wariness: neophobia and dietary conservatism

Beyond the variations in toxin resistance and sensory capabilities, predators also vary in their behaviour. Predator behaviour is a key aspect to the evolution of both colouration and secondary defences, as once predators have encountered a prey, how they deal with it is crucial to the prey's survival or consumption (Endler, 1991). Once identified as prey, predators may be wary to approach and consume the prey due to neophobia and/or dietary conservatism, collectively known as dietary wariness (Marples and Kelly, 1999) – see Chapter 3. Food neophobia is a well-studied behavioural trait found in many taxa, wherein predators are hesitant in attacking a novel food for a short period of time (Barnett, 1958), due to fear of a novel object, or to innate biases, for example against classic warning colouration (Caldwell and Rubinoff, 1983). Dietary conservatism is the longer-term process of predators refusing to incorporate novel foods into their diets (Marples and Kelly, 1999).

There is much evidence for the existence of dietary wariness and its occurrence in many groups of animals. Neophobia has been observed in mammals including humans, birds, amphibians, fish, and occasionally in invertebrates (reviewed in Crane and Ferrari, 2017). Dietary conservatism, a more recently identified phenomenon, has mostly been studied in birds (Marples and Kelly, 1999; Marples et al., 1998; McMahon and Marples, 2017; McMahon et al., 2014) and fish (Richards et al., 2011, 2014; Thomas et al., 2010), but it is likely that more research will show its presence in numerous other taxa yet examined. Interestingly, although neophobia is often common throughout the population of predators studied in experiments, conservatism is usually found only in a subset of the group, for example it was found in only about a quarter of sticklebacks (Richards et al., 2011).

Dietary wariness is closely connected to the evolution and maintenance of prey signals and defence, as it is yet another mechanism by which aposematism might evolve (Lee et al., 2010). Hesitancy to attack (neophobia) and reluctance to consume (dietary conservatism) can both contribute to the survival of novel morphs, and several researchers have also expanded this to the evolution of aposematism itself (Lee et al., 2010; Thomas et al., 2003). Neophobia is usually short-lived and overcome quickly, but even so may benefit novel morphs enough for them to persist (Speed, 2001). The longer-lasting dietary conservatism, which can be a weeks or months-long aversion, has also been postulated to allow for the initial evolution of aposematism itself (Lee et al., 2010; Marples and Mappes, 2011; Speed, 2001).

One evolutionary problem with regards to novel morphs is the assumption that novel conspicuous prey will be predated at higher rates due to frequency-dependent selection (Chouteau et al., 2016; Endler, 1988). There is some evidence for this, for example blue tits attacked a novel morph, in the form of a clay model, much more than the established ones (Lindström et al., 2001). Despite this, the evolutionary paradox of novel morphs can be alleviated by the existence of dietary wariness. For example, in a ‘Novel World’ setup with avian predators, Marples and Mappes (2011) also found that new conspicuous morphs faced the highest level of attacks. However, in this experiment the authors also found that a subset of birds exhibited dietary conservatism and were reluctant to consume the novel prey. This evidence that dietary conservatism can allow for the fixation of novel morphs was also found in European robins, where novel prey morphs reached fixation several times, and more frequently so when they were the classic aposematic colours of red and yellow (Thomas et al., 2003). Thomas et al. (2010) found similar results in fish, where the novel prey colours persisted in a third of tested prey populations. The evidence shows firmly that wariness can allow for the evolution and maintenance of rare novel morphs. Even if not all predators in a population exhibit neophobia or dietary wariness, the reluctance or hesitation of some predators to attack can allow these variations in prey signals to persist.

3.5. Behaviour: the ‘Go-Slow’ hypothesis

If a predator has found a conspicuous, toxic prey and captured it, perhaps overcoming any immediate neophobia, the next stages as described by Endler (1991) are subjugation and consumption. One theory, mentioned previously (Section 2.7) is the ‘Go-Slow’ hypothesis by Guilford (1994). As with dietary wariness, in the ‘go slow’ theory predators are more wary of conspicuous prey individuals. However, dietary wariness theory does not encompass differences in consumption, whereas in ‘go slow’ theory predators attack and are more cautious when consuming the prey individual, often more slowly and carefully than they would a cryptic prey.

Predation that is slow and careful includes the cautious attempted consumption of food, and can include taste rejection, or the release of prey due to an unpleasant taste. For example, Skelhorn and Rowe (2006b) found that birds could reject prey based on taste, and even discriminated accurately between prey of differing levels of chemical defence. This hypothesis would then generally mean that automimics and Batesian mimics are at a selective disadvantage compared to those conspicuous prey animals that are defended. However, if the ‘go-slow’ hypothesis is ubiquitous, then how do variable defences persist?

Ruxton and Speed (2006) reviewed this question, and concluded that there are multiple ways in which this variability can persist, including varying predation pressure, predators not investing in discrimination between prey, predation pressure increasing with more automimics, and constraints on the use of defence. Constraints on defence and varying predation pressure have been discussed in previous sections (Sections 2.6 and 3.1 respectively), and both are supported with experimental evidence. Frequency dependence of automimicry or Batesian mimics has long been modelled (Brower et al., 1970; Guilford, 1994), and there is evidence that predators are sensitive to the frequency of automimics in groups of prey (Gamberale-Stille and Guilford, 2004), and this can allow for the persistence of automimics (Skelhorn and Rowe, 2007a).

Taste rejecting takes valuable time and could mean costs to the predator in terms of less foraging time, more time being conspicuously out in the open, or the potential of ingesting toxins. To avoid these risks, some predators may either avoid all conspicuous prey, and some may not choose to taste reject prey, instead consuming indiscriminately. There is experimental evidence in support of predators not always discriminating between prey defence levels, as in some experiments with chicks, where they do not always learn to avoid differently defended prey (Halpin and Rowe, 2010).

3.6. Avoidance learning

As has been discussed (Section 2.3), avoidance learning is a key part of aposematism research. Predators learning to avoid defended, conspicuous prey underpins the models and theoretical framework of the evolution and maintenance of aposematism (Ihalainen et al., 2007; Johnston et al., 1998; Rowland et al., 2017; Skelhorn and Rowe, 2006c). As well as being critical, learning is also a very complex field that can be challenging to navigate, with even the definition of learning differing between researchers (Skelhorn et al., 2016).

Predators tend to learn to avoid conspicuous, defended prey better – usually defined as faster and retaining the memory for longer - than cryptic prey, even if the cryptic prey is defended (Alatalo and Mappes, 1996; Gittleman and Harvey, 1980; Roper and Redston, 1987; Roper and Wistow, 1986). Avoidance learning is therefore a key mechanism for the evolution and maintenance of aposematism (reviewed in Skelhorn et al., 2016). There has been research spanning decades examining avoidance learning and producing much experimental evidence for it in a wide range of taxa (Prudic et al., 2007b; Roper and Redston, 1987; Roper and Wistow, 1986). For a few examples: in birds, chicks learn to avoid unpalatable prey when they were conspicuous, not when they were cryptic (Halpin et al., 2008), and great tits learned faster when fed aposematic versus cryptic instars of the same poisonous caterpillar (Sillén-Tullberg, 1985). In other taxa, bats learnt to associate warning clicks with bitter mealworms but not with regular mealworms (Bates and Fenton, 1990), lizards remembered red unpalatable food items for longer than green (Ko et al., 2020), and praying mantises learnt to avoid brighter toxic bugs faster than less bright individuals (Prudic et al., 2007b). These studies increase the importance of aposematism as not just toxicity but also the advertisement of it, as avoidance learning is more effective when the signal and defence work in combination.

The classical view of avoidance learning as a driver of aposematism is changing and improving with the acknowledgement of more variation in learning than was previously thought. Predators can differ in learning both within a species (Powell and Taylor; Rowland et al., 2017), and as groups in different predator mixes (Hotová Svádová et al., 2010), and these variations can reinforce variation in prey aposematism (Endler and Mappes, 2004).

Within a species, one way that predators can maintain toxin diversity is if they do not distinguish between levels of toxicity in their prey, and either consume or avoid all individuals regardless of their specific level of chemical defence. This indiscriminate foraging has been found in some systems, for example chicks learnt to avoid prey equally in one experiment despite prey varying up to threefold in toxicity (Chouteau et al., 2019). Why predators would avoid all individuals equally may depend on them generalising defences after previous experiences; in the case of the chicks, the authors assumed that high levels of chemical defence in some individuals must have evolved for a different selection pressure than anti-predator defence. A lack of learning could also come from toxin saturation, as predators then may learn to avoid all species containing that toxin, even though they differ visually; Halpin et al. (2012) found this experimentally with starling predators and manipulated mealworm prey. In one study, European starlings were found to learn to avoid defended mealworms better when their defences were variable, rather than when the mealworms were consistently the same level of defended (Barnett et al., 2014).

The opposite result was found in jumping spiders, which did not learn to avoid milkweed bugs raised on their toxic host plant milkweed, instead continuously attacking and taste rejecting them while showing signs of poisoning (Bramer et al., 2018). Spiders also attacked milkweed bugs raised on sunflower seeds, which were not toxic, but they did consume those individuals. Although any differences in toxicity in the milkweed-raised bugs was not measured, if any variation existed it did not affect spider learning, as there was none. In these examples of indiscriminate predators, the result is that prey individuals with lower toxicity levels could persist in a population, as they face no difference in survival rate from more defended conspecifics. In these cases, either pressure from other predator groups could maintain the benefits of toxicity in a species (see Section 3.1), or the defence could serve other purposes (see Section 2.6).

Predators displaying indiscriminate consumption or avoidance toward aposematic species could therefore maintain toxin diversity in their prey communities.

Predator species also vary in their learning abilities within their ecosystems and local predator communities (Hämäläinen et al., 2021). As seen in the examples so far, learning has been shown across many taxa, and although most research originally focused on vertebrates there is now plenty of evidence for invertebrate avoidance learning as well. This is still an expanding field, and there are many species for which there is yet no indication of their learning abilities; there is also the reverse problem, in that studies showing negative results, i.e. no learning, will be less prevalent in the literature due to the publication bias towards results supporting a hypothesis (Jennions and Møller, 2002; Leimu and Koricheva, 2004). These two factors mean that for most predator species, their ability to learn to avoid prey is either known to be present or unpublished. Nevertheless, from what is known and published variation in learning across species is definitely present, and could lead to less prey investment in defence and signal. This could occur if a prey species faces a varied predator community, with some learning and some non-learning species; in this case it may not always be evolutionarily beneficial to be aposematic, as non-learning predators may dilute the advantage. This has been discussed and modelled by Endler and Mappes (2004), and it would be an interesting area for future researchers to directly test, as there are few clear examples where learning differences in predators was specifically known as the cause for variable prey. One example which does show this is the same case study of the mantids, birds, and hibiscus harlequin bugs, as this study shows variation in prey signals according to the prevalent local predator community (Fabricant and Herberstein, 2015; Fabricant and Smith, 2014).

Another area of expanding research is the interactions and variations in learning from a social context, as avoidance learning also can vary according to social information availability and use. Social learning is a long-studied but constantly expanding area of research, and there is growing evidence that observing conspecific individuals can have significant effects on the observer’s learning patterns (Heyes, 1994). This has especially been found in birds, with great tits learning to avoid firebugs (*Pyrrhocoris apterus*) faster when they had observed another bird having an adverse interaction with one (Landová et al., 2017). Similar results have been found in blue tits, who could also learn from video playback about novel aposematic prey, and learnt more efficiently after viewing the clips (Hämäläinen et al., 2020). These studies, and several others showing similar social learning of avoidance behaviours in chicks (Johnston et al., 1998; Skelhorn, 2011) and blackbirds (Mason et al., 1984), are likely just the beginning of discovering the importance of social transmission of information about aposematic prey. It may greatly increase the benefits of aposematism, if a prey faces more predators with social systems that allow for this learning, than if the individual faces many predators who must all learn independently of each other. Every attack is a risk for a prey animal, and social transmission could decrease it substantially, but as with any variation in predator behaviour, it depends on the local ecological community.

3.7. Combining avoidance learning and honest signalling research

Beyond these intriguing expansions in the study of avoidance learning, there are also specific questions that researchers are starting to answer with further research. In this thesis, a relevant question is whether predators learn more effectively when the prey species are honestly signalling. There are studies looking at predator discrimination of visual differences in signal through visual modelling (Arenas and Stevens, 2017; Llaurens et al., 2014), with no predation trials completed. There are also papers examining live predator responses to prey with varying signals or different morphs; these often show more conspicuous prey facing higher levels of predation (for example Noonan and Comeault, 2009; Stuart-Fox et al., 2003), although prey variability sometimes has no effect on predator discrimination (Amézquita et al., 2013; Rönkä et al., 2018). In learning, this increased discriminability can lead to enhanced predator learning with greater luminance (Prudic et al., 2007b) or colour intensity (Gamberale-Stille and Tullberg, 1999), although differences in signal do not always affect learning rates (Sandre et al., 2010) and indeed it can vary depending on predator against the same group of varying prey (Hotová Svádová et al., 2010). However, in all these studies the prey varied in signal but were either consistent in toxicity, or models with no toxicity included. For examining the assumptions and expected results of the resource competition model, it is critical to study the predator response to variations in both signal and defence in a prey species.

There is some published research in this area, with interesting results. Ihalainen et al. (2007) tested bird predators against groups of prey with both variable and unvarying signals and with moderate, mixed, and high defence levels. Their results showed that when facing variable prey, birds did not learn any more slowly than when facing uniform prey, indicating no disadvantage to a mixed group. The authors also found that, contrary to most aposematism theory, variation in signal benefited moderately unpalatable prey from attack. Birds also were similarly wary in a memory test with aposematic prey, regardless of if their previous experience was with mixed unpalatable or highly unpalatable prey. Altogether this paper suggests that, at least in their system of naïve great tits, variable signal and defences in their prey were not overly regarded or discriminated between.

Given the increasing interest and developing research in quantitative honest signalling in aposematic prey (reviewed in Summers et al., 2015; White and Umbers, 2021), it would be beneficial for the field to have more published studies of experiments testing predator learning against honest and dishonest prey. As signalling theory in general is dependent upon the receiver understanding signals (Guilford and Dawkins, 1991), and the resource competition model of honest signalling specifically depends on predator discrimination (Blount et al., 2009), there is much scope for future empirical evidence. With experiments of predators facing honestly and dishonestly signalling prey, the field would gain evidence to support or undermine the theory and determine whether prey variability in signals and defences really matters to predators.

4. Part 3: Model Organisms

4.1. Cardiac glycosides

Cardiac glycosides (CGs), including the toxin classes cardenolides and bufadienolides, are a diverse group of steroids derived from triterpenoids found primarily in plant species, where they have evolved in response to natural selection exerted by herbivores (Agrawal et al., 2012; Dobler et al., 2012; Zhen et al., 2012). CGs are also sequestered from host plants by some herbivores, where they have evolved as a chemical defence in response to natural selection by predators. The interaction between host plants, specialised insect herbivores, and the predators of these insects have played a central role in our understanding of coevolutionary specialisation (Dobler et al., 2011, 2015, 2019; Malcolm, 1991).

Cardenolides are informally known as heart poisons and are a class of toxins falling under cardiac glycosides. Animals that eat cardenolides and are not resistant to the toxins rapidly exhibit signs of toxicosis, which can manifest as vomiting and malaise (Brower and Fink, 1985; Brower et al., 1968a; Fink et al., 1983). Cardenolides are toxic due to their deleterious effects on the transmembrane protein Na+/K+-ATPase (NKA) (Schoner and Scheiner-Bobis, 2007), a crucial enzyme with wide-ranging functions affecting cholesterol distribution (Chen et al., 2009), signal transduction (Cui and Xie, 2017), cardiac function (Dostanic et al., 2004), and blood pressure regulation (Dostanic-Larson et al., 2005). When ingested cardenolides reach cells, they bind to the polar residues of the αM1-M2 extracellular loop of the NKA and thereby inhibit its ion transport (Laursen et al., 2015; Schoner, 2002). Cardenolide consumption in non-adapted animals can result in death, for example oleander poisoning in geese, dairy cattle, dogs, and humans (reviewed in Langford and Boor, 1996).

The large milkweed bug *Oncopeltus fasciatus* (Dallas 1876), hereafter called milkweed bugs, are highly resistant to cardenolides. In a study with ouabain, a common cardenolide used in laboratories, bugs had an 100 % survival at 200 nmol injected per insect, as opposed to the desert locust *Schistocerca gregaria*, which had a 50 % death rate at only around 6 nmol (Moore and Scudder, 1986). Milkweed bugs are specialist consumers, piercing and sucking the content of seeds from milkweed plants throughout their lives (Burdfield-Steel and Shuker, 2014). These insects have evolved resistance to cardenolide poisoning via mutations in the cardenolide binding pocket of the NKA (Bramer et al., 2015). These mutations consist largely of amino acid substitutions in the αM1-M2 extracellular loop of the protein’s α-subunit, preventing cardenolides from binding to it and lowering the sensitivity – this is called target site insensitivity (Bramer et al., 2015; Moore and Scudder, 1986). Target-site insensitivity to cardenolides shows striking patterns of molecular convergence, allowing resistant species to consume cardenolide-containing host plants or prey (Karageorgi et al., 2019; Ujvari et al., 2015). Various prey and predator taxa have evolved this resistance, including monarch butterflies (Holzinger and Wink,

1996) and garter snakes (Mohammadi et al., 2017). In this thesis, cardenolides are the basis for the ecological system studied, specifically through the variations in its sequestration by milkweed bugs, and the effects of this variation on an invertebrate predator.

4.2. The large milkweed bug *Oncopeltus fasciatus*

The large milkweed bug *Oncopeltus fasciatus* is a true bug (Hemiptera) species within the Lygaeidae family. Milkweed bugs have long been studied as a model organism for various questions (Feir, 1974), for example in migration (Aldrich et al., 1999; Attisano et al., 2013) and pheromones (Aller and Caldwell, 1979; Zhang and Aldrich, 2003), but especially about aposematism (Berenbaum and Miliczky, 1984; Feir, 1974; Hill, 2006; Prudic et al., 2007b). Unlike some members of the Lygaeidae family that are cryptic and elusive, milkweed bugs are conspicuous - at least to human eyes – with orange/red and black wings as adults, and orange/red and black bodies in their larval stages, where they are called nymphs. This reddish colouration is suggested to be from pteridines (Bartel et al., 1958; Hudson et al., 1959) and the black is from melanin (Liu et al., 2016). Milkweed bugs develop through five larval instars (L1-L5) before moulting to adulthood, when they develop wings. During their flightless immature stages, especially L1-4, bugs aggregate to feed on milkweed seeds, often in large groups of up to 30 individuals (Burdfield-Steel and Shuker, 2014; Sauer and Feir, 1973). At all life stages milkweed bugs feed exclusively on seeds and seed pods from species from the genus *Asclepias* (Duffey and Scudder, 1972), or milkweed. *Asclepias* is a broadly distributed genus of plants whose species synthesise cardenolides, toxins in the class of cardiac glycosides that have evolved in response to herbivory (Agrawal et al., 2012; El-Mallakh et al., 2019). Milkweed bugs are able both to bypass this plant defence as well as sequester these poisons for themselves (Burdfield-Steel and Shuker, 2014) as protection against predators, shown to be effective against several predator classes (Berenbaum and Miliczky, 1984; Bramer et al., 2018; Pokharel et al., 2020). They also feed out in the open on the stems on their host plants so are also behaviourally conspicuous (Burdfield-Steel and Shuker, 2014). Together this evidence of sequestration, colouration, and behavioural traits makes milkweed bugs a clear example of an aposematic species.

Milkweed bugs store cardenolides from feeding on milkweed seeds in a specialised layer of cells beneath the outer layer of their epidermis (Bramer et al., 2017). This cardenolide-rich secretion is released through specific thinner areas of their epidermis that break open when physically stimulated to do so (Scudder and Meredith, 1982). Milkweed bugs also release these secretions when raised on nontoxic host plants, but the liquid then does not contain cardenolides (Isman, 1977). *Asclepias* species vary greatly not only in cardenolide profile and content (Züst et al., 2019), but also in terms of flowering and setting seeds, and likely various other components (Kephart, 1987). Although milkweed bugs sequester a similar array of cardenolides in terms of polarity, regardless of the species of *Asclepias* they fed on (Moore and Scudder, 1985), they sequester varying total amounts of cardenolides depending on their sex (Isman, 1977) and *Asclepias* host species (Duffey and Scudder, 1974). Field-caught bugs show large variation in cardenolide content, in terms of digitoxin equivalent, unexplained by the difference in cardenolide content of their host plants, with bugs sequestering from 0 to 300ug (Isman et al., 1977).

There has been less attention given to quantifying colour variation across milkweed bugs. Rodríguez-Clark (2004) found no differences between males and females in pronotum colouration, although this study measured differences on a human-determined scale. Davis (2009) used more objective image analysis, and found that females had darker black sections on their wings. The author also found that larger bugs had deeper orange colouration and darker black areas, i.e., in terms of colour saturation, on their wings. Despite limited published research, these studies give us insight into the variation possible in milkweed bugs in their wing colouration. The underlying mechanism for such variation could theoretically come from any of the known reasons for signal variation (see Section 2.5 and Briolat et al., 2018a), i.e. immune function, sexual selection, quantitative honesty, etc.

The resource competition model suggests that prey variation in colour and toxicity is explained by a trade-off caused by the oxidative stress cost of producing defences. Given that milkweed bugs vary in both colouration and toxicity, they present as an appropriate model species in which to test this model. Even more so as there is also mixed evidence that milkweed bugs face a cost to sequestering cardenolides; bugs that vary in sequestration do not differ in terms of size or body weight (Isman, 1977), but Pokharel et al. (2021) found a life history cost in terms of reduced fecundity - milkweed bugs laid fewer eggs when fed on a more toxic diet. In addition to the potential physiological/life history costs associated with consuming cardenolides, there are also molecular costs of target-site insensitivity in terms of negative pleiotropic effects on the protein's ATPase function. When the milkweed bug NKA mutations were induced in a fruit fly, the enzyme was less efficient (Dalla and Dobler, 2016).

O. fasciatus is an ideal lab species: they are easy to rear, can be raised in large numbers under standardised conditions, their diets can be varied from toxic (cardenolide-containing) to nontoxic through either artificial diets or different host plant seeds, and their pigments and sequestered compounds are known. This allows me to examine the causes and consequences of variation in both colouration and toxicity, and determine how their signals and defences combine to deter predators most effectively.

4.3. The praying mantis

Praying mantids are a group of generalist invertebrate predators with more than 2,000 species, ranging across wide swathes of the globe (Prete et al., 1999). They are known as praying mantises, mantises, or mantids, and have fascinated humans for millennia with their unique posture, ferocious habits, and cannibalistic tendencies (Prete and Wolfe, 1992). Although a charismatic and interesting group of insects that range widely in habitat and behaviour, they are generally characterised as being ambush predators, or sit-and-wait hunters that do not stalk prey but rather wait for it to approach (Prete et al., 1999). Mantids have long been studied in ecological research with predation as the main focus, although usually only larger species that fall within a few select genera, including *Tenodera* (Carle et al., 2018; Corrette, 1990; Eisenberg et al., 1981), *Mantis* (Rathet and Hurd, 1983; Reitze and Nentwig, 1991), and *Hierodula* (Battiston et al., 2018; Mebs et al., 2016; Sugiura, 2021). In my experiments I used the Giant Asian mantis *Hierodula membranacea*, a large species that has been studied for its sexual cannibalism (Birkhead et al., 1988) and anatomy (Kerry et al., 1987; Rosner et al., 2017). As there are fewer studies using *H.*

membranacea in predation and toxin tolerance, except Mebs et al.'s (2016, 2017, 2019) research that will be discussed, this introduction will be general, encompassing all of the commonly used experimental mantid species.

Mantids are primarily visual hunters, a useful trait when studying visual warning signals in prey. Although their actual visual system is not fully understood, it is thought they are mostly adapted to daylight (Kral, 2012), and can see the world in three dimensions (stereopsis) (Nityananda et al., 2016). The spectral sensitivities of two *Tenodera* species are known (Rossel, 1979; Sontag, 1971), but it is still unsure whether or not mantids have monochromatic or dichromatic vision, and therefore whether they can see in colour (see Discussion in Prudic et al., 2007). However, their vision-based behaviour is very well studied (for examples, see Barry et al., 2015; Corrette, 1990; Kral, 2012). Although their visual hunting is the most reviewed in the literature, mantids can also smell (Maxwell et al., 2010; Prete et al., 1992), hear (Yager and Hoy, 1986, 1989), and taste (Carle et al., 2015; Tuffnell and Rowe, 2011). Smell and taste are very likely used in foraging and predation. Although chemosensing is used by various mantid species to detect pheromones (Hurd et al., 2004; Maxwell et al., 2010), mantids can also be repelled by volatile compounds (Noge et al., 2012; Prudic et al., 2008) and attracted to them; Chinese mantids were shown to actively choose a covered bag of banana over one of plastic beads, repeatedly trying to eat them (Prete et al., 1992). Mantids can also taste reject prey (Berenbaum and Miliczky, 1984), and have been shown to recognise bitterness (Carle et al., 2015). As many aposematic species exhibit multimodal signalling (Ratcliffe and Nydam, 2008), a model predator group that can respond to varied visual, olfactory, and gustatory characteristics is ideal.

Although hearing, which most mantids can do, is more likely to have evolved for evading bat predation rather than their own hunting (Triblehorn and Yager, 2005; Triblehorn et al., 2008), this sense evolved prior to the evolution of bats (Yager and Svenson, 2008) so could potentially have other functions. Some mantids also hear lower frequencies, and it is unknown as to why, with predation being one possible explanation (Yager, 1996).

Mantids also have an interesting research history with toxins and toxic prey, as many species can consume certain toxins readily but avoid others, with no clear pattern. For example, Chinese mantids can consume newts containing tetrodotoxin (TTX), excreting it through their digestive systems with no adverse effects (Mebs et al., 2016), but they reject beetles sequestering cucurbitacins (Ferguson and Metcalf, 1985). The same species also consumes cardenolide-sequestering monarch caterpillars (Rafter et al., 2017a), but rejects the cardenolide-sequestering large milkweed bug (Berenbaum and Miliczky, 1984). The California mantid species *Stagmomantis californica* is vulnerable to the aldehyde defences of the giant mesquite bug (Prudic et al., 2008), but the Australian mantid *Hierodula majuscule* readily eats the aldehyde-defended hibiscus harlequin bug (Fabricant and Smith, 2014). These mixed results look at first glance to be the result of using numerous species, but the mixture of toxin tolerance and intolerance in just the Chinese mantid show that the story must be more complex.

Although mantids are have evolved and exhibit behavioural adaptations to avoid poisoning, such as avoidance learning (Gelperin, 1968) and specialist prey handling behaviours like gutting (Rafter et al., 2013), how mantid species can physiologically tolerate some toxins is an open question with little direct evidence. In the case of cardenolides, TTX, and the bitter substance quinine, Mebs. et al. (2016, 2017, 2019) have shown that the toxins pass through the mantid gut unchanged, meaning the digestive system may have evolved structures and barriers to prevent absorption of the poisons, possibly through the secretion of a peritrophic matrix. Also relating to the digestive system, Tinker and Ottesen (2018) examined three mantid species' gut microbiomes and suggested that the variation in bacteria present may be due to their possible role in countering toxic prey. These hypotheses are buoyed by evidence that consumption is key to avoiding toxicity, as when Mebs et al. (2016) injected mantids with TTX they rapidly died. However, if the gut somehow is impermeable to these poisons, it must not be to others, as consumption of other toxins leads to poisoning (Berenbaum and Miliczky, 1984; Prudic et al., 2008).

With this fascinating body of research about mantids, they represent a useful model predator to investigate predation against aposematic prey: they use several sensory modalities, they are large and relatively easy to rear and handle, and they are sensitive to some toxins but not others. Mantids also have a long history as model predators in aversion learning experiments, usually against other invertebrate prey (Berenbaum and Miliczky, 1984; Carle et al., 2018; Rafter et al., 2013). In my choice of *H. membranacea* as a model predator for the large milkweed bug I have followed a path taken by many researchers before me who also saw these benefits, and my experiments will build upon their results.

4.4. Mantids and milkweed bugs

Scientists have long used both the large milkweed bug as well as praying mantids as objects of study, and for more than 50 years, researchers have also been investigating the interactions between these insects. Gelperin (1968) was the first to publish results on the topic, in a short paper detailing how a mantid (*Mantis religiosa*) learnt to avoid milkweed bug nymphs when offered them sequentially. This was somewhat surprising at the time, as research on learning in insects was less common, and the general scientific view was that there was not much evidence that insects could learn at all (Alloway, 1972).

