

Don't Eat Me! Variation in Warning Signals in an Australian Moth

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Abstract

Aposematism is an anti-predator strategy, using a conspicuous warning signal. Predators learn to avoid defended prey, and this incurs selective benefits to aposematic individuals. Variation is seen in most aposematic species, which does not fit with the current model of stabilising selection.

This study explores contributions to variation in warning signals in an Australian moth genus, *Amata*. The *Amata* genus is a highly cryptic one, so DNA barcoding is used to identify freshly collected specimens using the CO1 gene and compared with species descriptions. Using collected specimens, variation in the warning signal was quantified, and then correlated with likely explanatory variables. Genetic barcoding confirmed two separate genera were collected from 11 locations in New South Wales; *Amata* and *Eressa*. From the genus *Amata*, two species were positively identified, as well as several individuals from the *A. nigriceps* spp. complex. Analyses on selected signal variables gave support to sexual selection as a possible explanation for warning signal variation. Contrary to predictions, variation was not explained by climate or the diversity of local bird predators. Future studies on this interesting *Amata* system could include specific predator responses to the warning signals, as well as more in-depth genetic work on the *A. nigriceps* complex.

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Declaration

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Mariella Herberstein: input on study design, data analysis and writing

Julia Riley: input on data analysis

Kate Umbers: input on data analysis

All other research described in this report is my own original work.

A handwritten signature in black ink, consisting of a large, stylized 'G' followed by 'E' and 'B' with a star-like flourish at the end.

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Chapter One

Hoist the colours: Aposematic signals used as warning flags to avoid predation

Abstract

Aposematism is an anti-predator strategy, whereby a defended prey species advertises its unprofitability with a conspicuous warning signal. Predators learn to avoid aposematic prey after an initial unpleasant experience, and this in turn incurs selective benefits to aposematic individuals, such as increased survival rates. Until recently, warning signals were theorised to be under stabilising selection, and thus, should not vary. However, variation in warning signals is seen in most, if not all, aposematic species. This chapter reviews the history of our understanding of aposematism, the cost-benefit dynamics of warning signals, and the current understanding of variation occurring in warning signals that contradicts current theory. I also look at the effects of variation on predator learning, and mimicry complexes. This review highlights critical ecological and evolutionary features of aposematism and why it is such an effective anti-predator defence strategy.

1 | Introduction to Aposematism

The idea that animals use warning signals as a defence strategy was first described by Alfred Russel Wallace in response to a question posed to him by Charles Darwin (Wallace, 1867). Darwin pondered as to why some lepidopteran larvae displayed brilliant colouration, and how these sexless invertebrates contradicted his idea that sexual selection drives the evolution of conspicuous colours in the animal kingdom. Wallace proposed that these conspicuous colours acted as a ‘warning flag’ to predators, and that they possibly signalled a distastefulness that would otherwise be individually insufficient for protection against predation (Wallace, 1867). Darwin was famously impressed by the suggestion (Poulton, 1890). The term aposematism was officially coined by Poulton in his book, ‘The colours of animals’ (1890), from the Greek *apo*, which means ‘away’, and *sema*, which means ‘sign’ (Rojas, Valkonen & Nokelainen, 2015).

Aposematism is a strategy used by prey species, in which a two-fold defence system is employed to warn predators of unpalatability, or unprofitability (Wallace, 1867; Darwin, 1874; Poulton, 1890).

This strategy involves a primary defence, usually consisting of bright, conspicuous colours, sounds, or odours, and the secondary defence which generally involves a chemical and often toxic substance but can also include morphological traits such as spines or irritant hairs, or behavioural attributes such as aggressive or threatening postures (Ruxton, Sherratt, & Speed, 2004; Rojas, Valkonen, & Nokelainen, 2015). These two defences are often coupled with conspicuous behaviour, such as slow flight, obvious movement or posture that will contrast more obviously against the environmental background (Rojas, 2017). These conspicuousness traits aid in teaching predators to avoid these aposematic and unprofitable species (Rojas, Valkonen & Nokelainen, 2015).

In this review, I will assess the evolution and function of aposematism. Whilst this has already received some attention (see Ruxton, Sherratt & Speed, 2004, for example), I expand on previous work by highlighting the important factors, such as conspicuousness and the cost-benefits that lead to aposematism being such an effective prey defence system. Specifically, I explore predator avoidance learning and how variation in signals effects this learning, and the new idea that variation in warning signals is a frequent and normal occurrence (Briolat, Burdfield-Steel, Paul, Ronka, Seymoure, Stankowich & Stuckert, 2018), contradictory to long-held theory. I will also review warning signal diversity and form, to highlight the prevalence of aposematism as an anti-predator strategy.

2 | Cost-benefits of warning signals

Aposematism incurs selective benefits to the individual, such as increased survival rates (Halpin, Skelhorn & Rowe, 2008). To be effective, warning signals should achieve two functions: they should firstly be effective enough to be noticed by predators and to distinguish that species from those that are undefended; and secondly, should only be able to be used by those defended species fit enough to bear the cost of being detected (Sherratt & Beatty, 2003). Defended individuals have a higher chance of surviving an attempt at predation when conspicuous and therefore have lower mortality rates than cryptic species (those that blend in accurately with their surroundings) (Halpin, Skelhorn & Rowe, 2008). Further, aggregating individual populations of aposematic prey species have higher survival rates, and thus greater reproductive success (Mappes & Alatalo, 1997). Being defended also has the selective benefit of allowing an individual to forage and collect resources in full view of predators (Speed, Brockhurst & Ruxton, 2010).

Warning signals should be easily detectable and easily identifiable to work at their greatest efficacy (Sherratt, 2002), particularly by multiple predators that might vary in spectral sensitivities or perceptions (Arenas, Troscianko & Stevens, 2014). Convergent evolution has resulted in aposematic animals using red, yellow and orange, coupled with black as the most common warning colours (Endler & Mappes, 2004; Arenas, Troscianko & Stevens, 2014). These colour combinations are believed to be the most common due to their ability to be highly contrasting against most natural backgrounds, and visibly stable under most lighting conditions (Endler, 1992). However, warning signals are not limited to bright colours; they can include markings, spines, sounds or odours, or a combination of these to create multi-modal signals, the evolution of which can be seen in a broad range of aposematic species (Rowe & Halpin, 2013).

Warning signals are thought to be honest indicators of defence due to their cost, as conspicuousness increases detection and predation rates by naïve predators (Sherratt, 2002). Much research has highlighted conspicuous colours being an honest indicator of toxicity (Sherratt & Beatty, 2003; Blount, Speed, Ruxton & Stephens, 2009; Arenas, Walter & Stevens, 2015). For example, lady beetle toxicity correlates with their colour (Arenas, Walter & Stevens, 2015). However, the reverse is also true in the case of Batesian mimicry, where undefended prey mimic defended species (Summers, Speed, Blount & Stuckert, 2015). Theoretical models have predicted that once an aposematic prey species has successfully deterred a predator species, that selection on the prey species should relax resulting in energy saving through reduced chemical defence, and thus erode signal honesty (Blount et al., 2009). However, the trade-off in investment between chemical toxicity and colour pigment development is an added cost to the individual, thus making honest indication of defence a more likely strategy than signal dishonesty (Blount et al., 2009). Consequently, colouration and pattern markings are reliable indicators of individual quality in several taxa (Pérez-Rodríguez, Jovani & Stevens, 2017).

The presence of predators in an environment determines the type and strength of anti-predator defences. Due to the costs of maintaining warning signals, an absence of predators would likely see these defences relaxed, if not eliminated (Blumstein & Daniel, 2005). Global decreases in predator species abundance and diversity can lead to significant changes in community and trophic structures with the flow on effect of altering prey species behaviour (Palkovacs, Wasserman & Kinnison, 2011; Hollings, McCallum, Kreger, Mooney & Jones, 2015). Perhaps counter-intuitively, lower diversities of predators in simple communities induce more accurate mimicry in defended prey species (Ihalainen, Rowland, Speed, Ruxton & Mappes, 2012), however, predator release in some systems

are known to make extensive changes to the morphology and ecology of prey species (Palkovacs, Wasserman & Kinnison, 2011). Seasonal changes in predator community structure also influence the selection for anti-predator defences. For example, different times of the year can yield changes in the diversity of conspicuousness in lepidopteran larvae, depending on when naïve insectivorous bird predator fledging's enter the community (Mappes, Kokko, Ojala & Lindström, 2014).

3 | Evolution of aposematism

Aposematism has convergently evolved in several groups of animals, including insects, frogs and reptiles (Rojas, Valkonen & Nokelainen, 2015). Due to its diverse taxonomic spread, the question of how aposematism evolved has been greatly postulated in the literature. There has been particular interest in what aspect of the defence system evolved first: did conspicuousness develop before, or after unpalatability? Both may have gradually increased simultaneously (Alatalo & Mappes, 1996). Did gregarious behaviour influence conspicuousness, or were solitary individuals the first to be so obvious? Solitary individuals displaying conspicuous signals would undoubtedly experience higher predation rates at first, due to predator naivety (Ruxton, Sherratt & Speed, 2004). It is however, generally agreed that warning signals have evolved over time, and improve predator avoidance learning, and not the other way around (Alatalo & Mappes, 1996; Härlin & Härlin, 2003), but several different hypotheses of the evolutionary reasoning behind warning signals have been put forward (Table 1). It is generally recognised that due to the convergent nature of this anti-predator strategy, there are many possible pathways in which aposematism could have evolved (Speed & Ruxton, 2005).

Table 1 Summary of hypotheses of the evolution of warning signals and conspicuousness in defended prey

Hypotheses of Aposematism Evolution	References
Unpalatability selected for gregariousness, which in turn selected for a warning signal	Alatalo & Mappes, 1996
Defended prey evolved warning traits to be distinguished from undefended prey	Sherratt & Beatty, 2003
Visibly defended prey (spines) evolved warning colours to reinforce primary defence	Speed & Ruxton, 2005

Warning colours evolved in cryptic defended species	Halpin, Skelhorn & Rowe, 2008
Aposematism evolved to avoid predators and expand niches and resource collection	Speed, Brockhurst & Ruxton, 2010

Bright, conspicuous colours are the most obvious examples of aposematism, and colour in animals have been extensively reviewed (see Poulton, 1890; Kemp et al., 2015; and Cuthill et al., 2017). The evolution of colour vision may have sparked the use of colour in signalling by plants and animals (Osorio & Vorobyev, 2008). Conspicuous colours occur in several species of defended prey, and quite a few of them have become model species in which to study the evolution of aposematism, such as the bright colours found in Dart poison frogs. As previously mentioned, colours such as red and orange occur extensively in aposematism, and particularly in insects (see Rowe & Halpin, 2013, for an extensive list of aposematic insects and descriptions of their multi-modal signals). Amphibians, such as Poison dart frogs in the *Dendrobates* genus often show brilliant blues and greens as part of their warning signal, a trait tied in with sexual selection (Rojas, 2017). A possible explanation for the evolution of these reoccurring bright colours is that they have developed based on pre-existing colour biases held by predators (Ruxton, Sherratt & Speed, 2004).

The idea of conspicuousness conjures up an image of bright, overt colours, but it can also describe characteristics such as defensive spines, or obvious markings and movement that can attract notice easily without being brightly coloured. Spines can act as both the primary warning signal, and the secondary defence and are additionally effective in combination with chemicals (Speed & Ruxton, 2005). However, the question of how the coevolution of spines and chemicals occurred remains unanswered (Speed & Ruxton, 2005). Features such as eyespots in lepidopteran species perform dual functions, in both adding an element of conspicuousness to the individual, and by provoking a startle or intimidation response in predators (Stevens, Hardman & Stubbins, 2008). These eyespots are said to mimic those of vertebrate predators, such as owls, and could be an important factor in the evolution of this conspicuous defensive trait (De Bona, Valkonen, López-Sepulcre & Mappes, 2015). The zig-zag patterns of the venomous European viper (genus *Vipera*), not only cause a disruptive colouration through movement which reduces the animal's detectability, but whilst stationary acts as an aposematic signal to potential predators (Valkonen, Niskanen, Björklund & Mappes, 2011). Conspicuous behaviour can also act as part of the warning signal of defended species. Defended neo-tropical butterflies have slow and laborious flight behaviour to allow

potential predators time to notice their aposematic signals, in contrast to undefended butterflies which fly erratically and swiftly to avoid predation (Chai & Srygley, 1990).

In terms of how aposematic signals evolve, one of the most text-book examples of predator defence strategies is the 50-million-year-old co-evolutionary arms-race between microbats and moths (Conner & Corcoran, 2012). Microbats use high-frequency echolocation for predation, while moths have the ability to hear these frequencies and take evasive action to avoid the bats, and the bats adapted by changing their frequencies out of the range of the moth's audibility range (Conner & Corcoran, 2012). But in an interesting twist, some aposematic moth genera, such as those within the family Erebididae (the Wasp and Tiger moths) have evolved the ability to produce ultrasonic clicks with their tymbal organs, which act as a warning to bats, signalling their toxicity (Corcoran, Conner & Barber, 2010). This trait is also hypothesised to 'jam' the bats echolocation sonar to degrade their predation precision (Corcoran, Barber, Hristov & Conner, 2011). The mechanism of co-evolutionary traits can also be seen in predator species that have evolved to withstand toxic defences (Marsh & Rothschild, 1974).

4 | Predator learning

Aposematism works on the premise that predators will learn to associate warning signals with unprofitability and avoid prey species displaying those signals in future (Ruxton, Sherratt & Speed, 2004). Using one or more conspicuous signals enhances predator avoidance learning (Lindstedt, Lindström & Mappes, 2008). To fully optimise this lesson, the predator must have an unpleasant experience following the initial predation attempt and associate the warning signal (be it bright colours, odours, or sounds, etc.) with that unpleasant experience (Ruxton, Sherratt & Speed, 2004). The signal also needs to imprint on and improve the predator's memory (Roper & Redston, 1987). Several factors can expedite this avoidance learning. For example, unlearned colour biases, such as innate avoidance of the colour red, can play a role in successful avoidance learning in predators (Halpin, Skelhorn & Rowe, 2008; Raška, Štys & Exnerová, 2017). Multi-modal signals are thought to work best to facilitate learning; these include having colours, odours and behaviours that can be perceived from afar, or after detection occurs (Ratcliffe & Nydam, 2008), however other studies have not supported the additive effects of multicomponent defences on advancing learning rates (Lindstedt, Lindström & Mappes, 2008). Contrasting strongly against backgrounds (Alatalo & Mappes, 1996) and strong signals with conspicuous colours also increase the preservation of the

signal association (Raška, Štys & Exnerová, 2017). Predator vision is in many circumstances difficult to ascertain, and it is not known if most predators can perceive slight variations in signal strength (Arenas, Walter & Stevens, 2015).

It is important to remember that prey species are generally subject to multiple forms of predation (Fabricant & Smith, 2014). Most studies focus on vertebrate predators, particularly birds, as being the main visually-orientated predator of insects, amphibians and reptiles; but other studies have focused on arthropod predators, which have different, yet specialised visual systems (Fabricant & Herberstein, 2014). Warning signals that are perceptible to multiple groups of predators are found in aposematic species such as tiger moths that reflect signals in the ultra-violet (UV) spectrum and produce ultrasonic clicks (Ratcliffe & Nydam, 2008). These moths are not only preyed on by birds that perceive the UV signal, but commonly hunted at night by microbats that can perceive the clicks via echolocation (Conner & Corcoran, 2012).

