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# Evolution of Signal Diversity: Predator-Prey Interactions and the Maintenance of Warning Colour Polymorphism in the Wood Tiger Moth *Arctia plantaginis*

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“Nothing in biology makes sense except in the light of evolution”
- Theodosius Dobzhansky, 1973

“Look deep into nature and then you will understand everything better”
- Albert Einstein

“ …or not”
- Katja Rönkä

# ABSTRACT

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Yhteenveto: Peto-saalissuhteet ja paikallisen värimuuntelun evoluutio aposemaattisen täpläsiilikkään (*Arctia plantaginis*) populaatioissa
Diss.

Aposematic organisms avoid predation by advertising defences with warning signals. The theory of aposematism predicts warning signal uniformity, yet variation in warning coloration is widespread. The chemically defended wood tiger moth *Arctia plantaginis* shows both geographic variation and local polymorphism in warning coloration. In this thesis, I studied whether predation by local avian predators is driving the evolution of wood tiger moth warning colours. The close relatives of the wood tiger moth designated here to genus *Arctia* do not show similar colour polymorphism. The wood tiger moth is thus apparently under evolutionary radiation and provides a natural laboratory for observing current selection and studying the mechanisms leading to population divergence. We found evidence of positive frequency-dependent selection as predicted by aposematic theory, but the direction and strength of selection varied geographically. Variation in predator behaviour and the quality and abundance of alternative prey affected selection on wood tiger moth warning colour. Experiments with wild-caught blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*) indicate, that colour is of foremost importance in prey discrimination and avoidance generalization. Birds did not generalize their learned avoidance among morphs, but could generalize between vaguely similar prey species showing similar warning colours. I conclude, that a) the evolution of wood tiger moth warning coloration is driven by predation of local avian predators, b) the direction of selection is affected by variation in predator and prey qualities at different levels (from individuals to communities), which can contribute to the maintenance of polymorphism, and that c) the strength of selection can vary spatially and temporally, and be counterbalanced or overruled by other evolutionary processes, promoting variation in warning colour.

Keywords: Aposematism; generalization; mimicry; polymorphism; predator-prey interactions; warning signal evolution.

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## LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-IV.

I Rönkä, K., Mappes, J., Kaila, L. & Wahlberg, N., 2016. Putting *Parasemia* in its phylogenetic place: a molecular analysis of the subtribe Arctiina (Lepidoptera). *Systematic Entomology* 41: 855–853.

II Rönkä, K., Valkonen, J., Nokelainen, O., Rojas, B., Gordon, S., Burdfield-Steel, E., Tasane, T. & Mappes, J., 2017. Warning signal polymorphism despite positive frequency-dependent selection. Manuscript.

III Rönkä, K., De Pasqual, C., Mappes, J., Gordon, S. & Rojas B., 2017. Colour alone matters: no predator generalization among morphs of and aposematic moth. Accepted for publication in *Animal Behaviour*.

IV Rönkä, K., Mappes, J., Kiviö, R., Salokannas, J., Michalis, C. & Rojas, B., 2017. Can multiple-model mimicry explain warning signal polymorphism in the wood tiger moth? Submitted manuscript.

Table of author contributions to the original publications. Smaller contributions are stated in the acknowledgements of the original papers.

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KR = Katja Rönkä, NW = Niklas Wahlberg, JM = Johanna Mappes, BR = Bibiana Rojas, JV = Janne Valkonen, ON = Ossi Nokelainen, SG = Swanne Gordon, EB = Emily Burdfield-Steel, LK = Lauri Kaila, TT = Tönis Tasane, CP = Chiara de Pascual, JS = Jenni Salokannas, RK = Ronja Kiviö, CM = Constantine Michalis

# 1 INTRODUCTION

## 1.1 Prelude: Why are not all wood tiger moths red and why is it worth studying?

The wood tiger moth *Arctia plantaginis* (Linnaeus, 1758), formerly *Parasemia plantaginis* (see I for taxonomic revision), is a widespread, but rarely seen species, like many other tiger moths (Erebidae: Arctiinae). What has made the wood tiger moth a much studied model system in experimental evolution (e.g. Stevens and Ruxton 2012) is its extraordinary variation in coloration both within and between populations throughout its Holarctic distribution (caterpillars: Lindstedt *et al*. 2008, adults: Hegna *et al*. 2015). Variation in organisms' phenotypes has fascinated botanists and zoologists around the world for centuries and formed the basis for the classification and study of biodiversity. The principles for biological nomenclature that Carl Linnaeus used when describing the essence of *A. plantaginis* and many other species in the 18th century are still used in modern taxonomy (Simpson 1961). Later, with the evolutionary theory formulated in the 19th century by Charles Darwin (1859), it became possible to reconstruct the tree of life based systematically on characters shared by common ancestry.

In explaining the proximate questions on "how" and ultimate questions on "why" biodiversity has evolved, we often refer to the adaptive functions of characters, e.g. how the giraffe has grown a long neck in order to reach leaves at treetops (but see Simmons and Scheepers 1996 for an alternative explanation). It is good to remember, however, that evolution is a process, and does not require any conscious decisions by the organisms (Dawkins 2016). Evolution occurs when organisms 1) reproduce; 2) vary in phenotype; 3) have differential fitness (i.e. differential survival and reproductive success) depending on the phenotype; and 4) inherit qualities from their parents, so that those qualities that made it possible for the parents to have higher reproductive success are passed on to their offspring (Darwin 1859, Ridley 2003). Subsequently, the fittest phenotypes, such as the giraffes with the longest necks, become more and more common in the population over generations. Several mechanisms such as mutation, recombination and gene flow between taxa can create variation, while evolutionary processes such as random genetic drift (Lande 1976) and non-random selection sort the heritable variation. Selection could be induced by e.g. an extreme weather event that changes the biophysical environment of an organism, causing directional selection towards a new, adaptive phenotype (Grant and Grant 2002).

However, nature is filled with examples of variation not easily explained by the current theory – which is interesting, as it gives us opportunities of refining the biological law of evolution just as any law of natural sciences. Some characters, such as the peacock's tail, seem to defy evolution at first sight, as they are most likely costly to bear. However, as characters are inherited through descent, sexual selection can sometimes overrule selection for survival – if females prefer to mate with males with big tails, big tails are selected for despite causing reduced survival (Darwin 1871, Dawkins 2016). Analogically, the spectacular colours of tropical moth caterpillars, found by Henry Walter Bates, seemed puzzling to early evolutionary biologists. Conspicuousness would surely make them more visible to potential predators, and there could be no sexual selection for the phenotypes of immature life-stages are not capable of reproduction. Instead, Alfred Russell Wallace and Darwin suggested that the conspicuous colours of these caterpillars could have a warning function, advertising their toxicity to potential predators.

The theory of aposematism, i.e. prey showing warning signals that predators can learn to associate with their unprofitability and subsequently avoid, has been developed ever since (Poulton 1890, Cott 1940, Ruxton *et al.* 2004, Mappes *et al.* 2005, Rojas *et al.* 2015). Aposematism is thought to be beneficial for both prey and predators – prey can benefit from shared predator education cost trough signal sharing and predators can save energy and reduce risks by not attacking defended prey. Due to predator learning, aposematic organisms are under stabilizing purifying selection against novel phenotypes, leading to signal uniformity (Müller 1879, Endler and Greenwood 1988). Signal sharing between defended species, i.e. Müllerian mimicry, and between a defended model and an undefended mimic, i.e. Batesian mimicry (Bates 1862), are textbook examples of evolutionary adaptation as a consequence of purifying natural selection.

Colour variation in the aposematic wood tiger moth is another unsolved evolutionary puzzle. The moth shows conspicuous colours, such as white, yellow, orange and red combined with black patterns, as do many aposematic organisms. What is paradoxical about this, is that the moth warning colour varies not only between, but also within populations, which is unexpected, if predation is driving the evolution of warning colours towards local signal uniformity as predicted by the theory of aposematism. There is some evidence indicating that a morph with reddish hindwings occurring in Caucasus is the ancestral form (Hegna et al. 2015, I). Red is often shown to be an efficient warning colour against various avian predators (e.g. Gamberale-Stille and Tullberg 1999, Exnerová *et al.* 2006, Lindstedt *et al.* 2011, Svádová *et al.* 2009).

Why, then, has warning colour variation arisen, and how is local polymorphism maintained?

The theory of modification by descent was first formulated to understand how biodiversity came to be, but now this foundational work allows us to examine how biodiversity is maintained. There is a wide variety of possible explanations to the maintenance of phenotypic polymorphism (e.g. Gray and McKinnon 2007), but the relative contributions of different selective pressures maintaining warning colour variation are unknown (Stevens and Ruxton 2012, Chouteau *et al*. 2016). Studying variation in aposematic signaling has yet to offer new insights and detailed information on how natural selection by local predators works, how different selective pressures interact and which evolutionary pathways can lead to diversification. Species such as the wood tiger moth, which are currently under evolutionary radiation, provide a natural laboratory for observing evolution in action, and studying the mechanisms leading to population divergence. New technologies combined with accumulated information on predator visual systems allow new approaches to studying fundamental questions on evolution of coloration (Cuthill *et al*. 2017).

Knowing why the wood tiger moths are not all red, just for the sake of knowing, might seem to add little value to the ordinary life of an average taxpayer. Studies of ecology and evolution are however important, as we are currently in a biodiversity crisis, where extinctions occur at an alarming rate (Ceballos *et al*. 2015), and natural ecosystems are in danger of collapsing. Functional ecosystems are essential for example in food production (Costanza *et al*. 1997). Understanding the mechanisms that maintain variation, the processes leading to adaptation, and detailed ecological interspecies interactions can aid in choosing what and how to conserve with limited conservation resources (Moritz 1994, Stockwell *et al*. 2003). Improved understanding on the signaling functions and evolution of coloration has and will, also likely prove useful for e.g. clothing, military, security or medical applications (Cuthill *et al*. 2017).

## 1.2 Selection on warning colour

### 1.2.1 Predation and aposematism

A predatory event begins when the predator detects a potential prey, and is followed by the predators' decision to pursue the prey and to include it in its diet. This in turn is affected by the predators' capability to discriminate between different types of prey, and to remember previous experiences (Guilford and Dawkins 1991). Prey survival strategies against predation associated with coloration are divided roughly in two, which are rather the extremes of a continuum: camouflage and aposematism. Camouflaged or cryptically coloured organisms try to escape predation by blending into their backgrounds or resembling inedible objects common in their habitats, thus avoiding being detected or recognized as prey by the predators (Merilaita *et al*. 2017, Nokelainen and Stevens 2016).