During the 1980s and 1990s a body of research explored the relationship between milkweed bugs and mantids developed. As most studies were from the United States, the mantid species most commonly used was *Tenodera sinensis*, the Chinese mantid, a relatively recent invasive species (circa 1800s) to the United States. In these papers, mantids were shown to rapidly reject *Asclepias*-raised milkweed bugs within a few trials, and in most papers mantids were reported to show direct signs of poisoning like vomiting, mouth wiping, and taste rejecting the chemically defended prey (Berenbaum and Miliczky, 1984; Paradise and Stamp, 1990, 1991a, 1993). These papers vary in the instar of prey used, with Gelperin (1968) using adult mantids but milkweed bug nymphs, and both Berenbaum and Miliczky (1984) and Paradise and Stamp (1991a) using nymphs of both mantids and bugs. Research also varies in what foodplant the milkweed bug nymphs are reared on – many use *A. syriaca*, a milkweed species with a lower concentration of cardenolides than *A. curassavica* (Züst et al., 2019), another species sometimes used as a host plant (Prudic et al., 2007b), and Gelperin (1968) did not report the host species used.

During the 21st century, the interest in milkweed bugs and mantids has continued. Prudic et al. (2007) used adults milkweed bugs reared on *A. curassavica*, and adult Chinese mantids to test how differences in luminance affected mantid predation on bugs. Mantids were assigned into two treatments where milkweed bugs were painted with different luminance contrasts, low (0.19 contrast index) or high (0.57 contrast index). In their experiments, mantids learnt to avoid bugs within ten encounters, and the more luminant bugs were learnt to be avoided more rapidly than the less luminant ones.

This history may seem conclusive, with mantids always learning to avoid the bugs and the bugs always being distasteful. Together with experiments with the monarch butterfly (*Danaus plexippus*), where mantids removed the guts of the monarch caterpillars (Rafter et al. 2013, 2017a, 2017b) this suggests that mantids always avoid cardenolides. However, whether different species of mantids differ in their learning, which is one hypothesis for warning signal variation, is understudied. Furthermore, whether mantids learn differently about their milkweed bug prey when they are raised on different *Asclepias* species and differ in visual signals and chemical defences had not been studied. To my knowledge, no paper has yet directly compared learning in mantids when encountering bugs raised on different *Asclepias* species.

5. The Main Questions Posed in this Thesis

The topic of this thesis covers both the prey and predator sides of the coevolutionary equation, and centres on the topic of variation in prey signals and defence, and predator behaviour in response to this variation. I used my chosen model species of the large milkweed bug and praying mantids, and structured my questions around the resource competition and dietary wariness models as potential mechanisms for prey variation. With this system and framework, I attempted with my experiments to answer the following questions:

5.1. Does the resource competition model explain the variability in both signal and defence in the large milkweed bug *Oncopeltus fasciatus*?

In Chapter 2, I examine whether the resource competition model can explain the known warning signal variation in milkweed bugs. I use the large milkweed bug (*Oncopeltus fasciatus*) to test whether antioxidants that can impart colour trade against their availability to prevent self-damage caused by toxin sequestration. I investigated if (1) the sequestration of cardenolides is associated with costs in the form of oxidative stress; and (2) that oxidative stress can reduce the capacity of individuals to produce warning signals. I raised milkweed bugs on artificial diets with increasing quantities of cardenolides and then examined how this affected signal quality (brightness and colour intensity) and toxicity (sequestered cardenolides) across different life stages. I then related the expression of warning colours to indicators of oxidative state (Blount et al., 2009), – oxidative lipid damage (malondialdehyde), the antioxidant enzyme superoxide dismutase, and total glutathione content, a measure of capacity to handle oxidative stress.

5.2. What is dietary wariness?

In Chapter 3, I briefly summarise the concept of dietary wariness, a term including both neophobia and dietary conservatism. As discussed in Section 3.4, dietary wariness is crucial to the survival of aposematic prey species, as it encapsulates the hesitation of predators to consume conspicuous prey once it is captured (neophobia), and the longer-term reluctance to incorporate novel prey into their diets (dietary conservatism) (Marples and Kelly, 1999). This wariness can increase the rate of survival for aposematic prey, a result shown both theoretically (Lee et al., 2010) and experimentally (Marples et al., 1998; Thomas et al., 2010). This short review was published in 2021 in Current Biology.

5.3. Does the generalist invertebrate predator *Hierodula membranacea* show differences in attack behaviour and learning with milkweed bugs that vary in defence?

In Chapter 4, I measured neophobia, dietary wariness, attack behaviour, and avoidance learning with praying mantids and milkweed bugs that were reared on three diets: sunflower seeds (*Helianthus annus*), *Asclepias incarnata*, and *Asclepias curassavica*. As discussed in Section 3.4 and the short review Chapter 2, both dietary wariness and avoidance learning are key parts of how predators can vary in their responses to prey, and how this can then maintain diversity in prey aposematism. Although all bugs release secretions regardless of diet, milkweed bugs sequester different profiles and amounts of cardenolides when raised on different milkweed host plant species. By raising them on two species of milkweed (*Asclepias* spp.) as well as a control group on sunflower seeds, I created a set of varying prey.

I first measured signs of neophobia and dietary conservatism in mantids facing the nontoxic milkweed bug prey, in terms of how long until first attack, and how long to consume 16 milkweed bugs in total. I then assessed avoidance learning, in measuring how many trials it took for mantids to consume 36 toxic milkweed bugs, and if predation changed over trials, and whether they showed signs of taste rejection. By running the experiment over multiple weeks and months I was able to track the entire progression of predation in behavioural terms, from the first attack to either acceptance or avoidance in the diet.

Although there has been evidence of avoidance learning in praying mantids with milkweed bugs, my experiment is unusual in using *Hierodula membranacea*, and raising the milkweed bugs on different *Asclepias* species as well as sunflower seeds. This chapter continues the long history of research with these model species.

Chapter 2: Connections Between Oxidative Stress, Signal, and Toxicity in the Large Milkweed Bug (*Oncopeltus fasciatus*)

Author contribution statement:

Cecilia Heyworth: experimental design, milkweed bug rearing, artificial diet preparation, image collection, image analysis, oxidative stress analysis, statistical analysis, writing.

Prayan Pokharel: milkweed bug rearing, artificial diet preparation, image collection, cardenolide content analysis, data visualisation, contributed to writing.

Abstract

Aposematism is a form of qualitative honest signalling, as conspicuous warning signals are associated with secondary defences. However, in some aposematic species, the intensity or brightness of their signal correlates either positively or negatively to their toxicity. One proposed hypothesis to explain these patterns is the resource competition model, which suggests a trade-off in which the pigment molecules themselves are the limiting resource. As biological pigments are also antioxidants, aposematic individuals may allocate them either to their coloration (signal quality), or to combat the oxidative stress induced by sequestering or synthesizing toxins.

The large milkweed bug (*Oncopeltus fasciatus*: Heteroptera, Lygaeinae) sequesters high amounts of cardenolides to ward off natural enemies. I raised milkweed bugs on artificial diets with increasing quantities of added cardenolides. In each larval stage until adulthood, I measured their signal quality (brightness and redness) and toxicity (sequestered cardenolides). Additionally, I measured oxidative stress through biochemical assays for lipid peroxidation (malondialdehyde, or MDA), superoxide dismutase (SOD), and total glutathione content (GSH).

I predicted an increase in toxin sequestration to correlate to increased oxidative stress, and my results were mixed. Increased sequestration had no effect on the amount of MDA or SOD but led to a decreased amount of total glutathione (GSH).

I found no direct correlation between signal expression quality (in terms of luminance or redness and toxicity) and measured cardenolide sequestration individuals. However, warning signals were explained by an interaction between antioxidant availability and sequestration. Individuals reared in the diet with the highest concentration of cardenolides that had low levels of total glutathione produced less bright warning signals, whereas individuals with high levels of total glutathione produced increasingly bright warning signals. There was no correlation in the other dietary groups. I also found across all treatments that older individuals were less bright, less red, and proportionally more toxic.

Given these results, I suggest that the most toxic individuals were limited in luminance by their increased oxidative stress, indicating a connection between these three aspects possibly mediated by the resource competition model. I also found that individuals likely have different signalling strategies according to age, potentially connected with their different behaviours, mechanisms of defence, and amounts of cardenolides sequestered.

1. Introduction

Aposematism describes the link between a warning signal and a secondary defence, such as chemicals used as internal poisons or external secretions (Ruxton et al., 2018). Aposematic animals are usually identified from their conspicuous colouration (Cott, 1940), such as the red, yellow, and black colours of organisms like poison dart frogs, wasps and coral snakes (Ruxton et al., 2018). This signalling is described as qualitatively honest, as the colouration or other signals are connected to chemical defences (Summers et al., 2015). Signalling theory predicts uniformity in warning signals, as predators learn more easily to avoid one signal rather than several (Ihalainen et al., 2012), and predator learning depends on the associative strength of deterrent encounters with conspicuous prey (Emlen, 1968; Ihalainen et al., 2007; Luedeman et al., 1981; Skelhorn and Rowe, 2006b, 2006c). However, variation in the warning signals and chemical defences of aposematic taxa is widespread, both within populations and across groups of closely related species (Blount et al., 2012; Davis, 2009b; Grill, 1999; Holloway et al., 1995; Ruxton et al., 2018).

In some cases warning signals and chemical defences are positively correlated indicating a type of quantitative honest signalling (reviewed in Summers et al., 2015; White and Umbers, 2021), for example in adult ladybirds (Bezzerides et al., 2007), ladybird eggs (Winters et al., 2014), paper wasps (Vidal-Cordero et al., 2012), and strawberry poison dart frogs (Maan and Cummings, 2012). Similarly, brightness also correlates positively with defence across several species of opisthobranchs (Cortesi and Cheney, 2010). Negative correlations also exist in natural systems, for example more toxic poison dart frogs can be the least conspicuous (Darst et al., 2006; Wang, 2011), and no correlation was found to support these quantitative honest signalling theories in six-spot burnet moths (Briolat et al., 2018b). The positive correlations are interesting, because warning signals are not commonly considered honest signals (Guilford and Dawkins, 1993), as in sexual selection where such quantitative honest signalling is known (Zahavi, 1975), for example widowbird tails (Pryke and Andersson, 2005). Discussion around these existing correlations has allowed for alternative hypotheses for the positive correlations through automimicry (Svennungsen and Holen, 2007) and go-slow sampling (Guilford, 1994; see summary in Summers et al., 2015). Several theoretical studies have also offered explanations why the most toxic individuals should be least conspicuous, as they can survive predation attempts, and therefore are able to avoid the cost of producing warning signals (Leimar et al., 1986; Ruxton et al., 2009; Speed and Ruxton, 2007). In general, the mechanism or mechanisms underpinning these signal-defence relationships remain unknown and represent a growing area of research.

Handicap signal theory suggests that, in producing warning colours, an animal’s limited resources are depleted, hence resources to cope with toxins also decrease (Zahavi and Zahavi, 1999). This leads to the prediction that colouration and defences must both use some limited, shared resource, but previous theory did not elaborate on an exact candidate. The resource competition model (Blount et al., 2009), provides a potential currency for a trade-off between defence and signal to occur. In the framework of Blount’s model, it is envisaged that toxin sequestration and the production and maintenance of warning signals compete for access to a shared physiological resource - antioxidants. This is because sequestration, modification, and storage of allelochemicals may be oxidatively stressful for chemically defended organisms, and pigments such as carotenoids, flavonoids, melanin, and pteridines that deactivate reactive oxygen species (ROS) may need to be partitioned between warning signals and protection against oxidative stress. Blount's model also assumes that individuals vary in the resources they acquire from their environments, so that those with more resources can afford both to sequester more toxins and produce more pigment. Hence, if toxicity and pigmentation are mutually limiting, those individuals in best condition, with the most resources will be the most toxic and most pigmented. However, in high resource environments, the model can also predict a negative correlation between toxicity and warning signals, as with an abundance of antioxidizing pigments, it would be more beneficial to be toxic and cryptic. This, however, has not yet been examined experimentally.

Here I test this using a model aposematic system. The large milkweed bug, henceforward called milkweed bug, *Oncopeltus fasciatus* (Hemiptera, Lygainae). *O. fasciatus* are conspicuously patterned red and black insects, and feed on the seedpods of their host plant milkweed (*Asclepias spp*.; Burdfield-Steel and Shuker, 2014; Feir, 1974). Milkweed plants produce cardenolides - toxic compounds classed as cardiotoxins - as a defence against herbivory (Brower, 1969; Roeske et al., 1976). However, milkweed bugs 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 (Bramer et al., 2017; Duffey et al., 1978; Scudder and Meredith, 1982). Milkweed bugs vary in the amount and profile of the cardenolides they sequester in natural systems, even when feeding on the same host species (Isman et al., 1977). The intensity of their colouration also varies in the wild (Davis, 2009b; Rodríguez-Clark, 2004). The primary red pigments determining colouration in *O. fasciatus* are pteridines such as xanthopterin, isoxanthopterin, and 2-amino-4-hydroxypteridine, and pterins such as erythropterin (Bartel et al., 1958; Good and Johnson, 1949; Hudson et al., 1959). These pigments have been shown to have antioxidant properties (McGraw, 2005), making the milkweed bug a suitable system to test the resource competition model.

Because directly controlling antioxidant availability is challenging experimentally, I modulated the quantity of diet-derived toxin available to the individual to test whether (1) the quantity of sequestered secondary defence by milkweed bugs is associated with oxidative stress; and (2) whether oxidative stress reduces the capacity of milkweed bugs to produce warning signals displays. I used an artificial diet to modulate dietary toxins while controlling for host plant differences. I raised *O. fasciatus* on diets with an increasing amount of cardenolides and analysed the bugs' colour and oxidative stress levels. I also quantified the amount of cardenolides sequestered as a measure of toxicity. I predicted that independently of treatment group there would be a positive correlation between levels of cardenolides sequestered by milkweed bugs and oxidative stress, and a negative correlation between levels of oxidative stress and colouration. If highly toxic prey reduce investment in signals because they cannot bear the oxidative cost of investing highly in both traits, I predict that individuals with the highest levels of oxidative stress should have the lowest investment in signals, and that this trade-off should be strongest in the treatment exposed to the highest levels of food-plant cardenolides (i.e. a treatment x oxidative stress interaction effect on signalling). If, however, signal reduction in highly toxic prey is solely a response to effects of prey conspicuousness on predator behaviour, I would predict a negative relationship between toxin levels and conspicuousness, but no relationship with oxidative stress. By using a controlled artificial diet, and a model species that naturally varies in both signal and toxicity, in this study I can rigorously test the assumptions of the resource competition model, as well as contribute to the growing literature examining quantitative honest signalling in aposematic species.

2. 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. Milkweed bugs 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. I started my rearing experiment using third larval stage (L3) bugs, as these are large enough to handle. I split my experiments into three batches. In each batch, I divided 60-100 L3 *Oncopeltus* nymphs from a breeding colony into four treatment groups of 15-25 individuals each.

I reared milkweed bugs (N = 192) on four dietary treatments, three with increasing amounts of added ouabain and digitoxin, and one as a control diet with no added toxins. I followed Pokharel et al. 's (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 toxins (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. S3)) 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., 1977). The groups were reared into 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 replenished when dry, usually once per week.

2.2. Photography and colour analysis

I checked the insect boxes daily and monitored their moulting. I took photographs of *O. fasciatus* individuals at the approximate end of each larval stage L4 and L5 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. I 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. S1) 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 bugs in each dietary treatment group at each life stage were randomly selected for photography. I 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). I took three pictures with increasing exposure times (0.2, 0.33, and 0.77) with an aperture of 1.3 x for each filter, i.e. six pictures per insect. After photography, the bugs were placed into labelled Eppendorf tubes, flash-frozen in liquid nitrogen, and stored at -80 °C.

Photographs were analysed using micaToolbox (Troscianko and Stevens, 2015) in ImageJ software 1.51 (NIH, Bethesda, MD, USA). 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 and to covert the camera's RGB values to linearized and device-independent sRGB. Because the bugs reflect negligible amounts of ultraviolet (UV) light I used only photographs in the visible spectrum. In each photograph I delineated consistent indicative regions for one red section on the bugs' wings (Supplementary Fig. S2) and then used the micaToolbox to measure the red, green, and blue and L, A, and B values. I calculated redness of the bugs in sRGB as (R-G)/(R+G), and luminance as reflectance in the red channel.

2.3. Homogenisation of samples

Frozen milkweed bugs were weighed, then placed immediately into 2 mL Eppendorf tubes. Due to their smaller size, L4 nymphs were pooled into groups of two. Bugs were homogenised in a 5% (w/v) of PBS buffer solution (pH 6.6, 50 mM, with 1 mM EDTA) in a FastPrep™ homogenizer (MP Biomedicals, LLC, US) at 10 m/s for 15 s. Tubes were centrifuged at 16,000 x g for 4 min, and the clear homogenate supernatant was transferred to a new 2 mL Eppendorf tube. Three further aliquots were taken from each homogenate and placed into individual Eppendorf tubes. 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 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. Thirds, for HPLC analysis, 100 µL of the homogenate was transferred to a new 1.5 mL Eppendorf tube. The fourth, and last remaining homogenates was used for malondialdehyde (MDA) analysis. All aliquots and homogenates were frozen at -80 °C. All aliquoting was done on ice, with the centrifuge and homogeniser cooled to 4 °C.

2.4. Determination of oxidative stress

Oxidative stress describes the formation or presence of reactive oxygen species in excess of the available antioxidant buffering capacity, thereby damaging proteins, lipids, carbohydrates and so on. I performed three oxidative stress assays from the aliquoted homogenates: total glutathione (GSH), superoxide dismutase (SOD), and malondialdehyde (MDA). These assays were chosen to obtain a broad view of possible biomarkers of oxidative stress. 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 and 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 (Malstrom et al., 1975). MDA is formed by the β-scission of peroxidised polyunsaturated fatty acids, and therefore is a definitive marker of oxidative damage (Lapenna et al., 2001).

2.4.1. Total glutathione content (GSH)

The GSH assay was performed from kits according to the manufacturer’s protocol (Cayman Chemical, Item 703002), diluting the samples 1:2 to fit the absorbance values within the range of the standard curve. Briefly, I deproteinated homogenate samples with metaphosphoric acid as previously described, and 40 samples were aliquoted in duplicate into a 96-well plate, along with glutathione disulfide standards (Item 703014). A mixture composed of cofactor mixture (Item 703016), reconstituted 5,5’-dithio-bis-2- (nitrobenzoic acid) (Item 703012), MES buffer (2-(N-morpholino) ethanesulphonic acid) (Item 703010), reconstituted enzyme mixture (Item 703018), and water was added to each well, and the plate was incubated in the dark on an orbital shaker for 25 min. The plate was then read at 410 nm in a plate reader (Molecular Devices Spectramax M2, Molecular Devices, CA, USA), and I subtracted the absorbance values from a blank plate to standardise values. I calculated the average absorbance of each duplicated sample. I then calculated the corrected absorbance by subtracting the value of the control with no added sample or standard. Any samples that had a negative value after this calculation were disregarded, as indicating kit failure, and the entire plate was re-read. I plotted absorbances of standard values against their known GSH value to obtain a calibration curve for determination of the total GSH for each sample.

2.4.2. Superoxide dismutase (SOD)

SOD assays were performed using kits from Cayman Chemical (Item 706002). I added mannitol to 210 mM and sucrose to 70 mM sucrose per g tissue to the homogenates prior to them being frozen. After diluting the samples (1:50) to ensure that SOD activity fell within the range of the standard curve, I transferred homogenate samples and a dilution range of the SOD standards to a 96-well plate. 200 µL of radical detector was added to all wells, followed by 20 µL xanthine oxidase to initiate the reaction. The plate was covered and incubated for 30 minutes on a plate shaker. The absorbance was read using a plate reader at 450 nm, and the readings from a blank plate were subtracted to standardise. SOD was calculated by first averaging the absorbance for each set of duplicate wells. I then divided the average absorbance of each standard by the absorbance of the control standard, which had no SOD standard present. These linearised rates were plotted as a function of the known SOD activity of each standard. I then used this standard curve to calculate the SOD activity of samples. The unit obtained by this calculation is defined as the amount of enzyme required to catalyse dismutation within half of the superoxide radical, per mg of bug.

2.4.3. Malondialdehyde (MDA)

To measure MDA concentration I used an Agilent HPLC (Agilent Technologies, Santa Clara, CA, USA), using a modified version of Agarwal and Chase's method (2002; Nussey et al., 2009). I transferred a 20 µL aliquot of each homogenised sample into screw-cap Eppendorf tubes and added 20 µL butylated hydroxytoluene, 40 µL 2-thiobarbituric acid (TBA), and 160 µL phosphoric acid (0.4M), vortexed the tubes for 2 s, and heated them at 100°C for one h. Samples were centrifuged for one min at 13,300 x g, cooled for 5 min on ice and 160 µL n-butanol was added to each tube. Samples were vortexed for 10 s, centrifuged for 3 min at 12,000 x g at 4 °C. I took a 100 µL aliquot of the upper butanol phase and transferred them to a HPLC vial. I injected 20 µL of sample into the HPLC setup equipped with a Hypersil™ ODS C18 column (5µm, 100 x 4.6 mm, HSA-212-510R, Fisher Scientific, USA) and used methanol buffer (40:60 v/v) as the mobile phase running isocratically over 3.5 min at a flow rate of 1 mL/min at 37 °C. The buffer was an anhydrous solution of potassium monobasic phosphate (50 mM) at pH 6.8 (adjusted using 5M potassium hydroxide solution). Fluorescence detection was performed at 515 nm (excitation) and 553 nm (emission) (RF2000; Dionex Corporation, USA). A standard curve was generated in a parallel assay, using serial dilutions of 5 µM 1,1,3,3-tetraethoxypropane (which hydrolyses to produce MDA) in 40% ethanol.

2.5. Chemical analysis of sequestered cardenolides in *O. fasciatus* individuals

To analyse the amount of sequestered cardenolides in the sample aliquot, I freeze-dried to remove the water content, and vortexed the sample in 1 ml HPLC-grade methanol containing 0.01 mg/ml of oleandrin (PhytoLab GmbH & Co. KG, Germany) as an internal standard. To extract cardenolides, I 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. I dissolved the remnants 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). I 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 and Zalucki, 1996). Finally, I estimated the amount of cardenolides per sample by comparing the sum of all cardenolide peak areas to the area of the internal standard.

2.6. Statistics

All analyses were conducted using R statistical software, through RStudio software (Version 1.2.1335, RStudio Inc.). I used general linear models (GLMs) through the base R function *lm* to examine the relationships between dietary treatment, signal in terms of luminance and chromaticity, sequestration, and oxidative stress measures SOD, GSH, and MDA. Homogeneity of variances was evaluated by visual inspection of residual plots. In models where there was no reported batch effect, and the inclusion of batch did not improve the model as determined by Akaike Information Criterion (AIC) values, batch was removed as a fixed effect. Results were compared using estimated marginal means in the package emmeans (Lenth, 2020). As running models as mixed linear models with batch as a random effect led to them being overfitted, instead general linear models were run, and batch effects reported when present. Data was visualised using JMP® Pro 15 statistical software (SAS Institute, Cary, NC, US).

3. Results

3.1. Effect of diet on sequestration

I raised milkweed bugs (N = 192) on four dietary treatments, three with increasing amounts of added ouabain and digitoxin, and one as a control diet with no added toxins. All bugs on experimental diets sequestered cardenolides and all on control diets had no toxins (Fig. 1), and the amount sequestered by individuals increased significantly with increasing toxin in the diet (planned contrasts, emmeans; Control vs. Low, $p < 0.001$; Low vs. Medium, $p < 0.001$; Medium vs. High, $p < 0.001$). There was also a batch effect (planned contrasts, emmeans; 1-2, $p = 0.158$, 2-3, $p = 0.026$, 2-3, $p < 0.001$).

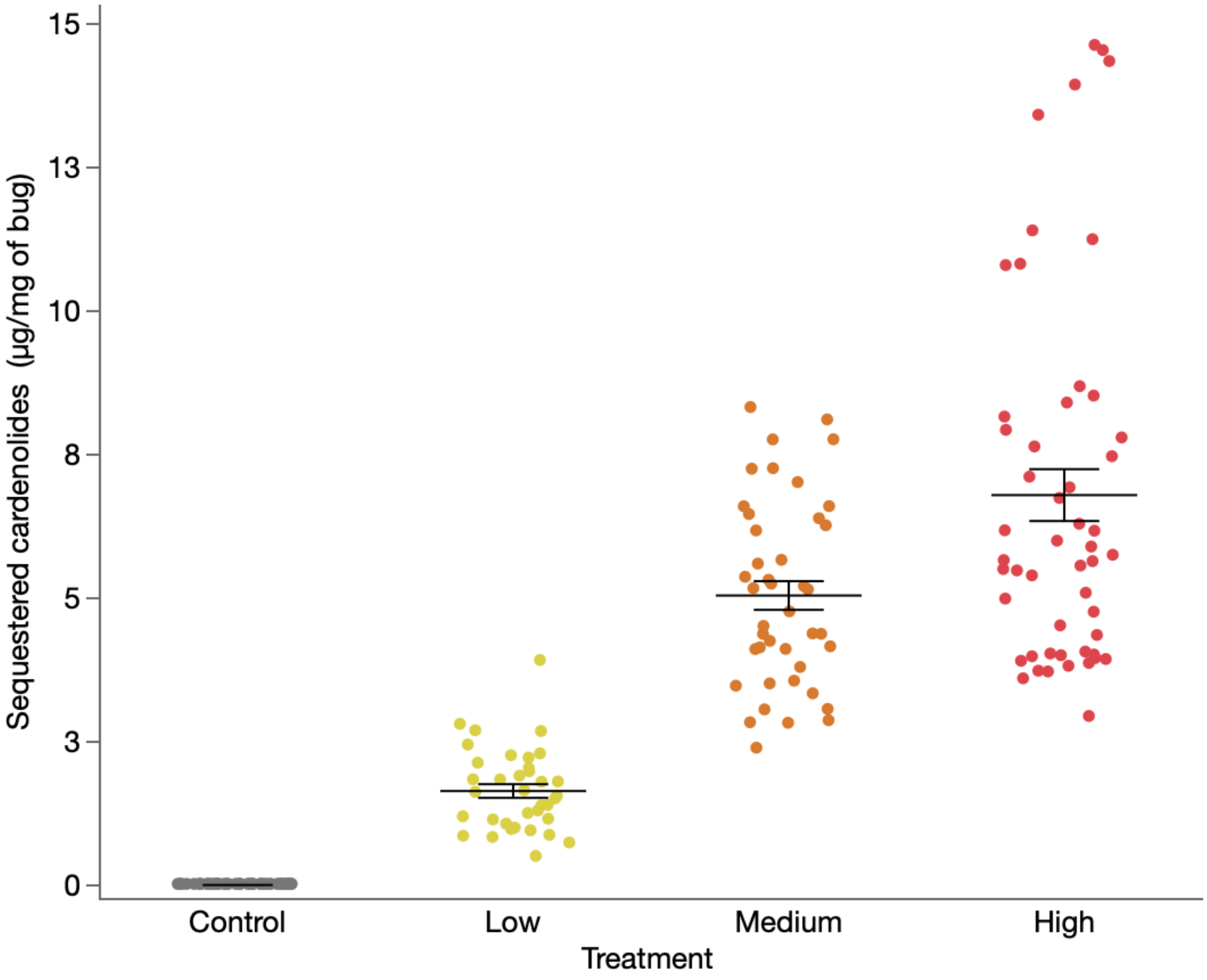

Figure 1. Cardenolides sequestered (μg/mg) by *Oncopeltus fasciatus* individuals when raised on different dietary treatments. Treatments shown are Control (grey), Low (yellow), Medium (orange), and High (red). Control, Low, Medium, and High diets had 0 mg/g, 2 mg/g, 6 mg/g, 10 mg/g equimolar ouabain and digitoxin added respectively.

3.2. Cardenolide sequestration and oxidative stress

I predicted that an increase in sequestered cardenolides would correlate to an increase in oxidative stress, measured through malondialdehyde (MDA), superoxide dismutase (SOD), and total glutathione content (GSH). Bug concentrations of MDA were not significantly affected by treatment (Fig. 2; planned contrasts, emmeans; Control – Low, p = 0.825, Control – Medium, p = 0.784, Control – High, p = 0.948). Although there was no significant difference between MDA concentrations in larval stages L4 and L5 (planned contrasts, emmeans; p = 0.942), MDA levels increased when the bugs reached adulthood, and again when they became older adults (planned contrasts, emmeans; L5-A1, p = 0.001; A1-A3, p < 0.001). There was no correlation between individual toxin level and MDA across individuals (Fig. 3a; estimate = -0.001 +/- 0.003, t = -0.46, p = 0.65).