5 | Chemical toxicity

Aposematism is not confined to targeting visually-orientated predators, as emitted odours can also be used as a deterrent from a distance, or as a contact chemical defence (Weldon, 2013). These chemicals can be sequestered from the species' associated food plant, or synthesized *de novo* (Nishida, 2002) and can contain highly complex mixtures of chemicals (Ruxton, Sherratt & Speed, 2004). For example, *Arctiidae* moths have several chemical defences, including toxic, unpalatable compounds sequestered within the haemolymph from plant allelochemicals, such as cardenolides and lichen phenolics (Nishida, 2002). They also possess a pyrazine warning odour, that warns olfactorial predators, much the same way that the conspicuous colours serve to warn visually-oriented predators (Rothschild, Moore & Brown, 1984).

Chemicals serve as the punishment, or unpleasant experience for the predator, although it is hard to define exactly what 'unpalatable' might be (Mallett & Joron, 1999). Different predators will find different chemicals acceptable or not; quails (*Coturnix japonicus*), for example, will readily consume the larvae of the Monarch butterfly, whereas blue jays (*Cyanocitta cristata bromia*) rather starve than risk a second experience (Marsh & Rothschild, 1974). Aposematic species will often combine several chemicals for defence to broaden the effect of unpleasantness across multiple predators (Marsh & Rothschild, 1974). However, some predators adapt to these chemicals and can even sequester the prey defences. Common garter snakes (*Thamnophis sirtalis*) consume the toxic rough-

skinned newt (*Taricha granulosa*) and, can usurp the newt's toxins for their own defences (Williams, Hanifin, Brodie & Brodie, 2012). There are several costs associated with maintaining chemical defences, for example, finding available food materials, generating and maintaining internal storage for the toxins, and the possibility of, and prevention of, autotoxicity (Ruxton, Sherratt & Speed, 2004), but none so great a cost as a predator that is immune to the chemical defence.

6 | Mimicry

If a prey species is not already aposematic, a common strategy is to mimic one that is. Mimicry is the ability of a species (the 'mimic') to display as similar as possible morphological and behavioural characteristics of a defended species (the 'model') to share selective benefits of survival (Ruxton, Sherratt & Speed, 2004). Mimicry is thought to reduce benefits to model species, unless mimetic species are also defended to some extent (Ruxton, Sherratt & Speed, 2004). There are different forms of mimicry- the extremes of which are Müllerian and Batesian mimics.

Müller's theory was based on the idea that defended and unrelated prey species that occupy the same habitat could evolve to share morphological similarities with each other (Müller, 1878); this resemblance would result in shared mortality costs associated with predator learning but increase survival rates for both models and mimics (Borer, van Noort, Rahier & Naisbit, 2010; Rowland, Hoogesteger, Ruxton, Speed & Mappes, 2010). This is an example of positive frequency-dependence selection, where a phenotype's fitness increases as it becomes more common in an environment (Ruxton, Sherratt & Speed, 2004). Müllerian mimicry can have a mutualistic net benefit for all mimic species involved by increasing survival rates (Rowland, Hoogesteger, Ruxton, Speed & Mappes, 2010). Probably the most studied Müllerian mimic is the neotropical butterflies from the genus *Heliconius* (e.g. Marsh & Rothschild, 1974; Mallet & Gilbert, 1995; Langham, 2004; Baxter et al., 2008; Reed et al., 2011). Several species within this genus form mimetic rings, in which defended species, often together with undefended species, have converged on similar patterns and colours, and cohabit in the same geographic location (Mallet & Gilbert, 1995). Less-defended mimics, or quasi-Batesian mimicry, are speculated to act as parasites within these complexes, siphoning protection, whilst simultaneously degrading it (Rowland, Mappes, Ruxton & Speed, 2010).

Batesian mimicry involves undefended and palatable prey mimicking the characteristics of aposematic prey (Bates, 1862), and when co-habiting the same space as the model species, confer protection to the mimics (Mallet & Joron, 1999). However, the presence of Batesian mimics slows

down the avoidance learning by predators (Ruxton, Sherratt & Speed, 2004). Interestingly, it is common to find Batesian mimics that imperfectly mimic their models, which contradicts the idea that mimics need to closely match their models to gain the protective benefits (Speed & Ruxton, 2010). Explanations for imperfect mimicry include evolutionary lag and costs and trade-off associated with mimicking morphological and behavioural traits (Speed & Ruxton, 2010).

Mimicry-rings are common in complex systems, where each member shares protection and mortality rates through mimicking aposematic signals (Pekár, Petráková, Bulbert, Whiting & Herberstein, 2017). Modelling suggests that mimetic rings are potentially encouraged by the presence of undefended (Batesian) mimics (Franks & Noble, 2003). There is evidence for increased predation rates when Batesian mimicry within a mimicry complex dilute the effect, which reduces the shared protection (Speed, 1999). Multiple-model mimicry involves an aposematic species whose different morphs mimic other defended species (Rönkä, Mappes, Michalis, Kiviö, Salokannas & Rojas, 2018). This polymorphism occurs in many butterfly species, such as the *Papilio*, *Danaus*, *Hypolimnys* and *Heliconius* genera and is influenced by factors, such as sexual selection (Joron & Mallet, 1998), or even escaping from an over-crowded use of the same mimetic form (Smith, Owen, Gordon & Owiny, 1993), resulting in a diversity of the warning signal.

7 | Variation

Up until very recently, it was thought that warning signals were under positive-dependent, or stabilising selection (Ruxton, Sherratt & Speed, 2004). This concept posits that the frequency of a warning signal type should be maintained and that any variation or morph of that type should experience a higher rate of predation due to predator naivety (Figure 1). This results in a population with reduced phenotypic diversity and suppressed variability (Lindström, Alatalo, Lyttinen & Mappes, 2001). Theoretically, variation should be low in aposematic species thereby enhancing successful predator avoidance learning and maximising the retention of memory and recognition (Arenas & Stevens, 2017). However, variation commonly occurs within and between aposematic species, and in most, if not all species, variation is the norm, rather than the exception (Briolat et al., 2018). Several explanations have been put forward to explain the persistent variation in aposematic species. These include both biotic factors, such as trophic levels and abiotic factors, such as environmental factors (Fabricant, Burdfield-Steel, Umbers, Lowe & Herberstein, 2018).

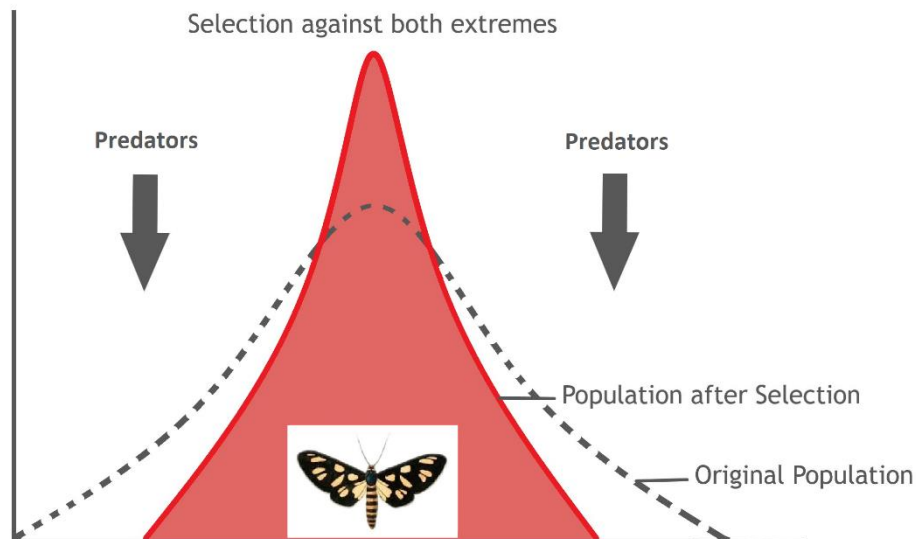


Figure 1 The theory of stabilising selection says that there should be predator-forced selection on prey that favours intermediate variants, reducing phenotypic diversity and suppressing variability

7.1 | Biotic factors influencing signal variation

As predation risk drives the evolution of warning signals, therefore the removal of predators ('predator release') should lead to a reduction in selection for anti-predator behaviour within the community (Blumstein and Daniel, 2005). Therefore, predator release could explain signal variation between populations of the same aposematic species. However, predators also drive variation within populations due to many factors, that include composition of predator community within the population (Ihalainen, Rowland, Speed, Ruxton & Mappes, 2012), individual predator motivation, for example, how hungry or desperate the predator might be (Carle & Rowe, 2014), or the predator's susceptibility to toxic defences (Endler & Mappes, 2004). In some cases, intrapopulation variation in warning signals may decelerate or confuse the predator, resulting in an attack delay. For example, jumping spiders decrease their rate of attack when presented with multiple colour morphs of aposematic firebugs *Pyrrhocoris apterus* (Raška, Štys & Exnerová, 2017). Predator aversion learning needs to occur before a predator knows to avoid aposematic species, and therefore novel morphs will challenge that learning process. Some predators innately have biases against certain colours and along with neophobia, can force selection of warning signal variation by allowing new morphs to persist (Hegna, Saporito & Donnelly, 2013).

Within populations, sexual dimorphism, as a consequence of sexual selection, can result in overall signal variation. For example, sexual selection in *Dendrobates tinctorius* poison frogs has driven the

evolution of bluer arms in males and more yellow arms in females in an apparent interaction between sexual and natural selection (Rojas & Endler, 2013). Strong female preference for brighter colours in male poison frogs in island population of *D. pumilio* contrasts with weaker preferences on mainland populations of the same species, which can lead to divergence (Maan & Cummings, 2009). Male-male competitive behaviour is affected by variation in male dorsal brightness in *D. pumilio*. Brighter males access rival population territories and behave more aggressively and have higher rates of fitness, leading to a promotion of brightness in populations (Crothers, Gering & Cummings, 2011).

Populations may also maintain signal polymorphism if each colour pattern is coupled with another favourable trait (Rojas, Devillechabrolle & Endler, 2014). For example, population of poison frogs *Dendrobates tinctorius* with polymorphic aposematic colouration, expressed specific escape behaviours matched to the warning signal. The more interrupted the colour pattern, the more erratic and unpredictable their changes of direction (Rojas, Devillechabrolle & Endler, 2014). The combination of pattern and movement influenced the predator's ability to assess the prey's trajectory and created the impression of a static position allowing the prey to escape attack (Rojas, Devillechabrolle & Endler 2014). This pattern-movement combination can be seen easily whilst comparing unpalatable animals that have a steady and obvious movement behaviour, with a palatable species that uses erratic and fast avoidance behaviours (Rojas, Devillechabrolle & Endler 2014).

7.2 | Abiotic effects on warning signals

In addition to biotic factors, abiotic factors are likely to play a stronger role in inter-population variation in warning signals, such as the geographic signal in pattern diversity in *Heliconius* mimetic rings (Baxter *et al.*, 2008). Abiotic factors such as temperature and other climatic variables can vary substantially across a species' range, and they have been suggested to cause colour variation between populations. For example, hibiscus harlequin bugs (*Tectocoris diophthalmus*) are redder in subtropical and tropical regions and bluer in temperate regions (Fabricant *et al.*, 2018), correlating with temperature and rainfall. Habitat loss and land clearing are major issues facing biodiversity with human involvement effecting every trophic level (Strong & Frank, 2010). Variation between populations of aposematic species can occur due to the flow on effect of resource limitations over geographic ranges (Blount *et al.*, 2012).

Of course, correlational patterns like these require experimental manipulation to identify the underlying mechanism. Accordingly, harlequin bugs reared in lower temperatures displayed more iridescence than those raised in higher temperatures (Fabricant et al., 2018). Similarly, a laboratory rearing experiment of Monarch butterfly larvae revealed that cold reared individuals had greater melanin production and thus were blacker and had less yellow pigmentation than individuals reared in warmer conditions (Solensky & Larkin, 2003). With the on-going threat of climate change, temperature manipulation experiments such as these give the opportunity to observe how species react to climate-induced change and what types and levels of variation might result.

8 | Conclusion

After decades of research into how predators learn to avoid aposematic signals, we are now turning our attention to appreciating the considerable variation in signals. Variation in warning signals can occur for many different reasons, whether it be predator selection, sexual selection, climate or geographical impact, or combination of those. Biotic factors such as predation, and abiotic impacts such as temperature and precipitation can influence colour expression, leading to intra-specific phenotypic divergence, as well as convergence between species. With our rapidly changing climate and landscape, it is important to study the effects of these changing environments on species interactions as it has implications for maintenance of biodiversity and ecosystem function. Similarly, predators are being lost from the environment at alarming rates with effects on all trophic levels. Aposematic species could be useful bioindicators, in changing ecosystems. Further research is needed to fully understand the mechanisms behind the observed variation in warning signals, with the hope it will allow us to better understand and mitigate further disruption to these natural systems.

Chapter Two

What's *Amata* with this moth? Quantifying variation in warning signals in the genus *Amata* (Lepidoptera: Erebidae) to determine the driving causes

Contributions

Georgina Binns: data collection, data processing and analysis, and writing

Jim Mclean: data collection and statistical assistance

Julia Riley and Andrew Allen: statistical assistance

Mariella Herberstein: study design, statistical assistance and feedback on writing

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Abstract

1. Warning signals protect defended prey from potential predators. The most effective warning signals consist of bright, conspicuous colours that aid in predator avoidance learning. Whilst these signals should theoretically be under stabilising selection and should not vary, variation in warning signals occurs in most aposematic species.
2. The wasp-moths, *Amata spp.*, are classically aposematic diurnal moths that possess red-orange spots against a black background and show conspicuous flight behaviour.
3. To quantify variation in *Amata spp.*, freshly caught specimens from 11 sites in New South Wales, Australia were genetically identified using the CO1 gene, and their warning signals quantified and analysed. Two genera were among the collected specimens; *Amata* and *Eressa*. From the *Amata* genus, two species were positively identified, along with several individuals belonging to the *Amata nigriceps spp.* complex.
4. Focusing on the *Amata nigriceps* complex, I analysed several warning colour traits, such as the number of red wing spots. I found that sexual selection is likely to contribute to variation in warning signals: females had 12 spots and males had 11.
5. However, contrary to predictions, neither climate nor predator diversity had a significant effect on warning colour traits. More in-depth genetic exploration into this species complex is needed to further tease apart identification, as well as intra-specific variation, while predator trials will identify how predators react to variation in *Amata* moth warning signals.

1 | Introduction

Warning signals in prey species signal to potential predators that attempted predation is likely to be unprofitable (Wallace, 1867; Speed, Ruxton, Blount & Stephens, 2010). These signals often consist of bright, conspicuous colours that occur alongside secondary defences, such as unpalatable and often noxious chemicals (Caro, 2017), and this two-fold defensive warning is known as ‘aposematism’ (Poulton, 1890). Primary defences such as conspicuous colours and the accompanying secondary defences are often coupled with other indicators such as odours or sounds, generating a multimodal signal to multiple predators (Ratcliffe & Nydam, 2008). Warning signals aid in predator avoidance learning, thereby reducing mortality rates for aposematic prey species (Lynn, 2005). Theoretically, warning signals should be under stabilising selection, reducing variation in warning signals (Figure 1), as this would dilute the recognition of warning signals by

predators and hinder subsequent learning (Endler & Mappes, 2004; Borer, van Noort, Rahier & Naisbit, 2010; Raška, Štys & Exnerová, 2017).