In contrast, aposematic organisms advertise their defenses against potential predators with warning signals, which often include conspicuous coloration (Ruxton *et al* 2004, Rojas *et al*. 2015). The term *Aposematism* translates from Greek into "away sign" (Poulton 1890). Thus, warning coloration has an important signaling function between the prey and potential predators as signal receivers. Warning coloration is expected to be under strong selection by predators, because it directly affects individual survival. From the predators' point of view, avoiding ingesting toxins or even avoiding spending energy in futile attack attempts is clearly beneficial. If the warning signal can provide a reliable cue of unprofitability, then predators will benefit from being able to detect such cues and use them to discriminate between edible and inedible prey. According to foraging theory, predators trade-off their time used foraging between exploration gathering information on prey profitability, and exploitation based on the information already gathered (Sherratt 2011). To optimize their time use, predators will therefore select for more salient warning signals, i.e. for those cues that are learned more quickly, or easy to remember.

Signal saliency is the property that makes a given signal stand out from other signals. Saliency depends on the qualities of the signal itself as well as of its receiver's (e.g. sensory system, perceptual and cognitive capabilities of the predator), and on the environmental conditions under which the signalling occurs. Saliency facilitates learning, and thus predators learn to avoid more salient warning signals faster than less salient ones. Features that facilitate detectability, discrimination, learning and memorisation of the signal in future encounters are e.g. signal size and conspicuousness (Roper and Redston 1987). Conspicuousness is affected by contrasts in hue or luminance within the warning signal itself, as well as against its background (e.g. Aronsson and Gamberale-Stille 2009, 2012). In nature, aposematic prey are often visible on natural brown or green backgrounds and warning signals often include bright colours, such as red, yellow, white or iridescent blue/green combined with a contrasting black pattern (Poulton 1890, Ruxton *et al*. 2004).

Warning signals can take other modalities, such as behaviour, sound, odour or taste, but coloration is thus far the most studied (Ruxton *et al*. 2004). Examples of warningly coloured prey include chemically defended insects (butterflies: Merrill *et al*. 2015, moths: Conner 2008, ground bugs: Sillén-Tullberg *et al*. 1982, beetles: Marples *et al*. 1994), crustaceans (Mäthger *et al*. 2012), nudibranchs (Winters *et al*. 2017), snakes (Smith 1975, Valkonen *et al*. 2011), amphibians (e.g. frogs: Rojas 2017, salamanders: Hensel and Brodie 1976) and even birds (Dumbacher *et al*. 2008), mammals (Stankowich *et al*. 2011) and plants (Lev-yadun 2009). Humans are able to discriminate colours rather well, but different types of predators vary in their visual perception abilities. For example, most birds' colour vision is based on four single cone types, meaning that they can see ultraviolet wavelengths, and oil droplets, which aid in discriminating colour, providing them a far better colour vision than mammals (Bennet and Théry 2007). Colours are seen as light reflected from the coloured patches reaches the viewer’s eye and can be measured as the light intensity (photon lux) at each wavelength spanning the full spectrum of light (Endler 1990). Birds see spectral wavelengths ranging from 300 to 700 nm, but the intensity of light reaching the viewer’s eye is also affected by ambient light conditions (e.g. time of day) and qualities of the transmission medium (e.g. air, fog). Theories of warning coloration functions have traditionally stemmed from human point of view – thus their validity needs to be confirmed experimentally.

Adding levels of complexity to the evolution of coloration, warning signals can also have multiple components, like the warning colour combined with black patterning, with each component contributing to the warning function. As different predators use different cues for prey recognition, warning signals often consist of multiple modalities in addition to colour (Guilford and Dawkins 1991, Rowe and Halpin 2013, Ratcliffe and Nydam 2008) and these different modalities can also interact. Different cues may also function at different steps of the predatory event, prior to, during or after the attack (Ruxton *et al*. 2004). A colour pattern may be aposematic when examined close, but blend into the background when viewed from a distance (Tullberg *et al*. 2005, Barnett and Cutthill 2014). Warning colour can also induce unlearned innate avoidance (Caldwell and Rubinoff 1983, Lindström *et al*. 1999), fear of the new, i.e. neophobia (Coppinger 1970) or dietary conservatism (Rabinowich 1968, Marples and Kelly 1999). These “special effects” of warning signals could somewhat counterbalance the cost of being easily detected by predators (Skelhorn *et al*. 2016). Predators may also rely on some cues, discarding others, due to limitations in their cognitive abilities. The cognitive processes behind attack decisions, however, are not well understood (Skelhorn *et al*. 2016). For example, it is now known that predators can use social information on prey palatability (Landová *et al*. 2017, Hämäläinen *et al*. 2017), which can have important implications to the evolution of warning signals (Thorogood and Mappes in press).

When tasting the prey, predators gain information on its nutritional value and defense level, and can either learn to use them as a source of nutrition or avoid them in future encounters (Skelhorn and Rowe 2006, 2007). Avoidance learning occurs when predators form a negative association between prey warning signal and the negative experience with prey defense as they sample the prey. Once predators have learned the association, they can generalize their learned avoidance to other prey sharing a similar cue (or cues) to avoid unprofitable predation attempts. The protection gained by aposematism is thus higher against educated predators, which already have experienced the negative outcome of attacking and/or consuming defended prey. Predator behaviour is often described with learning curves that are assumed to reach an asymptotic phase, where the predator has completely seized attacking. However, experienced predators have been shown to sometimes continue sampling the aposematic prey even after avoidance learning (e.g. Barnett *et al*. 2007, Marples *et al*. 1994). Sampling aposematic prey might be profitable under some circumstances, for instance, if the warning signal is not a reliable cue of prey profitability. In Batesian mimicry systems, undefended prey mimics the warning signal of a defended prey. Thus it is profitable for the predators to sample also warningly colored prey. The more common the undefended Batesian mimics are, the more the predators will sample of both mimics and models (Lindström *et al*. 1997, Rowland *et al*. 2007, Jones *et al*. 2016).

Attacking can result in rejection or consumption of the prey. As the prey may vary in their level of palatability, predators may use either a "go-slow" strategy, where they sample the prey cautiously, or "taste rejection", which happens during the attack (Guilford 1994, Skelhorn and Rowe 2006). Escaping predation could take place at any time during this process, but the further the process proceeds, the more costs it incurs to both predators and prey, in form of e.g. risk of damage on both parties. Aposematism is most effective when the prey itself can survive a predator attack, but it can also work via kin selection, where related individuals carrying similar phenotypes suffer a lower predation risk, especially in the case of gregarious prey (Järvi *et al*. 1981). Aggregation itself can also reduce the predation risk of an individual aposematic prey (Riipi *et al*. 2001).

A predator's motivation to attack an aposematic prey is affected by its nutritional state (Barnett *et al*. 2007, 2011), but also by its assessment of future prospects (Skelhorn *et al*. 2016). According to optimal foraging theory (Stephens and Krebs 1986), predators weigh their attack decisions based not only on the quality of a certain prey, but its quality in relation to other prey available. However, not much is known about how the predators estimate future food availability. Also, despite being distasteful or even toxic, aposematic prey are a source of nutrition for the predators. Specialist predators may be immune to a certain prey's defence (Brodie and Brodie 1990), or learn to handle them to avoid ingesting the toxins (Valkonen *et al*. 2012). Thus, predators may assess their attack decisions based on their physiological state, i.e. toxin load vs. starvation, and the availability and quality of alternative prey. The level of defense may vary between individuals of the aposematic prey, as well as between the aposematic prey and other prey. Thus, the aposematic prey does not necessarily need to be toxic to avoid being attacked. In some cases a mere distastefulness or agile fleeing ability may be enough because, when given choice, predators will selectively forage on the most palatable prey items (Davies 1977). The stronger the defence, however, the higher the cost of attacking is for predators (Skelhorn and Rowe 2006).

### 1.2.2 Density- and frequency-dependence

The traditional view of predator learning assumes that predators sample a fixed number of the defended prey before they stop attacking them (Müller 1879). This assumption provides clear predictions of purifying selection in the form of positive frequency-dependence on warning signals. Rare forms are disfavored by selection because predators have not learned to avoid them. Aposematic prey thus have strength in numbers, and the per capita predation risk decreases with increasing abundance. Given that the association between prey warning signal and defence has to be learned, the benefits of signal sharing depend on both the rate of encountering the signal (Müller 1879) and signal honesty (i.e. is the negative association enforced or decreased during the encounter; e.g. Speed and Turner 1999). The rate of encountering the signal depends on both frequency of the signal amongst all available prey and prey abundance, and is thus both density- and frequency-dependent (Müller 1879, Heino *et al*. 1998). There is a strong line of evidence for selection against rare or novel warning colour patterns by avian predators (e.g. Mallet and Barton 1989, Kapan *et al*. 2001, Noonan and Comeault 2009, Chouteau and Angers 2011), but positive frequency-dependent selection is not always found in the wild (Endler 1986), and warning colour variation is widespread amongst aposematic organisms (Joron and Mallet 1998, Briolat *et al*. in review).

The assumption of a fixed number of sampled prey has been criticized, as individual predators and different predator species vary in their speed of learning, and other, unlearned, features of predator decision making have been acknowledged (Skelhorn *et al*. 2016). Models taking into account the variability in predator and prey community, the spatial distribution of predators and prey, and predator behaviour, however, may or may not allow for polymorphism to be maintained (Speed 1999, Kokko *et al*. 2003, Endler and Rojas 2009). An even bigger problem causing disparity between models of selection and observed variation in the wild is, that the selection on warning colour is most often studied in the realm of phenotypic change at short time scales, i.e. as changes in phenotypic frequencies. The frequencies however vary in the wild not only due to selection but also because of temporal and spatial variation at the population level as the densities of both predators and prey vary. Extending the theory of frequency-dependent selection on aposematism to account for density-dependence is not straightforward (Heino *et al*. 1998). Perhaps for this reason, the actual densities of the focal prey and their predators are often ignored, not to mention the density of alternative prey in the community (but see Kokko *et al*. 2003). Prey density can have surprising effects on predator behaviour, as even defended prey may become preferred by predators when common (Mappes *et al*. 2005), and aggregations of aposematic prey can decrease their per-capita predation risk due to increased conspicuousness of the signal (Riipi *et al*. 2001). In order to take into account the temporal and spatial variation in ecological communities to the evolution of warning colour, a more comprehensive population dynamics modeling is needed.