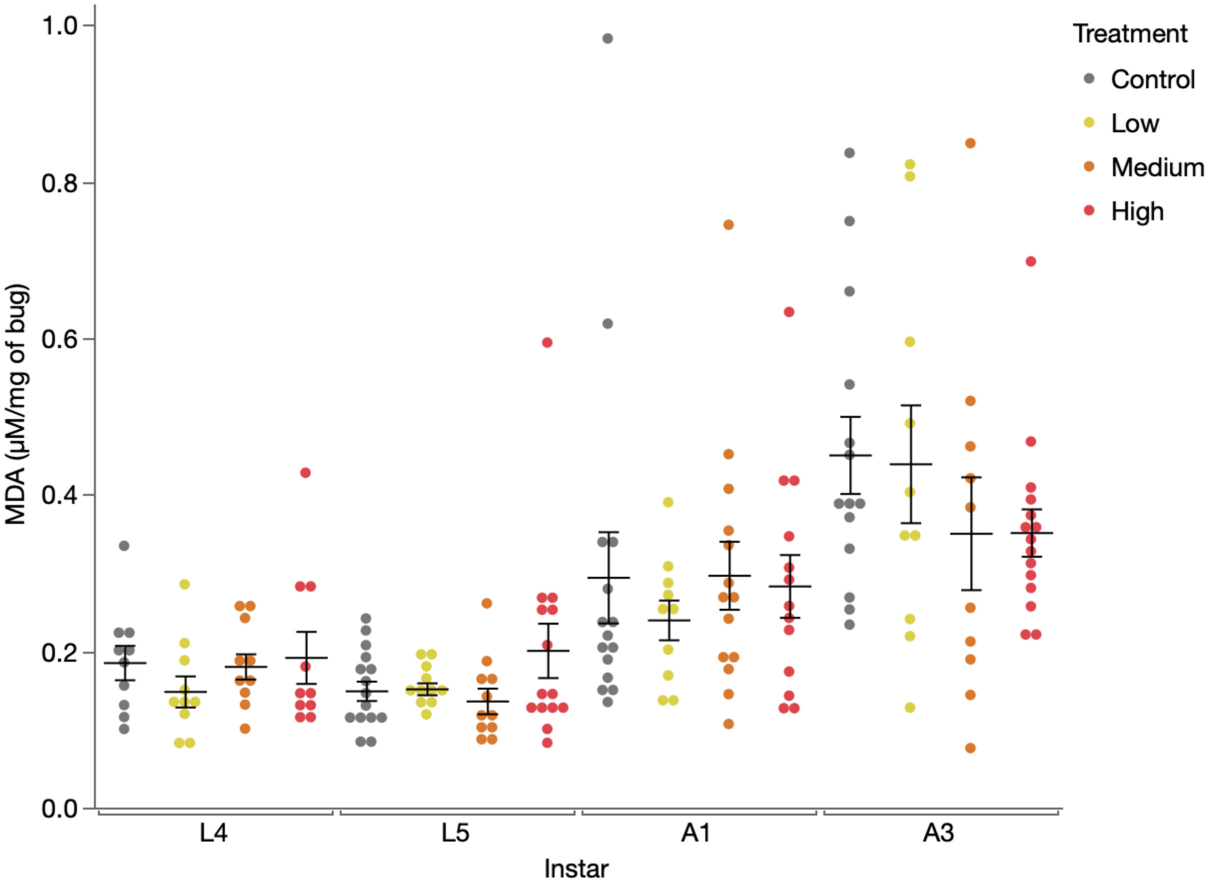

Figure 2. Malondialdehyde (MDA) level (µM/mg bug) in *Oncopeltus fasciatus* individuals when raised on different dietary treatments. Control, Low, Medium, and High diets had 0 mg/g, 2 mg/g, 6 mg/g, 10 mg/g equimolar ouabain and digitoxin added respectively. L4, L5 represent the larval stages 4 and 5. Adult 1 were recently moulted adults, and A3 were adult individuals one week older than this.

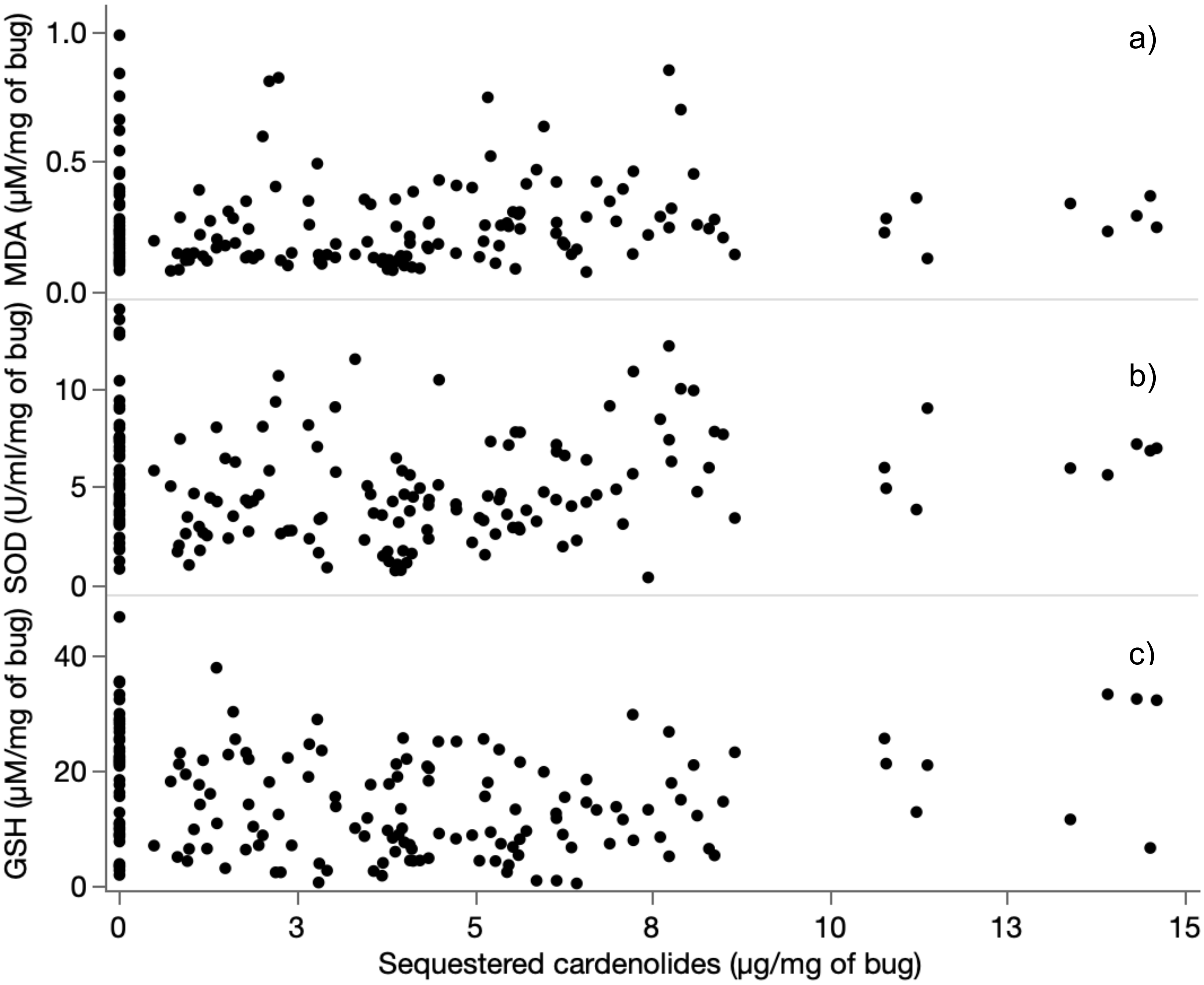

Figure 3. Oxidative state of individual milkweed bugs measured as a) Malondialdehyde (lipid peroxidation), b) super oxidase dismutase (antioxidant enzyme) and c) Glutathione (antioxidant availability) and the individual amount of sequestered cardenolides measured as ug of cardenolides per mg of bugs.

Dietary treatment did not have a significant effect on levels of SOD (Fig. 4; planned contrasts, emmeans; Control - Low, p = 0.927, Control – Medium, p = 0.725, Control – High, p = 0.215). There were life stage differences, with highest levels in L4 and A3, which were not significantly different (Fig. 4; planned contrasts, emmeans; L4 - A3, p = 0.8), and lower amounts in L5 and A1. Although L5 and A1 were not different (planned contrasts, emmeans; L5 - A1, p = 0.567), there were differences between the larval stages (planned contrasts, emmeans; L4 - L5, p < 0.001). There was also no significant effect of individual sequestration on SOD activity (Fig. 3b; estimate = -0.015 +/- 0.058, t = -0.26, p = 0.798).

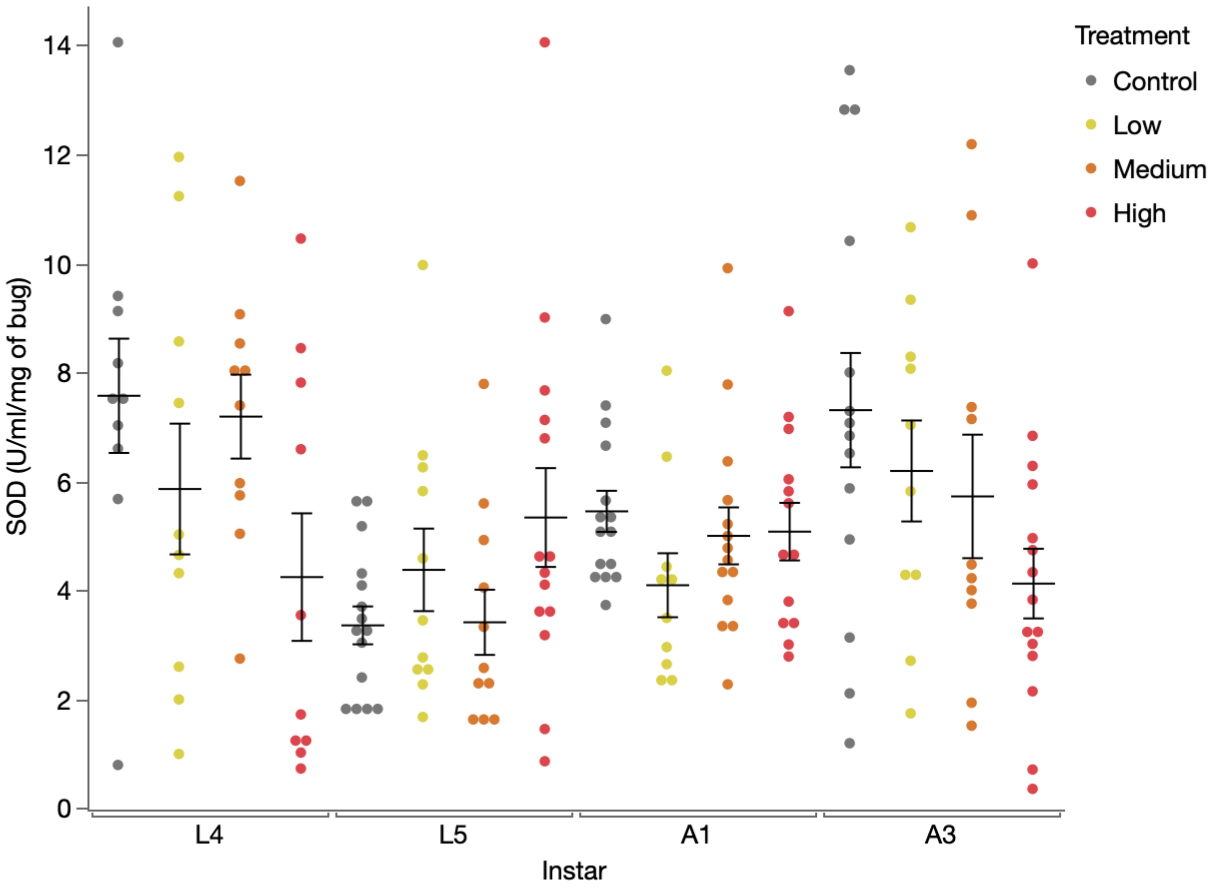

Figure 4. Superoxide dismutase (SOD) level (U/mL per milligram bug) in *Oncopeltus fasciatus* individuals when raised on different dietary treatments. Control, Low, Medium, and High diets had 0 mg/g, 2 mg/g, 6 mg/g, 10 mg/g equimolar ouabain and digitoxin added respectively. L4, L5 represent the larval stages Level 4 and Level 5. Adult 1 were recently moulted adults, and A3 were adult individuals one week older than this.

There was a tendency for an increase in sequestered cardenolides to be associated with a decrease in GSH (Fig. 3c; estimate = -0.3274 +/- 0.1736, t = -1.89, p = 0.061). Dietary treatments had a similar effect, with more toxic dietary treatments Medium and High correlated to lower GSH values than Control (Fig. 5; planned contrasts, emmeans; Control - Low, p = 0.529, Control - Medium, p = 0.046, Control - High, p = 0.004), although there was no difference between Medium and High (planned contrasts, emmeans; Medium - High, p = 0.917). Larval stage comparisons showed no difference between L4 and older adult (A3) bugs (planned contrasts, emmeans; L4-A3, p = 0.532), but decreased GSH in L5 (planned contrasts, emmeans; L4 - L5, p < 0.001) and increased in A1 (planned contrasts, emmeans; L4 - A1, p = 0.016).

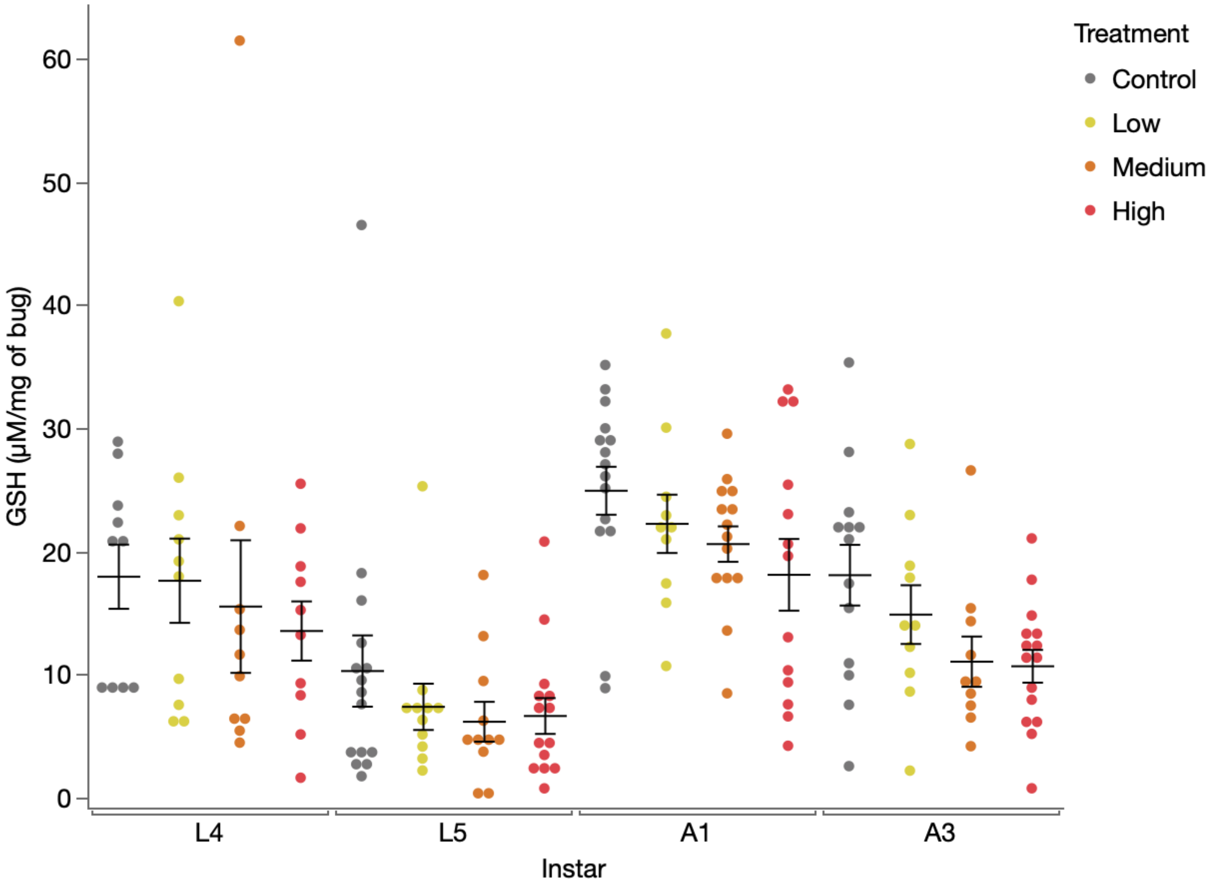

Figure 5. Total glutathione content (GSH) level (µM/mg) in *Oncopeltus fasciatus* individuals when raised on different dietary treatments. Control, Low, Medium, and High diets had 0 mg/g, 2 mg/g, 6 mg/g, 10 mg/g equimolar ouabain and digitoxin added respectively. L4, L5 represent the larval stages Level 4 and Level 5. Adult 1 were recently moulted adults, and A3 were adult individuals one week older than this.

3.3. Oxidative Stress and Signal Quality

With regards to luminance, I focused my analysis on the oxidative stress factor GSH, as the only oxidative stress measure that was determined to be affected by the level of sequestration. Across all treatment groups as GSH increased, luminance decreased, but this was not significant at the alpha 0.05 level (F = 3.82, p = 0.052). I found a significant two-way interaction between treatment and GSH (Fig. 6; estimate = 0.075 +/- 0.032, t = 2.37, p = 0.019). Milkweed bugs were brighter with increasing quantity of GSH, whereas those in the other treatments showed no change in luminance.

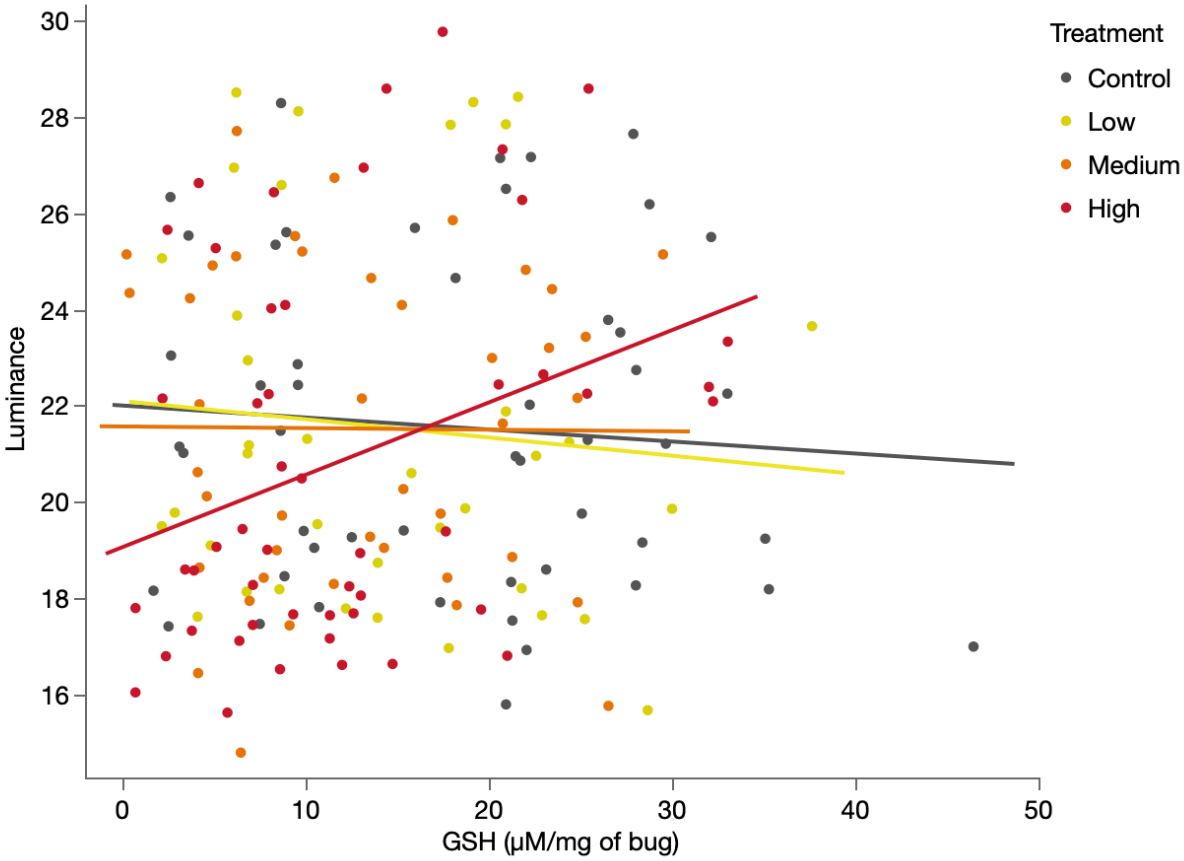

Figure 6. Luminance and capacity to handle stress (GSH) of *O. fasciatus* individuals raised on different dietary treatments. Control, Low, Medium, and High diets had 0 mg/g, 2 mg/g, 6 mg/g, 10 mg/g equimolar ouabain and digitoxin added respectively.

3.4. Sex Differences

Only adult individuals were sexed, and sex differences were examined in sequestration, signal quality, and oxidative stress. Adult male and female bugs did not differ in their rates of sequestration (estimate = 0.29 +/- 0.83, t = 0.36, p = 0.722), in their brightness (estimate = 0.074 +/- 0.37, t = 0.198, p = 0.84), or redness (estimate = 0.002 +/- 0.003, t = 0.67, p = 0.50). However, males and females did differ throughout all oxidative stress assays (Fig. 7). Males had higher levels of MDA (estimate = 0.165 +/- 0.037, t = 4.49, -p < 0.001), and batch 1 had lower levels of MDA than batch 2 (p = 0.004), lower levels than batch 3 (p = 0.042) and there was no difference between batches 2 and 3 (p = 0.367). Males faced higher SOD than females (estimate = 2.27 +/- 0.52, t = 4.43, p < 0.001) and batch 1 had higher levels of sod than batch 2 (p = 0.012), no difference in levels to batch 3 (p = 0.869), and batch 2 had lower levels of SOD than batch 3 (p < 0.001). Males had higher levels of GSH than females (estimate = 5.69 +/- 1.81, t = 3.14, p < 0.001).

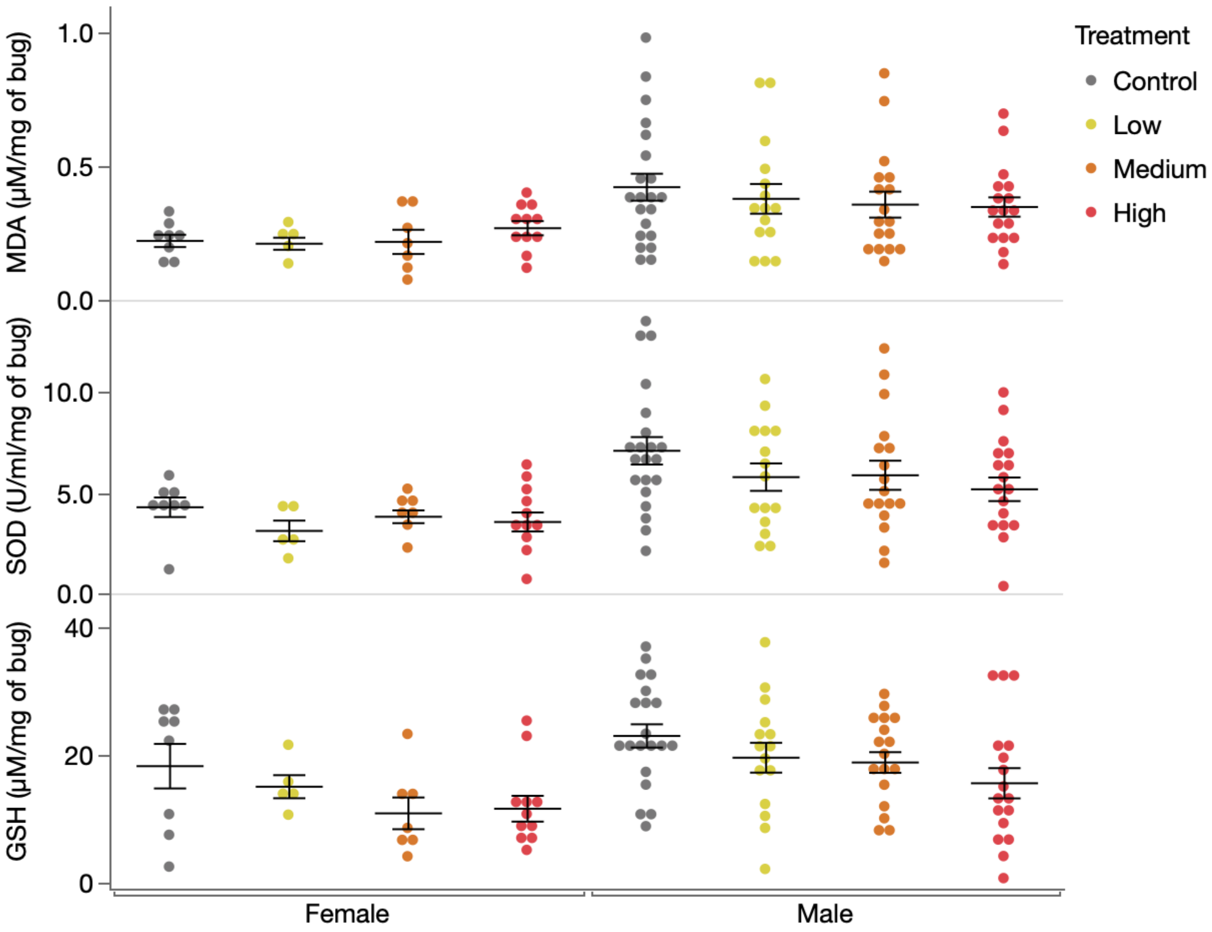

Figure 7. Sex differences in oxidative stress measures across adults for *Oncopeltus fasciatus* individuals. Control, Low, Medium, and High diets had 0 mg/g, 2 mg/g, 6 mg/g, 10 mg/g equimolar ouabain and digitoxin added respectively. Adult 1 were recently moulted adults, and A3 were adult individuals one week older than this.

3.5. Cardenolide sequestration, signal quality, and life stage

The resource competition model predicts a correlation between individuals' toxin level, and signal quality, measured in brightness or intensity of colour, but my data shows no such relationship (Fig. 8 and Fig. 9). Luminance showed no significant association with cardenolides sequestered across all individuals (estimate = -0.02 +/- 0.03, t = -0.657, p = 0.512) or when comparing groups in different dietary treatments (planned contrasts, emmeans; Control - Low, p = 0.969, Control - Medium, p = 0.575, Control – High, p = 0.768). Batch three was significantly brighter than batch 1 (p < 0.001) and batch 2 (p = 0.017), there was no difference in brightness between batch 1 and 2 (p = 0.214).

As with luminance, chromaticity had no relationship with toxicity in terms of how much individuals sequestered (estimate = -1.833e-05 +/- 3.614e-04, t = -0.051, p = 0.96) and there were no differences in redness according to dietary treatment (planned contrasts, emmeans; Control - Low, p = 0.480, Control - Medium, p = 0.724, Control - High, p = 0.799).

The life stage of the individuals did affect colour, with a trend of older larval stages becoming progressively less bright, although there was no difference between L5 and A1 (planned contrasts, emmeans; L4 - L5, p = 0.001, L5 - A1, p = 0.964, A1 - A3, < 0.001) (Fig. 8). Older larval stages were also less red, although with only a trend towards a significant difference between the two sequential measurements of adults (planned contrasts, emmeans; L4 - L5, p < 0.001, L5 - A1, p < 0.001, A1 - A3, p = 0.053) (Fig. 9).

The data also show an increasing toxicity with age, with the oldest adult bugs sequestering more cardenolides per milligram than larval instars (planned contrasts, emmeans; L4 – A3, p = 0.019, L5 – A3, p = 0.014), but with no significant differences between the two larval stages (planned contrasts, emmeans; L4-L5, p = 0.998), or the two adult groups (planned contrasts, emmeans; A1 - A3, p = 0.926) (Fig. 8 and Fig. 9).