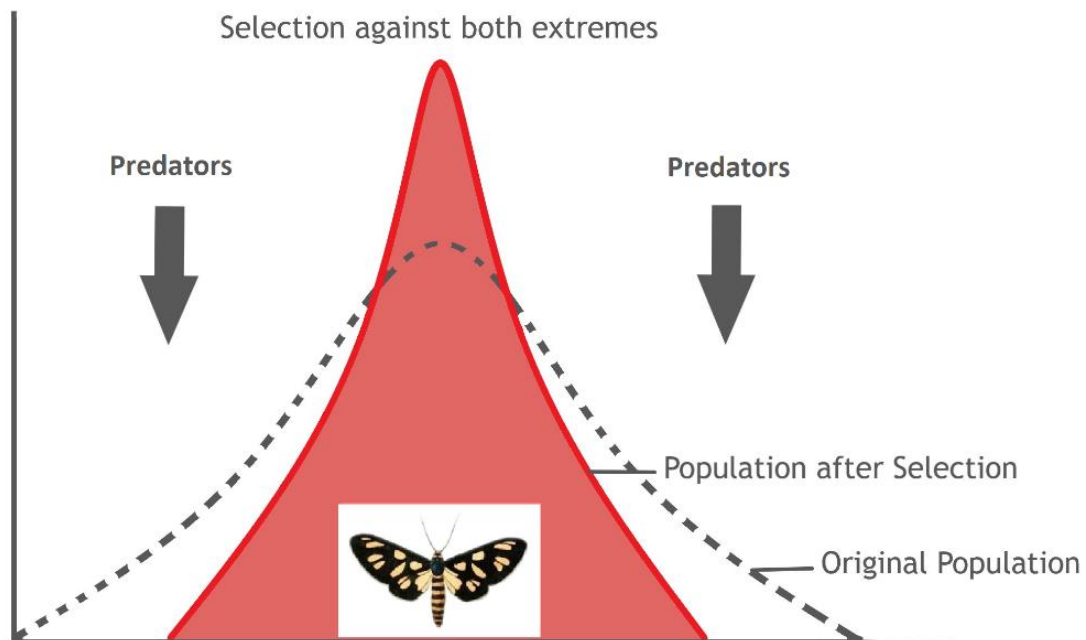


Figure 1 The theory of stabilising selection states that selection against the extremes found within a population should occur, reducing variation. *Amata* moth illustration by Boisduval, 1829

Contrary to theoretical prediction, variation in warning signals is frequently observed. For example, a recent review established that warning signal variation occurs in nearly every taxon that displays aposematism, due to subjection from many biotic and abiotic selective pressures (Briolat, Burdfield-Steel, Paul, Rönkä, Seymoure, Stnakowich & Stuckert, 2018). Several explanations for how this variation might arise have been offered, including a ‘shifting balance’ between changes in environmental factors, predators, pathogens or resource availability (Fabricant, Burdfield-Steel, Umbers, Lowe & Herberstein, 2018). These include abiotic factors, such as temperature and rainfall, that were found to have a strong impact on the phenotypic diversity of the aposematic hibiscus harlequin bug, *Tectocoris diophthalmus*, due to temperature sensitivity in melanin expression (Fabricant et al., 2018). This variation was notably different between populations and is referred to as ‘polytypism’, or variation occurring between populations of the same species (Crothers & Cummings, 2013). The expression for colour was found to be linked to the rearing temperature in the larval life stage of the Monarch butterfly, *Danaus plexippus*, with lower temperatures increasing

the proportion of black colouration (Solensky & Larkin, 2014). Variation might also be brought about by the tension between natural and sexual selection (Rojas & Endler, 2013), as seen in the striking sexual dimorphism displayed in many lepidoptera species. The *Papilio* swallowtail butterflies are classic examples of this evolutionary drive by sexual selection, due to the males often possessing bright colours to attract and signal to females; this in turn can be easily intercepted by predators, leaving them open to predation, and further driving selection in dimorphic signals (Allen, Zwaan & Brakefield, 2011).

The effects of warning colouration and pattern on predator learning have been effectively covered in the literature (Roper & Redston, 1987; Ruxton, Sherratt & Speed, 2004; Lynn, 2005; etc.). Interestingly, aposematic pattern similarity within a mimicry population did not increase the rate of predator learning in wild-caught bird predators (Rowe, Lindström & Lyytinen, 2004), however, it was noted that predators learned to discriminate visually based on the colour of prey species (Halpin, Skelhorn & Rowe, 2013). Bird predators can change their strategies and predate aposematic prey when palatable prey is scarce and hard to find (Carle & Rowe, 2014). Previous bird predator trials have found that conspicuousness is particularly important for strengthening predator recognition of defended prey and intensifying the duration of the learned memory of the unprofitable stimulus (Roper & Redston, 1987). Conspicuousness satisfies the main assumptions of aposematism, in that the signal needs to be recognisable, memorable and consistent, to assist and accelerate predator aversion learning (Ruxton, Sherratt & Speed, 2004).

The lepidopteran genus *Amata*, or 'wasp moths' (Noctuoidea: Erebidae: Arctiinae: Syntomini) contains at least 36 described species occurring in Australia (Common, 1990). The species within this genus are taxonomically cryptic, and their distributions overlap, making them difficult to distinguish from each other (Common, 1990). *Amata* moths are classically aposematic. They are chemically defended by pyrazines (Rothschild, Moore & Brown, 1984; Weller, Jacobson & Conner, 1999), display the characteristic conspicuous colouration associated with aposematism; bright orange spots and stripes against a contrasting black (Endler & Mappes, 2004), and exhibit a slow, almost sluggish flight which might enhance their conspicuousness (Srygley, 2004). Their stripes and diurnal behaviour give them a passing similarity to wasps, which may also enhance their warning signals (Common, 1990).

The aim for this study is to understand the level of variation found in warning signals, using *Amata* spp. as an aposematic model. My first question is whether variation in warning signals is quantifiable; I expected to see variation occurring between species, and limited variation occurring

within species, according to frequency-dependent theory (Ruxton, Sherratt & Speed, 2004). To test this hypothesis, I identified collected individuals, by DNA sequencing of the Cytochrome c oxidase I (CO1) gene. To measure variation in warning signals, colour metrics such as RGB values, and the number of wing spots and their relative size have been quantified to assess the level of variation in those traits. I am further interested in explaining the potential underlying causes of this variation, which may be due to the presence of cryptic species within the genus, sex differences, or geographic variation linked to climate and predator diversity variation. To test these ideas, I compared aposematic traits between sexes and I further hypothesise that conspicuousness and variability in warning colour is affected in predictable ways by variation in predator landscapes and predation risk, as well as variation in climate and abiotic factors. Specifically, when the predator landscape includes a lot of specialists that exclusively prey on insects, I expect conspicuousness to be favoured with little variability (Endler & Mappes, 2004). Similarly, we should see increased melanin expression with decreasing climatic variables such as temperature and precipitation (Fabricant et al., 2018).

2 | Materials and Methods

2.1 | Specimen Collection

Live *Amata* spp. specimens were collected from various sites across the New South Wales east coast, Australia, between November 2017 and April 2018 (Table 1). Individuals were mostly collected opportunistically in urban areas and surrounds, with butterfly nets or collection containers. Specimens were euthanised by placing into -30°C overnight. Specimens were then pinned, dried and labelled, and kept in a display box in a dark cupboard to reduce any altering effects of natural or artificial light. The box included naphthalene moth balls to remove risk of infestation. Notes on morphological characteristics were recorded for all freshly caught specimens. Individuals were sexed via the morphology of the tip of their abdomen (males have a ‘spatula’ shaped tuft, whereas females have a ‘tucked in’ genital opening).

Table 1 *Amata* collection sites in NSW, Australia

Location	Lat/Long
Macquarie University	33°46’25 S 151°06’55 E
Macquarie Park	33°46’38 S 151°07’45 E

Appin	34°11'58 S 150°47'08 E
Lane Cove West	33°48'16 S 151°08'47 E
Wahroonga	33°43'24 S 151°07'01 E
Heathcote	34°05'06 S 151°00'29 E
Bar Beach	32°56'24 S 151°46'04 E
Wyoming	33°24'25 S 151°21'36 E
Newport	33°39'23 S 151°08'47 E
Taree	31°53'22 S 152°16'40 E
Wardell	28°57'00 S 153°28'00 E

2.2 | 'The *Amata* disaster'

Whilst visiting the Australian National Insect Collection (ANIC) in Canberra, ACT, I was advised that “no entomologists want to work on *Amata*, due to their taxonomy being in such a mess” (Y. Su, pers. comms., 13th February 2018). According to Dr. Su, the descriptions for several *Amata* species are contested among researchers, as the holotypes at the Museum of Natural History in London have not been preserved in a useful state for identification. The original Latin description of *Amata annulata* (Fabricius, 1775), initially placed in the genus *Zygaena*, is not extensive, and a subsequent classification into *Syntomis* by Boisduval in 1829 contradicts the original description on several morphological characteristics, whilst omitting others (Boisduval, 1829). It is possible that the two descriptions are describing two different species. Then in 1876, the genus was again reclassified to *Hydrusa* (Butler, 1876) before systematists settled on *Amata* in the early 1900s. A similar story can be told about the description and re-descriptions of *A. aperta* (Walker, 1865; Meyrick, 1886). Recent work on the *Amata* genus suggests that *Amata nigriceps* could be a group containing several subspecies, and that it requires more in-depth elucidation before a ‘definitive identification can be made’ (Marriott, 2014, pg. 29).

2.3 | CO1 gene sequencing for species identification

Due to the cryptic taxonomy within the *Amata* genus, species identity needed to be established using molecular tools. I used the CO1 gene to resolve identification via genetic barcoding. The CO1 gene has been a popular choice by researchers for genetic identification in forensic entomology (Meiklejohn, Wallman, Cameron & Dowton, 2012) and other invertebrates (Folmer, Black, Hoeh, Lutz & Vrijenhoek, 1994). It has been successfully applied to Lepidoptera (Hajibabaei, Janzen, Burns,

Hallwachs & Hebert, 2006; Hausmann, Haszprunar & Hebert, 2011), and more specifically, Erebidae (Zahiri et al, 2012), Arctiinae (Zaspel, Weller, Wardwell, Zahiri & Wahlberg, 2014) and *Amata* (Lu, Su, Luo, Zhu & Wu, 2013). There has been much debate on the efficacy of the CO1 gene for barcoding purposes (Dawnay, Ogden, McEwing, Carvalho & Thorpe, 2007) for various reasons, including the presence of nuclear mitochondrial pseudogenes (numts) (Song, Buhay, Whiting & Crandall, 2008) and the suitability of using other genes to detect sub-species (Schneider, Legal, Dierl & Wink, 1999). However, I selected CO1 since the *Amata* specimens sampled from ANIC previously had been sequenced using the CO1 gene for the Barcode of Life Data System (Ratnasingham & Herbert, 2007), and those sequences could be used for comparison for this project.

2.4 | Mitochondrial DNA genotyping and analysis

Samples of 42 freshly collected specimens were taken for DNA sequencing. Three legs from each specimen were used for DNA extractions. A Qiagen DNeasy blood and tissue kit (Qiagen Australia) was used, following the manufacturers recommended protocol for DNA extraction from insects with the following minor modifications: samples were ground using a micropestle in a 1.5mL Eppendorf microtube after the Buffer ATL addition step; samples were incubated at 56°C for 30 minutes at 400rpm in an Eppendorf Thermomixer for lysis, and 100µl of Buffer AE was used for elution of the DNA for increased concentration.

For CO1 amplification, the primers LepF (5'-ATTCAACCAATCATAAAGATATTGG-3') and LepR (5'-TAAACTTCTGGATGTCCAAAAATCA-3') were used to target 690-bp fragments of the gene (Hajibabaei et al, 2006). All Polymerase Chain Reactions (PCRs) were performed using GoTaq Green Master Mix (Promega, Madison, USA). LepF and LepR thermocycling conditions required the initial denaturation of 1 min at 94°C; 6 cycles of 1 min at 94°C, 1 min 30 s at 45°C, and 1 min 15 s at 72°C; 36 cycles of 1 min at 94°C, 1 min 30 s at 51°C, and 1 min 15 s at 72°C with a final extension step of 5 mins at 72°C. PCRs were performed through an Eppendorf Mastercycler (Eppendorf, North Ryde, Australia). Gel electrophoresis was performed using a 2% gel made from 1.0g of agarose gel, 50mL of Tris(hydroxymethyl)aminomethane (TBE) and 2µl of SYBR Safe® DNA gel stain. A Bioline 100bp HyperLadder™ (Bioline, Eveleigh, Australia) was used for lane reference and 5µl of each sample was applied to the gel wells. Electrophoresis was performed using a Bio-Rad Power-Pac 300 at 100v/100mA for 30mins and a digital UV photo was taken using a Carestream Gel Logic 2200 PRO Imaging system (Carestream Health, Inc., USA), used in conjunction with the accompanying Carestream Molecular Imaging software, v. 5.0.4.44 (see Figure S1 in Supplementary Material). PCR

samples were sent to The Ramaciotti Centre at the University of New South Wales, Sydney, Australia for both forward and reverse sequencing, using the Sangar Sequencing Method and the centre's Core Prep Plus service with Exosap clean up. The nucleotide sequences were then aligned, trimmed and analysed for divergence/similarity through Geneious®, v11.1.5 (<http://www.geneious.com>), and compared using NCBI's BLAST (www.ncbi.nlm.nih.gov/BLAST/) and the Barcode of Life Data Systems to identify sequences to closest species match.

2.5 | Image analysis

Methods used here are similar to those used previously for assessing intra-specific colour variation (Bergman & Beehner, 2008). Digital photographs of all specimens were taken with a Nikon D90, fitted with a UV+NIR cut filter lens, using a Manfrotto MHXPRO-3W tripod, along with a Spectralon® Reflectance Standard for recalibration of images. Images were taken indoors; thus, artificial lighting was used consistently with a T8 LED 7-watt tube light. Raw Nikon Electronic Format (NEF) photos were processed using ColorChecker® Passport v1.1.1 (X-Rite, Inc.) and Adobe Lightroom CC® v1.3 for colour-calibration and camera profiling respectively and saved as Joint Photographic Experts Group (JPEG) files. These images were then white-balanced and cropped in Adobe Photoshop CC® v19.1.3 and the fore- and hindwings were 'cut out' for analysis. For each moth, the left-side wing was chosen, unless damaged or badly angled, in which case, the right-side wing was used. The number of wing spots was counted on the fore- and hindwing of the chosen side. In cases where the wing spot crossed over a vein and were separated by black (for example, the basal patch on the hindwing in Figure 2), these were counted as separate patches. Wing images were run through PAVO, a colour adjacency package (Maia, Eliason, Bitton, Doucet & Shawkey, 2013) in Rstudio, v3.4.2 (R Core Team, 2015) to obtain colour proportion metrics, which relate to spot size and number. All statistical tests are run in Rstudio, unless stated otherwise.

It was speculated that *Amata* species may possess an ultraviolet (UV) reflecting cue as part of their warning signal (Lyytinen, Alatalo, Lindström & Mappes, 2001), and UV images were taken of historical samples from the Australian Museum, Melbourne Museum and the Australian National Insect Collection. However, it was determined that these sampled moths do not possess this signal cue. An example of these images can be found in the Supplementary Material (Figure S2).