### 1.2.3 Mimicry and alternative prey

The survival benefit gained by signal sharing also readily explains why some species converge to resemble other defended species, forming mimicry rings (Müller 1879, Sherratt 2008, Stuckert *et al*. 2014). Once the protection is established, mimicry is prone to cheating both within and between species. Variation in prey defences can be caused by differential resource allocation, but also by dietary constraints (e.g. Ojala *et al*. 2005, Lindstedt *et al*. 2010). Predators also vary in their capability of tolerating different compounds occurring in chemical defences, as well as in handling defended prey (e.g. Veselý et al. 2017, Exnerova *et al*. 2003). Together, these sources of variation lead to a wide spectrum of palatability amongst prey communities, rather than to a strict division into unpalatable and palatable prey (e.g. Brower *et al*. 1968, Speed 1999). Variation in palatability will create changes in the direction of selection, as the prey interactions change from mutually beneficial to parasitic and vice versa.

Parasitic co-prey interactions have been suggested to select for warning signal polymorphism. When the proportion of edible Batesian mimics increases, predators benefit from sampling, and the risk of being eaten increases for all prey sharing the same signal. This decrease in protection may cause selection for the better-defended model to evolve away from the shared phenotype (Ruxton *et al*. 2004). If different Batesian mimics co-occur with the defended prey in different areas, the defended species may diversify into several allopatric forms, i.e. purifying selection may lead to polytypism. If individuals of different morphs then migrate between populations, depending on the level of gene flow and how the colours are inherited, local polymorphism could occur.

The importance of positive mimetic interactions between aposematic prey to biodiversity is not fully understood (Gross 2008, Elias *et al*. 2008). A theory of quasi-Batesian mimicry, where a less-defended species mimics a more strongly defended model, has been suggested to explain warning colour polymorphism (Mallet and Joron 1999). Due to the density-dependence of mimetic protection, the less-defended quasi-Batesian mimics (as well as Batesian mimics) are predicted to have a tendency to become polymorphic (Speed 1993). Empirical evidence for quasi-Batesian systems is however scarce (but see Rowland *et al*. 2010), and the assumptions made of predator behaviour in modeling mimetic relationships, such as the rates of learning and forgetting at different prey densities, may not be accurate (Mallet and Joron 1999). The remarkable case of *Heliconius numata*, which has several sympatric morphs mimicking different Müllerian models (Joron *et al*. 2011), shows that polymorphism can occur also when the mimic is not more palatable than the model (Arias *et al*. 2016a). The maintenance of multiple morphs however still remains a puzzle, as multiple-model mimicry does not necessarily provide an evolutionarily stable equilibrium under purifying selection.

Even less is known of about how the abundance of non-mimetic alternative prey affects these mimetic interactions. Amongst the biotic factors creating selection, predator-prey interactions are intensely studied, but not often taking into account the whole ecological community (Ruxton *et al*. 2004). The evolutionary outcomes from co-evolutionary arms race between prey defences and predators might be surprisingly much affected by the abundance of alternative prey or variation in the level of defence (or honesty) within the prey community. For example, increasing abundance of edible prey (in relation to predators) is expected to decrease the attack risk for all aposematic morphs, especially if predators optimize their foraging by choosing non-aposematic prey

(Davies 1977, Lindström *et al*. 2001a, 2004, Barnett *et al*. 2007). The abundance of non-mimetic edible prey has been shown to allow for less accurate mimicry (Ihalainen *et al*. 2012), and for mutual benefits for unequally defended prey (Rowland *et al*. 2007). This could mean that prey with even vaguely similar appearance, or moderate and variable level of defence, may benefit from converging to the same signal, or relax selection on warning colour if the predators generalize widely between aposematic phenotypes.

### 1.2.4 Generalization and the adaptive landscape

The benefit of signal sharing between aposematic prey, both within and between species, relies on predator generalization behaviour. In the context of aposematism, generalization occurs when predators transfer their learned avoidance from one stimulus to other similar stimuli (Guilford and Dawkins 1991, Gamberale-Stille and Tullberg 1999). Narrow generalization means selection against any deviation of the learned signal, whereas broad generalization means that predators consider a wide range of stimuli around the learned signal as similar, and thus may allow imperfect mimicry and even multiple morphs to coexist (Lindström *et al*. 1997, Kazemi *et al*. 2015). Predator wariness may affect the breadth of generalization, and as stronger defences are likely to cause more wariness, broader generalization should allow for more imperfect mimics the more toxic the model species is. Generalization may also occur more easily towards one stimulus dimension than another, i.e. be asymmetric (Ham *et al*. 2006). Asymmetric generalization can be caused by the physical properties of predator's visual system etc., or by biases in perception.

The concept of an adaptive landscape can help understand how different selective pressures can cause variation in warning coloration (Wright 1982, Mallet 2010, Chouteau and Angers 2012, Arias *et al*. 2016b). A decrease in predation caused by an effective warning colour, such as red, can be considered as a peak in the adaptive landscape. If the appearance of individuals changes gradually, in order to move from this peak to another adaptive peak, i.e. another efficient warning colour, such as yellow, it needs to cross a valley of non-adaptive (i.e. increased predation) phenotypes. If the predators generalize widely, say from red to an orange phenotype, that is intermediate between red and yellow, then the non-adaptive valley becomes shallower and less difficult to cross. If predator generalization is asymmetric, the changes in prey phenotype can more easily happen in one direction than another within the adaptive landscape.

## 1.3 The maintenance of aposematic polymorphism

Warning colour variation is widespread amongst aposematic organisms despite the expectation of strong purifying selection leading to signal monomorphism (Briolat *et al*. in review). The maintenance of several warning signal morphs within the same population (in high enough frequencies not explained solely by recurrent mutations) is an on-going evolutionary puzzle. Many hypotheses have been suggested and tested, but the relative contributions of each of the selective processes are not well known (Chouteau *et al*. 2016, Mallet and Joron 1999). The problem has yielded much interest among evolutionary biologists and population geneticists, because the same mechanisms can ultimately lead to diversification and speciation. It is also somewhat unclear, under which circumstances speciation will occur. Warning signal polymorphism is particularly interesting, because some of the most commonly found mechanisms maintaining polymorphism do not seem to apply in the case of warning signals, which are under purifying selection against any deviation from the common signal. For example, on the contrary to positive frequency-dependent selection (FDS), negative FDS favouring the morph with the lowest frequency would naturally balance morph frequencies in a population. Negative frequency-dependence could occur due to sexual selection or via predation, if predators form a "search image" for a common cryptic palatable prey and thus attack less of the rare forms (Allen and Greenwood 1988, Endler and Greenwood 1988).

Another mechanism maintaining polymorphism is heterozygote advantage. While heterozygotes (i.e. individuals with two different alleles of the same gene) have a higher fitness than homozygotes, the latter are also maintained in the population due to genetic recombination during sexual reproduction. In aposematic organisms, however, heterozygotes with intermediate phenotypes deviating from the established warning signal morph are expected to be selected against. The trait causing heterozygote advantage would thus have to be something non-visual to work (Mallet and Joron 1999). There are indeed a number of genetic mechanisms (observed often in mimetic systems), which cause tight linkage of the gene regions coding for the phenotype, making them less easily broken apart during recombination. Tight linkage caused by e.g. supergenes, few or specific loci and chromosome inversions as well as patterns of allelic dominance hierarchy (Le Poul *et al*. 2014) can all minimize the expression of intermediate phenotypes during hybridization. The evolution of such mechanisms is perhaps not surprising, as they will benefit both individual's survival and spread of the genes. There is ongoing debate over how much of the observed polymorphism is driven by selection at the genetic level versus selection at the individual or population level. Surely, conflicting selection at different levels of organization, between populations or between generations can contribute to warning signal variation, but the question is, to what extent?

Coloration can affect its bearer's fitness also in many other ways than protecting it from predation. Colour can have multiple functions, e.g. the melanin pigment can take part in immunological responses (Dubovskiy *et al*. 2013), help organisms in thermal regulation by radiation absorbance (Trullas *et al*. 2007) or protect tissues from UV-radiation (Ortonne 2002) and desiccation (King and Sinclair 2015). The different functions of coloration may act in opposition to each other, and be restricted by resource limitations, causing evolutionary trade-offs (Lindstedt *et al*. 2009, Hegna *et al*. 2013). Especially in the case of holometabolous insects it is obvious that the functions of coloration vary throughout individuals' ontogeny. This can also cause trade-offs in the efficacy of warning colour between the different lifestages (Lindstedt *et al*. 2016). Coloration is produced by structures or pigments, which can be either produced or acquired from diet (Shawkey and D'Alba 2017). Dietary restrictions due to e.g. differences in food availability during the larval stage could therefore produce variation in adult warning coloration. It could also be, that even aposematic prey have multiple strategies to avoid predation. For example, as aposematic prey can have many different types of predators, including naïve predators, it may be beneficial for aposematic prey to appear cryptic when viewed from a distance, and only reveal warning colour upon attack to startle the predator (Sargent 1990), i.e. show deimatic display (Olofsson *et al*. 2012, Umbers *et al*. 2017).

As discussed above, the coloration of adult moths can have important intraspecific signaling functions e.g. in sexual selection in addition to the warning signaling function. Therefore, if female wood tiger moths prefer to mate with white males, the white males could occupy an adaptive peak caused by differential mating success, whereas the yellow males could occupy an adaptive peak caused by increased survival. It has been suggested, that when purifying selection by predators is not strong, other selective forces could overrule, oppose or counterbalance it, allowing for example sexual selection to take over (Maan and Cummings 2008). Opposing selection pressures are however not likely to produce adaptive peaks of exact same height, and thus the stronger selection will eventually favor one signal over the other (Wright 1982). Conflicting selection can however slow down the loss of alleles, especially, if combined with temporal variation in the strength (or direction) of selection altering the peak heights (Mallet and Joron 1999).

When natural selection is weak, even neutral selective processes such as genetic drift or a founder effect may cause isolated populations to diverge. Geographic isolation and divergent evolutionary trajectories of isolated populations is perhaps the most widely supported pathway of evolutionary diversification, also in aposematic animals (e.g. Mallet 2010, but see Jiggins 2008). For example, according to Wright's (1982) shifting balance theory, movement from one adaptive peak to another occurs most easily in isolated populations. Genetic drift will change the appearance of a population, which then evolves to a new adaptive peak due to natural selection. Local variation may then result from previously isolated populations getting back to contact with each other or as a result of continuous gene flow between populations. If the new peak is higher than the peaks of other populations, the new appearance can spread to other populations or the individuals may interbreed with individuals from other populations, causing new adaptions to spread. A peak in the adaptive landscape could also be caused by some other quality of the prey than coloration, but if coloration is affected by the same quality, then a new colour form may arise. This could occur, if the genes producing the warning signal morph would also code for some other feature affecting the individual's survival or reproductive success (i.e. have pleiotropic effects).