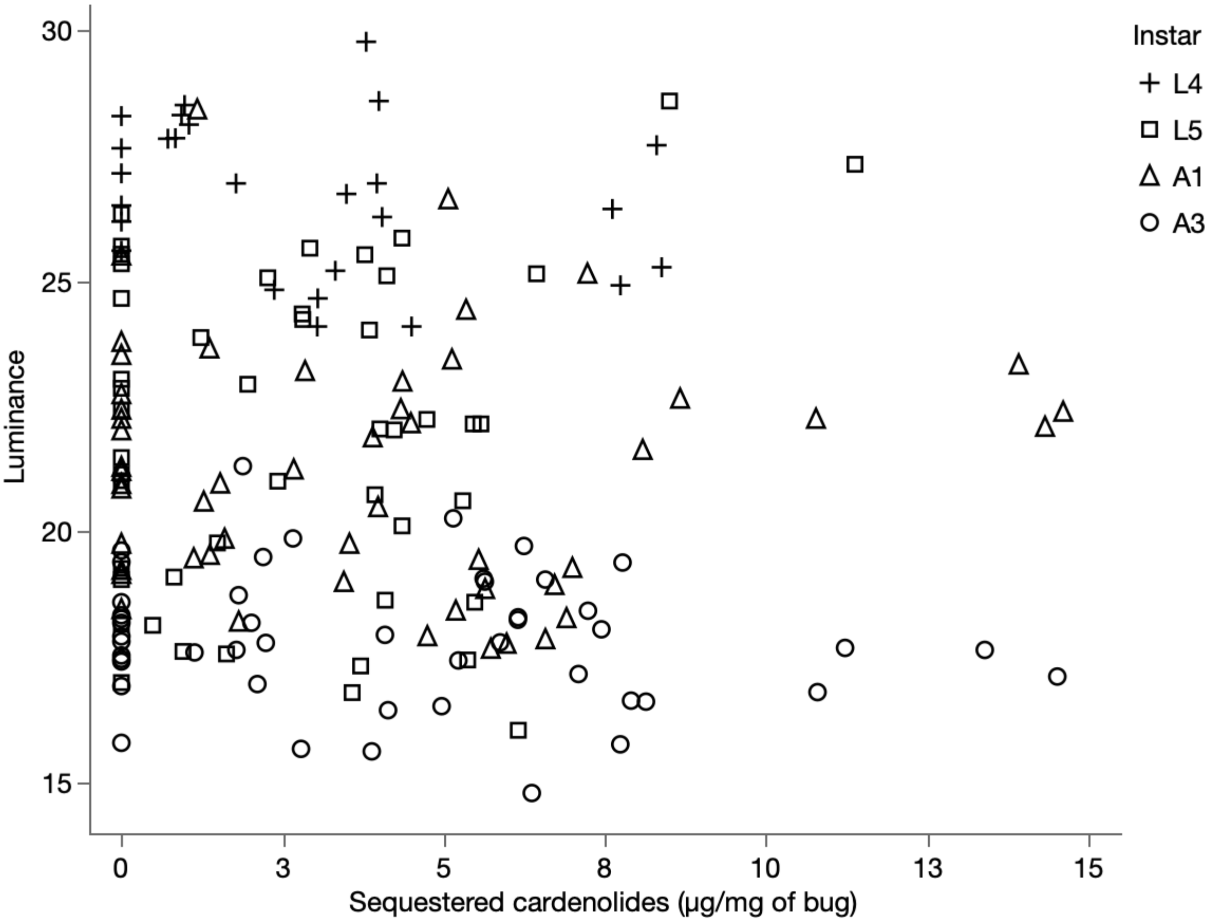

Figure 8. Luminance in *Oncopeltus fasciatus* individuals, according to the amount of cardenolides they sequestered. L4 and L5 represent larval stages Level 4 and Level 5, and A1 and A3 refer to recently moulted adults and adults a week old.

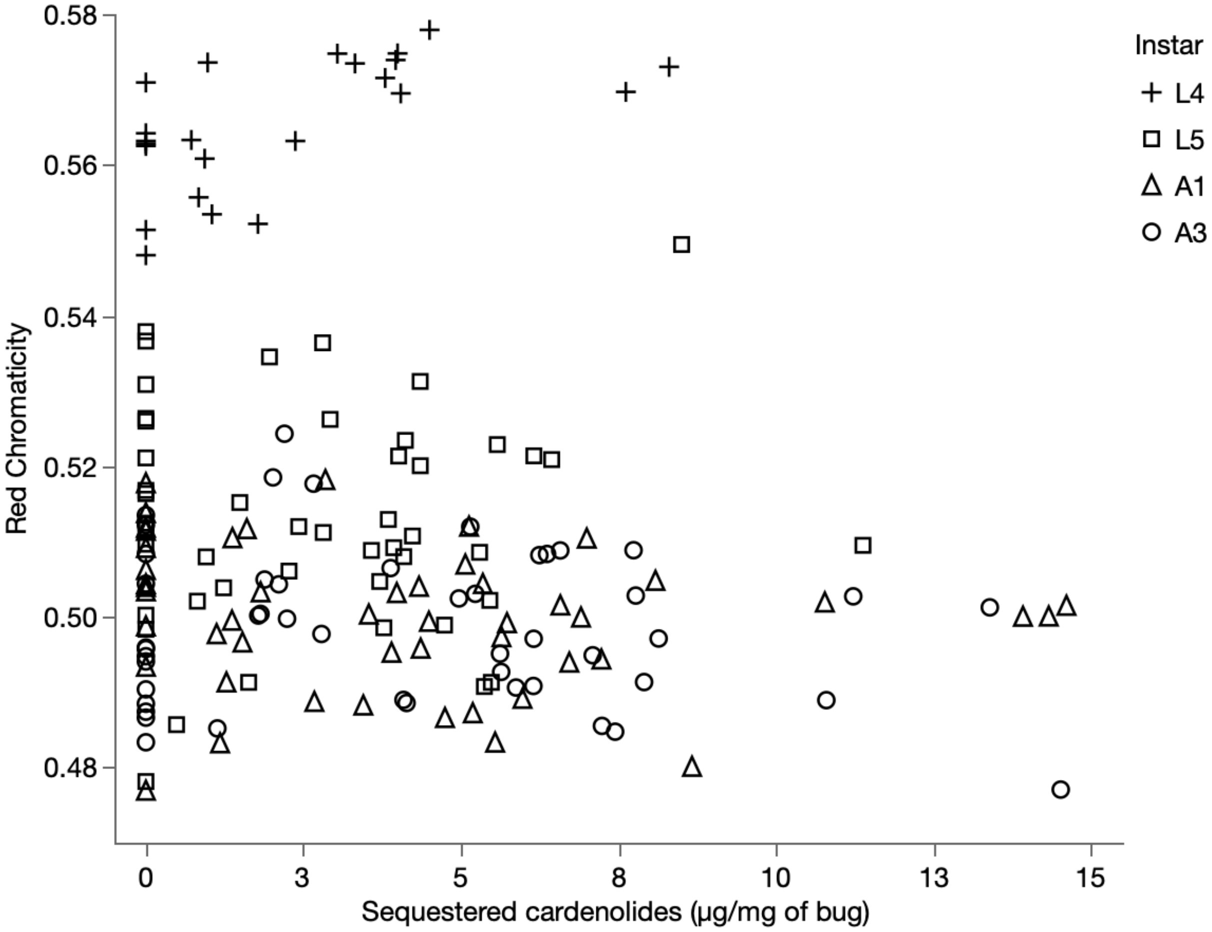

Figure 9. Redness, in terms of chromaticity, in *Oncopeltus fasciatus* individuals, in relation to the amount of cardenolides they sequestered. L4 and L5 represent larval stages Level 4 and Level 5, and A1 and A3 refer to recently moulted adults and adults a week old.

4. Discussion

I reared large milkweed bugs on an artificial diet with increasing concentrations of cardenolides and found that individuals that sequestered higher concentrations of cardenolides experienced decreases in the antioxidant molecule glutathione, measured as total glutathione content (GSH). Although there is some evidence that cardenolides can have a negative effect on milkweed bug fecundity (Pokharel et al., 2021) my results are some of the first to show a physiological cost of sequestration in milkweed bugs, and is similar to the costs that also found in monarch butterflies (Blount et al., in review). I also found that bugs in the high cardenolide treatment showed reduced investment in signal brightness when facing the highest levels of oxidative stress in terms of GSH. This clear connection between stress and brightness mediated through GSH indicates that the individual bugs cannot bear the oxidative cost of investing highly in both colouration and chemical defence. My results are the first experimental evidence of a mechanistic link between oxidative stress, warning signals and chemical defences in large milkweed bugs.

4.1. Dietary treatment and sequestration effects

Large milkweed bugs raised on an artificial diet with increasing amounts of cardenolides sequestered these toxins proportionally to their diet, with bugs feeding on the highest concentration diets sequestering the most cardenolides. Most milkweed bugs in the sub-family Lygaeinae possessing cardenolide-resistant Na/K ATPases sequester cardenolides proportional to the cardenolides present in the diet (Duffey et al., 1978; Pokharel et al., 2021). My data corroborates with the literature suggesting sequestration is a dose-dependent process.

If the resource competition model is relevant for *O. fasciatus*, I would expect greater sequestration to cause an increased oxidative stress burden. In this study, I did not find any increase in damage caused by increased sequestration through lipid peroxidation MDA, or changes in oxidative state measured by the enzyme SOD, but there was a significant decrease in the antioxidant glutathione. GSH is an especially relevant measure of oxidative state for this specialist species feeding on toxic host plants, as glutathione is an antioxidant molecule involved in detoxification processes (Enayati et al., 2005). Additionally, GSH is well-known for detoxifying other phytochemicals such as aristolochic acid (Gao et al., 2021) and sulforaphane (Villa-Cruz et al., 2009). I speculate that the bugs combat the oxidative stress so effectively with GSH that no measure of damage is shown in MDA or SOD.

This relationship between sequestration and GSH indicates that bugs were less able to deal with stress when more toxic, and is evidence of an oxidative cost to sequestration, adding to previous research showing possible metabolic (Dalla et al., 2017) and fecundity costs (Pokharel et al., 2021) in *O. fasciatus*. It is often assumed that sequestration of chemical defences produces physiological costs, suggesting a competition for resources with other functions (Roitberg and Isman, 1992; Ruxton et al., 2018). However, Zvereva and Kozlov (2015) revealed in their meta-analysis that chemical defences of herbivorous insects had no significant costs for herbivores, and it is expected that the costs of *de novo* synthesis of defensive compounds are higher than the costs of sequestration of plant toxins (Fürstenberg-Hägg et al., 2014). My findings suggest that there is a cost of sequestration in *O. fasciatus* in terms of oxidative stress, which would further imply that sequestration impacts the life-history traits (Pokharel et al., 2021).

4.2. Oxidative stress relationship to visual signals

As total glutathione was the only oxidative stress measure affected by sequestration, and therefore potentially relevant in a resource competition framework, I examined further any relationship between GSH and signal quality. Although there was no correlation between GSH and redness (i.e. chromaticity), I found that bugs in the most toxic (i.e. highest cardenolide content) dietary treatment with high levels of GSH produced increasingly bright warning signals.

Other researchers have found similar results in different taxa, for example in a poison frog species, resource-limited froglets (in terms of food) were found to have a negative correlation between luminance and oxidative stress (Flores et al., 2013). As there was an opposite correlation found in froglets with more food, the authors suggested a tradeoff induced by reactive oxygen species (ROS) that constrained the ability of the froglets to produce bright signals in the low food group.

The resource competition model (Blount et al., 2009) suggests a trade-off between the limiting resource – the antioxidant molecules being used for combatting stress - and visual signals. *O. fasciatus* pigmentation is from pterins (Bartel et al., 1958), and is synthesised *de novo*, and their toxicity (i.e. chemical defence) is from diet-derived cardenolides. This makes it challenging to understand the biochemistry underlying their variations in colouration and toxicity, as it is unknown whether these two processes interact or are related. However, my results suggest that there is a tradeoff in individuals producing both bright colouration as a signal and sequestering larger amounts of cardenolides, and that this tradeoff is mediated by oxidative stress in terms of total glutathione content. Those bugs with less resources may not be able to invest in pigments if they sequester more cardenolides, whereas those with abundant antioxidants can afford to invest in both signal and toxicity. In which case, the correlations that I see in nature may reflect the oxidative capacity of a species.

As I only found this result in the higher toxicity dietary treatment and did not find overall correlations between signal quality and sequestration among all dietary groups, this suggests that the tradeoff is only apparent when bugs are pushed to sequester large amounts of cardenolides. Future experiments could repeat or expand upon this finding by raising *O. fasciatus* individuals on a higher range of cardenolide dietary treatments, where this tradeoff may also lead to broader correlations across all groups.

4.3. Sex differences

I found both life stage differences in oxidative stress - SOD was highest in L4 and A3 insects, over all treatment groups. GSH was lowest in L5 and A3, and potentially this decreased capacity for stress could relate to either the imaginal moult for L5 and ageing in older adults. In contrast, MDA was lowest in the first and second larval stage and increased from the third larval stage to adults irrespective of the treatment group, indicating a life stage effect on lipid damage. MDA increasing with age has been found in other taxa, e.g. houseflies (Sohal et al., 1981) and rats (Yoshikawa and Hirai, 1967), and a relationship has previously been found between aging and MDA in *O. fasciatus* (McArthur and Sohal, 1982). Although I found no effect on MDA from increased sequestration, milkweed bugs can react to other toxins in this way: exposure to titanium dioxide and aluminium oxide nanoparticles through tarsal contact increased lipid peroxidation in *O. fasciatus* (López-Muñoz et al., 2019).

I also found sex differences in adults from the oxidative stress assays; males faced higher SOD and MDA, and lower GSH content than females, indicating a higher oxidative burden overall. Intraspecific competition could lead to this increase in oxidative stress faced by males, as they often physically fight over females (personal observation, Pokharel and Heyworth).

The extent that sexual selection has a role in *O. fasciatus* colouration remains unclear, but as I found no sex differences in brightness or redness, it seems unlikely to have a large influence. I also found no differences in sequestration between male and female adults.

4.4. Life stage differences in sequestration and signal quality

O. fasciatus individuals were brighter and redder as younger larval stages, and duller and less red as they aged; they also sequestered proportionally more as they aged, especially into adulthood. These trends could reflect older bugs being on the toxic diets for longer, and to fully clarify this I would need to place the life stages on the dietary treatments for the same amount of time. However, as there was no sequestration difference between younger and older adults it is unlikely that the relationship between time and sequestered cardenolides is linear. Instead, the most likely explanation for these trends appears to be different anti-predator strategies.

Milkweed bugs feed on seeds or seedpods that have fallen on the ground, exposing themselves to vertebrate predators such as birds (Aldrich, 1988), and nymphs tend to cluster around seed pods, while adults tend to disperse (Sauer and Feir, 1973). There are also documented aggregation pheromones found to be released by *O. fasciatus* nymphs (Aller and Caldwell, 1979). I hypothesise that nymphs, being less toxic and smaller than adults, may aggregate to enhance their total toxicity, and to advertise their unpalatability more effectively. Aggregation has been correlated to aposematism across species (Cott, 1940; Ruxton et al., 2018; Tullberg et al., 2000). Although a study reported that there was no link between chemical defence when raised on different *Asclepias* species and aggregation across four different lygaeid bugs (*Spilostethus pandurus*, *Lygaeus equestris*, *Lygaeus simulans* and *O. fasciatus*) (Burdfield-Steel et al., 2013), it is possible that this association could emerge ontogenetically within this species. The experimental literature supports this hypothesis, as Ralph (1976) showed that clustering significantly improved the survival of *O. fasciatus* nymphs in natural conditions. In another related species, domestic chicks (*Gallus domesticus*) also attacked an individual *L. equestris* larva more than when exposed to a group of nymphs (Tullberg et al., 2000).

Nymphs also have different methods of chemical defence than adult *O. fasciatus*. In larval stages L1-L5, bugs release a defensive secretion from two dorsal glands (Games and Staddon, 1973), whereas adults possess a specialised vacuolated cell layer where they store their cardenolide-rich secretion, which is released via thin areas of cuticle that break under pressure (Bramer et al., 2017; Scudder and Meredith, 1982). These differing strategies in behaviour and chemical defence deployment suggest that the anti-predator strategies of *O. fasciatus* nymphs and adults may target different predators. My findings that life stages also differ in toxin sequestration and signal quality support this, as predator communities vary in which signals and defences they consider when attacking. This has already been found in other Lygaeinae, as cardenolide sequestration by *L. equestris* (from *Adonis vernalis* seeds) protects against insectivorous birds but not against predatory lacewing nymphs (Petschenka et al., 2020; Pokharel et al., 2020).

4.5. Conclusion

Variation in colour and toxicity in aposematic species is found throughout nature (Briolat et al., 2018a; Speed et al., 2012), and the existence of such variation within species has presented an evolutionary puzzle for some time: if predators learn to avoid aposematic prey through uniformity of signals, how does such a variety of signal quality and chemical defence persist? There are many possible solutions, and likely there are multiple underlying factors dependent upon the ecology of the species, mechanism of sequestration, and predator community. With positive and negative correlations being found in numerous species across a wide range of taxa, Blount's (Blount et al., 2009) resource competition model is one possible framework. My experiment found evidence for an oxidative burden caused by sequestration in *O. fasciatus* in terms of GSH, as well as a tradeoff between signal brightness and stress in the most toxic bugs. There are also interesting life history variations in signal and defence. Together, these results suggest oxidative stress as a possible mediator in the relationship between signal quality and sequestration in *O. fasciatus*.

5. Supplementary Figures

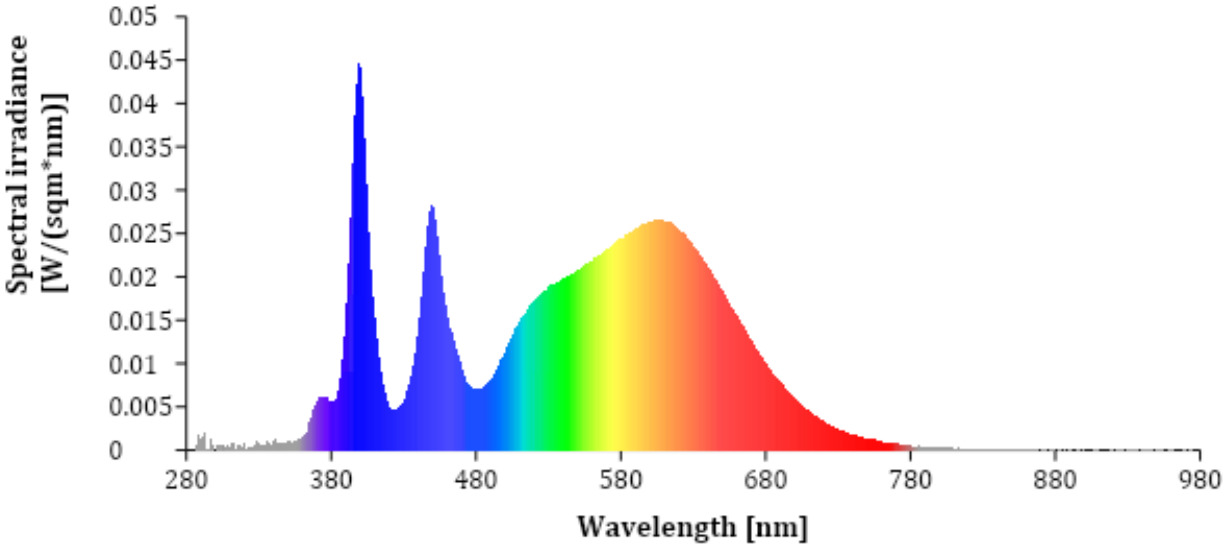

Figure S1. Reflectance spectra generated from the filter apparatus LEDs.

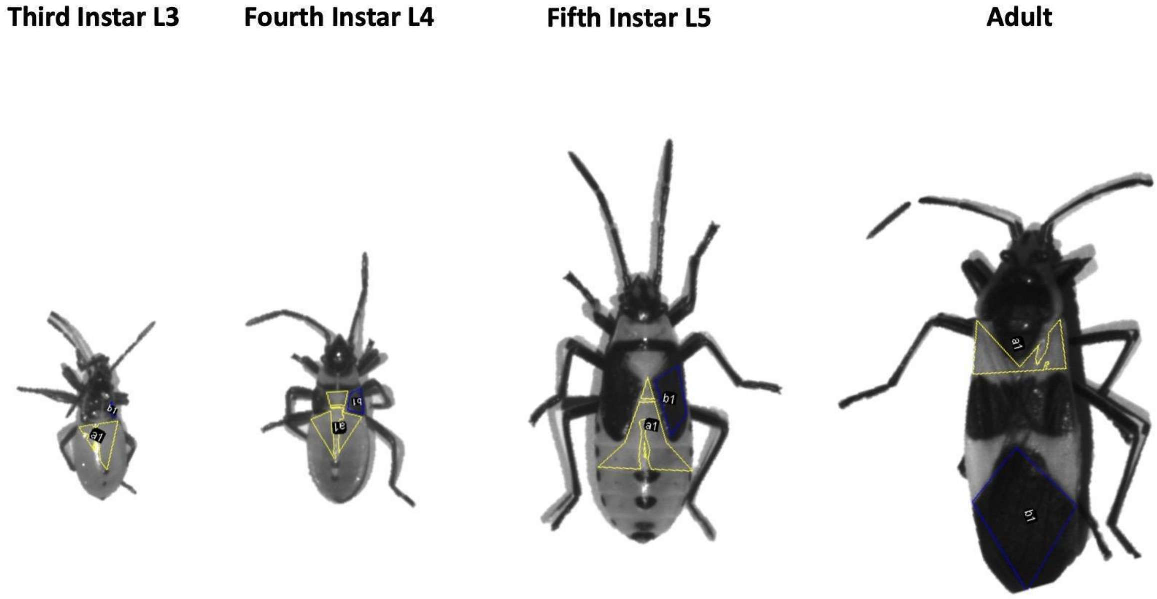

Figure S2. Regions of Interest selected in the different larval stages of *Oncopeltus fasciatus*. Yellow areas were the red selections used in analyses.

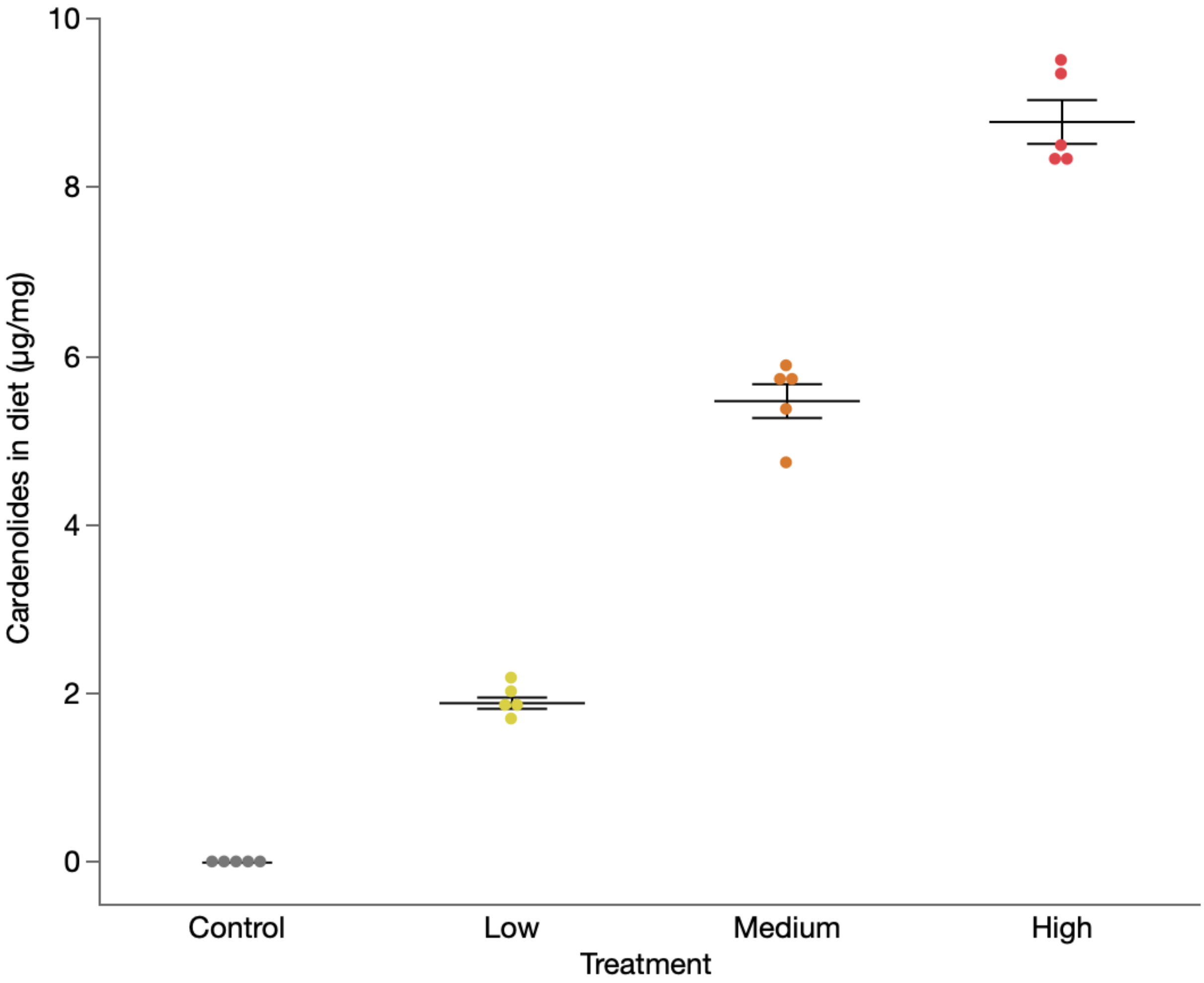

Figure S3. Amount of cardenolides in the artificial diet. Control, Low, Medium, and High diets had 0 mg/g, 2 mg/g, 6 mg/g, 10 mg/g equimolar ouabain and digitoxin added respectively.

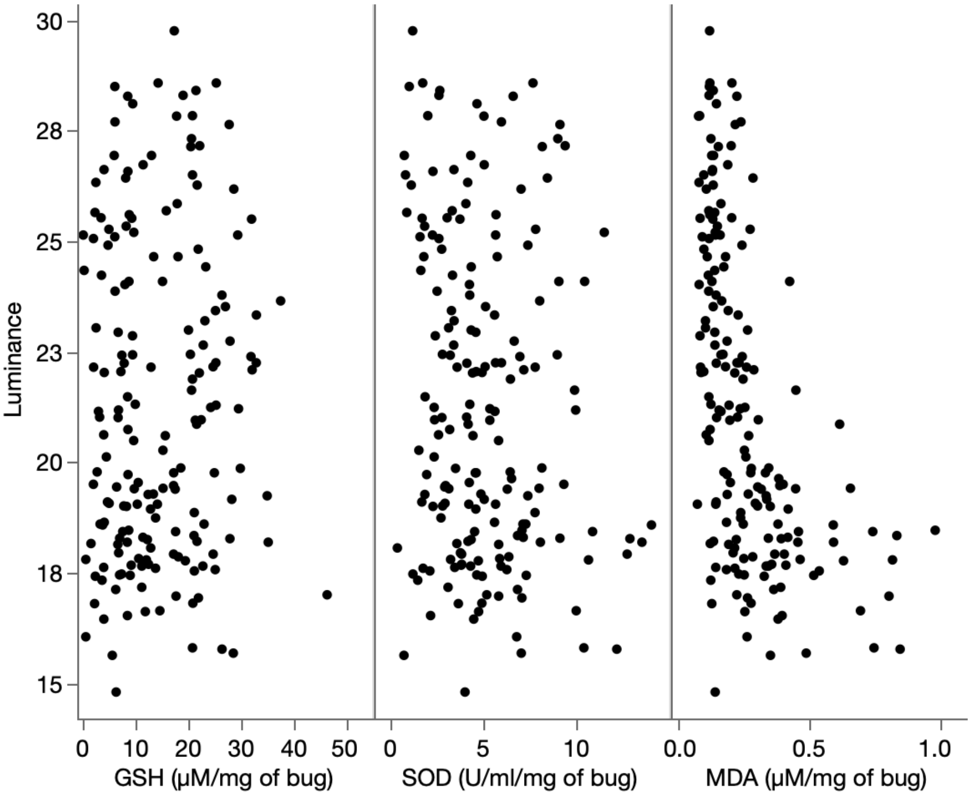

Figure S4. Oxidative stress measures with increased luminance in *O. fasciatus*.