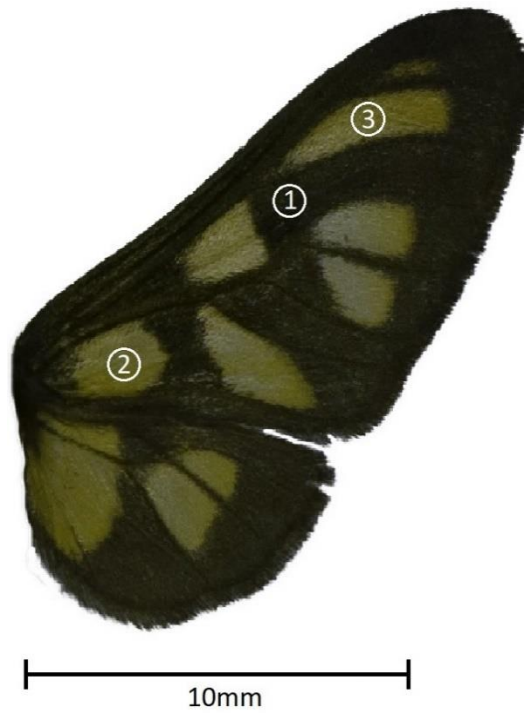


Figure 2 Sample of *Amata* sp. wing used for analysis, showing the three sample areas used to measure RGB values: (1) the ‘black patch’ located between the discal and the subapical regions on the forewing; (2) the red wing spot located closest to the base of the forewing (‘red base patch’); and (3) the red wing spot located at the closest to the apex of the forewing (‘red tip patch’). Note: on this wing example, an additional patch appears closer to the apex of the forewing; this patch only occurs in some individuals and was not used for RGB measurement for sample consistency

Colour was assessed via red, green & blue (RGB) values on a scale from 0, which represents black, the darkest value, to 255, which represents white, or the brightest value (Bergman & Beehner, 2008). RGB values were obtained through ImageJ® v1.51j8 (Schneider, Rasband & Eliceiri, 2012) using the RGB Measure function. Three mean values on each wing were taken, using a sample area of no less than 200 pixels: (1) the black patch located between the discal and the subapical regions on the forewing; (2) the red wing spot located closest to the base of the forewing; and (3) the red wing spot located at the closest to the apex of the forewing (Figure 2). Being most interested in the red colour of the patches, I then created a ratio of red and green values, known as R/G ratio, to be used for analysis. I used this ratio to reduce error rates as a result of multiple analyses using non-independent data (the individual values coming from the same patch of the same individual). The R/G ratio has shown to be useful indicator in turtles (Rocha, Saito, Silveira, De Souza & Ventura, 2008) and mammals (Bergman & Beehner, 2008), and whilst we don’t know how colour opponency

in birds works, previous work suggests this is universal mechanism across all tetrapods (Maturana & Varela, 1982; Bergman & Beehner, 2008).

2.6 | Warning colour variation

I estimated the level of variation in warning colours of recently collected *Amata spp.* specimens. Several metrics were analysed that could contribute to warning colour conspicuousness: 1) the number of red spots on their wings, 2) the proportion of red to black on the wings, and 3) the RGB values of the red spots. Variation was visualised using boxplots in ggplot2 (Wickham, 2016) and Raincloud plots (Allen, 2018) in Rstudio. To assess if there were significant differences in warning colour variables between the collected species, I first visualised variation using ggplot2 boxplots for *Eressa*, *Amata aperta*, *Amata nigriceps* and *Amata nigriceps spp. (complex)* (Figure 4 & 5). I then compared the average and the variance in the number of wing spots, the proportion of red and the 'base' wing patch R/G ratio between *A. nigriceps*, and *A. nigriceps spp. (complex)* using Rstudio base package. The red 'base' wing patch (Figure 2), was selected for analysis because it was hypothesised to be the most important patch for conspicuousness; the part of the wing closest to the body moves less than the tip of the wing, and therefore likely to be the most noticeable to predators.

2.7 | Model Analysis

In order to identify potential causes of warning colour variation across different populations, I obtained climate data and bird predator data for the location of 10 populations from which I collected *Amata* specimens (Table 1).

Climate Data

Bioclimatic variables were downloaded from WorldClim v2 (<http://www.worldclim.org>) with a resolution of 10 minutes (Fick & Hijmans, 2017). The bioclimatic variables are a set of biologically meaningful variables derived from the monthly temperature and rainfall values in the WorldClim dataset, defined for a spatial grid, and downloaded in GeoTiff format files. The variable value for each moth sample was obtained by locating the sample within the appropriate bioclimatic variable grid. Calculations were performed in RStudio using the raster package (Hijmans, 2017). Principle component analyses were performed in RStudio on a set of three temperature variables ('Annual

Mean Temperature', 'Max Temperature of Warmest Month', and 'Min Temperature of Coldest Month'), combined with seven variables representing precipitation ('Annual Precipitation', 'Precipitation of Wettest Month', 'Precipitation of Driest Month', 'Precipitation of Wettest Quarter', 'Precipitation of Driest Quarter', 'Precipitation of Warmest Quarter', and 'Precipitation of Coldest Quarter'). A correlation matrix was used due to having degree Celsius (°C) units for temperature, with millimetre (mm) units for precipitation.

Bird Predator Data

The number of bird predators found within a moth collection site were used to assess whether bird predators influence variation in warning signals. Insectivore (specialists) and non-insectivore (generalists) bird species were identified from the species list in Garnett et al. (2014), based on the expert opinion of Dr Hannah Rowland. The species names list, which use the taxonomy of Christidis & Boles (2008), were mapped to the species names used by Global Biodiversity Information Facility (GBIF) by querying the GBIF species API (<https://www.gbif.org/developer/species>). Several species names were manually corrected. Occurrence records were available within the downloaded data for 412 of the 517 generalist species, and for 31 of the 34 insectivorous birds. Occurrence records were downloaded from GBIF by querying for georeferenced records with no known coordinate issues within the orders Apodiformes, Caprimulgiformes, Charadriiformes, Ciconiiformes, Columbiformes, Coraciiformes, Cuculiformes, Galliformes, Gruiformes, Passeriformes, Psittaciformes and Strigiformes. Occurrences were spatially constrained to the general area of New South Wales, Australia. DOIs for the downloaded data are available in Supplementary Material (Table S1).

Occurrences were grouped into equal area square cells with sides of approximately 0.5 degrees (roughly 56 km). The Shannon diversity index (which is also the Hill number of order $q = 1$; Hill, 1973) was calculated for the species in each cell (Shannon, 1948; Chao, Gotelli, Hsieh, Sander, Ma, Colwell & Ellison, 2014). To account for the variable sampling coverage of different areas, sample coverage was calculated (formula 4a; Chao & Jost, 2012) then cells with a coverage < 95% were discarded from the data. Shannon diversity was then extrapolated or rarefied (Hurlbert, 1971) to a coverage level of 98%, which allows for meaningful comparisons between cells with different levels of sampling completeness. Calculations were performed in RStudio using the iNEXT package (Chao et al., 2014; Hsieh, Ma & Chao, 2016).

General Linear Mixed Effect Models

To test whether environmental climate or bird predator diversity is causing variation in warning signals, general linear mixed models (GLMMs) were used with the response variables (number of wing spots, proportion of red, and 'base' wing patch R/G ratio), with the predictor variables of climate (PC1, PC2 and PC3), bird insectivore and non-insectivore predator diversities, per collection site. The models were fitted using the Generalised linear models function in IBM SPSS Statistics v20.0 (IBM Corp.). The two proportional variables, proportion of red, and the 'base' patch R/G values were transformed by natural log (ln) for modelling purposes. Due to unbalanced specimen sampling at most collection sites, the interactions of sex with the other 5 predictor variables could not be modelled. The climate variables used represented the collection locations; whilst it would have been more appropriate to remove sites where only one specimen was collected (for example, the most northern site, Wardell, NSW), the climate gradient would have been affected, reducing the climate range with which to determine variation. Linear regression graphs were made in RStudio with ggplot2.

3 | Results

In total, 104 individuals were collected between November 2017 and April 2018. Of those specimens, 32 were excluded due to marked or damaged wings, or incomplete collection data. Four individuals were not in the *Amata* genus, leaving 72 individuals for analysis.

3.1 | Barcoding analysis

CO1 gene sequencing was performed on 42 specimens. Of those sequences, two *Amata* species were positively identified, nine *Amata aperta* and eight *A. nigriceps*, a further four *Amata* species were isolated within the *Amata nigriceps* spp. group (one of those species contained 18 individuals), and four individuals belonged to the *Eressa* genus (Figure 3). Nucleotide sequence comparison of approximately 620pb showed the genomic divergence between species, and sub-species of the *Amata nigriceps* complex (See Table S2 for more extensive genomic divergence figures).

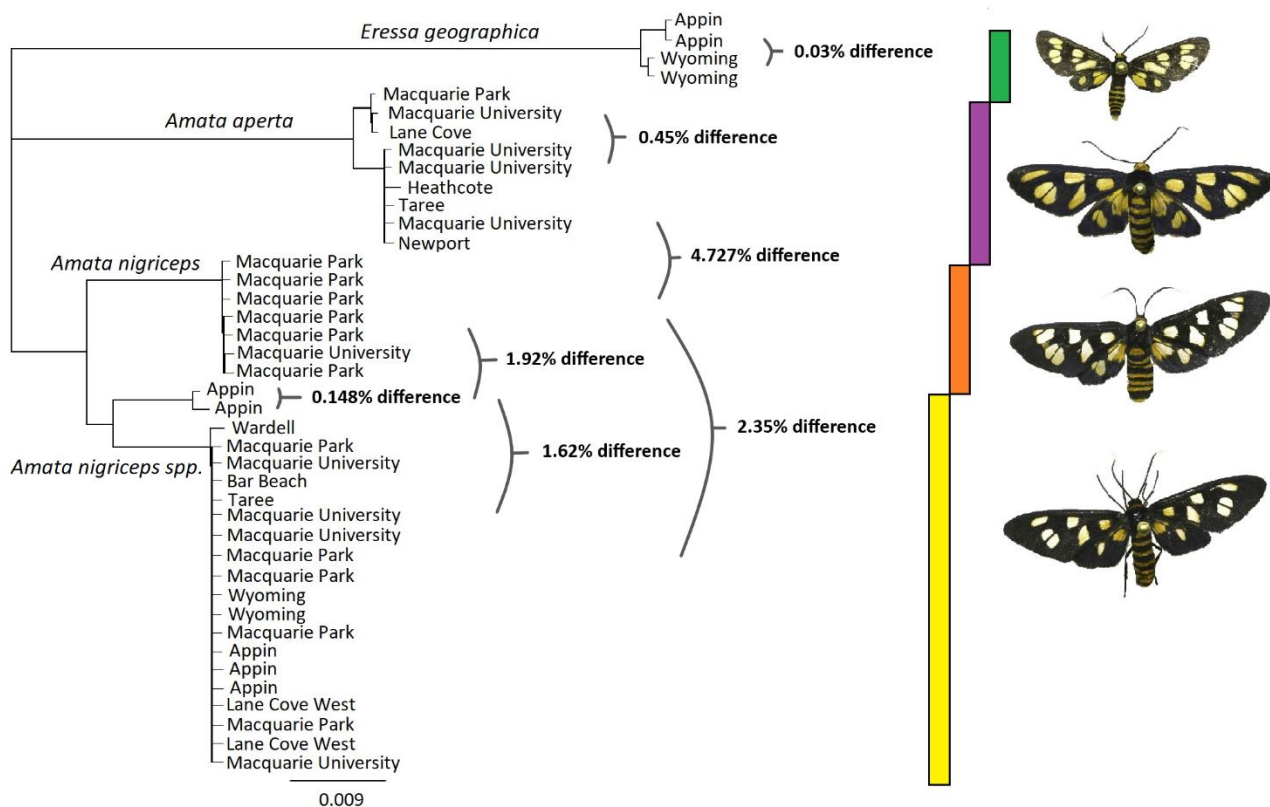


Figure 3 Phylogenetic tree showing the results of the CO1 barcode sequencing performed on freshly captured moths with their collection locations. Values indicate percent difference in CO1 between groups. 0.009 legend refers to number of nucleotide base-pair changes

Morphological characteristics were noted for all freshly collected individual specimens, to aid identification. Some major differences were observed as species-specific characteristics, such as completely black antennae (flagellum) belonging to *Amata aperta*, which is also a larger moth and possesses an orange spot located on the thorax (Table 2). By contrast, *A. nigriceps* and *A. nigriceps spp.* have a white-tipped antenna, no thorax spot and orange furred legs, and are visually indistinguishable from one other. *Eressa geographica* is a significantly smaller moth, has black legs, and the male possess a ‘feathered’ antennae.

The remaining specimens collected that did not have their CO1 gene sequenced were then assigned into the species groups according to these morphological characteristics. Any specimen possessing the white-tipped antennae and orange-furred legs were assigned to *Amata nigriceps spp.* complex.

Table 2 Summary of moths collected and their morphological characteristics

Genus	Species	Total collected	Distinguishing morphological characteristics
<i>Amata</i>	<i>aperta</i>	10	Black antennae with black apex; orange-yellow spots and markings; orange spot on thorax; orange collar connects to orange head; black hair on legs; wingspan approx. 34-42mm
<i>Amata</i>	<i>nigriceps</i>	8	Black antennae with white apex; red-orange spots and markings; orange collar; orange spot on frons (forehead); orange fur on legs; wingspan approx. 27-38mm
<i>Amata</i>	<i>nigriceps</i> spp.	50	Visibly indistinguishable characteristics from <i>A. nigriceps</i>
<i>Eressa</i>	<i>geographica</i>	4	Black antennae, feathery in males; orange-yellow spots and markings, spots often reduced in size and two spots joined on forewing near shoulder; orange spot on thorax; black fur on legs; wingspan approx. 22-25mm

3.2 | Variation between species

The number of wing spots varied across all species and ranged between 9-14 spots. Overall, *Eressa* and *Amata aperta* moths have somewhat fewer spots (Figure 4) and a larger proportion of red:black (Figure 5), but their small sample sizes, and obvious morphological differences did not allow for statistical comparison. Individuals classified as *A. nigriceps* had significantly more wing spots than those classified as *A. nigriceps* (complex) (t-test, $t_{8,9} = 3.160$, $p = 0.011$), while the variance in wing spots between these two groups was similar (F-test, $F_{7, 49} = 1.221$, $p = 0.618$, Figure 4).