In this thesis I focus in the role of predation, and particularly predator's tendency to generalize between different prey types, in maintaining warning signal diversity. The current theory of aposematism is based on purifying selection by predators, which intrinsically cannot maintain polymorphism. The strength and direction of selection by predators could however both vary in time and space (Mappes *et al*. 2005), especially when density-dependence is taken into account, which could allow multiple morphs to be maintained in the long run. For example, purifying selection could be relaxed, when specialist predators that can cope with prey defences are abundant (Valkonen *et al*. 2012), or if the predators generalize widely between different warning signals. Furthermore, generalization behaviour could be affected by prey density, due to for example predator satiation, and density interacting with morph frequencies as the frequency of more or less palatable prey in the prey community sharing similar warning signals changes. The importance of mimicry among aposematic prey and the cognitive processes behind predators' perception of warning signals and consequent attack decisions are currently not well known, although both affect the strength and/or direction of purifying selection towards warning signals. The direction of selection could also vary at small spatial scales, which may facilitate the maintenance of multiple morphs within a prey population. If the different morphs and their predators segregate to by microhabitats in their environment (Beccaloni 1997, Willmott *et al*. 2017), divergent evolutionary trajectories could even lead to diversification of colour in sympatry (i.e. within the same population). Predator responses to warning signals as well as potential positive interactions between co-prey are thus of high interest in current evolutionary biology.

## 1.4 Evolutionary history constraints

Evolution can only work on existing variation, and the range of variation can be somewhat limited by the evolutionary history of a species. Selective bottlenecks can wipe out genetic variation in certain characters, leaving mutation or interbreeding with other taxa as the only ways for producing new alleles. Thus, species' phenotypes may not be adapted to their current selective pressures, but rather represent remnant features incapable of change. In contrast, variation is rather easily produced in some features, such as the lepidopteran wings, due to their genetic- and physical structure (Ghiradella 1991, Beldade and Brakefield 2002). Lepidoptera are named after their scaled wings ("lepido" "ptera" from Greek). These wing scales can have different shapes and pigments, enabling the formation of various colour patterns on both upper- and lower sides of the wing membranes (Eliasson *et al*. 2005). Tiger moths (Arctiina) have been a particularly challenging group for systematic study because their wing patterns and colours, traditionally used for species identification, are highly variable

(Schmidt 2007, Weller *et al*. 2009) but lack reliable synapomorphies, i.e. shared derived characters supporting group monophyly. With modern molecular methods it is possible to find more characters for phylogenetic inference, making it possible to build more robust phylogenies. Robust phylogenies with high-resolution sampling are needed for using comparative methods to answer interesting evolutionary questions, such as the emergence of mimetic relationships among warningly coloured taxa (Simmons 2009). A phylogenetic hypothesis is also needed to determine whether colour polymorphism in the wood tiger moth is adaptive and under selection, or rather a remnant feature shared by common ancestry.

## 1.5 Aims of the thesis

The evolution and maintenance of warning signal polymorphism is an evolutionary puzzle, because current theory predicts warning signals to be under purifying selection by local predators (Ruxton *et al*. 2004). This thesis focuses on studying the roles of predator generalization and mimicry in maintaining warning colour variation in wood tiger moth populations. To this aim, four chapters are included in the thesis.

In the first chapter (paper I) I used molecular evidence to build a phylogenetic hypothesis for Arctiina in order to find the closest relatives for *plantaginis*, the only species in its former genus *Parasemia*. With a robust phylogeny it is possible to determine when the colour polymorphism observed in *plantaginis* emerged, and whether it is shared with other tiger moths. If hindwing colour polymorphism would have been an old derived character shared by many species, it could have been selected for earlier in the evolutionary history rather than being under current natural selection by predation.

In the second chapter (paper II) I asked whether local predators select for locally common morphs, as predicted by the theory of aposematism. Likewise, I examined whether geographic variation in predation could explain the observed warning colour distribution in four populations varying in their degree of local polymorphism. The strength and direction of selection by local avian predators was measured in large-scale field experiments using artificial moth models representing the white, yellow and red hindwing colour morphs (illustrated in Fig. 1 and Fig. 2 top row). I also monitored the local predator- and co-prey communities from the same sites to test whether changes in the predator community or the abundance and coloration of alternative prey would affect the attack risk towards wood tiger moth morphs.

In the third chapter (paper III), I tested whether predators in polymorphic populations generalize their learned avoidance between the three wood tiger moth morphs, thus relaxing selection towards warning colour, and allowing other evolutionary processes to maintain local polymorphism. I tested this in laboratory assays using the same colour morphs as in chapter II, and wild-caught blue tits (*Cyanistes caeruleus*) from a polymorphic population in Finland.

Finally, in the fourth chapter (paper IV) I looked for evidence of signal sharing between the white and yellow morphs of the wood tiger moth and four other moth species carrying a similar warning colour. Each of the wood tiger moth's colour morphs could gain protection from other prey species carrying similar warning colours, thus contributing to the maintenance of polymorphism (e.g. Joron *et al*. 2011). First, I tested the relative palatability of the potential models of the wood tiger moth by feeding them to great tits without visual cues. Second, I tested whether experience with a potential model would change bird behaviour towards the potential mimic by offering sequences of freeze-killed samples to both blue and great tits (*Parus major*).

# 2 METHODS

A wide selection of materials and methods were used to study selection on wood tiger moth hindwing warning colours. Relevant aspects of study species biology, methods for data collection, experimental protocols and statistical analyses used in this thesis are briefly explained below. More detailed methods can be found in the corresponding thesis chapters (papers I-V).

## 2.1 Study sites

Wood tiger moths are capital breeders (Tammaru and Haukioja 1996), i.e. do not feed as adults. Females eclose with their abdomen filled with eggs and ready to mate. The clutches of ~200 eggs hatch into polyphagous larvae, meaning that they feed on a wide variety of host plants, such as *Plantago* sp., *Rumex* sp., *Taraxacum* sp., *Tussilafo farfara* and *Vaccinium uliginosum* (Ojala *et al*. 2005). Therefore, the preferred habitats for wood tiger moth across the Holarctic are also rather variable, ranging from alpine tundra to lush meadows and pine-dominated bogs (Hegna 2013). The species seems to prefer warm microhabitats (Silvonen *et al*. 2014), sheltered from wind by forest edges or steep slopes (personal observation). Larvae overwinter usually at fourth instar and continue feeding in the spring until big enough to pupate (Ojala *et al*. 2005). Adults eclose in early summer, and their flight season peaks around the beginning of July in the Western Palearctic. Females are most often found luring males with pheromones on ground vegetation in late afternoons, in Finland along small forest roads nearby semi-open pine-dominated bogs (personal observation). Males are usually seen on re-settlement flights nearby ground vegetation during the day, and following pheromone traces in the light northern evenings, when the females are most actively calling (Rojas *et al*. 2015). Mating lasts for approximately 7 hours (Gordon, personal communication), often overnight, and the moths remain resting visibly on ground vegetation afterwards. All morphs can thus be seen together during daytime, when avian predators are active.

The study populations for this thesis were selected to represent different levels of hindwing colour polymorphism in the Western Palearctic, ranging from monomorphic yellow in Scotland to polymorphic Finland, where white, yellow and red morphs co-occur. Across its range, both geographic variation and local polymorphism in the hindwing coloration of the wood tiger moth are common (Hegna 2013). In all study populations, the study sites were selected to represent a range of forested, semi-open and open habitats where *A. plantaginis* was known or presumed to occur. In Georgia study sites were located 900 – 2300 m above sea level, as the *A. plantaginis* occurs there mainly in the mountains.

## 2.2 Observing predator and prey communities

Predatory events are very rarely observed in the field, but there is some indirect indication that birds are the most important source of natural selection on *A. plantaginis* colour morphs. Wood tiger moths are not active during the dark hours (Rojas *et al*. 2015), and lack the ability to produce ultrasonic clicks typical to tiger moths (Ratcliffe and Nydam 2008), making it unlikely that bats would be their main predators. Spiders have been shown to attack and consume moths caught in their webs but are not affected by wing colour (Burdfield-Steel unpublished data). As this thesis focuses on the adult coloration, I will not speculate on predators in the other life-stages, although interactions between selection on other life-stages and adult coloration can occur (Lindstedt *et al*. 2011).

When harassed by humans or birds, the adult moths may try to escape by flying high or dropping down to the ground. On ground the moths either fake death by tonic immobility, or dig deep into the vegetation (Honma *et al*. 2015, personal observation). In addition to this, the wood tiger moths protect themselves with two target-specific chemical defences (Rojas *et al*. 2017). A yellowish fluid containing two pyrazine compounds, is released from two prothoracic glands. Thus, upon attack, this fluid is in contact with the bird's beak and nostrils as it grabs the moth in its beak and squeezes the moth around the thorax. A light brown abdominal fluid is released from the end of abdomen, and is effective against invertebrate predators, such as ants (Rojas *et al*. 2017). Great and blue tits attack and consume the moths in the laboratory (Nokelainen *et al*. 2012, IV, Fig. 1B), and have been observed to attack them also in the field. Pied flycatchers attack and consume less wood tiger moths than blue tits (Rönkä and Mappes, unpublished data). This is perhaps because blue tits handle their prey leaving some parts uneaten, whereas flycatchers swallow moths as a whole (IV, Lyytinen *et al*. 1999).

In order to monitor local predator and prey communities at the same sites where natural selection on wood tiger moth morphs was examined (chapter II), I used modified transect count methods (Nokelainen *et al*. 2014, Pollard 1977). Birds were counted from a 25 m-wide section along the predation transects and identified to species level based on both vocal and visual observations by an experienced observer. Only insectivorous bird species were included in the analyses, and the variation in the bird communities was assessed with a principal component analysis (see detailed methods in chapter II). Similarly, diurnal Lepidoptera above one centimetre wingspan were counted along the same predation transects from a 10 meter wide section, identified to species or genus level, and classified into colour classes, and to be either conspicuous or inconspicuous, based on the prevailing coloration on upper wing surface. Birds were counted once during the predation experiment period, on still and dry weather when birds were actively singing. Lepidoptera were counted usually twice (1-3 times) before and after the predation experiment on a sunny, still and dry weather (never when the wind exceeded 6 on Beaufort scale, under 13 degrees and fully cloudy, or rainy weather).