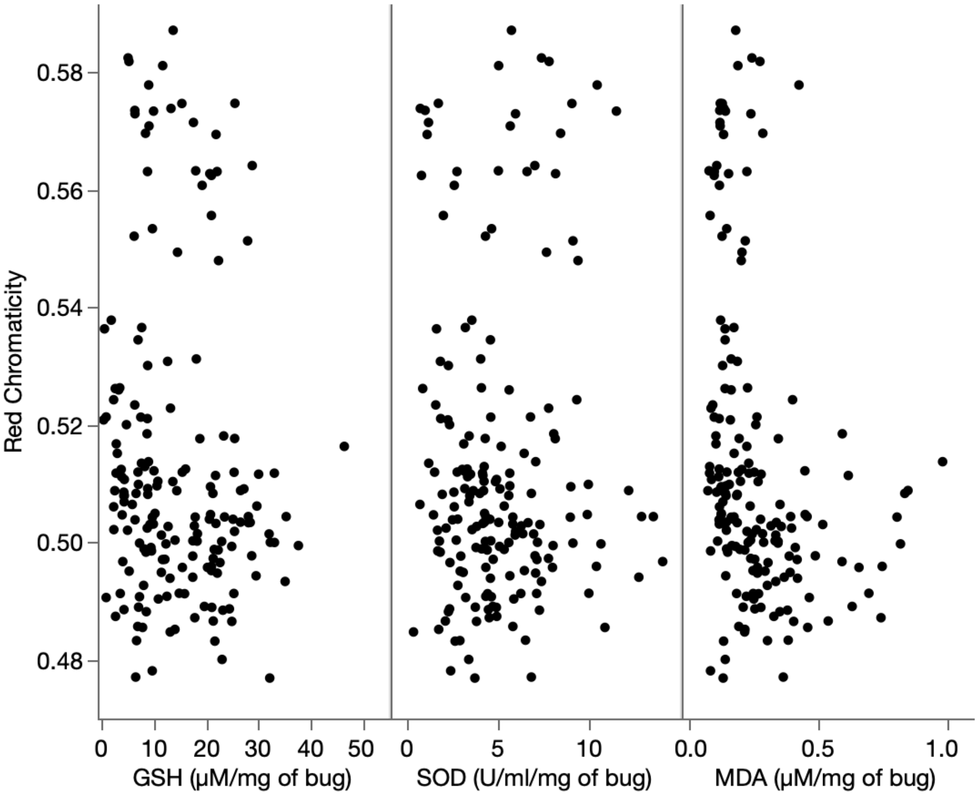

Figure S5. Oxidative stress measures with increased chromaticity in *O. fasciatus*.

Chapter 3: Dietary Wariness, Food Neophobia and Dietary Conservatism

H. Cecilia Heyworth, John Skelhorn, Hannah M Rowland

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Abstract

Dietary wariness, encompassing neophobia and dietary conservatism, is the observed phenomenon of animals exhibiting hesitation and reluctance towards novel food, even when palatable. This wariness has been shown to be prevalent across many taxa, and has been proposed as a potential mechanism for the evolution of novel morphs in conspicuous species, and of the initial evolution of aposematism itself. In this short review paper, I discuss the definitions of neophobia and dietary conservatism, including examples from predator-prey systems in laboratories. I also examine the factors determining levels of wariness, the proposed reasons behind the evolution of dietary wariness in animals, and the possible future research avenues for the field, with a list of recommended further reading. Dietary wariness is an important field in theoretical ecology, as it may shed light on the early evolution of aposematism and help to explain the emergence and maintenance of novel conspicuous morphs in a species. Wariness is also practically relevant, as manipulating wariness levels in domestic livestock and animals in captivity could have implications in agriculture and conservation work.

What is dietary wariness?

The term ‘dietary wariness’ describes animals’ reluctance to sample novel foods. It encompasses two processes: neophobia, a short-term refusal to touch the new food; and dietary conservatism, a longer-term hesitancy to fully incorporate this food into the diet. These are considered to be discrete processes because dietary conservatism is a complex multi-stage process that is resistant to deactivation and is often present in only a sub-set of individuals in a population. This is not thought to be true of food neophobia. However, neophobia may be more complex than is currently appreciated (see Figure 1), and there is little direct evidence that it is easier to overcome than dietary conservatism (see below). Food neophobia is near universal and has been documented in insects, fish, birds and mammals, but is absent in a small number of species. It has a genetic component in humans and birds, but the genes that influence it are poorly understood. Dietary conservatism is a more recently described phenomenon that has been observed in several species of bird and fish, but this list is likely to expand as research continues.

Why should animals reject perfectly good food?

Animals face a dilemma when encountering novel food: lacking knowledge of the food's palatability or toxicity, should they invest time exploring the novel food and take the risk that it could be toxic, or disregard it in favour of familiar foods of known value? Models of this exploration-exploitation trade-off suggest that wariness of novel food is the optimal strategy under a range of ecological conditions. They predict that animals should be wary when novel food is rare, and the cost of sampling toxic food is high. The benefits of sampling novel food outweigh the costs only when novel food is found at high densities, and under these circumstances animals should expand their diets to include novel food. These predictions are supported by findings that dietary wariness can be overcome by repeated exposure to novel food, which enhances the food's perceived density. However, there is no empirical evidence that dietary wariness enhances an individual's fitness, and mathematical models are yet to directly explore why levels of wariness vary so much among individuals.

What factors affect the expression of dietary wariness?

Food density is not the only factor that can influence the expression of dietary wariness. Any factor that decreases the cost or increases the benefit of sampling novel food should reduce dietary wariness, whilst factors that do the reverse should enhance it. For example, wariness is reduced when competition for food is high, or when familiar individuals are observed eating the novel food. It is enhanced when novel food is perceived to be high risk: when it is warningly coloured or causes conspecifics to perform distaste responses. An individual's physiological and energetic state (hunger levels, fat stores, toxin burden, current diet quality, and previous experience with food) are also likely to alter the costs and benefits of sampling novel food, which could explain why the expression of dietary wariness varies considerably among individuals. It has been suggested that neophobia is deactivated more easily than dietary conservatism, and that factors that affect one of these processes may not necessarily affect the other. There is some support for this latter hypothesis: in blue tits, the presence of a conspecific reduces dietary conservatism, but not neophobia. However, this is not the case in domestic chicks, and the results of other experiments testing these ideas are difficult to interpret as they measure wariness rather than dietary conservatism.

Why does any of this matter?

Determining how animals respond to novel food allows us to understand their diet breadths, and why they may choose imbalanced diets associated with adverse health and development outcomes. It helps us to predict how animals might adapt to both natural and anthropogenic environmental changes in food availability, and has important implications for signal evolution. Vertebrate predators that express dietary wariness avoid novel prey, which can allow mutations that cause warning signals to spread through populations of defended species. Understanding what factors influence the expression of dietary wariness will also allow us to manipulate its expression for welfare, commercial and conservation purposes. In agriculture, decreasing dietary wariness in hens could eliminate the reduced growth rates and outbreaks of injurious feather pecking associated with the diet changes common in commercial farming. It could also allow translocated animals of conservation concern to better integrate into novel environments that do not contain food that poses a significant health risk. When releasing animals into riskier environments, increasing dietary conservatism, if teamed with training on ‘safe’ food, could be used to reinstate natural levels of wariness that may have been lost in captivity. As we increasingly need to find sustainable food sources, the ability to overcome western societal wariness to alternative protein sources like insects will be crucial to ensuring future food security.

Where do we go from here?

Many questions remain about dietary wariness. Are neophobia and dietary conservatism distinct processes, and how should they be measured? How widespread are these traits and how are they affected by genetics, ontogeny and experience? What are the fitness benefits to being adventurous and wary, and how do these differ among individuals and species? Is dietary wariness part of a general anxiety trait, and can it be manipulated without changing other behaviours? Dietary wariness promises to be a fruitful area of research that will have important practical applications.

Where can I find out more?

Dovey, T.M., Staples, P.A., Gibson, E.L., and Halford, J.C.G. (2008). Food neophobia and ‘picky/fussy’ eating in children: A review. Appetite 50, 181–193.

Knaapila, A., Tuorila, H., Silventoinen, K., Keskitalo, K., Kallela, M., Wessman, M., Peltonen, L., Cherkas, L.F., Spector, T.D., and Perola, M. (2007). Food neophobia shows heritable variation in humans. Physiology & Behavior 91, 573–578.

Marples, N.M., and Kelly, D.J. (1999). Neophobia and Dietary Conservatism: Two Distinct Processes? Evolutionary Ecology 13, 641–653.

McMahon, K., and Marples, N. (2017). Reduced dietary conservatism in a wild bird in the presence of intraspecific competition. Journal of Avian Biology 48, 448–454.

Richards, E.L., Alexander, L.G., Snellgrove, D., Thomas, R.J., Marples, N.M., and Cable, J. (2014). Variation in the expression of dietary conservatism within and between fish species. Animal Behaviour 88, 49–56.

Sherratt, T.N. (2011). The Optimal Sampling Strategy for Unfamiliar Prey. Evolution 65, 2014–2025.

Figure 1

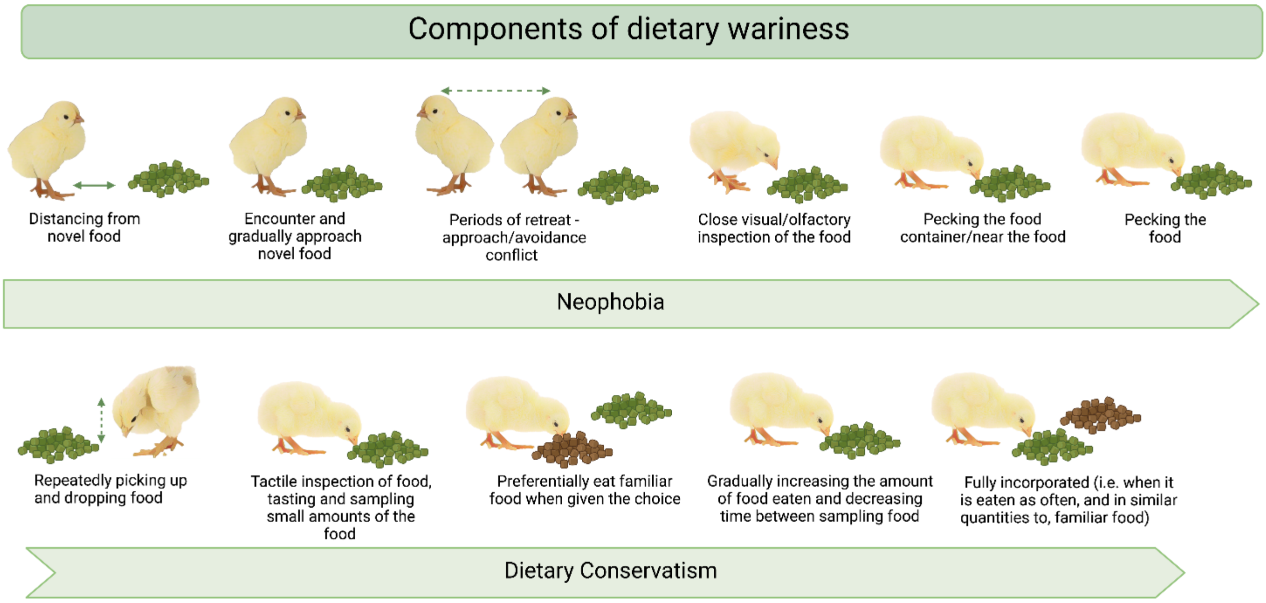

Figure 1. Top: the stages involved in overcoming neophobia. When faced with novel food, chicks initially distance themselves from it (left). As neophobia wanes, chicks move progressively closer to the food until they eventually touch it (right). At this point neophobia has been overcome. Bottom: the stages involved in overcoming dietary conservatism. Chicks begin by handling food carefully (left), slowly incorporate it into their diet, and eventually eat it at similar levels to familiar food (right). At this point dietary conservatism has been overcome. (Figure created using BioRender.com.)

Chapter 4: Praying Mantises Do Not Learn to Avoid the Large Milkweed Bug (*Oncopeltus fasciatus*) but May Suffer Long-Term Toxic Effects.

Abstract

Variation in aposematic prey is widespread, both in terms of signal and defence, but predator response to this variation is less studied. Milkweed bugs, which can be raised on different host plants (genus *Asclepias*), and the invertebrate generalist predator the praying mantid (*Hierodula membranacea*) provide an ideal model system to test predator responses. Previous research has shown mantids taste rejecting milkweed bugs when raised on *Asclepias*, and learning to avoid them within a few trials, but none have yet tested differing predation responses of mantids against milkweed bugs raised on different *Asclepias* species. I reared large milkweed bugs on two *Asclepias* species (*incarnata* and *curassavica*) that vary in cardenolide concentration, as well as a nontoxic control diet of sunflower seeds. Milkweed bugs varied visually when raised on different host plants, potentially showing honest signalling in the intensity of their pigmentation. In predation trials, mantids showed some neophobia, and females showed a range of tendencies toward dietary wariness, but none learnt to avoid the *Asclepias*-raised milkweed bugs. Despite this lack of avoidance learning, high mortality rates throughout the experiment implied that mantids exhibited chronic poisoning from cardenolide consumption. Our findings showed an unexpected lack of avoidance learning, differing from all previous mantid-milkweed bug literature, and suggest that future research should include signs of longer-term chronic cardenolide toxicity as well as acute reactions in learning experiments.

1. Introduction

Over millennia, prey species have evolved defences against their predators, both physical, like hairs (Lindstedt et al., 2008) and spines (Speed and Ruxton, 2005), and internal, in the form of toxins (Blum, 2012; Eisner and Meinwald, 1966; Nishida, 1994). Some animals with toxins advertise these defences with warning signals, an evolutionary strategy called aposematism (Ruxton et al., 2018; Sherratt, 2002). Aposematism provides advantages to the prey species through accelerating avoidance learning and enhancing memory in predators (Endler, 1988; Halpin et al., 2008), and it is thought that more conspicuous, uniform visual signals are most effective in this (Stevens and Ruxton, 2012). However, not all aposematic species have strong signals, with many varying in their signal quality and expression even within a population (reviewed in Briolat et al., 2018a). As suggested by the resource competition model, this variation can correlate to how defended a prey is, a relationship underpinned by the oxidative stress caused by development and maintenance of toxins (Blount et al., 2009). Although these correlations can be explained by resource allocation trade-offs, to support the model fully, more evidence is needed of whether predators recognise and differentiate between variable signals, and if this discrimination leads to changes in predation.

There is already significant research on predator responses to aposematic prey, especially with avian predators. Birds are more likely to handle aposematic prey more carefully than cryptic prey (Gamberale-Stille and Guilford, 2004; Wiklund and Järvi, 1982), and can taste-reject individuals based on their level of chemical defence to avoid poisoning (Halpin and Rowe, 2010; Skelhorn and Rowe, 2006a, 2006d). Predation by invertebrates, and specifically insects, is often overlooked in experiments in comparison, but evidence suggests that invertebrate predation can have a wide and powerful top-down effect on prey species (Snyder and Evans, 2006).

Oncopeltus fasciatus is an orange/red and black aposematic insect, with toxic cardenolides sequestered from their host plants, *Asclepias* spp. (Feir, 1974). With both naturally varying toxicity (Isman, 1977; Isman et al., 1977) and colouration (Davis, 2009a), as well as the ability to raise them on several different host species with differing cardenolide profiles and amounts (Züst et al., 2019), *O. fasciatus* present as an ideal species in which to measure predator responses to variation in diet. The natural predators of *O. fasciatus* are not often studied, but evidence from fieldwork has suggested invertebrate predators are common, including spiders, lacewing larvae, Nabidae family insects, katydids, assassin bugs, and other milkweed bugs (Sauer and Feir, 1972). Given this, it is unsurprising that invertebrate predators have often been used in predation experiments with *O. fasciatus*, including spiders (Bramer et al., 2018; Hill, 2006) and, for decades, praying mantises (Berenbaum and Miliczky, 1984; Gelperin, 1968; Prudic et al., 2007b).

Praying mantids (*Hierodula membranacea*) are relatively commonly used as model predators for aposematism experiments, and they are not deterred by some antipredator defences, e.g. the hibiscus harlequin bug (Fabricant and Smith, 2014) and newts containing the neurotoxin TTX (Mebs et al., 2016), but are deterred by others, for example cucurbitans in the swallowtail butterfly (Chow and Tsai, 1989). Mantids are useful predators because they have the ability to smell (Maxwell et al., 2010; Prete et al., 1992) and taste (Carle et al., 2015; Tuffnell and Rowe, 2011), but hunt primarily through visual cues (Yamawaki, 2017), making them a good predator to test the effects of aposematism. Their vision differs from birds, a common group used in predation assays, but like birds they can use prey brightness when learning and remembering about aposematic prey (Prudic et al., 2007b). I found differences in brightness in milkweed bugs in Chapter 2, so testing whether these visual differences have a fitness effect is warranted.

Research using praying mantids and milkweed bugs as a system started from more than 50 years ago, when Gelperin (1968) was the first to repeatedly feed *O. fasciatus* individuals to adult mantids, finding that the mantids learnt to avoid the bugs within a few trials. Further papers in the 1980s and 1990s confirmed these results and expanded upon them. Berenbaum and Miliczky (1984) were the first to use sunflower seed-raised milkweed bugs as a nontoxic control group, and their results also found that mantids learnt to avoid milkweed bugs within 10 trials. Paradise and Stamp (1990) used ground milkweed bugs in a prepared diet for younger mantids (third instar), finding that the more toxic diets acted to deter feeding. These same authors found similar levels of learning in mantids facing milkweed bugs, and also measured bug mortality, finding that bugs facing experienced mantids were more likely to survive (Paradise and Stamp, 1991a). Paradise and Stamp (1993) also looked at whether feeding nymphal mantids would affect their growth rate, finding that individuals eating more unpalatable bugs took longer to moult. Bowdish and Bultman (1993) were the first to manipulate the prey visual cues, finding that milkweed bugs painted with both orange and black were more likely to enhance avoidance learning than bugs painted a solid colour. This trend was continued into the 21st century, with Prudic et al. (2007b) painting milkweed bugs with grey paint of differing luminance, and finding that mantid avoidance learning was faster and remembered for longer when they faced the brightest bugs.

These papers vary hugely in their approaches and available technology, and even in the species of mantid and life stages. Most researchers have used *Tenodera sinensis* (the Chinese mantid), a common invasive species in North America (Rathet and Hurd, 1983), but the life stages of the mantid varies from nymphs of different instars (Berenbaum and Miliczky, 1984; Paradise and Stamp, 1991b, 1993) to adult (Prudic et al., 2007b). The milkweed bugs are also often raised on different host plant species, primarily *A. syriaca* (Bowdish and Bultman, 1993; Paradise and Stamp, 1990, 1993) or *A. curassavica (Prudic et al., 2007b)*. Milkweed bugs vary in their sequestration when raised on different host plants (Isman, 1977; Moore and Scudder, 1985) and are subjectively described as differing in colour, but this has never been formally quantified.

Some species of mantids appear to be poisoned after consuming milkweed bugs, and show stereotyped behaviours like mouth wiping, grooming, vomiting, and throwing the prey away (Berenbaum and Miliczky, 1984; Gelperin, 1968; Prudic et al., 2007b). In contrast, praying mantises can apparently consume both larvae and adults of the monarch butterfly, which also sequester cardenolides, with no adverse effects (Rafter et al., 2013), and mantids that consume more toxic monarchs actually gained more weight and laid heavier eggs than those that consumed less (Rafter et al., 2017a). This leaves the open question of what the underlying reason is for mantid distaste and avoidance learning of milkweed bugs.

Research on insect learning is an expanding area of interest, and exploring the effects of different host plants on the visual signals of prey and the responses of predators is important for understanding ecological interactions in nature. Although this has not yet been tested in *O. fasciatus*, Similar work has been done with other milkweed bug species. Pokharel et al. (2020), raised two species of milkweed bug (*Lygaeus equestris* and *Horvathiolus superbus*) on two different host plants (*Adonis vernalis* and *Digitalis purpurea*), and found that only *D. purpurea* raised bugs were protected against lacewing predation, despite them varying in the amount of cardenolides they sequestered. The authors concluded that these differences must be due to structural differences in the cardenolides leading to varying effects on predators. I propose that the varying chemical profiles of different *Asclepias* species could have similar effects on mantid predation.

In this experiment, I investigate how variability in cardenolide sequestration affects the survival of large milkweed bugs (*O. fasciatus*) when presented to praying mantids. By raising bugs for several generations on either sunflower seeds, *A. incarnata*, or *A. curassavica*, and then analysing their colour and mass differences, I quantified visual differences, and then performed predation assays to examine predator responses to different aposematic traits. Monarch butterflies sequester a lower quantity of cardenolides when raised on *A. incarnata* as compared to *A. curassavica* (Jones et al., 2019) so I predicted that mantids would learn to avoid bugs raised on *A. curassavica* faster than those raised on *A. incarnata*. This experiment is the first with the aim to record the differences in predator behaviour to *O. fasciatus* with regards to the differing phytochemistry of *Asclepias* species.

2. Methods

2.1. Insect species and rearing

All insect species were maintained in incubators on a 16:8 H day:night cycle, with the temperature set to 28 C during the day and 18 C at night, at 60% humidity.

Large milkweed bugs (*O. fasciatus*) were obtained from a long-term laboratory colony (originally from the United States) maintained on sunflower seeds. Milkweed bugs are a convenient model prey for these studies, as they are apparently distasteful when raised on milkweed (*Asclepias* spp., their natural host plants), but they can also be raised on nontoxic sunflower seeds (Helianthus annuus), which do not have cardenolide content (Isman, 1977). Milkweed bugs were reared in 30 x 20 x 20 cm plastic breeding cages, their lids covered with paper towels. Bugs were provided *ad libitum* with seeds, either *Helianthus annuus*, *Asclepias curassavica*, or *Asclepias incarnata* (all seeds from Jelitto®), and with water, supplied in 2 mL Eppendorf tubes sealed with dental cotton. Two generations were raised on the same diet before adults were used for photographing or predation trials.

Hierodula membranacea L4 nymphs were purchased from Mantiden & Mehr in September 2020, and further subadults were obtained from the same seller in March 2021. Nymphs were raised in 11 x 8 x 10.5 cm plastic cages, with a stick for resting on, and provided greenbottle flies for food twice weekly. When adults, mantids were kept in 20 x 20 x 20 cm plastic containers with mesh lids, with a stick for resting. Mantids were sprayed with water two to three times a week and fed an average of one fly a day prior to experiments. 15 male and 15 female mantids were used in the predation trials.

2.2. Photography and colour analysis

In April 2021, 14 adult bugs of each dietary treatment were selected for colour analysis. I photographed each individual insect using a Nikon D7000 digital SLR camera (Nikon, Tokyo, Japan) and a UV-Nikkor 105mm f/4.5s. I had fitted the lens with a ring light fixture that illuminated the bugs with LEDs emitting light with a wavelength between 380-780 nm. I used a Baader UV-IR blocking filter (Baader Planetarium, Mammendorf, Germany; permitting only visible spectrum light from 420 to 680 nm) to block UV, as previous experiments had shown that the bugs' elytra have no UV reflectance (see Chapter 2).

I sedated individual insects using CO_2 and photographed them with their elytra facing upwards, as they lay on a colour palette (ColorChecker Passport Photo 2, X-rite, Pantone©, Michigan, USA) alongside a 40% Spectralon® grey standard (Labsphere Inc., North Sutton, NH, USA). These colour standards allow the user to convert the camera's RGB values to linearized and device-independent sRGB. I took three pictures with increasing exposure times (0.2, 0.33, and 0.77) with an aperture of 1.3 x for each filter, i.e. three pictures per insect. I selected the picture with no overexposure for later analysis, based on visual inspection of the camera histograms.

I analysed the photographs in ImageJ software 1.51 (NIH, Bethesda, MD, USA) using micaToolbox (Troscianko and Stevens, 2015). For each photograph, the image was calibrated against the 40% standard, and consistent indicative regions were chosen for one red section on the bugs' wings. I marked this region in every picture, and measured reflectance in the red channel, which I refer to as luminance. I calculated chromaticity, or the redness value, by dividing the reflectance values as so: (R-G)/(R+G). As the natural predators of milkweed bugs remain largely unknown (see Pokharel et al., 2020) I chose not to model the reflectance measures by any predator visual system, but used instead an objective analysis of colour.

2.3. Predation Trials

2.3.1. Experimental cages and prey presentation

H. membranacea adults started trials roughly one week after their final moult. Before the trial, the mantids were moved from their regular cages to a modified experimental cage, the same width and depth, but with a height of 10 cm, to ensure the prey were encountered frequently and at short distances. At the beginning of each trial, a milkweed bug was weighed to three decimal places (a thousandth of a gram) and placed into the experimental cage with tweezers. After 30 minutes, any bug remains were weighed, and any live bugs removed. As pilot experiments had shown that male mantids lost motivation to hunt after four prey presentations over two days, mantids ran four trials over two hours. If the mantid did not consume all four bugs, four more trials were run the following day, so they had a maximum of eight trials per week in which to consume four bugs. The one to two days of trials were run once a week, and the mantids were given 3 flies outside of trials, ensuring they were hungry and motivated to hunt. Trials were filmed using Yi Home 1080p CCTV cameras, set up roughly 15cm away from the boxes, so that they could be observed during the trials without disturbance.

2.3.2 Dietary wariness trials

Predation trials were run from April to August 2021. I screened each mantid for its level of neophobia and dietary wariness. Dietary wariness has no exactly defined or universally accepted criteria and varies according to researchers; for example, it has been measured in experiments as the consumption of novel-coloured food on three successive trials (Marples et al., 1998), the time to eat three novel food items (Marples et al., 2007), or more than five consecutive pecks at novel food (Camín et al., 2016). The methods for testing dietary wariness also vary depending on the species assayed (Marples and Kelly, 1999). In my experiment I followed previous researchers in their categorisations. I defined the duration of neophobia as the latency to strike the novel prey, following Marples and Kelly (1999), here the first nontoxic milkweed bug. Marples et al. (2007) defined dietary conservatism as the time an individual takes to consistently eat novel food, so in my experiment I defined dietary conservatism as the time (in number of trials) to eat 16 bugs. The overall duration of dietary wariness therefore incorporates both the duration of neophobic avoidance and the duration of avoidance due to dietary conservatism (Marples et al., 2007).

Mantids were placed in the experimental arena and presented with one non-toxic milkweed bugs (i.e. raised on sunflower seeds) using the trial setup described above. Each screening session lasted 30 minutes. If the mantid did not consume the bug it was removed from the cage and presented with a new bug in the next trial, to ensure that they were not avoiding the bugs simply because they had not noticed it. As all male mantids died during or shortly after the dietary wariness trials, only female mantids moved to the next stage, learning.

2.3.3. Learning trials

After the neophobia and dietary wariness assay, the 15 female mantids participated in the learning trials. Mantids coming from the dietary wariness trials were randomly assigned one of the three dietary treatments. Trials were run asynchronously, and when a mantid in one dietary group died, it was replaced with a new mantid coming from the dietary wariness trials. The control group (N = 5) continued to be presented with sunflower seed-raised milkweed bugs. The second were presented with bugs raised on *Asclepias curassavica* (N= 7), and the third were given *Asclepias incarnata*-raised bugs (N = 3). The milkweed bugs used in the mantid predation trials were massed before being released into the trial arena. 8 individuals died during the experiment: 2 in the sunflower seed bug group, and 6 in the *A. curassavica*-bug group. Mantids were presented with four milkweed bugs per day, twice a week for nine to ten weeks, until they had struck 36 milkweed bugs. Mantids were observed during all trials through cameras, and any unusual behaviours recorded.

2.4. Data analysis and statistics

All analyses were conducted using R statistical software, through RStudio software (Version 1.2.1335, RStudio Inc.). I used the packages tidyverse (Version 1.3.1), broom (Version 0.7.9) and dplyr (Version 1.0.7) to organise and categorise the datasets, and both base functions in R and emmeans (Version 1.4.5) to analyse the data. All distributions were evaluated for normality by visual inspection of the q-q plots, residuals, and by the Shapiro-Wilk W test. The data for milkweed bug luminance was log transformed because of positive skew. Bug mass was not normally distributed, and the data did not fit the assumptions of parametric models, so were analysed with a Kruskall-Wallis test and a Dunn's Kruskal-Wallis post-hoc test.

In the dietary wariness section, all 15 female mantids consumed the required number of bugs and completed trials, but only 4 of the 15 male mantids survived to complete the trials. For the wariness analysis and visualisation, only mantids that completed the trials are included. The data for the number of trials that mantids took to attack the first novel milkweed bug, my measure of neophobia, and the number of trials to attack 16 milkweed bugs, my measure of dietary conservatism, was not normally distributed and was analysed with Wilcoxon's rank sum tests. To test for a correlation between neophobia and dietary wariness I used a Pearson's correlation test.