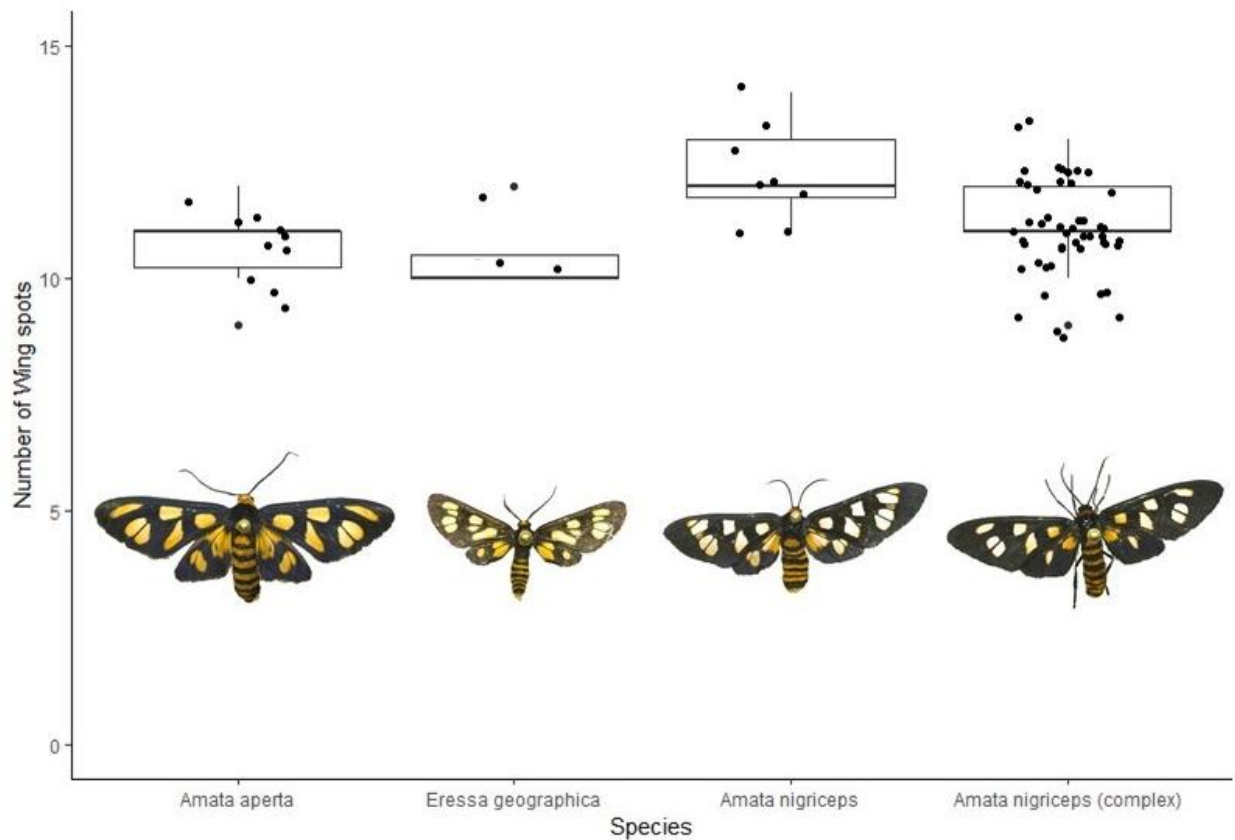


Figure 4 Boxplots illustrating the variation in number of wing spots on the fore- and hind wings of freshly collected moth specimens according to species, with raw data points

The proportion of red on the fore- and hindwings of collected moth specimens ranges from 10-27% overall (Figure 5). The proportion of red:black on the wing was not significantly different between *A. nigriceps*, and *A. nigriceps spp. (complex)* (t-test, $t_{9,4} = 0.610$, $p = 0.556$) and neither was their variance (F-test, $F_{7, 49} = 0.975$, $p = 0.920$).

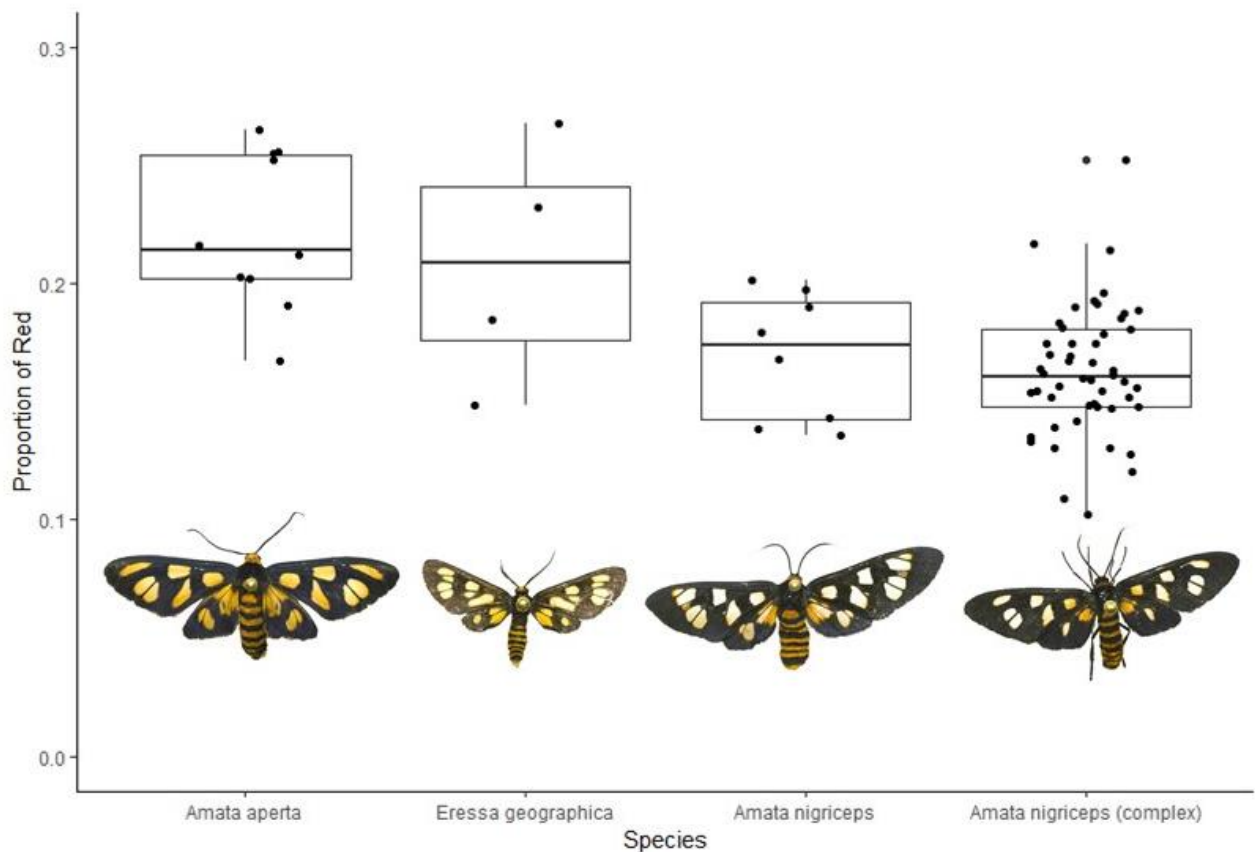


Figure 5 Boxplots illustrating the variation in the proportion of red, on the fore- and hind wings of freshly collected moth specimens according to species, with raw data points

The R/G ratios of the base patch varied greatly among species (Figure 6). *Amata aperta* had a larger colour ratio than *Eressa*, but no statistical comparison was done, once again, due to small sample sizes and morphological differences. The *A. nigriceps* spp. had both a significantly higher R/G ratio (t-test, $t_{32.9} = -6.852$, $p < 0.001$) and a greater variance (F-test, $F_{7, 49} = 0.108$, $p = 0.005$) than the *A. nigriceps* sample.

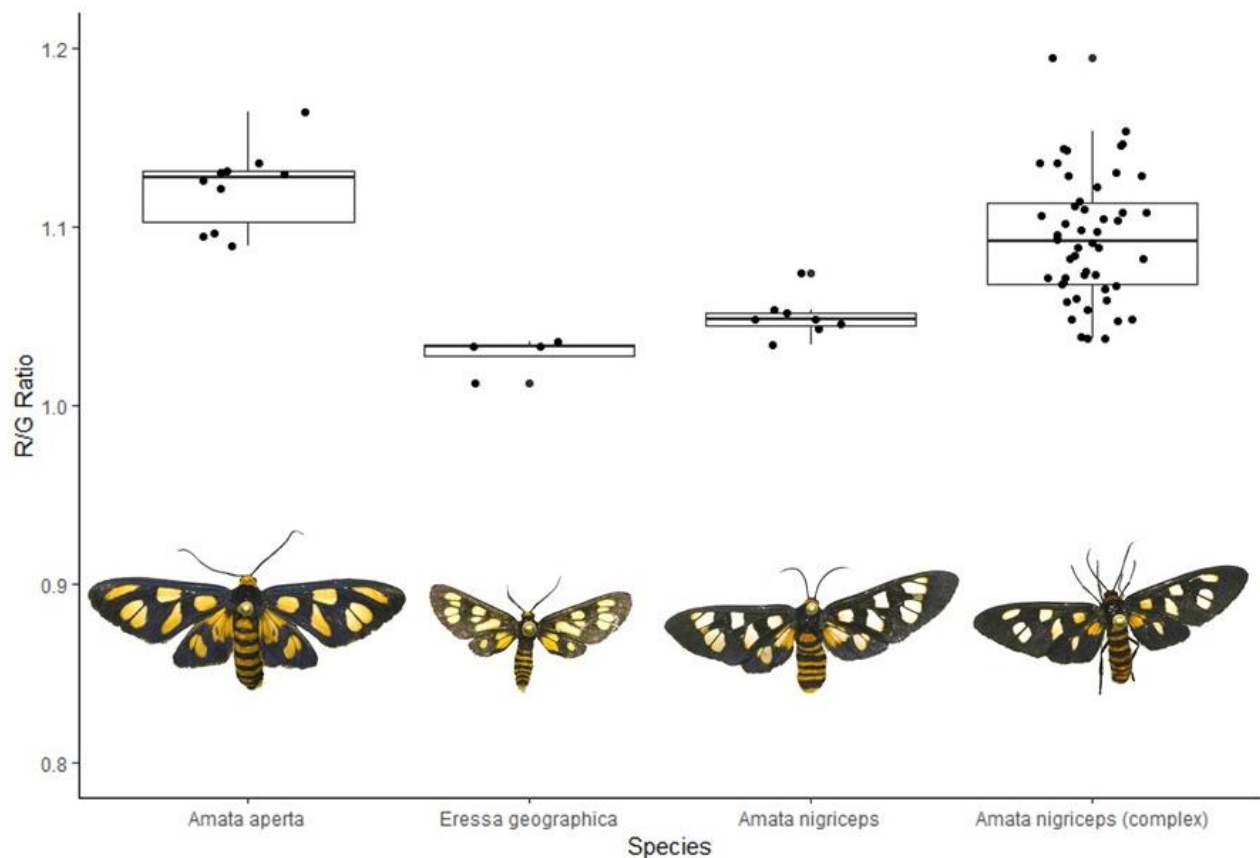


Figure 6 Boxplots illustrating the variation in R/G ratio values from the red ‘base’ patch of freshly collected moth specimens according to species, with raw data points

After consideration of the morphological differences and genetic results, and due to the limited number of specimens collected, I decided to focus further analysis on all the combined *Amata nigriceps* as a complex group. Both the positively identified *A. nigriceps* individuals and the ‘possible’ *A. nigriceps* spp. are included in this group (n = 58). There are, however, significant differences between *A. nigriceps*, and *A. nigriceps* spp. (complex), in both the number of wing spots, and the R/G ratio comparison. This is likely to be due to the small sample size of the *A. nigriceps* group, and even more likely to be influenced by a sex bias. The *Amata nigriceps* samples consisted of four females and four males, whereas the *A. nigriceps* spp. (complex) group contains 1:3 females to males’ ratio. Therefore, it is likely that the differences observed reflect sex biases in both groups rather than species specific differences. Thus, I feel it is warranted combining the two groups together for further analysis. Neither the *Eressa* individuals, nor the *Amata aperta*, were used in further analysis due to the small sample sizes and their obviously different morphological characteristics to *A. nigriceps*.

3.3 | Variation in warning colour traits

The number of wing spots in the *Amata nigriceps* complex varied from 9-14 spots (Figure 4). The proportion of red in this complex varied from 10- 26% of the fore- and hindwing area (Figure 5). I quantified the within-complex variation in the colour by assessing the red, green and blue values (RGB) taken from the forewings, specifically from one black patch and two red patches from each *Amata nigriceps* spp. (Figure 7). The individual R, G and B values for the black patch do not vary significantly from each other ($p > 0.05$). However, for R, G and B, the mean values for the base patch were significantly lower ($p < 0.0001$, in all cases) than those from the tip patches, meaning that the base patches were more saturated, or darker, than those at the tip of the wing.

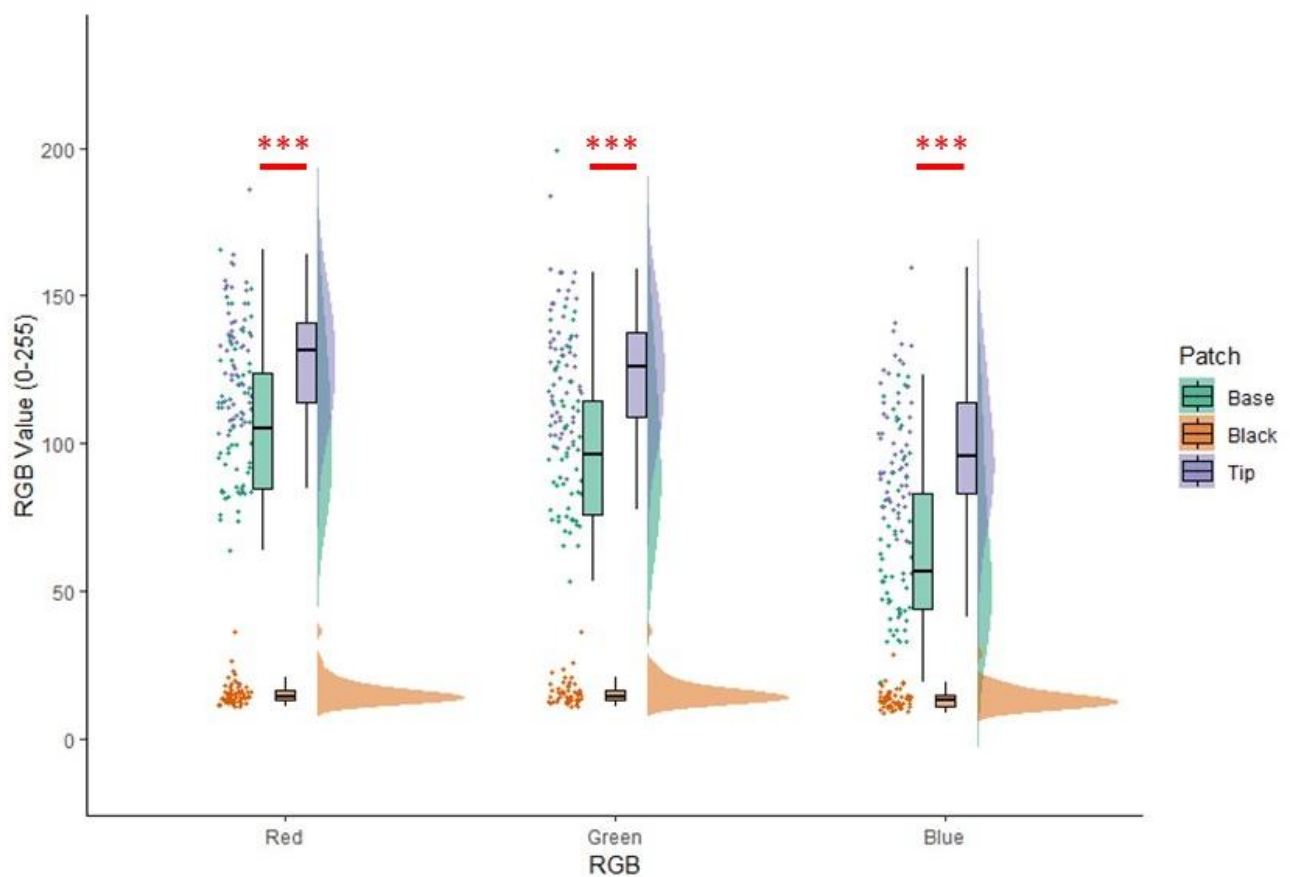


Figure 7 A comparison of variation in Red, Green and Blue “RGB” values between three spot patches located on the forewing of *Amata nigriceps* spp.; (1) the black patch located between the discal and the subapical regions on the forewing (‘Black’); (2) the red wing spot located closest to the base of the forewing (‘Base’); and (3) the red wing spot located at the closest to the apex of the forewing (‘Tip’). Raincloud plot also shows raw data points and distribution of variation. Asterisk ‘***’ represent significant difference ($p < 0.001$) between Base and Tip patches

The base patches, which are closest to the body, are likely to have most impact on the conspicuousness of the warning signal; as they experience less movement than the tip patches and possibly are the most informative for predators. For that reason, only the R/G ratio value for the base patch were used for further analysis.