## 2.3 Sample collection

### 2.3.1 Field survey and moth sampling

Data on the wood tiger moth's morph frequencies has been collected from museum samples and field monitoring data around the world (Hegna *et al*. 2015). Field survey data from years 2009-2015 was used to determine the local morph frequencies in Finland, Estonia, Scotland and Georgia in chapter II. The survey is based on pheromone trapping, using live laboratory stock females to lure males, complemented with netting at suitable habitats during the peak flight season. Moths used in the mimicry experiments (paper IV) were collected from the wild in Finland, except for *Pseudopanthera macularia*, which were caught in Estonia, and *Zygaena* sp., which were caught in Georgia.

For the phylogenetic analysis we aimed to collect all possible relatives of the wood tiger moth. A list of species to be sampled was based on existing literature and sent out to Lepidopterologists in order to gather DNA-samples from private and museum collections. Tissue samples (legs, larval protolegs or skin) less than three years old stored dry, frozen, or in alcohol were preferred to avoid degradation of DNA strains and gain high quality sequences of both nuclear and mitochondrial genes selected. Sampled individuals were also photographed for voucher identification and the sequences, together with voucher information, were stored to GenBank.

### 2.3.2 Laboratory stock and rearing of moths

In order to produce enough samples for experiments and to be able to control and/or manipulate the environmental and genetic variation in experimental animals, moths were reared in the laboratory in addition to field sampling. The wood tiger moth stock is originated from Finnish wild populations, and reared for three generations per year in a greenhouse at the university of Jyväskylä.

Moths overwinter as caterpillars in temperature regulated cool chambers at +2 degrees Celsius. Caterpillars were grown in sibling groups of ~30 until pupation. Rearing was done in plastic containers, where lettuce or leaves of *Taraxacum* sp. and *Plantago major,* when available, were added and uneaten food and excrements were cleaned daily. Pupae were moved to individually marked jars for colour morph monitoring and further use of the adult moths. For overwintering the larvae were divided in groups of ~10 to jars equipped with regularly moistened *Sphagnum*-moss. Matings were planned and backcrosses with wild-caught individuals were conducted yearly to avoid inbreeding.

Other species of moths used in mimicry experiments (chapter IV) were reared in an outdoor greenhouse in Jyväskylä in 2015-2016. Wild-caught parental individuals were mated and stored frozen in -20 degrees C after reproduction. The resulting offspring were reared together on natural food plants collected from the wild (IV). All species overwintered as pupae (some individuals overwintered for two seasons), and were killed and stored by freezing in -20 degrees C freshly after eclosion.

## 2.4 Molecular methods

The extraction of DNA from was done using the DNeasy Blood+Tissue extraction kit (Qiagen, Hilden, Germany) following the manufacturer's protocols in both University of Turku and University of Jyväskylä. For the samples processed in Jyväskylä the washing and eluting were done with a KingFisher robot (Kingfisher, Waltham, MA, U.S.A.) using MagAttract tubes and the programme Qiagen Blood. Walhberg and Wheat's (2008) laboratory protocols were followed for polymerase chain reaction (PCR) and primers. In cases of older samples not yielding enough PCR product for purifying by cutting from agarose gel, the PCR product was used as a template for another round of PCR with the same primers.

We sequenced seven nuclear gene regions (CAD, GAPDH, IDH, MDH, Ef1□, RpS5 and Wingless) and one mitochondrial gene (COI) including the barcode region. The barcode region was sequenced for part of the samples in Jyväskylä with Big-Dye terminator v3.1, Cycle Sequncing kit (Applied Biosystems, Carlsbad, CA, U.S.A.) and ABI 3130xl Genetic Analyzer (Applied Biosystems), whereas all other PCR products were sequenced in Macrogen Europe (Netherlands). The resulting DNA sequences were aligned manually using MEGA 5.2.2 (Tamura *et al*. 2011) or BIOEDIT (Hall, 1999) and stored in VOSEQ database (Peña and Malm, 2012) and Genbank. Samples with less than two successfully sequenced gene regions were excluded from the analysis, resulting in a total of 1000-5915 basepairs from 100 species in Arctiinae: Arctiini *sensu lato* and two outgroup species (Lithosiini: *Setina* sp. and Syntomini: *Amata* sp.).

To find the best-fit evolutionary model for our dataset, the Bayesian information criterion was used for the data partitioned by each codon position within each gene region in PARTITION FINDER v. 1.1.1 (Lanfear *et al.*, 2012). For Bayesian inference (BI) analyses we sampled every 1000 generations of 10 million generations and four chains (one cold and three heated) in two independent runs on MRBAYES v3.2.3 (Ronquist *et al*. 2012) on the Cipres science gateway (Miller *et al*. 2010). Visual inspection of log-likelihood stationary distributions including the last 75% of sampled trees, the final average standard deviation and the scale reduction factor was used to check for convergence of the two runs. A mixed evolutionary model was used and the parameters and models of evolution were unlinked between character partitions. A maximum likelihood (ML) analysis was run in RAXML-HPC2 (Stamatakis 2014) on XSEDE (Towns *et al*. 2014), and bootstrap support for ML nodes was calculated from 1000 replicate runs in Cipres science gateway.

## 2.5 Experiments with avian predators

### 2.5.1 Artificial moth models

Artificial moths were used in order to study the effects of hindwing hue only controlling for any individual variation in other characters of the moths, such as melanisation, pattern, palatability (chemical defence and nutritional value), odour, size, shape, behaviour and wing position. The model wings were printed double-sided with a calibrated printer (HP Colour LaserJet CP2025) and cut from waterproof paper (Rite in the rain ©, JL Darling Corporation, Tacoma, WA, USA). Pictures for the wings were taken from one wild-caught representative white *A. plantaginis* f. *hospita* male, and then assembled in Gimp 2.8.16 (GNU Image manipulation program) to three morphs by duplicating the left-side wing to both sides, and changing the hue of the hindwing colour to create yellow and red morphs keeping the pattern the same. To avoid any novelty-effect caused by forewing pattern, we also took a picture of a representative left forewing of *A. plantaginis interrupta* male from Georgia, which was used for preparing the models to be used in the predation experiment there. To ensure that the printed wing colours were similar to real moths wing colours we measured reflectance from both with an Ocean Optics Maya2000 Pro spectrometer and compared the resulting reflectance curves (III).

Bodies for the models used in the field (II) were made out of plasticine (Caran D'Ache Modela 0259.009 Black), which allows recording beak marks from avian attacks (Fig. 1C). Plasticine is also neutral in taste, which reduces the risk of local predators learning to predate on the bodies. Bodies were attached to the wings and the whole model attached on three trunks or big leaves on natural ground vegetation with a black-painted needle fastened with a small piece of masking tape. This ensured that the models were clearly visible to avian predators, at least from two directions, but would not fall of easily when attacked or during rain (Fig. 1A, C).

Bodies for the models used in laboratory assays were made of an edible pastry consisting of flour, lard and water or a pastry made inedible by adding 15% quinine solution instead of water. Chloroquine is commonly used in bird assays for it is practically odourless and very bitter in taste. Edible bodies were used to motivate the birds to repeatedly attack the models.

### 2.5.2 Field predation experiments (II)

Artificial moth models representing the white, yellow and red hindwing colour of the wood tiger moth were exposed to natural predation in the field to study if predation by local avian predators is driving the observed warning colour variation. Sixty models (20 of each colour) were placed on natural vegetation along 900 m long transects in a semi-randomized order so that models of the same colour were never next to each other. Models were set every 15 meters in Finland, Estonia and Scotland, in 15 locations each. In Georgia, models were set every 10 m along transects due to limited accessibility of the study sites above 2000 m in the Caucasian mountain range. Models were checked daily (except in Georgia where they were left to the field for several days before inspection and removal) for beak marks resulting from bird attacks on the plasticine bodies (Fig. 1C). Attacked and missing models were replaced with a model of the same colour in a nearby location to keep experimental morph frequencies constant. Clear beak marks in both original and replaced models were included as attacks in the predation risk analysis.

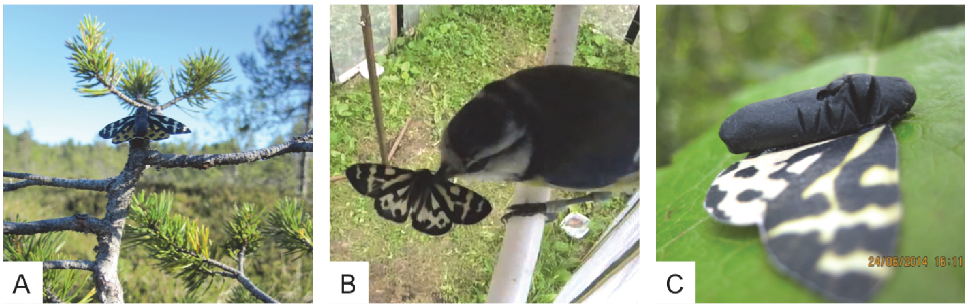

FIGURE 1 A) A yellow artificial moth pinned on a small pine in a boggy habitat, B) a live white wood tiger moth caught by a blue tit (*Cyanistes caeruleus*) and C) beak marks imprinted on the plasticine body of an artificial white wood tiger moth. Photographs by KR.

### 2.5.3 Laboratory feeding assays (III, IV)

The experimental design to test for avoidance generalization between the three wood tiger moth colour morphs consisted of three phases. First, as both neophobia and learned avoidance could prevent the birds from attacking novel morphs, 53 blue tits were familiarised with models of all three colours as edible (with pastry bodies). At the same time we were able to test for any initial bias or preference towards the colours by the wild-caught predators, and make sure that all possible biases were removed before the next phase. Second, we divided the 47 birds that ate the whole bodies of all models in three rounds of preference into three groups. Each group was offered one of the morphs (white, yellow, red) as unpalatable in sequential trials until they refused to attack three models in a row, but did attack the palatable prey offered in between models. In the third and final phase of the experiment, we tested whether the birds would generalize their learned avoidance of one of the colours to the other two, and thus avoid attacking them in the subsequent generalization trials. The two unlearned colours were offered simultaneously to the birds to test for any potential bias in generalization between the colours.