In the learning trials, only female mantids were included in the analysis, because the majority of male mantids died during the dietary wariness trials. To test for learning I used a mixed effects logistic regression in the R package lme4 (Bates et al., 2015) with the big diet and trial as fixed effects, and mantid ID as a random effect to control for the repeated experiments on the same individual. I calculated the proportion of bug that was consumed per trail by dividing the mass remaining by the mass of the bug presented. I analysed this using a linear mixed model in the R package lmer (Kuznetsova et al., 2017) with diet of the bug and trial as fixed effects and mantid ID as a random effect. Of the 15 female mantids who started the learning trials, 7 consumed all 36 milkweed bugs. As there was only one mantid who consumed the requisite number of *A.*

curassavica-raised bugs, that datapoint was excluded from analysis of bug mortality. I calculated mortality by dividing 36 bugs killed by the number of trials that it took an individual mantid to consume the 36 bugs. I analysed this Results in parametric tests were compared using estimated marginal means in the package emmeans (Lenth, 2020). Data were visualised using the R package ggplot2 (Version 3.3.5) and ggpubr (Version 0.2.5).

Of the 15 female mantids that started the learning experiments eight died during the experiment (Table 1). To test whether the diet of the bugs they were consuming was related to this mortality I analysed survival using the R package survival (Therneau et al., 2021) with the independent variable bug diet, and the time to death in weeks as the dependent variable.

3. Results

3.1. Effect of diet on milkweed bug luminance and colour

When raised on different diets, bugs differed in their visual appearance. Sunflower-raised bugs were the brightest and *A. incarnata*-raised bugs the least bright (Fig. 1). The luminance of bugs raised on *A. curassavica* was very variable, and there was no significant difference between these bugs and the other dietary treatments (*A. curassavica* – *A. incarnata*, estimate = 0.137 +/- 0.07, t = 1.95, p = 0.1374, *A. curassavica* – Sunflower, estimate = -0.122 +/- 0.07, t = -1.74, p = 0.2036). Sunflower-raised bugs were significantly brighter than *A. incarnata*-raised bugs (estimate = -0.259 +/- 0.07, t = -3.69, p = 0.0019)

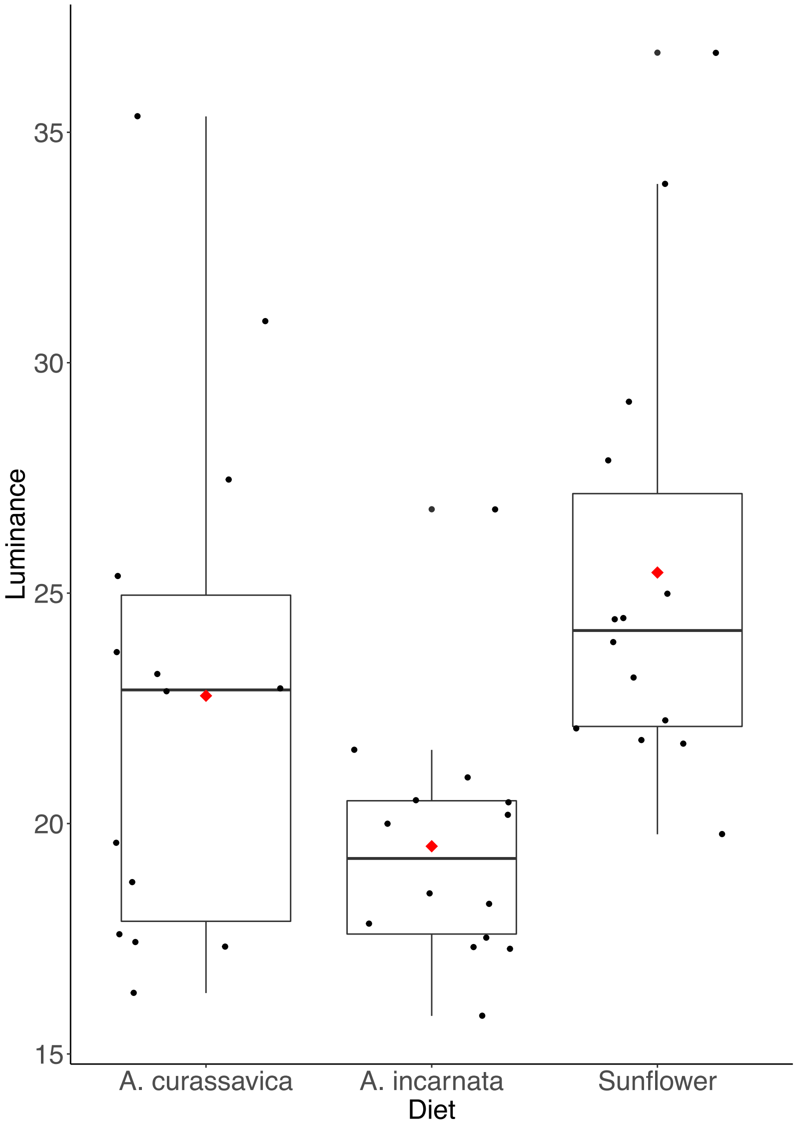

Figure 1. Luminance differences in *O. fasciatus* individuals when raised on different species of host plant (n = 14 for each diet). Horizontal bars represent the median values and the red diamonds represent the means. The upper bound of box shows the third quartile and lower bound shows first quartile of data, the upper whisker shows the largest value no further than the interquartile range multiplied by 1.5, and the lower whisker extends to the smallest value at most the interquartile range multiplied by 1.5.

In terms of redness, *A. curassavica*-raised individuals were the reddest, significantly more so than *A. incarnata* or sunflower seed-raised individuals (Fig. 2; *A. curassavica - A. incarnata*, estimate = 0.038 +/- 0.009, t = 4.107, p = 0.0006; *A. curassavica* – Sunflower, estimate = -0.0573 +/- 0.009, t = -6.15, p < 0.0001). There was no difference in redness between *A. incarnata* and sunflower-raised individuals (*A. incarnata* – Sunflower, estimate = -0.019 +/- 0.009, t = -2.045, p = 0.1150).

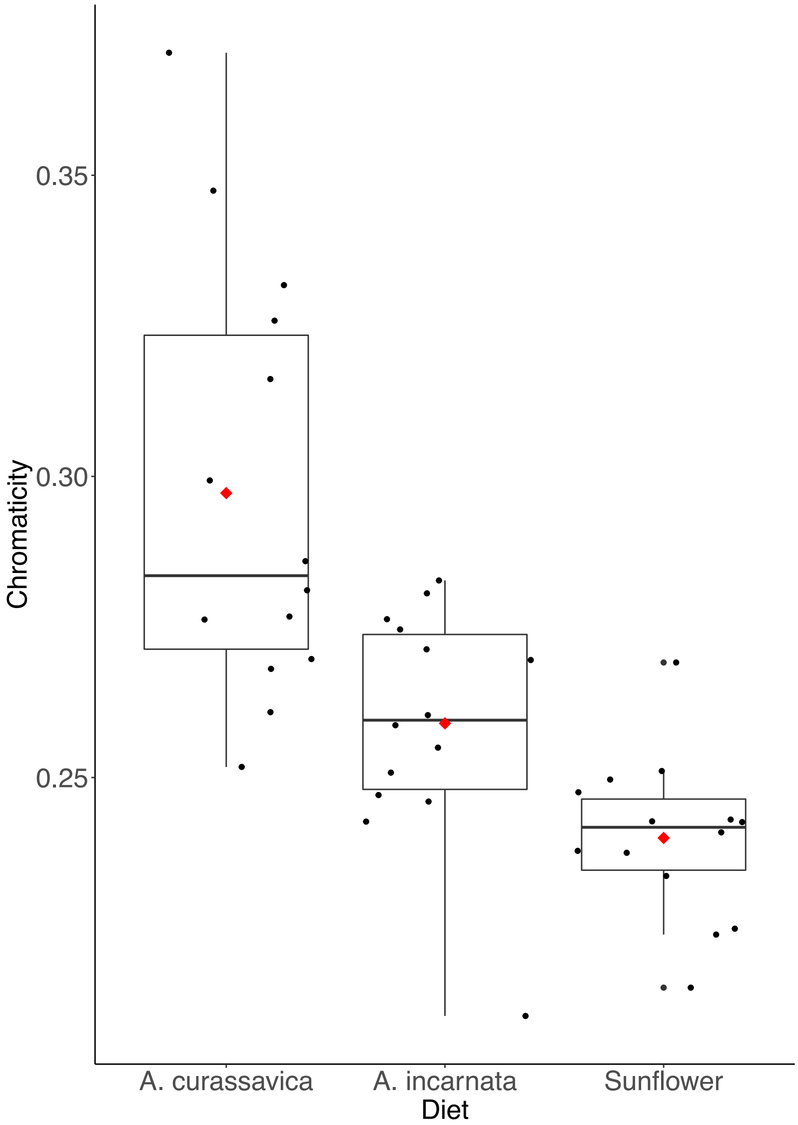

Figure 2. Chromaticity differences in *O. fasciatus* individuals when raised on different species of host plant (n = 14 for each diet). Horizontal bars represent the median values, and the red diamonds represent the means. The upper bound of box shows the third quartile and lower bound shows first quartile of data, the upper whisker shows the largest value no further than the interquartile range multiplied by 1.5, and the lower whisker extends to the smallest value at most the interquartile range multiplied by 1.5.

3.2. Effect of diet on milkweed bug weight

Bugs raised on different diets had different masses (Fig. 3; chi-squared = 80.78, df = 2, p < 0.001). *A. curassavica*-raised bugs had lower masses than those raised on *A. incarnata* (Z = -7.60, p < 0.001) or sunflower seeds (Z= -8.15, p < 0.001), and there was no difference between the masses of bugs raised on sunflower or *A. incarnata* (Z = 0.06, p = 0.99).

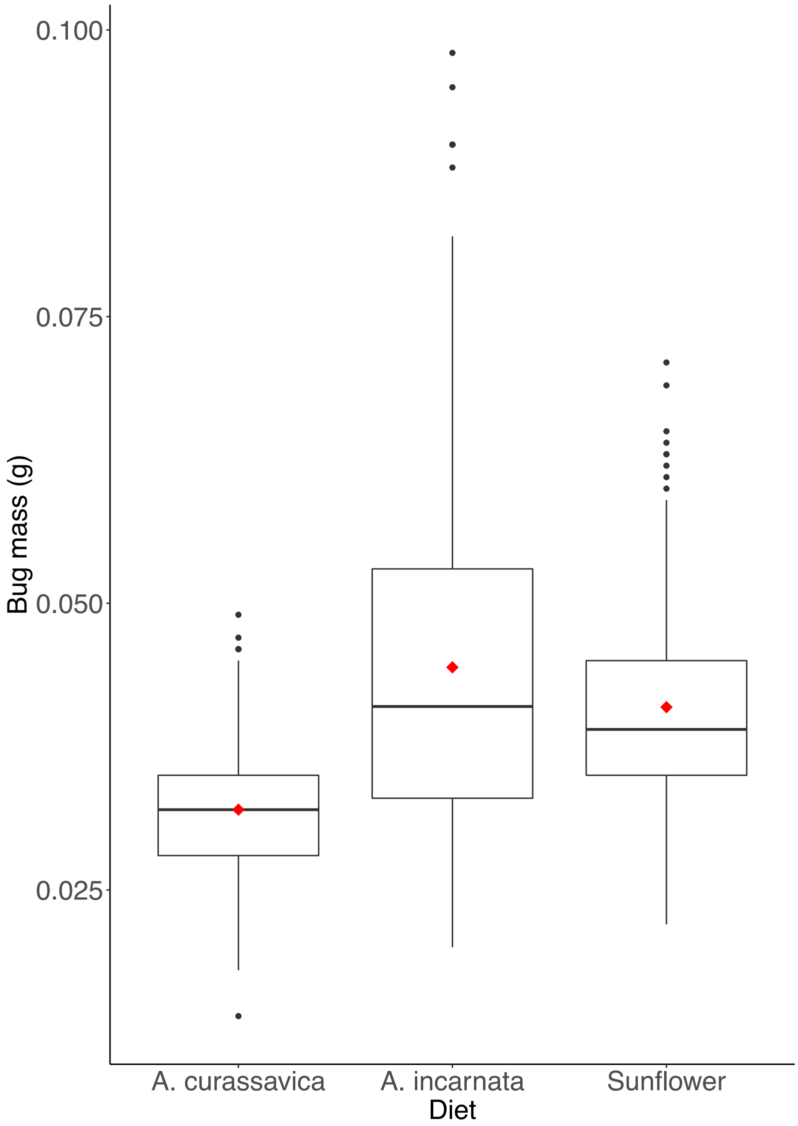

Figure 3. Mass in grams of adult *O. fasciatus* individuals when raised on different species of host plant. Horizontal bars represent the median values, and the red diamonds represent the means. The upper bound of box shows the third quartile and lower bound shows first quartile of data, the upper whisker shows the largest value no further than the interquartile range multiplied by 1.5, and the lower whisker extends to the smallest value at most the interquartile range multiplied by 1.5.

3.3. Mantid neophobia and dietary wariness

There was no significant difference in the number of trials that male and female mantids took to strike at the novel prey (Fig. 4; neophobia; Wilcoxon ranked sum test; p = 0.289). Two thirds (N= 10 out of 15) female mantids struck the milkweed bug within the first trial and approximately half (N= 7 out of 15) male mantids struck the bugs within their first encounters.

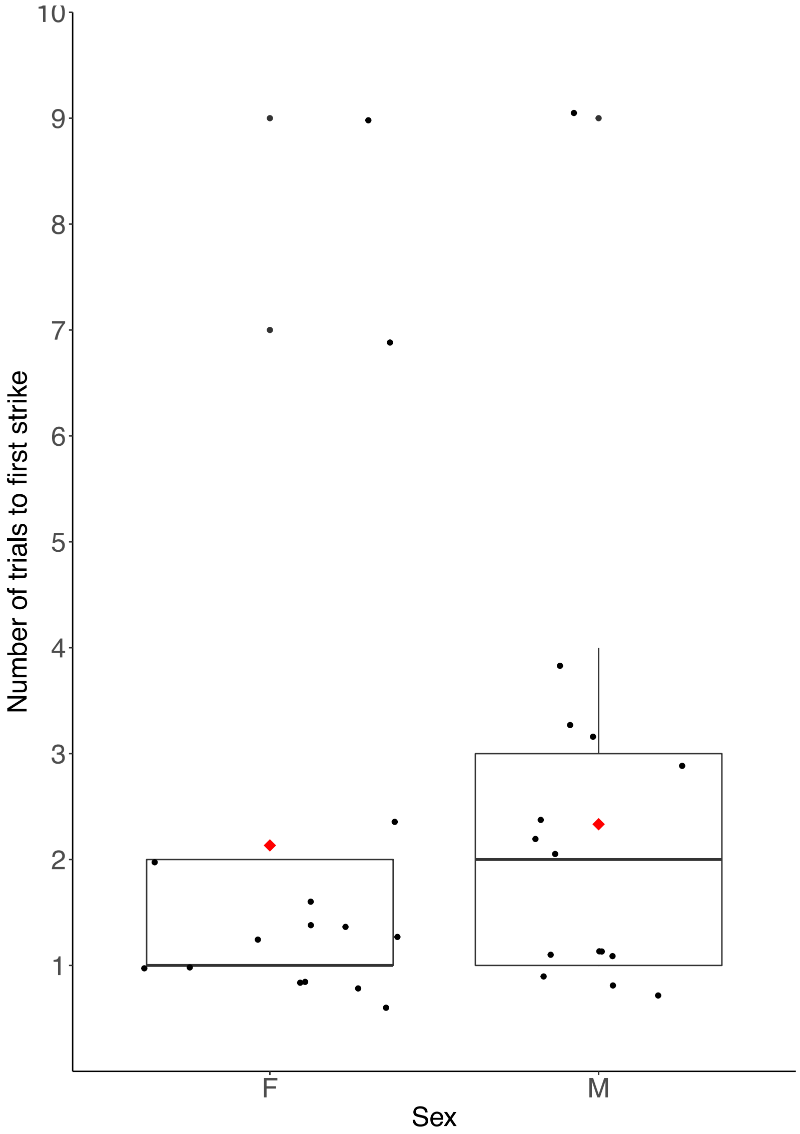

Figure 4. Neophobia in *H. membranacea*. The number of trials required for mantids to strike at the first novel prey (*O. fasciatus* individuals) by sex of the mantid, with F representing females, and M representing males. Each trial was 30 minutes long, and mantids consumed up to 4 bugs in a week, over a total possible 8 trials. Horizontal bars represent the median values, and the red diamonds represent the means. The upper bound of box shows the third quartile and lower bound shows first quartile of data, the upper whisker shows the largest value no further than the interquartile range multiplied by 1.5, and the lower whisker extends to the smallest value at most the interquartile range multiplied by 1.5.

Male mantids required significantly more trials to consume 16 milkweed bugs than females (Fig. 5) (Wilcoxon ranked sum test; p = 0.0054).

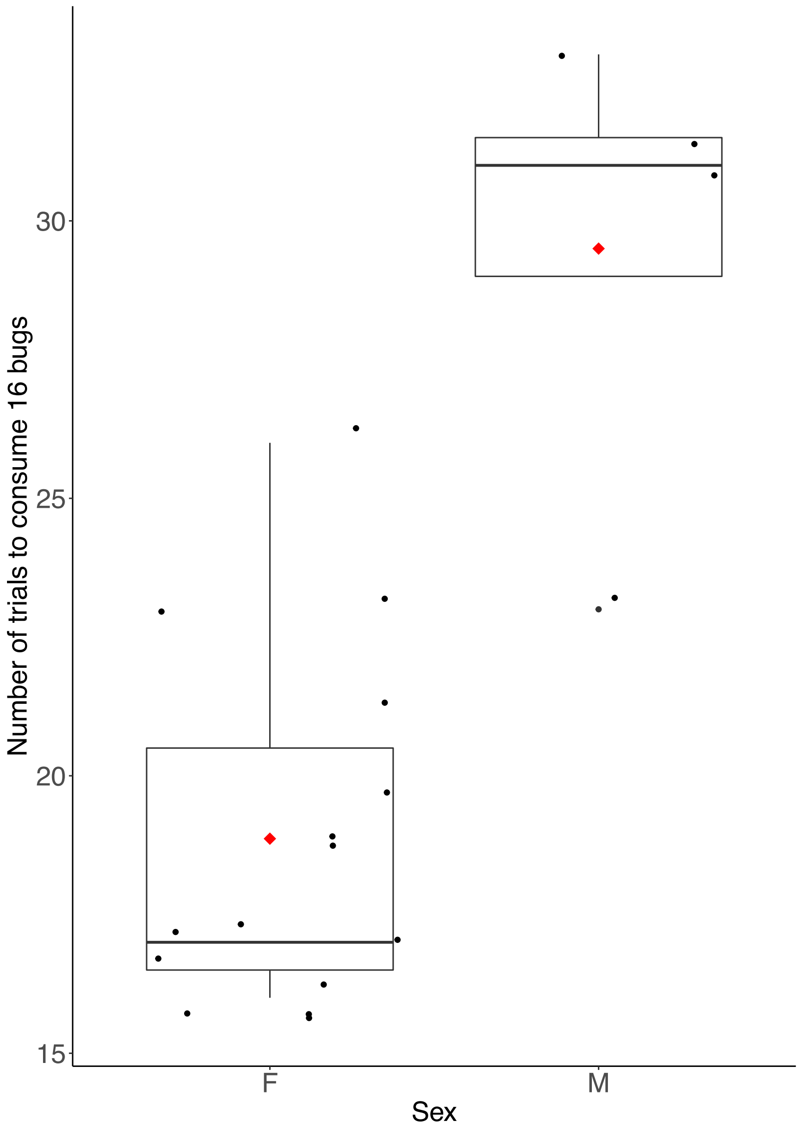

Figure 5. Dietary conservatism in *H. membranacea*. The number of trials required for mantids to strike at 16 novel prey (*O. fasciatus* individuals) by sex of the mantid, with F representing females, and M representing males. Each trial was 30 minutes long, and mantids consumed up to 4 bugs in a week, over a total possible 8 trials. Trials ran over several months. Horizontal bars represent the median values and the red diamonds represent the means. The upper bound of box shows the third quartile and lower bound shows first quartile of data, the upper whisker shows the largest value no further than the interquartile range multiplied by 1.5, and the lower whisker extends to the smallest value at most the interquartile range multiplied by 1.5.

3.4. Correlation between neophobia and dietary wariness

There was no correlation between the number of trials to first strike and to attack 16 prey individuals in total (t = 1.0079, df = 17, p = 0.3276) (Fig. 6), but there was a significant correlation in female mantids – those that took longer to attack the first milkweed bug also took longer to attack 16 prey in total (t = 4.0564, df = 13, p = 0.001). There was no significant correlation in males (t = 0.32733, df = 2, p = 0.77).

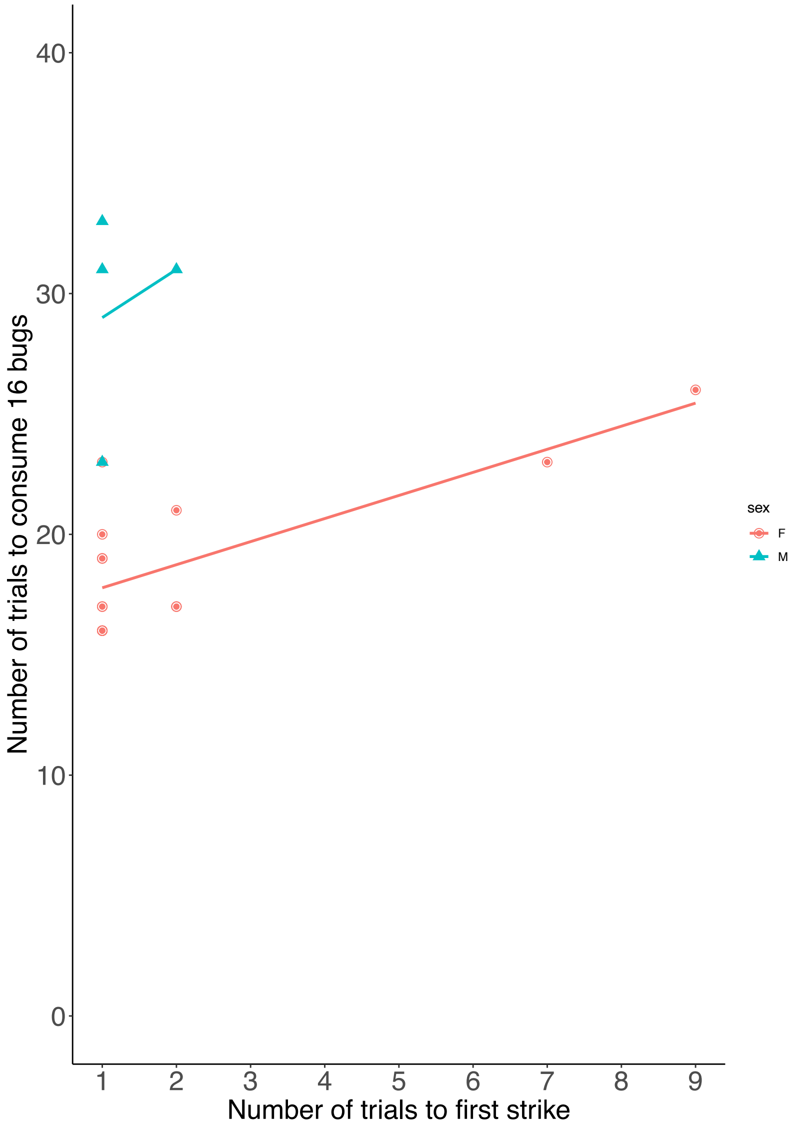

Figure 6. Relationship between neophobia and dietary conservatism tendencies in H. *membranacea*. Colours represent sexes, with red (F) representing females, and blue (M) representing males. Each trial was 30 minutes long, and mantids consumed up to 4 bugs in a week, over a total possible 8 trials. Trials ran over several months.

3.5. The effect of different diets on mantid avoidance learning

No mantids showed signs of poisoning through vomiting or mouth wiping in any trials, and no mantids died during a trial. Female mantids continued to strike at and consume milkweed bugs throughout their trials (Fig. 7). There was no significant change in the number of bugs attacked across trials (Fig. 7a; estimate = -0.007 +/- 0.01, t = -0.624, p = 0.532). Female mantids did not attack bugs on *A. incarnata* or sunflower diets differently (estimate = 0.580 +/- 0.536 p = 0.53), or *A. curassavica* and sunflower differently (estimate = -0.731 +/- 0.462, p = 0.25), but did strike at *A. curassavica*-raised bugs significantly less than A. incarnata (estimate = -1.311 +/- 0.55, t = 0.05, p = 0.045).

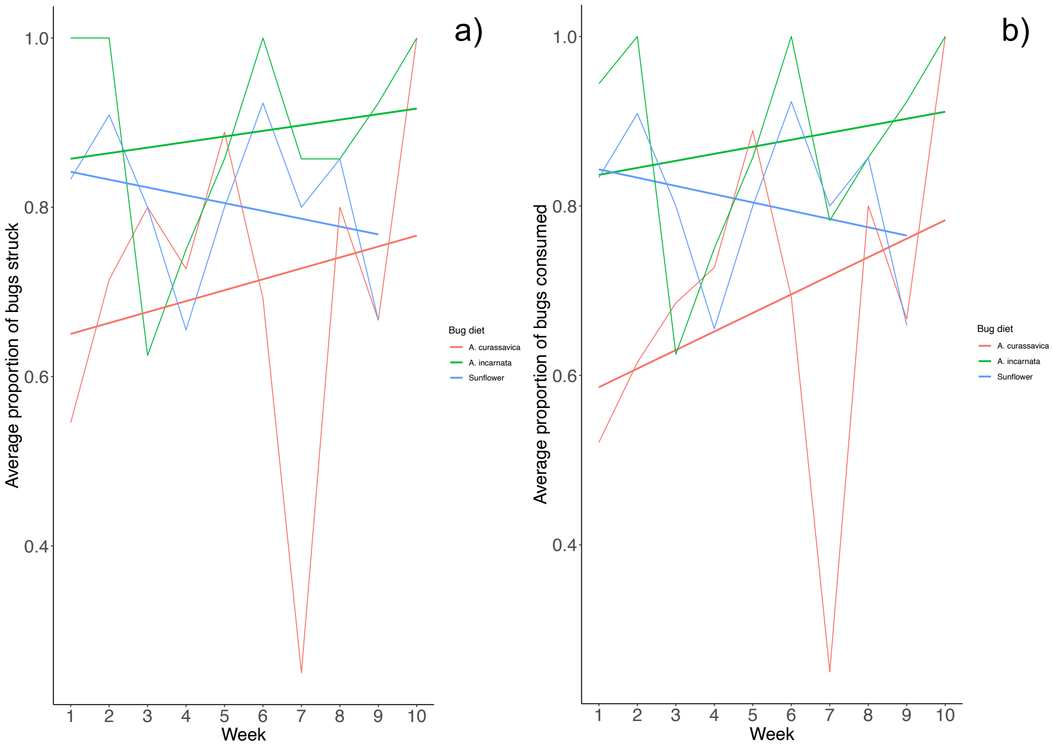

Figure 7. Attack and consumption behaviour of female mantids (*H. membranacea*) during the learning trials a) Average number of *O. fasciatus* bugs attacked per week; the red line represents *A. curassavica*-raised bugs, the green line represents *A. incarnata*-raised bugs, and the blue line represents sunflower seed (*Helianthus annus)*-raised bugs. Trendlines are in bold in the same colours. b) Average proportion of individual milkweed bugs consumed each week on each diet. Proportion consumed was calculated as mass consumed/mass presented for each group of mantids per week. Trendlines are in bold in the same colours.

The proportion of bugs consumed per trial did not differ significantly across trials (estimate = -0.001 +/- 0.002, t = -0.80, p = 0.42250. The proportion of *A. incarnata*, *A. curassavica*, and sunflower seed-raised bugs consumed per trial per week did not significantly differ (*A. curassavica* - *A. incarnata* estimate = -0.29+/- 0.14, t = -2.095, p = 0.1359; *A. curassavica* – Sunflower estimate = -0.22 +/- 0.12, t = -1.825, p = 0.2035; *A. incarnata* - Sunflower estimate = 0.072 +/- 0.14, t = 0.506, p = 0.87).