3.4 | Sexual dimorphism

A possible source of variation in *Amata nigriceps* warning colours could be sexual dimorphism. I found that females carried significantly more wing spots than males (Figure 8a, Table 3). Consequently, the proportion of red on the wing was greater for females than males (Figure 8c, Table 3), but R/G ratio values were greater in males than in females (Figure 8b, Table 3). By contrast, I found no difference between the sexes in how variable these warning colour variables were (Table 3).

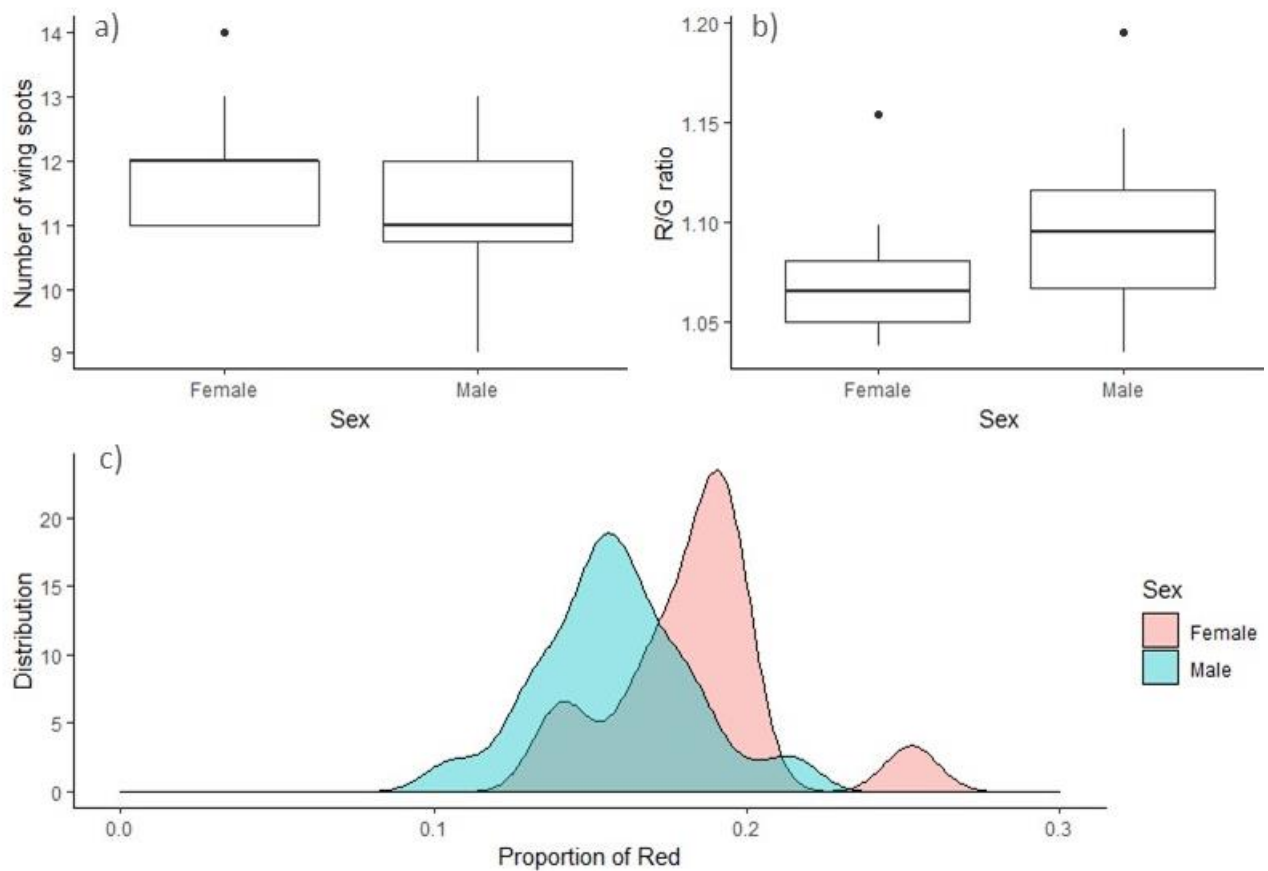


Figure 8 Variation in freshly caught *Amata nigriceps* spp. on a) the number of fore- and hindwing spots, b) 'base' patch R/G ratio, and c) distribution of proportion of red with a comparison between females and male moths (n = 58)

Table 3 Means and standard deviations comparing sexes in number of spots, proportion of red in fore- and hindwings, and ‘base’ patch R/G ratio values.

	Female	Male	Comparison of Means	Comparison of Variance
Number of spots	11.79 ± 0.89	11.00 ± 1.01	$t_{24.5} = 2.77$, p = 0.01	$F_{13, 43} = 0.78$, p = 0.64
Proportion of red	0.18 ± 0.03	0.16 ± 0.02	$t_{20.0} = 3.09$, p = 0.005	$F_{13, 43} = 1.26$, p = 0.55
Base patch R/G ratio	1.07 ± 0.03	1.09 ± 0.04	$t_{25.7} = -2.42$, p = 0.023	$F_{13, 43} = 0.71$, p = 0.514

3.5 | Climate and predator variables

The Principle Component analysis on ten climate variables returned three principal components (PCs) (See Table S2 in supplementary materials for loading values). PC1 explained 61.2% and was negatively associated with the precipitation of the wettest quarter; PC2 explained a further 24.5% and was negatively associated with the precipitation of the driest quarter; and PC3 added 12.9% to the proportion of variance and was negatively associated to the maximum temperature of the warmest month.

Generalised Linear Mixed Model

Three generalised linear mixed models were run, one for each of the three response warning signal variables, with six predictor variables (sex, PC1, PC2, PC3, insectivore diversity and non-insectivore diversity). The first GLMM, modelling the effect of the six predictor variables on the number of wing spots, was not significant (Likelihood ratio chi-square test $\chi^2_6 = 1.178$, $p = 0.978$), and none of the individual predictor variables had any significant influence on the number of wing spots (Table 4; Figure 9, Figure 10).

Table 4 The effect of sex, climate and predator diversity on the number of wing spots. GLMM represents best fit model in Poisson distribution; represents parameter estimates, SE, Wald Chi-square (Z), and significant p-values (in bold)

GLMM1 - Number of Wing spots				
Source	Estimate	Standard Error	Z (Wald χ^2)	p- value
Intercept	4.466	8.451	0.279	0.597

Sex	0.075	0.094	0.634	0.426
Precipitation of wettest quarter (PC1)	-0.030	0.157	0.037	0.848
Precipitation of driest quarter (PC2)	0.052	0.196	0.070	0.792
Max. temperature of warmest month (PC3)	-0.018	0.121	0.023	0.881
Insectivorous predator diversity	-0.154	0.592	0.068	0.795
Non-Insectivorous predator diversity	-0.012	0.673	0.031	0.861

GLMM2 tested the effect of the 6 variables on the proportion of red and revealed a significant model (Likelihood ratio chi-square test $\chi^2_6 = 33.794$, $p < 0.001$), however, sex was the only predictor variable to have any significant influence over the proportion of red (Table 5; Figure 9, Figure 10).

Table 5 The effect of sex, climate and predator diversity on the proportion of red (natural log). GLMM represents best fit model; represents parameter estimates, SE, Wald Chi-square (Z), and significant p-values (in bold)

GLMM2 - Proportion of red				
Source	Estimate	Standard Error	Z (Wald χ^2)	p- value
Intercept	3.865	3.374	1.312	0.252
Sex	0.161	0.397	16.375	<0.001
Precipitation of wettest quarter (PC1)	-0.085	0.063	1.816	0.178
Precipitation of driest quarter (PC2)	0.131	0.078	2.828	0.093
Max. temperature of warmest month (PC3)	-0.024	0.049	0.243	0.622
Insectivorous predator diversity	-0.413	0.238	3.011	0.083
Non-Insectivorous predator diversity	-0.035	0.027	1.638	0.201

The last model, GLMM3 tested the effect of the 6 predictor variables on the effect on the base patch R/G ratio. This model was marginally significant (Likelihood ratio chi-square test $\chi^2_6 = 12.744$, $p =$

0.047), and it was sex, once more, that had a significant influence on colour (Table 6, Figure 9, Figure 10).

Table 6 The effect of sex, climate and predator diversity on the ‘base’ patch R/G ratio (natural log). GLMM represents best fit model; represents parameter estimates, SE, Wald Chi-square (Z), and significant p-values (in bold)

GLMM3 - Base patch R/G ratio				
Source	Estimate	Standard Error	Z (Wald χ^2)	p- value
Intercept	0.840	0.801	1.099	0.294
Sex	-0.023	0.009	5.808	0.016
Precipitation of wettest quarter (PC1)	-0.008	0.015	0.314	0.575
Precipitation of driest quarter (PC2)	0.020	0.018	1.157	0.282
Max. temperature of warmest month (PC3)	0.000	0.012	0.001	0.973
Insectivorous predator diversity	-0.035	0.056	0.386	0.535
Non-Insectivorous predator diversity	-0.007	0.006	1.278	0.258

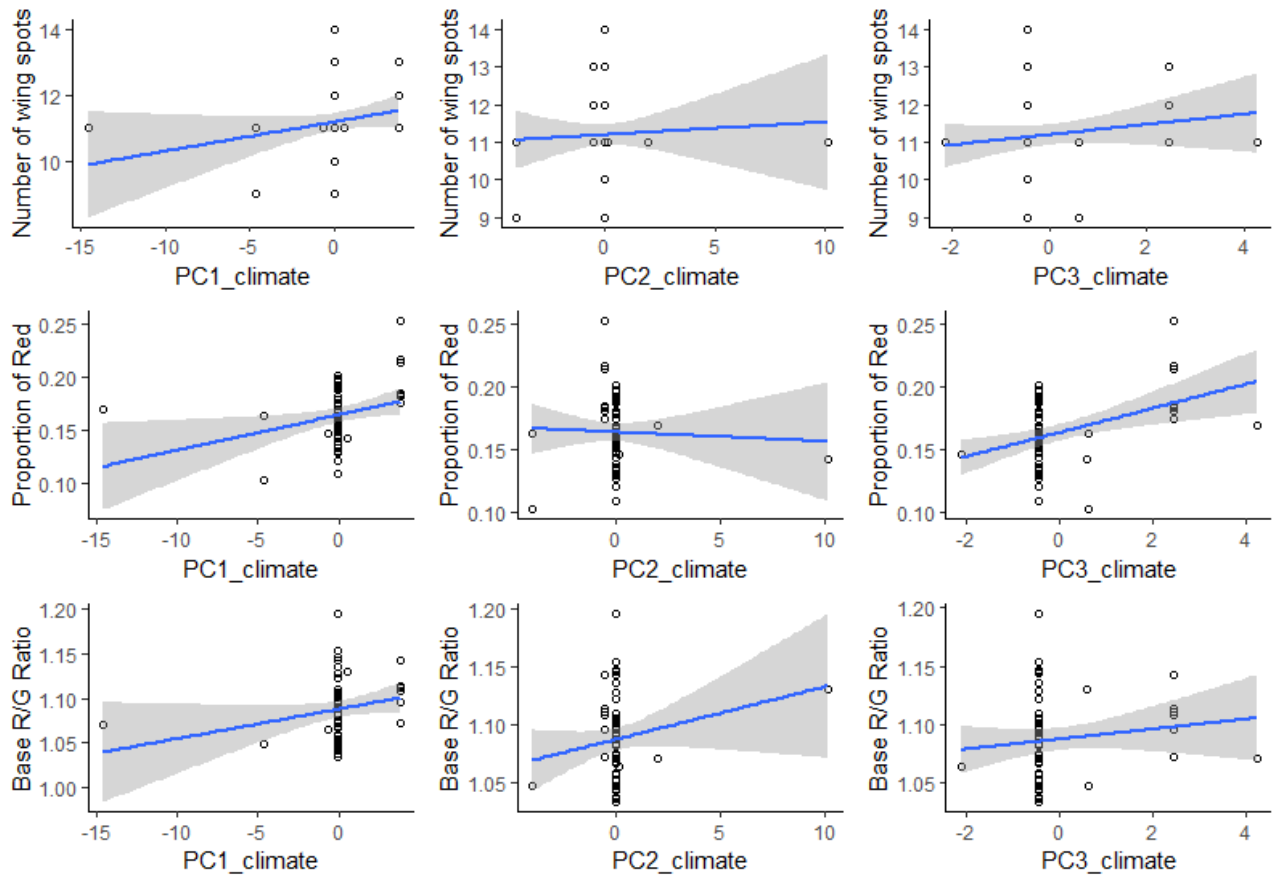


Figure 9 Linear models showing the relationship between the three warning signal variables (number of wing spots, proportion of red, and R/G ratio values), and the three predictor climate PCAs from the moth collection sites. Grey shading shows the 95% confidence intervals