The palatability of the white and yellow wood tiger moth male morphs and their potential mimics (white *Rheumaptera hastata* and *Lomaspilis marginata* or yellow *Arichanna melanaria* and *Pseudopanthera macularia*) was tested with great tits in two experiments. First, birds were presented samples of each species without visual cues against mealworm controls and moth controls, and second, birds were offered freeze-killed individuals of each species (with visual cues). To get rid of the visual cues, moth samples were freeze-dried. Left side wings of every third individual were stored for taking pictures for image analysis, and the rest were ground into powder in groups of three. Water was added to the samples to create a smooth paste before offering them to the birds. For the feeding assay, a small amount of the paste (20-70 mg depending on the experiment) was added to a custom-made cup, and sealed with parafilm. All eight samples were offered simultaneously to each bird (the four potential model species, yellow and white *plantaginis* morphs, positive control [i.e. a palatable prey - either *A. gamma* or mealworm] and negative control [i.e. a unpalatable prey - either *Zygaena* sp. or crushed mealworm mixed with 10% quinine solution]). Cup contents were weighed before and after the trials, and the proportion eaten was measured as a proxy for moth palatability. In the second experiment thawed moths were offered one by one as whole, set on a clean petri dish on a green-painted background, with hindwing colour visible. Here we also tested whether predator reactions towards one moth species would change after experience with the other by offering the birds four trials with the potential model species and a fifth trial with the potential mimic (and vice versa). The most promising mimetic relationship was also tested with blue tits to account for species-specific reactions.

In all experiments, birds were food-deprived for at least one hour before the trials to ensure motivation to forage. Bird's motivation to attack was controlled by offering palatable food (seeds or mealworms i.e. *Tenebrio molitor* larvae known to be highly edible to tits) between experimental trials. If the bird did not attack the edible food offered, it was considered unmotivated, and got a 10-minute break before continuing the trials. If the bird did attack the edible prey within a given time, the trials were continued. Therefore we could ensure that the bird's hesitation to attack the experimental prey offered was not due to lack of motivation to forage or satiation.

## 2.6 Image analysis with avian vision model (IV)

The development and increasing availability of digital imaging, image analysis methods and animal visual models has made it possible to study colour patterns taking into account what the natural non-human predators can and can not see (e.g. Endler and Mielke 2005). Image analysis was used in order to estimate how similar the putatively mimetic moth species sharing either a white or yellow warning coloration combined with black pattern look to avian predators. To this aim, a total of 94 pictures of dry moth samples were taken with a customized camera set made of a Samsung NX1000 camera with Nikon EL-80 mm lens and filters for UV and visible light imaging. To obtain colour values representing the true colours of the moths in the raw pictures, these were calibrated, normal and UV wavelength pictures were aligned, scaled, linearized and saved as a four-dimension file with Mica toolbox (Troscianko and Stevens 2015) using ImageJ (Abramoff *et al*. 2004). Colour values as seen by the avian predators were extracted from upper sides of the left hindwings outlined by hand in ImageJ. The cone distribution used for colour discrimination is known for only a handful of species, the blue tit amongst them (Hart *et al*. 2000).

For extracting texture values, a specified MATLAB code was used to set the moth outlines and 8000 pixels convolved with log-Gabor filters of four spatial frequencies and six orientations (0 to 150° in 30° increments). Such Gabor wavelets are used to describing substrate texture based on images, and they function in a somewhat similar manner to how simple cells in the visual cortex of mammals process visual information (Daugman and Kammen 1986). Here Gabor wavelets were used to create a texture space, based on the double cone output of birds, to compare wing pattern textures between the putatively mimetic pairs.

With the objective colour data, we asked how easily birds could distinguish a putatively mimetic species A from species B, first based on their overall appearance (i.e. both texture and colour) and second, based on hindwing colour only. In practice, logistic regression models were used to evaluate how easy it is to determine if a randomly selected pixel belongs to species A or species B, comparing both colour and texture and colour separately. To avoid overfitting of the model (i.e. including too many terms), we used 'leave-one-out cross-validation' (Lantz, 2013). Here, the model is fit several times to the dataset, excluding every time one individual of species A and one of species B. On each round, the excluded individuals are used for model validation (testing), and the process is repeated until each individual of the two species is left out once (Lantz 2013). Measures from Signal Detection Theory (Wickens 2002) were used to determine how well the classification model works. Discrimination (by the model in our case) can result in four outcomes: 1) a successful recognition of species A (a hit), 2) a successful identification of species B (a correct rejection), and two types of errors: 3) failure to recognize species A (a miss) and 4) classifying species B as species A (a false alarm). Based on these outcomes, several measures of classification success were calculated. Most importantly, the area under curve gives the probability of differentiating a randomly selected individual of species A from a randomly selected individual of species B, ranging between 0.5 (i.e. no better than random) and 1 (all individuals of species A can be perfectly discriminated from individuals of species B). In addition, a receiver operating characteristic curve was drawn on a plot of the proportion of hits (i.e. of correctly classified individuals of species A) against the proportion of correct rejections (i.e. correctly classified individuals of species B) to represent the trade-off between Type I (proportion of misses) and Type II error (proportion of false alarms). This plot is used to evaluate the balance between Type I and II errors, instead of conventional statistical methods evaluating only Type I error by p-values. This is because the question asked here is not whether the two species can be distinguished (i.e. null hypothesis testing), but rather how easy it is to do so.

# 3 RESULTS AND DISCUSSION

## 3.1 Putting *Parasemia* in its phylogenetic place (I)

Both Bayesian inference and Maximum likelihood analyses resulted in well-supported phylogenetic hypotheses with nearly identical topologies (I). *Parasemia* was placed within Arctiina *sensu stricto* in the genus *Arctia* **comb. nov.,** as sister to *Arctia festiva* (Hufnagel) with bootstrap support (BS) = 94 and Bayesian probability (BP) = 1 (Fig. 2). Based on our results with the most comprehensive sampling to date of Arctiina *s.s.*, we presented a taxonomic revision with 33 new genus-level synonymies (I). This was done in order to improve the informational value of the taxonomic nomenclature in this group at the genus level, which is essentially intended to combine groups of related species below the family level. Many of the tiger moth genera in Arctiina *s.s.* were previously described as monotypic (i.e. contained only one species each), partly due to lack of information of their phylogenetic systematics. Delineating which clades in a phylogeny should represent the generic level is somewhat subjective and not codified. We based our suggestion on four general criteria: genera should be 1) monophyletic (i.e. consist of all the descendants of a common ancestor), clearly supported in phylogenetic analysis, 2) distinct in terms of e.g. ecology, morphology or biogeography, i.e. evolutionarily relevant criteria, which are the drivers of evolution, rather than the characters found in DNA-sequences as a consequence of evolution, 3) reasonably compact and 4) comprise of phylogenetic units similar to other analogous genera.

The topology and examples of species placed in the genus *Arctia* together with *A. plantaginis* are illustrated in Fig. 2. Most of these species show sexually monomorphic yellow-to-red hindwing coloration, indicating that the sexually monomorphic continuous yellow-to-red hindwing colour variation shown by *A. plantaginis caucasica* in Georgia (Hegna *et al*. 2015) could be the ancestral form. Population genetics with microsatellite markers show clear separation of the Georgian population from the rest (Hegna *et al*. 2015). Although only two samples of wood tiger moth were included in the final analysis, several individuals around the Holarctic were also tested for chapter I, and the Georgian population was the only one separated from the others based on the nuclear and mitochondrial gene regions used. Without dated trees and detailed knowledge on the inheritance of hindwing coloration, it is however impossible to determine whether the white male morph is a more recent development than the yellow and red morphs. If it is true that the loss of red (and yellow) pigmentation in male hindwings is a new adaptation, the question of "why there is warning signal variation" would transform into "why white males have spread across the Holarctic", and "has this spread been adaptive?".

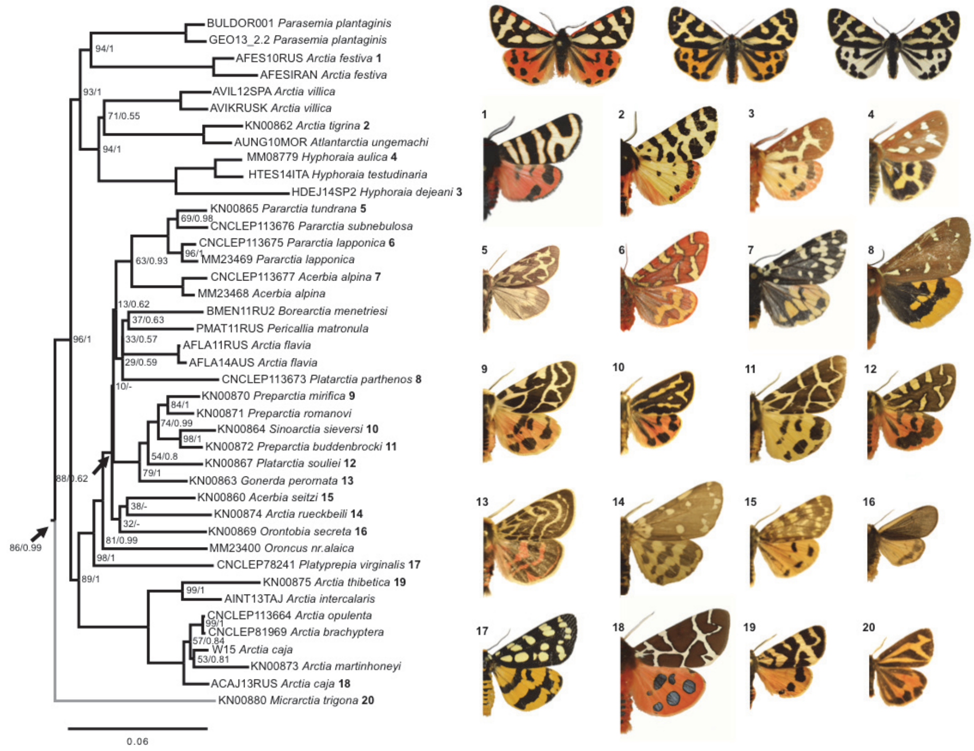

FIGURE 2 Topology and illustrations of species belonging to the revised genus *Arctia* (rooted to *Micrarctia*) based on phylogenetic data. Bootstrap/Bayesian posterior probability support values are given next to the nodes. The three wood tiger moth hindwing color morphs (red, yellow, white) with typical Georgian and European wing patterns are illustrated in the top row. Relative sizes of the moth pictures are loosely indicative of their true relative sizes. Photographs taken by KR at Finnish Museum of Natural History, Kari Kulmala (yellow and white *plantaginis*), Jocelyn Gill (8, 17) and Kari Nupponen (2, 5, 9-16, 19-20).