Between the mantids striking and consuming *A. incarnata*-raised individuals and sunflower seed-raised individuals, there was no significant difference in the number of trials it took for mantids to consume 36 milkweed bugs (Fig. 8; Wilcoxon rank sum test; p = 0.2). The effect of mantid predation on milkweed bug mortality rates is shown in Fig. 9.

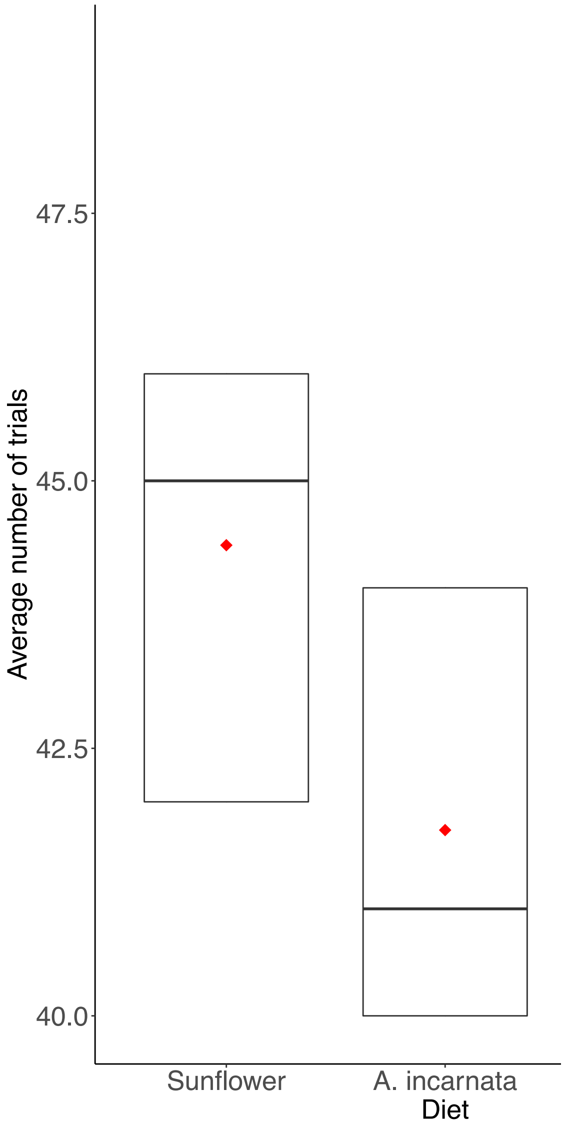

Figure 8. Number of trials taken for mantids (*H. membranacea)* to consume *O. fasciatus* individuals raised on different dietary treatments. Each trial was 30 minutes long, and mantids consumed up to 4 bugs in a week, over a total possible 8 trials. Trials ran over several months. Horizontal bars represent the median values and the red diamonds represent the means. The upper bound of box shows the third quartile and lower bound shows first quartile of data, the upper whisker shows the largest value no further than the interquartile range multiplied by 1.5, and the lower whisker extends to the smallest value at most the interquartile range multiplied by 1.5.

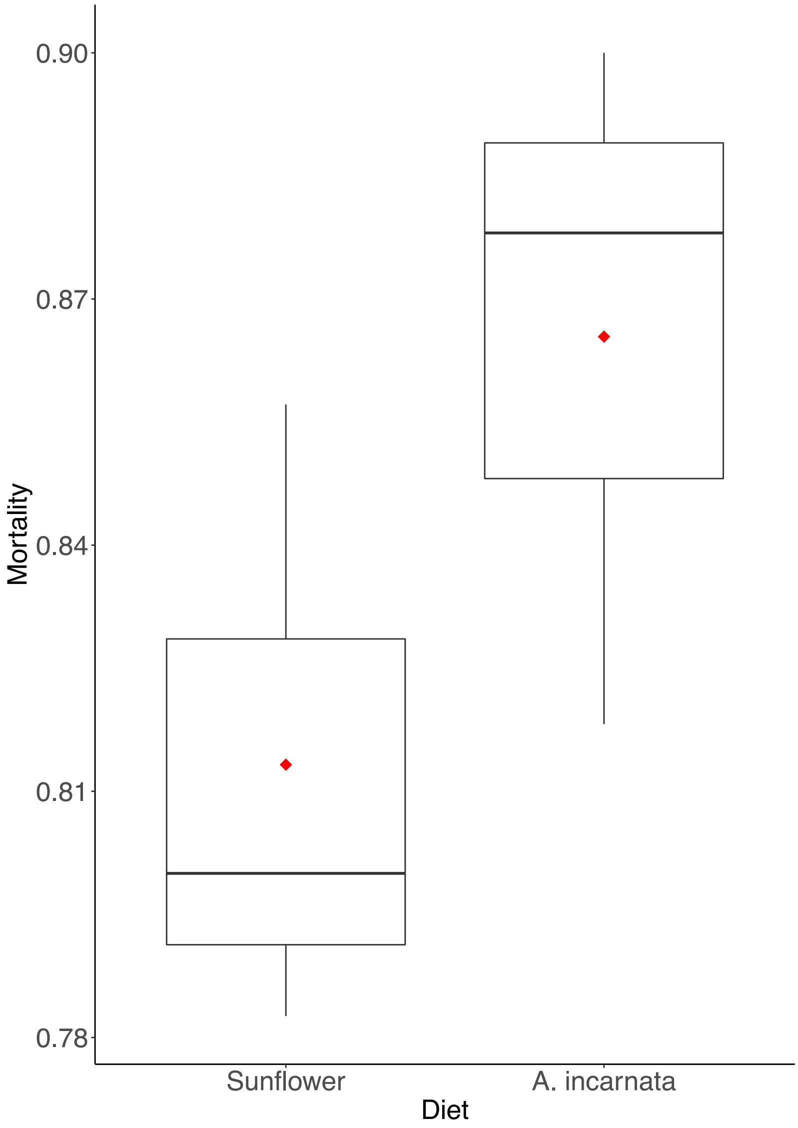

Figure 9. Milkweed bug mortality throughout the learning experiments with mantid predators. Horizontal bars represent the median values and the red diamonds represent the means. The upper bound of box shows the third quartile and lower bound shows first quartile of data, the upper whisker shows the largest value no further than the interquartile range multiplied by 1.5, and the lower whisker extends to the smallest value at most the interquartile range multiplied by 1.5.

3.6. Mantid survival

There was a significant difference in the survival of the mantids on different diets (Fig. 10; Chisq= 10.43 on 2 degrees of freedom, p= 0.005). Mantids that fed on *A. curassavica*-raised bugs survived significantly less than those fed on sunflower-raised bugs (-1.43e +/- 0.654, z = -2.18, p = 0.029). Mantids that fed on *A. incarnata*-raised bugs did not survive differently to those sunflower-raised bugs (5.80 +/- 2.95e+03, z = 0.00, p = 0.998).

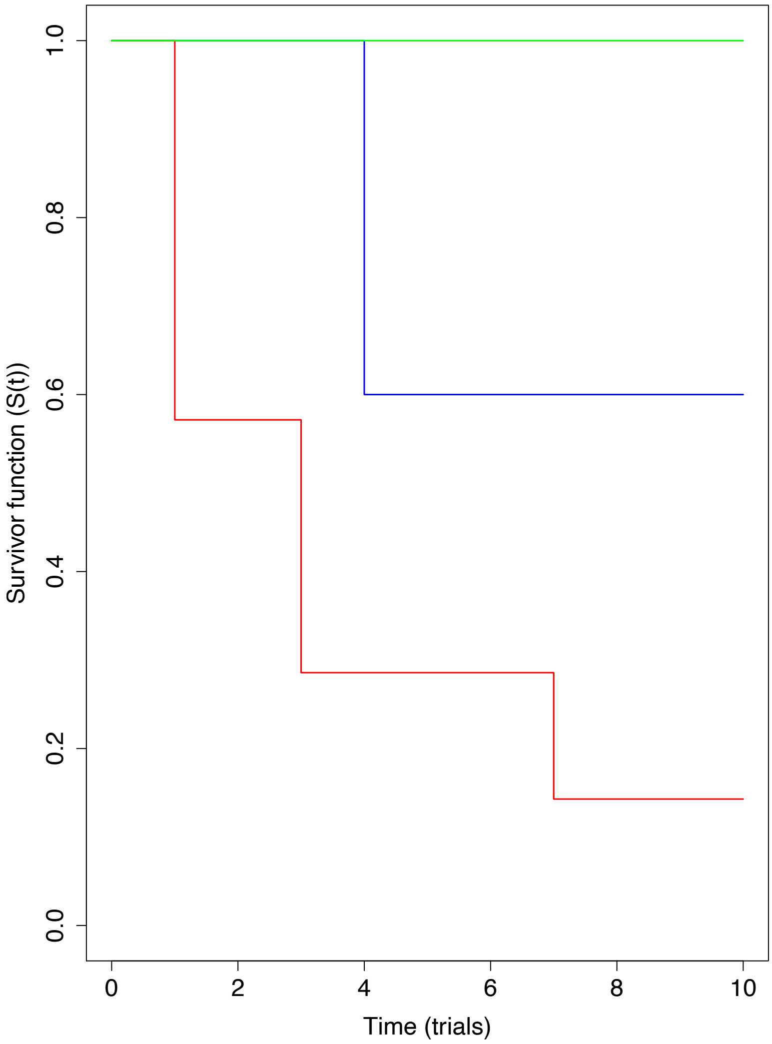

Figure 10. Mortality of female mantids during the learning trials. Colours represent the diet of the large milkweed bugs the mantids were fed with: red represents *A. curassavica*-raised bugs, green *A. incarnata*-raised bugs, and blue sunflower seed-raised bugs.

4. Discussion

In this experiment, I found visual differences in milkweed bugs (*O. fasciatus*) when they were reared on three different host plant diets, two species of *Asclepias* (*curassavica* and *incarnata*) and one nontoxic diet of sunflower seeds. To my knowledge, this is the first evidence of such differences in signals linked to host plant differences in this species. I then tested this variable set of visual signals and chemical defences of aposematic prey with a generalist invertebrate predator, the praying mantid (*Hierodula membranacea*). Mantids displayed neophobia when first presented with milkweed bugs for which they had no prior experience, and some evidence of sex-biased dietary conservatism. Evidence for the existence of dietary wariness in this species, and in any invertebrate, is minimal, and my research contributes to the growing evidence that dietary wariness exists in many taxa (Crane and Ferrari, 2017). I found that neophobia and dietary wariness were correlated in female mantids, and a range of more and less wary tendencies in individuals. This is previously unrecognised in mantids.

In learning trials, mantids did not learn to avoid the milkweed-raised *O. fasciatus* individuals as compared to sunflower-raised bugs, an anomalous result from the previously published literature. However, mantids did strike at *A. curassavica*-raised bugs significantly less than *A. incarnata*-raised bugs, and I also observed higher mortality in mantids feeding on *A. curassavica*-raised bugs than those raised on *A. incarnata*-raised bugs. These two results suggest that mantids may manage their toxin consumption, as has been found in vertebrate predators (Skelhorn and Rowe, 2007b), and that mantids may suffer from chronic poisoning from cardenolides, as opposed to the more commonly studied acute emetic effects (Berenbaum and Miliczky, 1984; Bramer et al., 2018; Brower et al., 1968b, 1982; Gelperin, 1968). My results suggest that predation trials over much longer periods may be vital for understanding both the short and longer-term effects of aposematic prey on mantid predation behaviour, and the evidence for dietary wariness indicates there is much more to be learnt about the foraging and toxin avoidance strategies in this group of predators.

4.1. Visual characteristics of the large milkweed bug

The visual appearance of prey is a critical aspect of the predator-prey relationship (Guilford and Dawkins, 1991), especially when the predator is primarily a visual hunter, and the prey relies on aposematism as their predator-deterrent strategy. Among numerous factors in appearance and behaviour, luminance (brightness), intensity of colour (chromaticity), and size could all affect predator behaviour in terms of detectability and discriminability. For example, a stronger signal, e.g. a brighter or more intense colour, could both increase visibility to predators and could induce a stronger learning response, if associated with secondary defences like toxins (Ruxton et al., 2018). This has been previously found in the large milkweed bug, as some praying mantises species learn to avoid more luminant individuals faster, and to remember distaste for this prey for a longer time (Prudic et al., 2007b).

Large milkweed bugs have been shown to vary in their wing colouration in the wild (Davis, 2009a), but to date there have been no studies on visual differences in captive-bred populations raised on different host plants. In my study, *O. fasciatus* showed significantly different visual signals when raised on different host plant species. In this experiment luminance was measured as red reflectance, which I take as a proxy of pigment production, as more red pigments present would mean a stronger red signal and reduced luminance. Redness, or chromaticity, was measured as the red signal proportional within the insect, a proxy of red pigment allocation. The differences in both luminance and chromaticity in my results indicate that diet had some direct or indirect effect on pigment production and allocation. Although sunflower seed-raised bugs were the brightest, sunflower is not a natural host plant for *O. fasciatus* but a laboratory diet developed to rear them without cardenolides (Gordon and Loher, 1968). The visual differences here may be explained by variation in nutritional quality of the diet, or the result of a specialist insect adapting to a less than ideal non-host plant. Between the *Asclepias* species there was no difference in luminance, although *A. curassavica*-raised bugs showed a much wider range of luminance compared to *A. incarnata*-raised bugs.

A. curassavica-raised bugs were significantly redder than *A. incarnata* bugs. I hypothesise that this could be a form of honest signalling in the milkweed bugs. *A. incarnata* is a temperate species that grows within or bordering swampy land, whereas *Asclepias curassavica* is a tropical species that flowers year-round. Both are natural host plants of *O. fasciatus* (Braman and Latimer, 2002; Davis, 2009a), and both synthesise cardenolides, but in different quantities. *A. curassavica* produces a much greater quantity of cardenolides and is assumed to then be more toxic to herbivore predators, whereas *A. incarnata* synthesises a very similar profile of cardenolides, but in a much smaller amount (Züst et al., 2019). As the milkweed bugs raised on *A. curassavica* were much redder than those raised on *A. incarnata*, they could be signalling their presumably higher concentration of cardenolides to potential predators.

In Chapter 2, I found a relationship between luminance, oxidative stress, and colouration in terms of luminance in *O. fasciatus*, with the most toxic bugs facing constraints that limited their ability to produce bright signals. My results in Chapter 4 indicate this same type of relationship could be present in natural populations of milkweed bugs, with bugs feeding on increasingly toxic host plants exhibiting a positive correlation between colour and sequestration as predicted by the resource competition model (Blount et al., 2009). Future studies could combine these signal quality examinations with a chemical analysis of the cardenolide quantity, to further examine this potential relationship.

In weight, *A. incarnata*-raised and sunflower-raised bugs were heaviest and *A. curassavica* bugs were lightest. Larger size has been associated with aposematism, and indeed in a cardenolide-sequestering insect, as the lubber grasshopper is assumed to compound its toxicity with its unusually large size as anti-predator defences (Whitman and Vincent, 2008). In this experiment, even the heaviest *O. fasciatus* were too small to be rejected by mantids, so the weight differences shown in my experiment were unlikely to convey an advantage on the heavier individuals.

The reason behind the size differences I found in these experiments is unclear. Previous studies have shown life history differences in milkweed bugs when raised on different diets (Chaplin and Chaplin, 1981), and have indicated that these differences could be caused by differences in seed quality. I found that *A. incarnata* was associated with higher body mass in the bugs, although previous studies have found the opposite effect (Chaplin and Chaplin ,1981). My findings that sunflower seed and *A. incarnata*-raised individuals were larger could indicate a fitness advantage, as larger body size is correlated with fecundity in many insects (Honěk, 1993) but could also suggest a substandard diet, wherein the individuals cannot process the seeds as efficiently, and accumulate lipids. Chaplin and Chaplin's (1981) results, as well as findings that cardenolide-sequestering monarch butterflies preferentially fed upon *A. curassavica* instead of *A. incarnata* in a mixed stand of both (Malcolm et al., 1986), indicate that *A. incarnata* may not be the most suitable host plant. In Pokharel's recent study (2021), milkweed bugs were found to have decreased fecundity on higher amounts of cardenolides in their diets, which could fit well with my results of larger size on the lower-cardenolide species.

4.2. Dietary wariness

There are two components to dietary wariness: neophobia, the initial hesitation to consume a novel food type, and dietary conservatism, a longer-term reluctance to incorporate a novel food into the diet (Marples and Kelly, 1999). Neophobia has been observed across a wide range of taxa (reviewed in Crane and Ferrari, 2017), e.g. in birds, amphibians, fish, and mammals, including humans (reviewed in Dovey et al., 2008), but invertebrate predator neophobia has rarely been studied (but see Vickers et al., 2021). This is a significant gap in research, as there are many invertebrate predators who hunt using visual cues (reviewed in Lim and Ben-Yakir, 2020), e.g. ladybirds (Hattingh and Samways, 1995), jumping spiders (Bednarski et al., 2012), and praying mantises (Yamawaki, 2017), and for whom novel food may pose threats through secondary defences, making neophobia a relevant possibility. It is especially important, as dietary wariness has been suggested as a mechanism by which aposematism could have evolved (Lee et al., 2010; Speed, 2001) and invertebrates are a much older lineage than the more commonly studied avian predators.

In this study, I measured neophobia by the number of trials it took for a mantid to strike at a milkweed bug. As the mantids had only previously consumed flies, the milkweed bug was a novel food in shape, colour, and taste. Neophobia was also not influenced by unpalatability as the bugs were raised on sunflower seeds and therefore did not sequester any cardenolides (Isman, 1977). My results indicated no difference in neophobic behaviour in male or female mantids, although neophobia was possibly present, as the average mantid first attacked the novel prey in the second trial, i.e. from 30-60 minutes. There was variation in this, with two thirds of the female mantids, and half the male mantids, striking within the first trial, i.e. 0-30 minutes. Evidence for neophobia in mantids is somewhat surprising, as in previous studies with mantid predators most researchers describe rapid, unhesitating strikes to novel prey (for example Berenbaum and Miliczky, 1984; Fabricant and Smith, 2014; Prudic et al., 2007). However, mantid neophobia is not unexpected, they are generalist predators that will likely encounter a wide variety of defended species, and showing caution when encountering new conspicuous prey, following theory like the 'go-slow' hypothesis (Guilford, 1994) of careful consumption, could be an advantageous strategy.

As my experiment ran over several weeks, I was also able to study differences in dietary conservatism. There is no defined time or amount of novel food consumed to define dietary conservatism, but it is usually distinguished from neophobia as being longer-lasting, from several weeks into months (Marples and Kelly, 1999). As each mantid could eat up to four milkweed bugs per week, I decided that a minimum of four weeks of acceptance, or 16 bugs consumed, would qualify the mantid as overcoming any dietary conservatism. In my experiment, males showed a much stronger reluctance to incorporate milkweed bugs into their diets, although with such high mortality in male mantids this is a difficult result to analyse in detail and should be viewed with caution.

There has been very little investigation into whether neophobia and dietary conservatism are correlated, especially in species that may show both. In this study, I found no correlation in males, but female mantids were more likely to show dietary conservatism if they also were more neophobic in the first set of trials. This correlation indicates that female mantids may be classified as more or less cautious consumers, with varying dietary wariness in the population. If this is true in natural populations of mantids it could affect fitness in the local aposematic prey, as more cautious females in a population would be more likely to allow a prey individual to escape. It would also be interesting to examine any differences in proportions of conservative consumers in mantids, and other invertebrate predators, as distinct proportions have been found in other predator groups like fish (Richards et al., 2014). Predation from invertebrates like mantids has strong, variable, and sometimes unpredictable effects on trophic levels (Fagan et al., 2002; Hurd and Eisenberg, 1990; Moran and Hurd, 1997), and dietary wariness in their populations would add another level to explain this variation. This is an area ready for much further exploration, as invertebrate dietary wariness is generally understudied (Crane and Ferrari, 2017), but may have significant effects on local aposematic prey populations.

This link in dietary wariness was only found in female mantids and may be associated only with female hunting behaviour. *H. membranacea* mantids show strong sexual dimorphism; male mantids can fly, and are much smaller and lighter than females (Birkhead et al., 1988). In other large Asian species, such as the Chinese mantid *Tenodera sinensis*, male mantids have been found to feed much less than females (Eisenberg et al., 1981), a trend that matches my experimental results. In *Tenodera angustipennis*, males were found to move around much more, regardless of their satiation rate, which the authors concluded was likely due to them focusing greater time and energy in searching for mating opportunities rather than hunting (Inoue and Marsura, 1983). These differences may explain my observations of greater reluctance to consume novel foods. If the males' priorities are to find and secure a mating opportunity, and gaining weight matters little, this might explain why males are less motivated to sample new foods and incorporating them into their diets. In Chinese mantids, females are more stationary and, when developing eggs, are often food limited (Eisenberg et al., 1981); it is likely that this is also true for *H. membranacea*. Females would then have much greater motivation to feed, as they require plenty of food to sustain themselves and their egg development (Prete et al., 1999), and may invest more in probing and consuming novel foods. It would be interesting to further these results in future experiments, by testing whether wariness in females exhibits a tradeoff with fecundity; this has been found in chickens, with the highest wariness in those individuals that produced less eggs annually (Rowland et al., 2017).

4.3. Learning

In my experiment, mantids received sequential prey, first nontoxic sunflower-raised, and in the next trials either the same dietary treatment of bugs or one of two milkweed-reared groups of bugs. This setup, dividing mantids into dietary treatments and giving them sequential presentations of the same type of prey, is similar to several previous papers examining mantid learning, all of which found mantids to taste reject milkweed-raised bugs, and to cease attacking them within a few trials (Berenbaum and Miliczky, 1984; Bowdish and Bultman, 1993; Gelperin, 1968; Prudic et al., 2007b).

In my experiment, unlike in previous experiments where mantids have been shown to vomit after attempted predation (Berenbaum and Miliczky, 1984; Bowdish and Bultman, 1993; Gelperin, 1968; Prudic et al., 2007b), I found no signs of the emetic effects associated with predators consuming cardenolides (Brower, 1969) observed during any of the trials, in any dietary treatment. Mantids did not attack *A. incarnata*-reared milkweed bugs differently to sunflower reared bugs, although there was a slightly lower strike rate toward *A. curassavica*-raised bugs, and there was no change in the attack behaviour over time. Between *A. incarnata* and sunflower bug dietary treatments, there was also no difference in the time mantids took to consume 36 bugs. Taken together, this evidence suggests that although mantids may have found *A. curassavica*-raised bugs slightly more distasteful or a greater toxic burden, overall mantids showed no avoidance learning, in the classical asymptotic learning-sense, of the *Asclepias*-raised milkweed bugs. My experiment required that mantids attack and consume milkweed bugs over a minimum of nine weeks of trials, significantly longer than previous experiments, and therefore stronger reason to conclude that there was no classical avoidance learning, as opposed to not having provided enough opportunity to learn. In my experiment, although not every female struck every bug, overall female mantids never stopped striking at both milkweed-raised and sunflower-raised milkweed bugs and showed no consistent signs of rejection.

There are several potential reasons for this, including a lack of toxicity, weak signals inhibiting learning, or differences in the species used. Although I did not chemically quantify the sequestration amounts in each milkweed bug, *O. fasciatus* are known to sequester cardenolides when raised on various *Asclepias* spp. host plants (Isman, 1977), and I followed previous studies in raising bugs for several generations on each species, to ensure consistency (Prudic et al., 2007b). It is therefore unlikely that this lack of learning came from the milkweed bugs not sequestering cardenolides at all. A more likely explanation is that there could be a lack of poisonous effects from the specific chemical profile, or the total amount, of cardenolides in *A. incarnata*-raised bugs. In similar milkweed bug species, host plant species (even when all were toxic) has been found to be crucial in that they can offer predator-specific protection, i.e. one host plant may be toxic to one predator but not others (Pokharel et al., 2020).

Another potential reason could be sunflower seed-raised bugs were significantly more luminant than *A. incarnata* bugs, and previous research has found these less bright signals are more difficult to learn for mantids (Prudic et al., 2007b). This weaker signal, combined with potentially less cardenolides than *A. curassavica*-raised bugs, could explain why learning differences were not evident. To fully clarify this, future studies could make total chemical profiles of the sequestered cardenolides in the bugs, as well as model the differences in visual characteristics of the milkweed bugs from the perspectives of mantids.

A third potential explanation for these differing results is the mantid species used; the majority of previous studies used the Chinese mantid (*Tenodera sinensis)* as the praying mantis model (for example Berenbaum and Miliczky, 1984; Bowdish and Bultman, 1993; Prudic et al., 2007), whereas my experiments used *Hierodula membranacea*. Although both species fall within the family Mantidae, and are both large, temperate or tropical Asian species, my results provide evidence that their toxin tolerance may differ, at least in terms of acute toxic effects. Mebs (2017) has shown *H. membranacea* to consume monarch caterpillars with no adverse effects, bolstering this argument.

It is interesting to consider what this lack of learning would mean to mantids living in nature. If weak signals are less effective against such invertebrate predators, then predation pressure from mantids could select for brighter and more conspicuous colouration, or alternatively more cryptic prey that are strongly defended with high amounts of toxins. Previous experiments with mantids facing a range of milkweed bug prey with different levels of brightness found this effect, with mantids learning brighter prey more effectively (Prudic et al., 2007b), and this could translate into natural scenarios as well, with mantid predation pressure selecting against weakly or dishonestly signalling prey.

Host plant variation in effectiveness against predators could also affect prey, if feeding on only specific species gives protection from the dominant class of predator. Then the local predator community could then exert selective pressure for or against feeding on specific host plants; similar results have been found in monarch butterflies, with predators preferably selecting those caterpillars feeding on less toxic species of host plants (Oberhauser and Solensky, 2004).

Milkweed bug behaviour could also affect mantid predation in the wild, as *O. fasciatus* are also gregarious (Aller and Caldwell, 1979; Burdfield-Steel and Shuker, 2014), although less so as adults, and it is possible that mantids would encounter them in groups. If they continued to attack and consume milkweed bugs despite their defence, they could potentially face internal poisoning. If mantids can tolerate the toxins with no adverse health effects, then learning to avoid aposematic prey would deprive them of necessary food. It has been found that *H. membranacea* can consume cardenolides and quinine, and that once consumed the toxins passed unaltered through their digestive systems (Mebs et al., 2017, 2019). If this happened in my experiment, then it is possible that the mantids consumed and excreted the toxins with no deleterious effects within the timescale of the experiment. However, mantids did show longer term signs of chronic poisoning, indicating that this tolerance does not last indefinitely.

4.4. Mortality, chronic poisoning, and future studies

Despite no clear signs of learning to avoid milkweed bugs in the mantids, there may be evidence of long-term effects of toxicity. My experiment was hampered by high mortality. I found that mantids survived for significantly less time when feeding exclusively on *A. curassavica* bugs. However, the small sample size requires cautionary interpretations of these results. Similar results were found by Reitze and Nentwig (1991), where male mantids readily consumed prey containing formic acid, and did not learn to avoid them, but died the following day.

A. incarnata is a cardenolide-synthesising species of milkweed with a cardenolide profile similar to *A. curassavica* (Rubiano Buitrago, unpublished data), yet no mantid on this diet died during the experiment. Mantids fed *A. incarnata* bugs also did not require a larger number of trials than those fed bugs raised on sunflower seeds to consume 36 prey individuals. It is of interest that, of the 8 mantids that died, 6 were being fed *A. curassavica*-raised milkweed bugs, and that the one mantid that survived on the *A. curassavica*-fed bugs also required 49 trials to consume the bugs, the highest number of trials of any mantid in the experiment. Together, this suggests that *H. membranacea* may be poisoned by *A. curassavica*-raised prey, but through chronic poisoning rather than acute toxicity, the latter of which has previously been reported in the literature for *T. sinensis*.

This toxicity is likely caused by the milkweed bugs sequestering much larger quantities of cardenolides from *A. curassavica*; while *A. incarnata* has a much lower quantity of these compounds. This has been demonstrated in its leaves and latex (Malcolm et al., 1986; Züst et al., 2019) and it is likely that it the same trend is followed in its seeds. If this is the case, then *H. membranacea* mantids can tolerate a certain level of cardenolides well, but beyond a certain threshold they face chronic poisoning from cardenolide consumption. Vertebrate predators have been observed to feed on cardenolide-rich butterflies in cycles of seven days that appear to allow them to manage their toxin burden (Brower and Calvert, 1985), and whether mantids also do this would be worthwhile to pursue. Future studies could examine the effects of chronic poisoning in invertebrate predators in much more detail, and in experimental setups designed to account for long-term chronic toxicity.