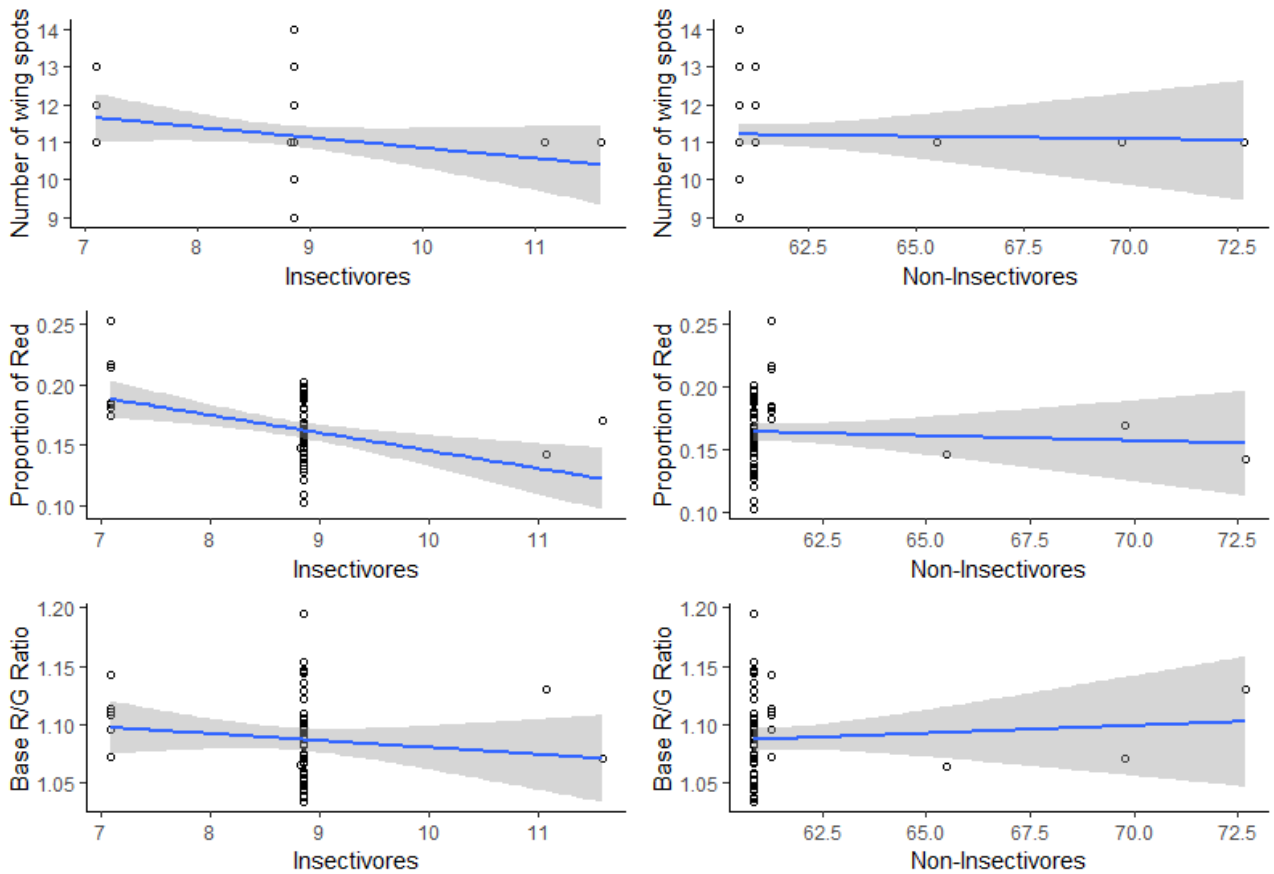


Figure 10 Linear models showing the relationship between the three warning signal variables (number of wing spots, proportion of red, and R/G ratio values), and two predator variables, Insectivores (specialists) and Non-Insectivores (generalists) from the moth collection sites. The predator variable represents the species diversity of each type of predator at the sampled moth collection site. Grey shading shows the 95% confidence intervals

4 | Discussion

Variation in warning signals, whilst no longer seen as unusual, still raises questions as to why it occurs, and what factors may influence the prevalence of variation. In this study, I attempted to quantify the variation found in the conspicuous colour metrics in freshly collected *Amata* moths, using the number of spots and proportion of red to black found on the fore- and hindwings, as well as RGB values. I further predicted that variation in predator landscapes and climatic variables would influence variation found to occur in warning signals. I discuss the implications of my findings below, along with the results of my genetic inquiry into the identification of these aposematic moths.

4.1 | Genetic species identification

In this study, the CO1 sequencing returned positive identification results for three species; two of which were 100% positively identified for *Amata aperta* and *A. nigriceps*, and the other as *Eressa geographica*. The remaining samples possibly belong to the *A. nigriceps* spp. complex, but due to a lack of extensive genetic work on *Amata* and the taxonomic disagreement, I was unable to resolve the more detailed identity of my specimens. Whilst some morphological characteristics, such as antennae apex colour, are useful for distinguishing between species, the characteristics within the *A. nigriceps* complex were virtually indistinguishable between individuals demonstrating why identification of these specimens has been historically difficult (Marriott, 2014). Genital morphology has been previously used to identify cryptic moth species (Skowron Volponi & Volponi, 2017), and that technique may well be useful in identifying the morphological differences in this complex in future along with further genetic work, including the use of molecular markers other than CO1, such as 16S rRNA (Schneider et al., 1999; Lu et al., 2013).

I applied a sequence divergence of <1% difference to determine species identity. This benchmark has previously been used for insects, such as the once-extinct Lord Howe Island stick insect, *Dryococelus australis* (Mikheyev, Zwick, Magrath, Grau, Qiu, Su & Yates, 2017). The genomic divergence I found within the *A. nigriceps* spp. complex suggests that at least three (sub) species (divergence > 1%) were collected within this complex (see Figure 3, and Table S2 in Supplementary Material for values). Despite this genomic divergence, I grouped them together for the analysis of warning signals due to their major similarities in morphological characteristics.

4.2 | Variation in warning signals

The variation in the three warning signal variables found between species was not unexpected (Figures 4-6). However, intra-specific colour variation could occur due to any number of reasons, including genetic disposition, natural selection, or climate variation (Sacchi et al., 2013). Within the combined *Amata nigriceps* complex, I found a substantial amount of variation in the number of wing spots. Variation in wing spot pattern is not uncommon in Lepidoptera. For example, the extensive variation in the warning colours of *Heliconius* butterflies is due to variable repeated elements of conserved gene sequences (Papa et al., 2008).

The proportion of red on the fore- and hindwings describes the amount of wing taken up by the red patches. Higher proportions of red can be attributed to a greater number of spots, or to larger spots.

The proportion of red varied between 10-26% in the *Amata nigriceps* complex, and this correlates positively with the number of spots (see Figure S3 in supplementary material), suggesting that in the *A. nigriceps* complex the increase of red is mostly due to an increase in the number of spots, rather than spot size. Nevertheless, without spot area measurement of the individual spots, it is difficult to confirm that the proportion of red is a function of spot number. Elements of colour patterns diversify and evolve independently of each other and consequently size, shape and position of wing spots or patterns vary considerably among individual Lepidoptera (Nijhout, 2001).

I found that the black patches on the wings did not show much variation in RGB values (Figure 6). This result was not unexpected as melanin production starts in the insect cuticle and melanisation is tightly linked with the cuticle sclerotization, resulting in consistent black pigment production (Matsuoka & Monteiro, 2018). However, the two red patches tested, significantly differed from each other in the RGB values. Pigmentation synthesis occurs late in the pupal stage, not long before the adult insect emerges (Nijhout, Maini, Madzvamuse, Wathen & Sekimura, 2003) and the colour pigment is pumped into the wing scale cells during enclosure (Nijhout et al., 2003). Red colours are likely to be carotenoids (Common, 1990) and other ommochrome pigments (Matsuoka & Monteiro, 2018). The base patch was more saturated, generating a more intense colour than the 'tip' patch. This could either reflect a dilution effect as the pigment is pumped from the body into the wing or be a result of selection. The spot nearest the body of the moth, the 'base' patch, may be important in the warning signal, or conspicuousness, as this area of the wing experience less movement than the tip patch. This idea is consistent with the observation that slow wing beats enhance the effect of warning signals in defended lepidopterans (Chai & Srygley, 1990).

4.3 | Sexual dimorphism in warning signals

Morphological variation between individuals naturally occurs within a species or population (Rojas, 2017). Variation between adult females and males of the same species generally occurs due to sexual selection, and in Lepidoptera, the sexual differences in colour and pattern are often profound (Allen, Zwaan & Brakefield, 2010). However, the difference between the two sexes in the *A. nigriceps* complex are not obvious to the initial inspection. Somewhat surprisingly, I found significant differences in all three colour traits between female and male *A. nigriceps* complex. Females generally had a greater proportion of red, more wing spots, and lower value in the red to green ratio than males (Table 3).

If we interpret these differences between males and females as greater conspicuousness for females, there may be several reasons why females may benefit from greater conspicuousness. It may be that colour in females are signalling not only to warn predators, but also to potential mates. While there is some evidence for sexual dimorphism in moth eyes, male choice is not likely to be responsible for polymorphisms in females (Henze, Lind, Mappes, Rojas & Kelber, 2017). Alternatively, females may be under stronger selection from predators than males. In polymorphic aposematic wood tiger moths, *Parasemia plantaginis*, females with red colouration were less likely to be attacked by great tit predators than females with orange colouration (Lindsteadt, Eager, Ihalainen, Kahilainen, Stevens & Mappes, 2011). However, males of this same species of wood tiger moth show widespread colour polymorphisms and experience higher predator rates from blue tits in white morphs over yellow morph forms (Nokelainen, Hegna, Reudler, Lindsteadt & Mappes, 2011). Whilst females of the *Amata nigriceps* complex expressed more conspicuousness-related warning signals than males, both sexes varied similarly in their warning signal expressions. This could be evidence of predation pressure acting alongside sexual selection (Nokelainen et al., 2011).

4.4 | Climate and predators explain variation

To explain the underlying causes of warning signal variation, I hypothesised that local climate and predator diversity would influence warning signals and thus explain differences between populations. I predicted an increase in red colouration and proportion with increasing temperature and rainfall areas as lower temperatures and precipitation generates greater melanin expression in exothermic invertebrates (Solensky & Larkin, 2014). I further predicted that when the predator landscape includes a high proportion of insectivorous predators, conspicuousness should be favoured with little variability in warning signals (Endler & Mappes, 2004). However, contrary to my predictions, neither climate variables nor predator composition explained the observed variation in warning colour traits between the sampled populations (Figure 9).

Conspicuousness aids in predator aversion-learning and helps predators to retain memory of the warning signals (Roper & Redston, 1987). If we assume that increased number and size of wing spots and brighter red-orange colouration represents greater conspicuousness, than my findings do not support my prediction of conspicuousness in the *Amata nigriceps* complex being favoured in the presence of increased insectivorous predator diversity (Figure 10). We also find that increasing diversity of generalists, who are less efficient at learning to avoid aposematic prey and therefore should select for decreased conspicuousness (Ihalainen, Rowland, Speed, Ruxton & Mappes, 2012),

have little effect on warning signals in the *A. nigriceps* complex. The GLMMs models once again found no significant effect of predators on these warning signal variables.

The non-significance of the models is most likely a result of sample bias. There were not enough individuals sampled in the most northern and most southern parts of New South Wales, thereby limiting the climate range and variation in local predator diversity. A greater contrast in spatial and climatic range could have given a more in-depth understanding of these influences. For example, variation in warning signal plasticity in hibiscus harlequin bugs were measured across three contrasting climatic regions (temperate, sub-tropical and tropical), with over 1100 specimens collected, resulting in strong correlations between climate variables and variation in colouration (Fabricant et al., 2018). An increased sampling size of *Amata* spp. moths may well have led to stronger conclusions in this study.

5 | Conclusion

The present study found that variation in warning signals occurs not only between species collected in the same communities, but also intra-specifically. My attempt to explain intra-specific variation based on local climate and predator diversity was not successful, contrary to predictions. However, the observed sexual dimorphism in the warning signals in *Amata nigriceps* complex, is somewhat surprising. Long-held theories of sexual selection impacting on natural selection suggest that sexual dimorphism is due to female choice and therefore, it is generally lepidopteran males that are more conspicuous. Yet, it appears that *Amata nigriceps* females are more conspicuous than males. This is unusual as it suggests females either signal to males, or require greater protection from predators, which would be intriguing to investigate further, however, conspicuousness itself depends on background contrast and the perception of predators, and those will need to be taken into account in future research. To unambiguously dismiss climate and local predator as influences on warning signals requires further work to increase samples size and sample distribution. The results of this study do however add to the increasing evidence of persistent variation in warning signals.

Final Conclusions

Exploring variation in warning signals in *Amata* moths

Knowledge gap

At the beginning of this year, I had little to no prior knowledge of *Amata* species moths, or their biology, or even knew much about aposematism and variation in warning signals. There is little in the literature about these moths, the last field guide to Australian moths was released in 2007 and the tiny paragraph about the *Amata* genus does not even correctly identify the specimen in accompanying photo, saying “They all appear to belong in a huge mimicry ring... the larvae are very poorly known but probably feed on many different plants.” (Zborowski & Edwards, 2007, pg. 183). We know that there are over 36 described species in Australia, with their range spreading across South-east Asia and beyond (Marriott, 2014), some *Amata* possess chemical toxicity (Marsh & Rothschild, 1974), and their taxonomy has been in a state of constant flux since the 1800s. They can also jam bat sonar with ultrasonic clicks (one of the first facts I found out about these moths, and the one that immediately attracted me to them!) (Connor & Corcoran, 2012).

I was invited to the Australian Museum, the Melbourne Museum, and to the Australian National Insect Collection (ANIC) in Canberra to take (over 1500) photographs of their *Amata annulata* historical specimens for this study. It wasn't until the final trip to ANIC where I fully grasped the enormity of the identification problem within the genus, these moths have been collected since the late 1800s all over Australia, but entomologists consistently had a difficult time sorting them into species. ANIC attempted to rectify this issue some years ago, by teaming up with the Barcode of Life Database, and using legs from historical specimens to extract CO1 gene for genetic identification purposes. The main problem, however, was correctly identifying the specimens in the first place, and Dr Youning Su from ANIC had told me he had spent upwards of two years attempting to sort and separate their specimens alone, and ended up setting them aside, incomplete. I decided that the only way to be able to ensure that specimens I had collected for analysis were the same species, was to do my own molecular analysis on them, something I have had minimal experience in past my undergraduate studies.

Learning curve

Not having any prior knowledge on the *Amata* ecology other than the fact they are diurnal and incomplete collection notes from the historical specimens, I planned a moth collecting field trip with the intention to sample at sites where historical specimens had been collected, with the intention to perform an analysis that compared the variation in their signal spatially and temporally. Planning around other requirements for the Master of Research (i.e. introductory seminar talk), I set off up the north coast of New South Wales in April 2018, about a month too late and returned from 11 collection sites with 9 individuals. The season appeared to be over. Luckily, I had been collecting the moths in my local area since the previous November and had approximately 100 specimens to work with in total.

I learned how to extract DNA from my specimens, and isolate the CO1 gene, perform the PCRs and prepare the samples to send off for sequencing. In the meantime, I had 1500 images of historical specimens from the East-coast of Australia to process, as well as taking and processing images of my fresh specimens. This involved learning to use five new image-processing software's, including Photoshop CC and Lightroom CC by Adobe.

When my sequences came back from the Ramaciotti Centre at the University of New South Wales, I then learned how to use Geneious software to align the sequences and create a phylogenetic tree to understand the results. My results led me to realise that the *Amata nigriceps*, which was my largest group, was likely to be a mimetic ring complex, which was also suggested by another entomologist (Marriott, 2014). Whilst I have no wish to cause conflict with the suggestion that the historical specimens have been misidentified, I have not been able to conduct my intended spatial and temporal comparison analysis due to the inability to confirm the identity of all the specimens, according to their external morphological characteristics, from my images alone. I hope to rectify this after I submit my Master's thesis and complete this analysis for future publication.

My analyses for this project also involved me becoming capable in the R language. Whilst I cannot profess proficiency as yet, I have far exceeded the level I began with at the beginning of the year. Further, I have gained a huge appreciation for the sheer amount of resources available on the internet, as well as my own statistical limitations.

Future Questions

The work I have done this year has only added to the questions I have for future research opportunities with some ideas on how to test these questions.

- How many species are in the *Amata nigriceps* complex, and are they sympatric or allopatric?

To assess the species question, much more sampling is needed, across a greater landscape gradient, to accomplish more in-depth molecular work. This includes more complex gene extractions of mitochondrial and ribosomal DNA markers. Population genetics could also determine relatedness and DArTSeq targeted genotyping would probably be an appropriate method to achieve this.

- What are *Amata* moths' main predators?
- Do some phenotypes experience more predation than others?
- Are females or males more or less likely to be attacked?

It is still very uncertain as to what predator types predate on these moths, in fact, the only animal I have ever witnessed eating them are spiders. Predator trials would be very useful to answer all of these questions, and the use of 3D models and image recorders such as Go-Pros would be essential in capturing predation attempts.

- Are *Amata* warning signals honest about their toxicity?