## 3.2 The role of predation in explaining warning colour variation

### 3.2.1 Geographic differences in predation (II)

Geographic variation in predation pressure on the different colour morphs was evident in the field experiments spanning four countries on the monomorphic-polymorphic continuum of colour variation in the Palearctic. Predation pressure was negatively correlated with local morph frequencies (GLMM, Z=-3.6, P=<0.001). In the monomorphic yellow Scottish population, the yellow morph had a significantly lower daily attack risk than the red and white morphs, while in the red-dominated Georgia red morphs were attacked significantly less than the novel white morphs, and the second most common yellow morph had an intermediate level of attacks (Table 1). In white-dominated Estonia the overall predation pressure was very low (on average less than 5% of the models were attacked daily), and no significant morph-related differences were found (Table 1). Accordingly, in Finland, where all three morphs co-occur, all morphs were attacked evenly (Table 1). These findings are in line with positive frequency-dependent selection by local predators, which have learned to avoid locally common morphs, and attack more on locally novel morphs.

TABLE 1 Estimated attack risk towards each wood tiger moth colour morph in each country. W= white (in the intercept), Y = yellow and R = red.

| Country | Morph colour | Estimate | Standard error | Z - value | p - value |
|---|---|---|---|---|---|
| Scotland | W | 2.71 | 0.22 | 12.38 | <0.001 |
| | Y | 0.41 | 0.17 | 2.48 | **0.013** |
| | R | -0.066 | 0.15 | 0.45 | 0.66 |
| Georgia | W | -3.02 | 0.22 | -13.63 | <0.001 |
| | Y | -0.26 | 0.19 | -1.38 | 0.17 |
| | R | -0.49 | 0.20 | -2.44 | **0.015** |
| Estonia | W | -4.02 | 0.23 | -17.66 | <0.001 |
| | Y | 0.13 | 0.25 | 0.50 | 0.62 |
| | R | 0.18 | 0.25 | 0.72 | 0.47 |
| Finland | W | -2.92 | 0.14 | -20.64 | <0.001 |
| | Y | 0.14 | 0.17 | 0.82 | 0.41 |
| | R | -0.15 | 0.19 | -0.81 | 0.42 |

Selection by local predators seems thus to explain the geographic variation in wood tiger moth coloration. The first principal component from principal component analysis conducted to catch the variation in abundances of insectivorous birds in different families observed across countries, explaining 43.2 % of the total variation, was the only component with a significant effect on predation risk of the three morphs (II). The first component was loaded most distinctly with Estonian bird community, characterized mainly by Sylvidae and Fringillidae. As the overall attack risk in Estonia towards all wood tiger moth models was very low, but the bird community was abundant, we assume that Sylvidae and Fringillidae were not attacking our moths, thus relaxing selection for colour morph. In the other extreme, the component was characterized with Paridae, which do attack the models and moths in both laboratory and field assays (e.g. III, IV, Lindstedt *et al*. 2011, Nokelainen *et al*. 2012, 2014). Nokelainen *et al*. (2014) found a similar effect on overall predation pressure on white and yellow wood tiger moth models in Finland and Estonia. In their analysis, the overall predation pressure on models was the lowest when the community (in principal component 2) was characterized by Laniidae and Sylvidae, and highest when characterized by Paridae and Sturnidae.

Nokelainen *et al*. (2014) found also a significant interaction between a third principal component and morph colour: when the bird community was characterized by Prunellidae, the yellow morph was attacked more, but when it was characterized by Paridae, the white morph had a disadvantage. In our analysis including Scotland and Georgia, in addition to Southern Finland and Estonia, we found that the bird community composition affected the attack risk of each morph differently in different countries (II). For example, when the bird community was characterized by dunnocks (Prunellidae), the yellow morphs were attacked least in Scotland but most in Finland. Therefore, population level differences between predators could be driving geographic variation in the direction of selection. Differences in predatory behaviour towards the same prey have been previously demonstrated between great tit (*Parus major*) populations in Finland and Bohemia (Exnerová *et al*. 2015). Exnerová *et al*. (2015) associated the observed differences with differences in birds' experience with the prey tested. This could also be true in our case, as the Scottish population is monomorphic yellow, whereas the yellow morph is the least common of the three in Southern Finland, with approximately 22-24 % of the males being yellow each year, thus offering fewer opportunities for the birds to learn it. The alternative prey community is also much more abundant and diverse during the wood tiger moth flight season in Finland compared to Scotland, which may hinder predator learning (Ihalainen *et al*. 2012).

Similarly to Nokelainen *et al*. (2014), we found geographic variation in the strength of selection towards wood tiger moth warning colour (II), which coupled with gene flow between populations, could explain how polymorphism is maintained in some populations. Genetic drift may also play a role in determining which morph is fixated in more isolated populations. The more polymorphic populations in Estonia and Finland seem better connected than the rather isolated island population in Northern Scotland and the mountainous Georgia. The Georgian population is genetically isolated from the others to some degree, supporting this conclusion. There is however genetic indication of the warning signal components of wood tiger moth coloration being under non-neutral selection (Galarza *et al*. 2014), and the monomorphic yellow population in Scotland clusters together with other wood tiger moth populations (I, Hegna *et al*. 2015). The overall predation pressure in Scotland is however very high, and thus any migrating individuals are likely quickly eaten (Nokelainen *et al*. 2014, II). In Estonia, with practically no avian predation, other selective forces are likely to drive the evolution of wood tiger moth hindwings. For example, the white morph has been found to have mating benefit, at least under stressful conditions (Nokelainen *et al*. 2012) or when high in frequency (Gordon *et al*. 2015), and is genetically dominant over the yellow morph (Galarza unpublished). Geographic variation in the direction of selection by local avian predators together with gene flow between the differently selected populations, together with the fact that also the overall strength of predation varies, could thus explain how polymorphism is maintained in some populations and not in others. The direction of selection could also vary at a much smaller scale, e.g. between habitats or bird territories. In the preference test (III), wild-caught blue tits in Finland selected certain morphs as first or last, but no bias was found at the population level. Movement of moths between predator territories and temporal changes in bird assortment could create enough spatio-temporal variation in selection for local polymorphism to be maintained (Endler and Rojas 2009, Nokelainen *et al*. 2014).

### 3.2.2 Generalization based on colour and potential for mimicry

The blue tits discriminated between wood tiger moth models based on hindwing colour (III) instead of generalizing based on the black patterning or other features of the models. Colour seems to be of foremost importance in prey recognition (III, IV, Terhune 1977, Aronsson and Gamberale-Stille 2008), although when colour is kept constant, avian predators can also discriminate based on pattern (e.g. Prokopová *et al*. 2010), or other cues of the prey (e.g. Karlíková *et al*. 2016). The wood tiger moth morphs hindwing colour varies rather independently of the forewing pattern, thus indicating that the forewing and hindwing coloration have evolved as a result of different selective pressures. It is common in Lepidoptera that the coloration on the upper and lower sides of wings have different functions related to behaviour - the upper sides of butterfly wings are often visible when the butterfly is basking and the coloration in the middle of the wings is important for thermoregulation, whereas the lower sides are mainly visible when the butterfly is resting, and are usually cryptic to avoid detection by predators (Eliasson *et al*. 2005). Lepidopteran hindwings are actually not needed for flying, but can be used in flight maneuvering (Jantzen and Eisner 2008) or for other functions, such as signaling to potential predators. Contrary to butterflies, the wood tiger moths tend to keep their hindwings partially visible when they are active and seen sitting on vegetation during the day, and fold them hidden under the forewings when resting and inactive. The forewing coloration may be disruptive, decreasing the probability of being noticed (Honma *et al*. 2015). Although aposematic coloration is selected to be conspicuous, even aposematic prey benefit from reducing their risk of being detected by uneducated predators. The disruptive effect is at its best viewed from a distance whereas the contrasted patterns may have a warning function observed up close. The risk of being detected however immediately increases once the moth starts to move. The warning function of the hindwing coloration is thus most likely important during movement, and during a predatory event. The wood tiger moths are not particularly good fliers, do not startle, and often do not try to hide from predators. Instead, when caught by a bird, they usually show tonic immobility (i.e. play dead) and release a defensive fluid from their prothoracic glands, situated right by the bird's nostrils when the bird has the moth in its beak. If the bird then releases the moth, they usually drop down on the ground, where they can also continue to escape by digging into the vegetation (personal observation). Predators may thus associate the colour they saw at flight and during the attack, with the aversive smell (and taste) of the defence fluid, and an unsuccessful predation attempt. The main function for the hindwing warning colour could be to enhance the memory consolidation of the negative experience and thus facilitate avoidance learning. Warning colours are often thought to function to facilitate avoidance learning and memory consolidation (e.g. Roper and Redston 1987, Speed 2000).

In chapters II and IV, we investigated whether mimetic relationships, i.e. shared warning coloration among alternative prey would affect predation on wood tiger moth morphs, thus potentially contributing to the maintenance of both geographic and local variation in warning colour. To a human observer, there are no perfectly accurate mimics of *A. plantaginis* occurring in Finland, but several co-occurring species are easily mistaken as yellow or white *A. plantaginis* morphs especially in flight, when searching for the moths in the field (Rönkä, Nokelainen, Mappes, Rojas, Gordon, personal observations). The mimetic resemblance between the white, yellow and red morphs and other black-and-white, black-and-yellow or black-and-red prey could be at its best during movement, because wing patterns blur when the moths are flying. The wood tiger moths are very conspicuous in flight, for example when looking for females or for a place to oviposit. Insectivorous birds often attack moths on the move (although blue tits and flycatchers tested in a semi-natural enclosure tended to rather attack resting moths than the flying ones, especially after catching the first few, Rönkä and Mappes, unpublished). The avian predators could also perceive patterns differently from us humans, in which case "vague resemblance" could be perfect mimicry to an avian predator (Dittrich *et al*. 1993).