There was also high mortality in male mantids from the consumption of apparently nontoxic milkweed bugs, bugs that had not consumed any cardenolides. One possible explanation for this is the high mortality of males once they reach adulthood, well-known in the hobby community, and likely the reason that many studies on predation in adult mantids only use females (for example Carle et al., 2018; Fabricant and Herberstein, 2015; Kramer, 1960).

Another potential explanation is the volatile signals, or possibly defences, of milkweed bugs. Mebs (2019) has previously suggested that mantids may be repelled more by the volatiles in milkweed bugs than the cardenolide content, and this has never specifically been examined in a predation experiment. Mantids have the ability to smell (Prete et al., 1992), and can sense and distinguish between groups of aldehyde compounds (Ezaki et al., 2021). Adult *O. fasciatus* individuals possess metathoracic scent glands, from which they release aldehydes when attacked, and some of the main components of their volatile profile, e.g. (E)-2-hexenal and (E)-2-octenal (Games and Staddon, 1973), have been found to repel several species of mantid, and even cause injury and death (Noge et al., 2012; Prudic et al., 2008), although in other cases mantids have been found to consume prey with aldehyde-based defences (Fabricant and Smith, 2014). If this is the correct explanation behind the male mantid deaths, it may have affected males more because they are smaller. Males also potentially have a lower toxin tolerance than females, as they have been shown to have a lower tolerance for bitter tastes (Carle et al., 2015), and bitterness has been associated with toxic compounds (Nissim et al., 2017).

4.5. Conclusion

In my experiments, I found that milkweed bugs showed significant variation in visual characteristics when raised on different milkweed species and sunflower seeds. Despite this, and regardless of cardenolide sequestration, mantids did not show evidence of avoidance learning. Although mantids continued to strike at and consume toxic milkweed bugs, the high mortality of mantids present throughout my experiments indicates a possible chronic poisoning caused by the bugs raised on *A. curassavica*, a milkweed species especially rich in cardenolides. Further studies designed to accommodate for chronic rather than acute toxicity would clarify these results, and determine whether the mantids' lack of learning would be a disadvantage or an advantage for their foraging and survival, and affect milkweed bug fitness.

General Discussion

Although the classic view of aposematism supports the idea that warning signals should be monomorphic based on the positive frequency dependent selection imposed by predators (Ruxton et al., 2018), it has long been acknowledged that aposematic animals across a wide range of taxa vary in both their signals (Bezzerides et al., 2007; reviewed in Briolat et al., 2018) and defence (Blum, 2012; Dixey, 1919; Eisner et al., 1967; Holloway et al., 1995; Marshall, 1908; Pasteels et al., 1983; reviewed in Speed et al., 2012), and on different scales, from within populations (Vidal-Cordero et al., 2012) to across groups of species (Cortesi and Cheney, 2010). How predators respond to this variation is less studied than the prey variation itself, but is just as interesting because predator responses to warning signals and chemical defences is the key to the theory and evolution and maintenance of aposematism. Throughout my thesis, I have focused my hypotheses and experiments on the theme of diversity in prey signal and defence, and the predator response to this variation. Here I will discuss the main questions I stated in Chapter 1 (Section 5), drawing together the evidence from my experimental results, and I will discuss new avenues for future researchers to consider.

1. Does the resource competition model explain the variability in both signal and defence in the milkweed bug *Oncopeltus fasciatus*?

In Chapter 2, I tested whether the large milkweed bug *Oncopeltus fasciatus*, which naturally varies in both toxicity in terms of sequestration (Isman et al., 1977) and warning signal colouration (Davis, 2009a; Rodríguez-Clark, 2004), follows the expectations of resource allocation trade-offs where variable access to resources may result in differential costs of signalling (Blount et al., 2009; Holen and 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). Alternatively, sequestration of toxins could impose metabolic costs in terms of oxidative stress.

Oxidative stress is molecular damage to various systems within the body, caused by the imbalance of reactive oxygen species (ROS) and antioxidant

defences (Chaitanya et al., 2016; Metcalfe and Alonso-Alvarez, 2010). Plant allelochemicals can be pro-oxidants, which when ingested can induce oxidative stress in the consumer (Ahmad, 1992). These negative effects caused by consuming or producing toxins has been suggested as a potential cost to chemical defence in organisms (Aucoin et al., 1991; Carroll et al., 1997; Eichenseer et al., 2002). As pigments commonly used in warning signalling, for example melanin, pterins, etc., can also be antioxidants,
the resource competition model by Blount et al. (2009) suggests that these antioxidant molecules can be a limiting resource. This constraint could cause an ecological tradeoff between producing colourful signals and combatting oxidative stress induced by sequestration or production of toxins. There have been many observational studies on correlations between toxicity and colour in accordance with the expected results from the resource competition model (for example Bezzerides et al., 2007; Briolat et al., 2018; Cortesi and Cheney, 2010; Vidal-Cordero et al., 2012), but less published information on the potential influence of antioxidant availability and oxidative stress on the development of aposematic displays (Flores et al., 2013; Ojala et al., 2005; Sandre et al., 2007b).

I reared large milkweed bugs *O. fasciatus* on an artificial diet with increasing concentrations of cardenolides and found that individuals that sequestered higher concentrations of cardenolides experienced decreases in the antioxidant total glutathione (GSH). My results suggested a cost for sequestration in terms of oxidative stress, as measured by total glutathione content (GSH). Milkweed bugs that sequestered the highest concentrations of cardenolides had the brightest warning signals when glutathione was highest, and reduced their warning signal conspicuousness as glutathione decreased, while those that sequestered the lowest levels of cardenolides invested equally in warning signal brightness with increasing glutathione, which provides a mechanistic link between GSH, luminance, and sequestration. In the most toxic *O. fasciatus* individuals, the most stressed bugs were also the least bright, indicating a constraint to their colouration mediated by oxidative stress. From these results I can conclude that there is evidence that the resource competition model could be relevant for this species.

My results contribute to the evidence for a cost of sequestration in my chosen model species, *O. fasciatus*. Recent meta-analyses from Zvereva and Kozlov (2015) have implied that chemical defences in herbivorous insects may not have costs, and in one species of moth that can both sequester and synthesise secondary defences, *de novo* synthesis was found to be more costly (Fürstenberg-Hägg et al., 2014). Large milkweed bugs are milkweed specialists, with resistant Na+ K+ ATPases ensuring they have an extremely high tolerance to cardenolides (Bramer et al., 2015; Moore and Scudder, 1986), so a cost to sequestration is perhaps not expected. However, my results support the theoretical predictions for the costs of toxin sequestration and/or production having some deleterious effects on aposematic species (Longson and Joss, 2006).

Similar evidence has been found in other specialist herbivores. For example, there is evidence that cardenolides can be a burden for monarch caterpillars in various ways: in terms of detoxification and modification (Agrawal et al., 2021), requiring behavioural adaptations to avoid poisoning from latex (Zalucki et al., 2001), and high amounts of cardenolides and nitrogen inhibiting caterpillar growth (Tao et al., 2014). In *O. fasciatus*, research has found they may experience molecular costs associated with their evolved insensitivity to cardenolides, with Dalla et al. (2017) showing that when the mutations from milkweed bugs were introduced in *Drosophila*, the NKA enzyme was less kinetically efficient (Dalla & Dobler 2016; Dalla, Baum & Dobler 2017). Recently, Pokharel et al. (2021) showed that milkweed bugs that sequestered more cardenolides have lower fecundity than less toxic individuals, although the more toxic individuals also showed an increased growth rate and adults raised on cardenolide-containing diet lived longer compared to individuals raised on cardenolide-free diets. My findings add to this small but growing body of literature, with findings for a physiological cost to sequestration in terms of oxidative stress for *O fasciatus*, and imply that oxidative state may be a key aspect of where costs lie for specialist herbivores. This is especially important for aposematic species, as the relationships between oxidative stress, sequestration, and signalling are still unexplored for many groups.

I found that when *O. fasciatus* individuals were raised on higher concentrations of cardenolides they had lower levels of total glutathione, which suggests that cardenolide sequestration uses up these antioxidant stores. My results therefore contribute to the growing body of literature exploring the possibility that warning signals are quantitative honest signals of chemical defences. Quantitative honesty is the gradient of signals (i.e. more or less conspicuous, measured in various ways) aligning with the gradient of chemical defence in a population, species, or group of species, either in a positive or negative correlation. These relationships are enforced by the limiting resource of pigments with antioxidant properties, which can act either to combat the oxidative stress caused by toxin production or sequestration, or to contribute to animal colouration. Such correlations have been found in numerous taxa, from within populations to across groups of related species (for examples see Blount et al., 2012; Cortesi and Cheney, 2010; Maan and Cummings, 2012; Wang, 2011), and is presumed to be maintained by predator discrimination of signals and defence variability.

The mechanistic underpinnings of these mostly correlational studies are understudied, and the lack of any correlations in some species (Briolat et al., 2018b) indicates that it may depend heavily on species, the chemical defences that they sequester/biosynthesise, and the pigment molecules they use for signal production. In my experiment the combination of sequestration causing an increase in oxidative stress in terms of glutathione content, and the most toxic individuals facing a constraint in luminance, suggest that antioxidant molecules are a viable mechanism for enforcing this tradeoff. This is especially likely as glutathione is an antioxidant molecule used in detoxification processes (Arias and Jakoby, 1976; Pastore et al., 2003), and has been found to be involved in detoxification of plant allelochemicals as well as insecticides in insects (reviewed in Enayati et al., 2005). Glutathione is also involved in the melanin synthesis pathway (Clark et al., 2010; Meister, 1994); although the red pigments in large milkweed bugs have been identified as pterins, pteridines also commonly act as cofactors of enzymes involved in the melanin synthesis pathway (Chen et al., 2015; Feirer and Fuqua, 2017). Given that glutathione was depleted with increasing concentrations of sequestered cardenolides, and individuals that had low levels of GSH produced less bright warning signals with increasing concentration of cardenolides, whereas individuals with high levels of GSH produced increasingly bright warning signals with increasing concentrations of cardenolides, I speculate that glutathione availability has a role in the biochemistry underlying the variation in colouration and toxicity in *O. fasciatus*. However, warning colours are usually regulated by more than one mechanism (Orteu and Jiggins, 2020; Wellenreuther et al., 2014), and this area of research warrants further biochemical study.

These variability in warning signal luminance in the high cardenolide treatment did not extend to other dietary groups, and there were no direct correlations between sequestration and luminance or chromaticity across all individuals in my experiment. From this, I suggest that the lower cardenolide diets in the experimental setup were not toxic enough to induce high levels of oxidative stress in the milkweed bugs, and that the tradeoff between oxidative stress and colouration may only be enforced when the bugs are raised on more cardenolide-rich diets. These results may then be elucidated further by raising the bugs on much more toxic diets, a range starting from my High treatment and becoming even more cardenolide-heavy. This higher range could reveal any broader correlations between sequestration, oxidative stress, and colouration that were not possibly in my experiment.

Further experiments could also use *Asclepias* species directly as dietary treatments, as they vary in cardenolide content (Züst et al., 2019), although this does introduce other variables like nutritional content that would need to be managed. Using the natural host plant of the milkweed bug would come with advantages, however, and would enable researchers to relate my findings to a more ecologically relevant setup. The same experiment as Chapter 2 with *Asclepias* dietary treatments instead of my lab-created artificial diets would reveal whether (1) the oxidative cost of sequestration is present in *O. fasciatus* when reared on their natural host plants, and (2) if this tradeoff between oxidative stress and colouration is found across all host species of milkweed, or only the most toxic.

There are numerous other avenues to investigate sequestration cost and oxidative stress in a broader sense for *O. fasciatus*. Milkweed bugs have a wide geographic range across North America, allowing for regional comparisons between different populations. Bugs show variation in life history parameters such as development time, clutch size, etc in populations separated by just 60 km (David Baldwin and Dingle, 1986), and life history differences have genetic correlations across more distantly spaced populations (Leslie, 1990). It is also likely that oxidative state depends on the combination of genetic, environmental and gene by environment interactions that determine an individual's condition. For example, monarch butterflies show patterns of local adaptation to their hostplants based on larval growth rate (Freedman et al., 2020), and show environmental and genetic interactions in sequestration ability (Freedman et al., 2021) which may reflect either a lack of evolutionary history with different species of *Asclepias*, or a physiological trade-off in sequestration ability; similar investigations could be made for milkweed bugs.

O. fasciatus also have both migratory and non-migratory populations that vary in their host plant use (Dingle, 1972; Dingle et al., 1980). Milkweed plants (*Asclepias* spp.) are also widespread, with different species present in different locales that vary in their cardenolide production (Züst et al., 2019) as well as life history traits like flowering and fruiting times (Kephart, 1987). Taking these two sources of variation together, an interesting question could be: do those milkweed bugs feeding regularly on the less cardenolide-rich milkweed species face higher costs from sequestration? Is there evidence for local adaptation? Is there variability in in resistance to cardenolides? Also, with regards to different populations, do migratory milkweed bugs face periods of increasing stress when moving to areas with more toxic milkweed species? This, combined with the stress from migration, could have deleterious life history effects.

I also found interesting life stage differences in the *O. fasciatus* bugs, with younger larval stages more bright and redder than adults, as well as with lower cardenolide sequestration. I suggest that this could be related to different aposematic strategies throughout their lives, with younger larval stages relying on aggregation to amplify their conspicuousness and aid predator avoidance learning. There is research backing this hypothesis, as gregariousness has been shown to increase the efficacy of aposematism both theoretically and in experimental tests (Despland, 2020; Hatle and Salazar, 2001; Riipi et al., 2001).

With regards to milkweed bugs, although there is older published research on how gregariousness helps *O. fasciatus* survival in a natural environment (Ralph, 1976), and laboratory predation experiments relating to gregariousness in a similar species (Tullberg et al., 2000), this is an area perfect for expansion. Further research could test directly whether the effect of aggregation enhances avoidance learning with various predators, for example lacewing larvae or mantid nymphs, and whether the solitary adult bugs are similarly protected. Modulating the nymphs' diets could pinpoint the exact benefits, if any, that aggregation gives the milkweed bugs, and at what point increased toxicity is sufficient to resist predation attempts.

Overall, Chapter 2 of my thesis contributes to the growing literature on the costs of sequestration in specialist herbivore insects, and adds to the evidence for oxidative stress being a possible mechanism in enforcing quantitative honest signalling in aposematic prey species. Future research could enlarge upon these findings by broadening the experiments to natural populations of milkweed bugs, examining the effects of migration and/or population differences, and by using *Asclepias* seeds as a dietary treatment.

2. Does the generalist invertebrate predator *Hierodula membranacea* show differences in attack behaviour and learning with milkweed bugs that vary in defence?

My results in Chapter 4 showed variation in milkweed bug visual appearance when raised on different species of host plants, with bugs raised on sunflower seeds being the brightest, and those raised on *A. curassavica* the reddest. As far as I am aware there is no published information on visual differences in milkweed bugs when raised on different host plants, making these observations novel. It has been published previously that different host species do affect milkweed bugs in other ways, for example in cardenolide sequestration (Isman et al., 1977) and life history traits like body size and growth rate (Chaplin and Chaplin, 1981; Isman, 1977). Given the wide variation in *Asclepias* species, including cardenolide content (Züst et al., 2019), fruiting, and flowering (Kephart, 1987), one possible underlying mechanisms for why there is signal variation in the herbivorous milkweed bugs could be honest signalling of differing cardenolide sequestration amounts. *A. curassavica* produces a much larger amount of cardenolides than *A. incarnata* (Züst et al., 2019), and as the bugs sequester proportionately to the cardenolides in their diet (see Chapter 2), individuals raised on *A. curassavica* could be signalling their increased toxicity with redder signals.

I found in Chapter 2 that difference in luminance can be mediated by GSH amounts in the most toxic bugs, and that the older adults when compared to larval stages were less bright and sequestered proportionally more cardenolides. Similar to these results, in Chapter 4 I found that bugs feeding on the more toxic host plant were less bright than those on the less toxic and nontoxic host plants. However, chromaticity results varied in the two experiments, as redness was also lower in the older, more toxic bugs in Chapter 2, but higher in individuals feeding on more toxic host plants in Chapter 4. This is potentially due to different diets, as in Chapter 2 I used commercially bought cardenolides in an artificial diet, whereas in Chapter 4 I used *Asclepias* seeds directly. Different *Asclepias* species seeds contain different cardenolide compounds as well as varying in other traits, like nitrogen content (Tao et al., 2014), and potentially amino acids or antioxidant concentrations. As the individuals feeding on *A. curassavica* diets were redder in Chapter 4, potentially the relationship between cardenolide content and colour is revealed through chromaticity when bugs are raised on their natural host plants. This relationship could still be mediated by oxidative stress, as I found for luminance in bugs raised on the artificial diet in Chapter 2 and as predicted by the resource competition model (Blount et al., 2009). The milkweed bugs raised on *Asclepias* seeds still fulfil the assumptions for the model, as the pigments determining colouration in *O. fasciatus* are pteridines (Hudson et al., 1959), which have the potential to function as biological antioxidants (McGraw, 2005). Future research to clarify this relationship in bugs raised on *Asclepias* seeds could analyse various measures of oxidative state, as well as chemically quantify the pigment and cardenolide content in individuals when rearing them on different natural host plants.

The gold standard for examining signalling differences in aposematic prey, especially when their predators are known or involved in the experiment, is to model prey appearance from the visual system of the predator (for a few examples see Arenas and Stevens, 2017; Llaurens et al., 2014; Stuart-Fox et al., 2003). This is increasingly common as a more accurate way of assessing predator perception of conspicuous signals and identifying whether a prey species warning signal is perceivable to its intended receivers. Time constraints in the experimental process in Chapter 4 meant that modelling the milkweed bug visual differences from the mantid perspective was not possible, but this would be a relatively simple way to improve this experiment. Future research could model the orange and black colouration of the individual bugs when raised on different diets into the monochromatic visual system modelling for mantids, similar to the work done with hibiscus harlequin bugs and the mantid *Hierodula majuscule* by Fabricant and Herberstein (2015).

Outside of visual signals, another area of potential research is the possibility of multi-modal aposematism in milkweed bugs. Although in my experiment I focused on the visual characteristics of the milkweed bug, like all true bug species *O. fasciatus* also release volatiles through a metathoracic scent gland (Aldrich, 1988). In milkweed bugs, this gland only develops as adults, and in larval stages bugs release volatiles through dorsal glands (Staddon, 1995). The volatile components released by adults consist mostly of aldehydes, including 2-hexanal and 2-octenal (Staddon and Daroogheh, 1981), and nymphs release a similar combination but slightly different, including 2,4-oxo-octenal and 2-heptenal (Games and Staddon, 1973). As adults, individuals also release methoxypyrazines within their exudate (Aldrich et al., 1997). It has been suggested that as the metathoracic scent gland is small in *O. fasciatus*, and the volatile output relatively low, that the volatile emissions of bugs are not effective in predator deterrence (Aldrich, 1988; Aldrich et al., 1999). Instead, it has been shown that milkweed bugs release volatiles for pheromones (Sheng, 1996; Zhang and Aldrich, 2003) and possible as conspecific warning pheromones (Aller and Caldwell, 1979). However, the potential for these aldehydes to have multiple functions should not be discounted. Several of the compounds released are known to have deterrent effects, and there is experimental evidence of them repelling potential predators (Noge and Becerra, 2015; Noge et al., 2012 Prudic et al., 2008). There has also been more recently a suggested from Mebs et al. (2019) that the milkweed bug volatile output could contribute to their aposematism.

My results do not provide any evidence for or against a volatile component in the milkweed bug suite of defences and signals, but it could be an interesting area for future research. Experiments could examine whether these volatiles differ when the bugs were raised on milkweed or sunflower seeds, as in a similar species insects raised on less toxic host plants had a larger volatile output, perhaps to compensate (Havlikova et al., 2020). Volatiles in sunflower seed-raised milkweed bugs appear not to deter mantids, as in previous experiments mantids readily consume them (Berenbaum and Miliczky, 1984; Bowdish and Bultman, 1993; Prudic et al., 2007b), but experiments could examine this more rigorously by creating sets of multimodal and unimodal prey and presenting them to predators. Milkweed bugs with their metathoracic scent gland removed, for example, could be tested against mantid predators, as well as sunflower-raised milkweed bugs that are sprayed with volatiles from milkweed-raised bugs. By creating a set of varying milkweed bug prey, the exact effect of the volatile component of milkweed bug defence against invertebrate predators could be more thoroughly investigated.

I also found that mantids showed evidence for dietary wariness, with potential neophobia across many individuals, and dietary conservatism especially present in males. In female mantids, neophobia and dietary conservatism correlated, indicating a range of tendencies towards more and less cautious consumers. There has been no published information in dietary wariness in mantids, and very little in other invertebrate predators (but see Vickers et al., 2021), making these results both novel and interesting. Dietary wariness in invertebrate predators is understudied (Crane and Ferrari, 2017), but should be an area ready for further research.

Predation pressure from invertebrate predators can be very important in an ecosystem (Blumenshine and Hambright, 2003; Crowder and Snyder, 2010), and the effect dietary wariness in invertebrates have on local aposematic prey could be substantial, especially if the level of wariness differs from other predator groups like birds. This is especially important for studying the origins of aposematism, which have been postulated as originating from dietary wariness (Lee et al., 2010); as invertebrates have existed much longer than avian predators, it seems likely that aposematism evolved prior to birds, in which case studying the initial evolution of aposematism should include invertebrate predators.

Invertebrate species also differ from avian predators in their sensory ecologies and hunting strategies, and many may hunt using chemical senses (Aartsma et al., 2019; Kamio and D. Derby, 2017; Pervez and Yadav, 2018), and may be more sensitive to novel volatile components than colours in their prey. Their differing hunting strategies and sensory ecologies could mean that their caution in attacking aposematic prey species may depend on different signals and defences than for vertebrate predators. There is some published research on dietary wariness in other sensory modalities, for example with novel auditory signals (see Siddall and Marples, 2011a) and smells; there is evidence for innate learning biases in chicks when they smell pyrazines, known deterrents produced by many insect species (Rowe and Guilford, 1996). Future research in dietary wariness with invertebrate predators should expand on this, examining predation effects on aposematic prey with both novel visual and volatile components, and ideally both in the field as well as in laboratory conditions.

My results in Chapter 4 also showed that mantids did not learn to avoid milkweed bugs. Within the context of mantid-milkweed bug literature, these findings stand somewhat as an anomaly. All previous predation experiments using mantid species and milkweed bugs have shown learning or aversion (for example Berenbaum and Miliczky, 1984; Bowdish and Bultman, 1993; Gelperin, 1968; Prudic et al., 2008), yet the mantids in my experiment at no point exhibited the classical asymptotic reduction in attack tendency, which would be a measure of learning to avoid milkweed bugs. They also did not exhibit the ‘classic’ emetic effects of cardenolide consumption known across several species(Berenbaum and Miliczky, 1984; Brower, 1969). These findings seem to fit more into the mantid and monarch caterpillar literature, where they have been found to readily consume the cardenolide-containing prey (Rafter et al., 2013, 2017a, 2017b), although in those experiments mantids showed no deleterious effects from cardenolide consumption. Perhaps more analogous are the results found that orb-weaver spiders also do not learn to avoid milkweed bugs, despite them finding the milkweed-raised individuals unpalatable (Bramer et al., 2018).

Experiments showing no avoidance learning in predators are challenging to find in published studies, but these negative results are just as important in contributing to the body of literature on the subject. My experiment is the first to show a lack of asymptotic avoidance learning in *H. membranacea* to cardenolide-containing milkweed bugs, but I did find that the mantids consistently attacked *A. curassavica*-raised bugs significantly less than the other dietary treatments. In Skelhorn and Rowe's (2007) experiments with European starlings, birds learnt to avoid defended prey but still attacked them at levels determined by their current toxin burden. It is possible that the mantids in my experiment were similar and based their attacks on *A. curassavica*-raised bugs from their current level of toxin burden. A future experiment could test this directly, by examining if the mantids discriminate between sequentially presented defended and undefended bugs with different colour signals. Or, like in the Skelhorn and Rowe (2007) paper, the mantids' toxin burdens could be manipulated to see if this reduces the number of defended prey that they ingested in the subsequent trial.

Despite the lack of asymptotic learning in my results, the mantids in my experiments did show potential signs of chronic poisoning, evidenced from the high mortality present throughout the experiment. The absence of acute poisoning and potential presence of chronic poisoning raises other questions in terms of mantid-milkweed bug interactions. If some mantid species face chronic poisoning from toxic prey, how do they avoid poisoning when foraging? There are many potential avenues to explore in answer to this question. Future experiments could measure chronic poisoning when mantids are fed on artificial diets laced with different cardenolides. A similar experiment with juvenile mantids by (Paradise and Stamp, 1990), who used ground milkweed bugs raised on only one species of *Asclepias*, found that higher proportions of milkweed bugs in the diet inhibited feeding and growth. A more comprehensive study could use a range of host species or cardenolides to examine exactly which diet of milkweed-produced cardenolide sequestered by the milkweed bugs causes chronic toxicity to the mantids.

The mantids in my experiments faced a single milkweed bug adult, whereas in nature they may be more likely to encounter aggregations of nymphs. How individual variation in defence and warning signals and how gregariousness of milkweed bugs affect mantid hunting could quite easily be tested. Mantid choice tests are challenging as their hunting behaviour includes fixating on one target sequentially (Yamawaki, 2000, 2003, 2017), however such olfactory and visual choice experiments have been published previously (Hoese et al., 2006; Prete et al., 1992). There is also some field evidence that mantids forage selectively and not just based on frequency dependence, i.e. consuming the most abundant available prey (deHart et al., 2015; Hurd et al., 2015). Knowing this, another possible angle would be choice tests using milkweed bugs and other conspicuous and palatable prey. Potentially the mantids in my experiment were poisoned as they had no choice between milkweed bugs and more palatable options.

Different mantid species are also known to be able to consume other types of toxins (see summary in Section 4.3), and one obvious unanswered question is: what is their mechanism for avoiding poisoning? As generalist predators, we may not expect any toxin mitigation at all as the effort taken to counter prey defences may be costly (Arbuckle et al., 2017; Kikuchi et al., 2021), but the evidence of some toxin tolerance in a range of species suggests some mechanism must be present. Mitigation of prey defences in mantids has rarely been examined in detail, and researchers have suggested impervious guts (Mebs et al., 2019), gut microbiomes (Tinker and Ottesen, 2018), prey handling (Rafter et al., 2013), etc. but there is a lack of focused research on the topic. Future experiments could examine whether injecting cardenolides into mantids causes death and could determine whether the gut is crucial to their survival, similar to how it does for TTX (Mebs et al., 2016). There has also never been a genome sequencing of any mantid species, but once this is completed for *H. membranacea* it would be possible to determine whether they have evolved target site insensitivity in their Na+ K+ Atpase, similar to other resistant species.

Most experiments to date have used only one or two species, especially *Tenodera sinensis* (for example Berenbaum and Miliczky, 1984; Bowdish and

Bultman, 1993; Prudic et al., 2007). Mantid genera have a wide geographical range, and can be temperature or tropical (Prete et al., 1999). It would be interesting to know whether toxin tolerance and/or avoidance learning has any relationship to their local ecosystem or climate.

3. Conclusions

This thesis examined several questions around variation in aposematism, through both signalling and chemical defences. I used a naturally varying prey species, the large milkweed bug, and a generalist invertebrate predator, the praying mantid. My experiments in Chapter 2 provided evidence of mechanistic underpinnings for antioxidant availability mediating resource allocation in defence and signalling in milkweed bugs, as predicted by the resource competition model, and showed interesting life history differences in aposematic strategies. In Chapter 3, I gave a quick guide review to dietary wariness, its importance in aposematism research, and how it can reveal new information about the evolution and maintenance of signalling. My results in Chapter 4 opened many new questions about the possibility of chronic cardenolide poisoning in mantids, and gave the first evidence for a lack of asymptotic avoidance learning in the mantid-milkweed bug literature.

Variation in signalling and defence in prey species remains an interesting area of research, with much yet to discover, and predator mitigation and behaviour regarding variable prey could reveal much about the evolution and maintenance of aposematism. Although weak signalling and automimicry have been seen as evolutionary paradoxes, for some prey species variability is apparently no disadvantage. For some species, signal variations can represent gradations in defence, honestly signalling to their carefully discriminating receivers, and for others, these differences in colour or pattern may simply not matter to their main predators. For each predator-prey relationship, both natural history as well as laboratory studies can elucidate the underlying mechanisms for these variations in aposematism, and the ways in which they affect predator foraging and consumption behaviour. With more research will come greater understating of aposematism, a diverse section of nature full of varying colours, patterns, smells, and tastes, along with the predators encountering them.

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