Answering this question involves chemical testing of freshly caught specimens, comparisons between species and sub-species of the *A. nigriceps* complex, and rearing experiments in the lab on different food sources. Little is known about what the larvae eat, and what is effects different food plants have on their adult toxicity.

- Just how conspicuous is their flight behaviour?

I am interested in comparing the flight behaviour of different *Amata* species using trajectory and movement analyses.

- Can they all speak microbat? Is it all at the same frequency?

Microbats species use different frequencies for echolocation, and I am interested to know whether *Amata* moths in Australia also vary in their ultrasonic click frequencies. This would involve capturing these sounds on ultrasonic detectors.

In Summary

It has been a busy and productive year. Whilst not everything went according to plan, I thoroughly appreciate the need to be adaptive in science. I have thoroughly enjoyed my first adventure into research, the diversity of skills I have learned along the way, and the wonderful and thoughtful academics I've had the fortune of meeting. The mystery of these little aposematic moths still has a fair amount of unravelling needed, but I hope to continue to be a part of their disentanglement. I'd like to once again, thank my supervisor Mariella Herberstein for being such a wonderful mentor.

Supplementary Material

Figure S1 Electrophoresis image of completed PCRs with Lepidoptera-specific primers, LepF and LepR. Bioline HyperLadder [™] 100bp in the left most lane.

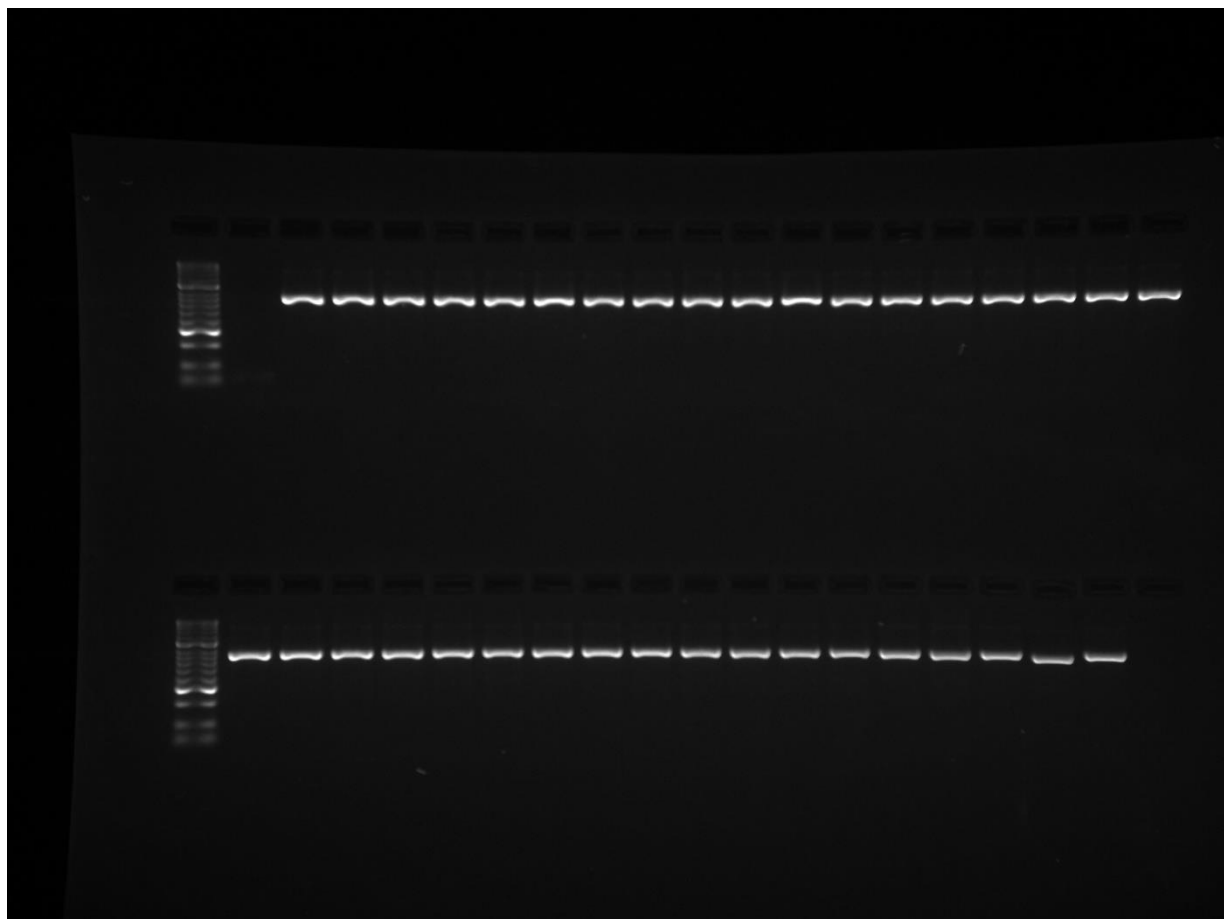


Table S1 DOIs for bird diversity data downloaded from Global Biodiversity Information Facility (GBIF).

Bird Taxon	DOI citation	Number of records
Apodiformes	GBIF.org (26th July 2018) GBIF Occurrence Download https://doi.org/10.15468/dl.6cumao	43804 records from 43 published datasets
Caprimulgiformes	GBIF.org (26th July 2018) GBIF Occurrence Download https://doi.org/10.15468/dl.6euuvm	37550 records from 44 published datasets
Charadriiformes	GBIF.org (23rd July 2018) GBIF Occurrence Download https://doi.org/10.15468/dl.fy0ovv	765597 records from 63 published datasets
Ciconiiformes	GBIF.org (26th July 2018) GBIF Occurrence Download https://doi.org/10.15468/dl.zwkvqn	8506 records from 19 published datasets

Columbiformes	GBIF.org (26th July 2018) GBIF Occurrence Download https://doi.org/10.15468/dl.gwbemg	631342 records from 54 published datasets
Coraciiformes	GBIF.org (26th July 2018) GBIF Occurrence Download https://doi.org/10.15468/dl.qrcgiv	440999 records from 61 published datasets
Cuculiformes	GBIF.org (23rd July 2018) GBIF Occurrence Download https://doi.org/10.15468/dl.7f5hey	199088 records from 46 published datasets
Galliformes	GBIF.org (23rd July 2018) GBIF Occurrence Download https://doi.org/10.15468/dl.obl9du	71858 records from 46 published datasets
Gruiformes	GBIF.org (26th July 2018) GBIF Occurrence Download https://doi.org/10.15468/dl.dtqupk	300411 records from 49 published datasets
Passeriformes	GBIF.org (23rd July 2018) GBIF Occurrence Download https://doi.org/10.15468/dl.3rcinn	9790874 records from 84 published datasets
Psittaciformes	GBIF.org (23rd July 2018) GBIF Occurrence Download https://doi.org/10.15468/dl.mgoxue	1866063 records from 71 published datasets
Strigiformes	GBIF.org (26th July 2018) GBIF Occurrence Download https://doi.org/10.15468/dl.ixeijs	77897 records from 55 published datasets

Table S2 Table of CO1 genomic divergence of freshly sampled moths of New South Wales, Australia

Specimen ID	#1579- #1568 A.nigriceps Confirmed	#1007 A.nigriceps Possibly humeralis	#1564 A.nigriceps Possibly humeralis	#1611 A.nigriceps Possibly phepsalotis	#1547-#1550 A.nigriceps Possibly other	#1566+#1567 E.geographica Confirmed	#1597+#1598 E.geographica Confirmed	#1582-#1603 A.aperta Confirmed	#1554-#1602 A.aperta Confirmed
#1579- #1568 A.nigriceps Confirmed		1.92%	2.07%	2.51%	2.36%				4.73%
#1007 A.nigriceps Possibly humeralis	1.92%		0.15%	1.77%	1.63%				
#1564 A.nigriceps Possibly humeralis	2.07%	0.15%		1.92%					
#1611 A.nigriceps Possibly phepsalotis	2.51%	1.77%	1.92%		0.15%				
#1547- #1550 A.nigriceps Possibly other	2.36%	1.63%		0.15%					5.02%
#1566 + #1567 E.geographica Confirmed							0.03%		
#1597+#1598 E.geographica Confirmed						0.03%			
#1582- #1603 A.aperta Confirmed									0.45%
#1554-#1602 A.aperta Confirmed	4.73%				5.02%			0.45%	

Table S3 Components and their associated variables for the PCA performed on temperature and precipitation. These values were extracted from BioClim and represent the associated value at each moth collection site. +/- values indicate a positive or negative association with each variable within the component. The proportion of variance for each component is also shown. Values in bold show those variable with greatest effect in component.

Climate Variable	PC1	PC2	PC3
Annual mean temperature	-0.864	0.211	-0.453
Max. temperature of warmest month	-0.739	0.393	-0.539
Min. temperature of coldest month	-0.925	-0.019	-0.339
Annual precipitation	-0.927	-0.079	0.367
Precipitation of wettest month	-0.856	0.293	0.419
Precipitation of driest month	-0.352	-0.800	-0.468
Precipitation of wettest quarter	-0.945	0.213	0.219
Precipitation of driest quarter	-0.295	-0.945	0.116
Precipitation of warmest quarter	-0.942	0.193	0.216
Precipitation of coldest quarter	-0.622	-0.733	0.200
Proportion of Variance	0.6117	0.2446	0.1288

Figure S2 Ultraviolet (UV) image taken of a historical *Amata nigriceps* spp. specimen from the Melbourne Museum.

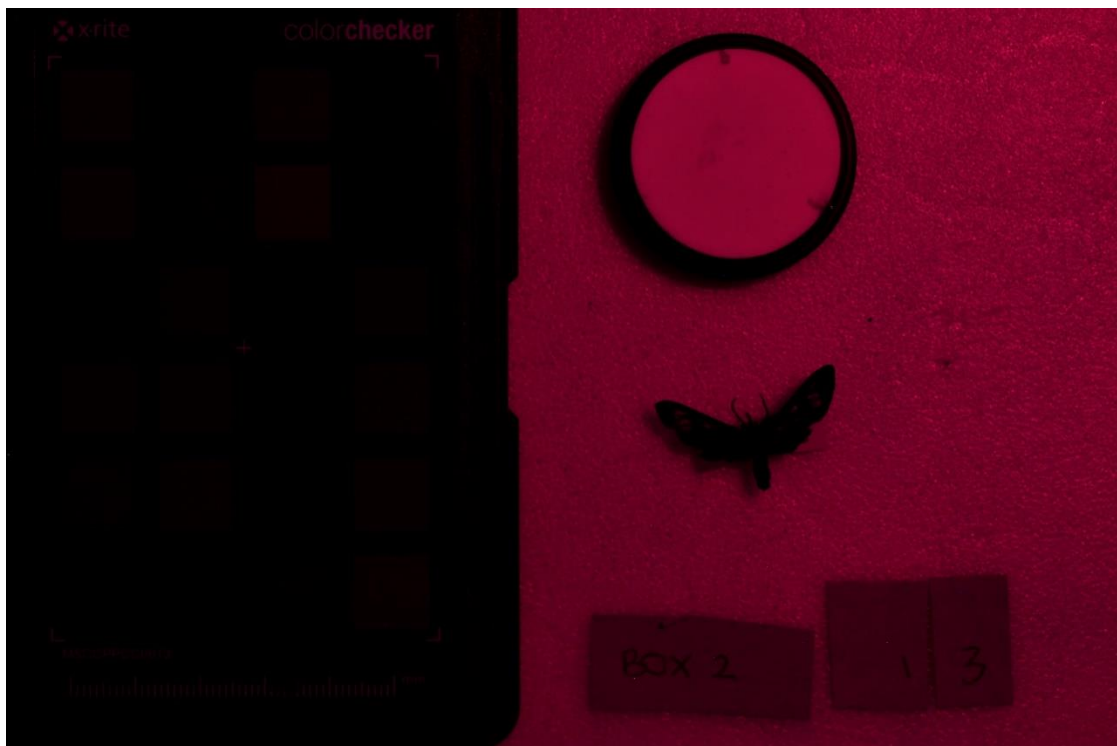
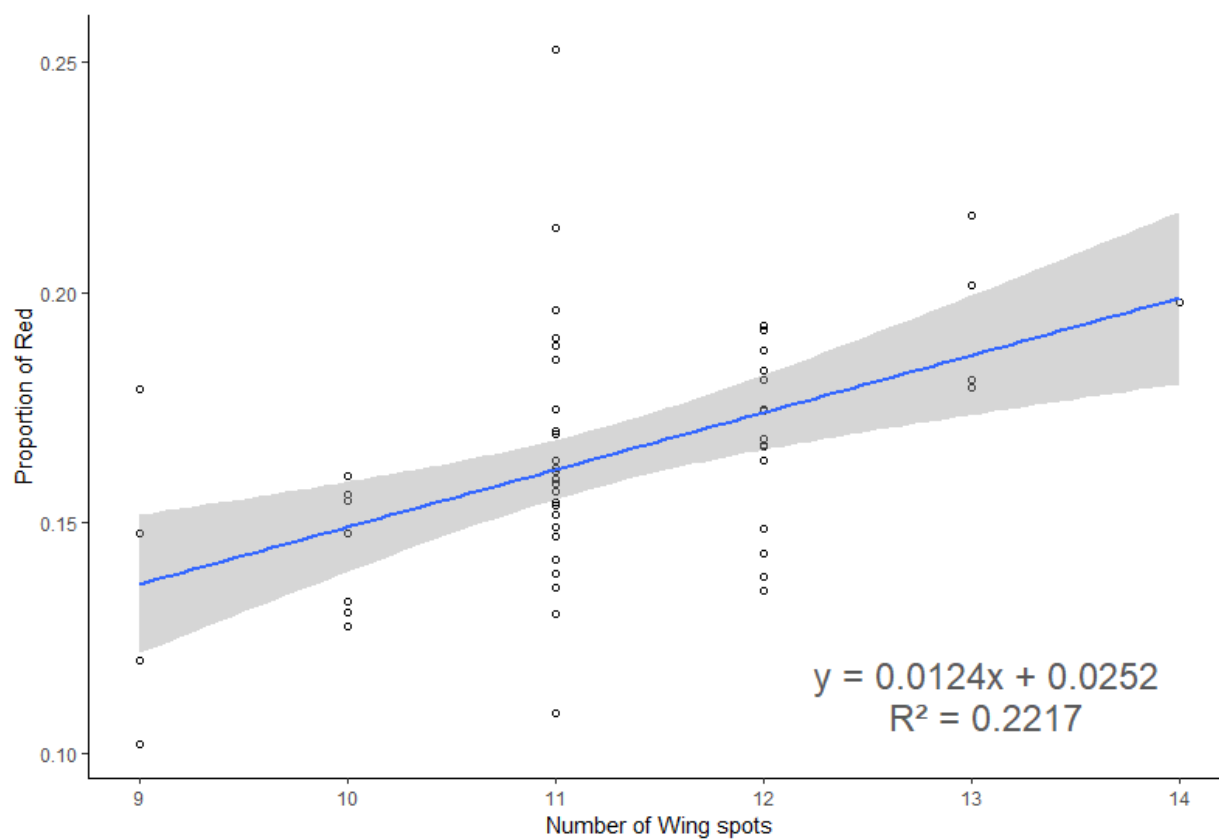


Figure S3 Linear correlation graph illustrating the relationship between the number of wing spots and the proportion of red on the fore- and hindwing of freshly collected *Amata nigriceps* complex specimens



References

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