Because predators often have limited time to make their attack decision in the wild due to risk of being preyed upon themselves, or the prey escaping or being eaten by others, they may rely on using only one or few cues for prey recognition (e.g. Chittka and Osorio 2007, Aronsson and Gamberale-stille 2012). They could either generalize widely among typically warningly coloured prey, or they could learn about warningly coloured prey trough experience and categorize them first based on simple rules, and only later learn about the details of each prey type (Chittka and Osorio 2007). If birds categorize prey based simply on warning colour, or generalize their learned avoidance based on colour *per se*, even vaguely similar defended species could benefit from sharing a similar coloration. The most accurate mimetic systems (e.g. *Heliconius* butterflies, Merrill *et al*. 2015) are usually found in the tropics, where predator-prey communities are relatively stable, and the predators have plenty of time to learn about the prey species present in their territories. However, at higher latitudes, such as in Finland, almost all insectivorous birds are migratory and the residential birds often move territories between years. There are also seasonal changes in the predator community, as the proportion of naïve predators peaks during the breeding season (Mappes *et al*. 2014). In addition, seasonal stochasticity in weather can cause variation in which Lepidoptera and which predators are present in the community at any one given time. Therefore, the predators in the high latitudes are constantly in the process of learning about their prey community. Likewise, a larger proportion of them may be using only simple categorizations about cues signaling for prey palatability or, alternatively, they might be prone to generalize more widely than predators in more stable communities.

Assuming that birds use colour as the primary cue for prey palatability, we hypothesized, that the local polymorphism of yellow and white males in Finland could be maintained if both morphs gain protection by sharing their warning coloration with other defended prey (IV). Multiple-model mimicry, where each morph gains benefit from sharing its phenotype with different sympatric Müllerian models, has been shown to maintain local polymorphism in the tropical aposematic butterfly *Heliconius numata* (Brown and Benson 1974, Joron *et al*. 2011). According to image analysis and blue tit vision model, the birds have trouble distinguishing two of the putative co-mimics (models and the corresponding morph) based on hindwing colour, but could perfectly distinguish between the putative models and the *A. plantaginis* morph of other hindwing colour (IV). We did not find a potential model for the white wood tiger moth morph, as both *L. marginata* and *Rheumaptera hastata* were found more palatable to great tits than *A. plantaginis*. Great tits also quickly attacked and consumed *P. macularia*. On the contrary, *Arichanna melanaria* was found to share a similar hindwing colour and to be as unpalatable (IV) to the birds than the yellow *A. plantaginis*, thus showing the highest potential for a Müllerian mimetic relationship.

In chapter II, we hypothesized, that the geographic variation in colour morph frequencies, i.e. which morph is favored in which location, could be explained by differences in the abundances of defended alternative prey sharing a similar warning colour. Although widespread, the wood tiger moth is not amongst the most abundant Lepidoptera (II), and also not extremely toxic (IV), which makes it likely to benefit from sharing predator education costs with other warningly coloured prey. Mimicking several different models could also allow the wood tiger moths to occupy a wider distribution range. No signs of signal sharing with local prey community were however found – the abundance of white, yellow or red alternative prey did not significantly affect the attack risk of the corresponding wood tiger moth morphs across countries

(II). As we grouped the alternative prey according to colour only, it is likely that each group was composed of prey varying in their level of defence. For example, the alternative prey classified as yellow could thus consist of both Müllerian and Batesian mimics of the yellow morph, or not share their warning signal with the yellow morph in the eyes of bird predators. However, recent experience with freeze-killed moths with a yellow hindwing colour did change great tit responses towards the yellow morph, even if the birds could see differences in their coloration (IV). In the laboratory, the attack latency towards the yellow *A. plantaginis* was significantly shortened by resent experience with the relatively palatable black-and-yellow *P. macularia* compared to the attack latency towards yellow *A. plantaginis* without any previously offered moths (Wilcoxon rank sum test, $W = 166$, $p = 0.0039$). Interestingly, the yellow morph was relatively least attacked in the field in Scotland where *P. macularia* was not present (II). *P. macularia* was observed during the predation experiment in 2014 in Estonia, Finland and Georgia (II).

In the field experiment, we found that the increasing abundance of alternative prey of other coloration than white, yellow or red, i.e. the likely non-mimetic brown, beige, orange, blue and grey species, reduced attacks towards the white and the red morph but increased attacks towards the yellow morph (II). The lowered predation risk on white and red morphs could be explained by a simple dilution effect, where the per capita mortality risk is lowered when alternative prey is abundant, as the predators get satiated. However, I am uncertain why the daily attack risk towards the yellow morph is greater as abundance of non-mimetic alternative prey increases. One potential explanation could be, that specialist predators capable of handling also defended insects are common in sites where lepidopteran prey are common, and because the yellow morph is particularly conspicuous against the natural green backgrounds (Lindstedt *et al*. 2011, Nokelainen *et al*. 2012), they are targeted more by the specialists than the other two morphs. If the abundance of non-mimetic alternative prey affects particularly the survival of yellow *A. plantaginis*, it is another factor potentially contributing to the maintenance of different morphs.

### 3.2.3 Variable bird responses towards wood tiger moth morphs

In the field, no one colour morph was favoured over the others across countries, indicating that all three colours can be effective warning signals against wild predators (II, see also Ham *et al*. 2006). As stated above, no population level bias towards any of the colours was found in laboratory trials with blue tits either (III). A vast majority of blue tits tested (37/45) attacked the other two morphs after learning to avoid one morph, showing no generalized avoidance between the colours (III). There were, however, some morph-related differences in bird responses. Blue tits took fewer trials to learn to avoid the red morph compared to the yellow (and white) morphs (Cox regression model, $Z = 2.17$, $P = 0.03$), indicating that red is a more salient warning signal than white or yellow, at least in laboratory conditions with artificial lighting (III). Lindstedt *et al*. (2011) also found lower predation risk towards red female wood tiger moths compared to orange ones. In addition, some blue tits showed a tendency to generalize between white and yellow, whereas only one individual left white and yellow models untouched after learning to avoid red (III).

When white and yellow wood tiger male morphs were offered to great tits without visual cues, the birds found both equally unpalatable (IV). Offering freeze-killed moths, with visual cues (IV), however, revealed some morph related differences. Earlier, blue tits have been found to hesitate significantly longer before attacking yellow than white live wood tiger moths during a single encounter (Nokelainen *et al*. 2012). The great tits seemed to also hesitate longer towards the yellow than the white morph during a first encounter, but the attack probability (within four trials lasting 5 minutes each) increased towards the yellow morph in further trials whereas it slightly decreased towards the white morph (IV). The birds' attack latencies and the probability to attack in subsequent encounters are likely affected by both morph colour and chemical defence. Recent experiments with white and yellow male morphs suggest, that the two morphs may be using different lines of defence (Rojas *et al*. unpublished). The defensive fluid of the yellow morph seems to be more repellent in terms of odour (Rojas *et al*. 2017), but the white morph seems to taste worse (Rojas *et al*. unpublished). This is in line with our findings: great tits tended to eat smaller proportions and did significantly more beak cleaning after eating the white males, and the probability of attack tended to decrease compared to the yellow morph during four subsequent trials (IV). Beak cleaning rate is a good proxy for unpalatability, but the birds seem to do this behaviour for multiple reasons, including cleaning sticky substances from their beak, and as a response to both unpleasant taste and unpleasant odour. More specific experiments would thus be needed to disentangle whether it is colour, odour, taste or a combination of these that the birds are mainly responding to when handling each morph.

Bird individuals and species can also vary in their reactions towards similar prey. Both blue and great tits increased their attacks on yellow *A. plantaginis* over four subsequent trials (IV), but the blue tits ate smaller proportions of them than the great. Great tits attacked less *A. melanaria* as the trials proceeded, but blue tits reduced instead the proportions eaten (although both kept eating the mealworms offered), indicating that blue tits may have been using a taste-rejection strategy whereas the great tits may have rejected prey also based on the visual cues. Tits may be especially good in handling defended prey, as they handle them by holding the prey against a perch with their feet and take out the wings with their beak. They eat selectively the soft (fat) body parts and often leave the wood tiger moths' defensive glands containing the defensive fluid uneaten. On the contrary, pied flycatchers (*Ficedula hypoleuca*) are more reluctant to attack live wood tiger moths than blue tits (Rönkä and Mappes, unpublished), perhaps because they swallow their prey whole, thus ingesting all toxins (Lyytinen *et al.* 1999). Juvenile inexperienced and adult experienced predators can also differ in their reactions towards aposematic prey (Lindström *et al*. 1999, Exnerová *et al*. 2006, Mappes *et* *al*. 2014, Veselý *et al*. 2017). Exnerová *et al*. (2003) found different reactions to aposematic and non-aposematic prey of insectivorous and partly granivorous passerine bird species, and suggested that this was related to their level of experience with insect prey. Different insectivorous passerine birds also showed variable responses towards cockroaches equipped with warning colours in Turini *et al*.'s (2016) experiment, although previous experience with non-modified cockroaches made most of them attack also the modified prey.

Taken together, birds' decision to attack and consume aposematic prey is highly dependent on their previous experience (or lack of experience, e.g. Lynn 2005). In the generalization experiment (III), the birds had first learned to consume all morphs, whereas in the avoidance learning they associated one of the colours to a negative experience. Thus, their earlier positive association with edibility may have overruled their tendency to generalize their learned avoidance from one colour to the other two. Initially, the birds hesitated long before attacking the models (III). This indicates, that wariness of natural predators, caused by e.g. neophobia, dietary conservatism or aggregation of the prey may well contribute to the maintenance of all three morphs in more natural settings. In chapter III, it was necessary to get rid of any initial avoidance in order to test for generalization based on colour only. This was not done in chapter IV, as we were interested in the effects of co-mimics on morph survival rather than the confounding mechanism behind predator behaviour. The wild-caught birds may or may not have had previous experience of the moths tested, as is the case in natural predator communities. As the four trials with one moth species did affect bird reactions towards another moth species, we conclude that alternative prey of even vaguely similar appearance is likely to affect bird responses towards different wood tiger moth morphs. In natural communities, predators also often forage in groups and may learn from each other. Observing disgust behaviour by a conspecific can also bias their responses towards warningly coloured prey (Skelhorn 2011, Landová *et al*. 2017).

## 3.3 Multiple strategies in multi-predator world

In this thesis I focused on studying the selective pressures caused by avian predators on the warning colour of wood tiger moth hindwings. Predator's decision to attack and consume a prey may however depend on other cues as well. Comprehensive information about the ecology, interactions and defences of species are needed to study relative importance of evolutionary processes by which the warning colour variation is selected for. Thus, more studies on the relative contributions of each signal component and ecological context are required to understand the predator-prey interactions in more detail.

Experiments with live moths and bird predators have shown, that wood tiger moths can survive bird attacks relatively unharmed (Rönkä and Mappes, unpublished). On the other hand, the birds can also consume quite a few moths without any considerable ill effects (e.g. IV, Rönkä and Mappes, unpublished). Thus, I suggest that the wood tiger moth defense is mainly based on a surprise effect that the bird experiences, when it grabs the moth in its beak and tastes the defense fluid and/or smells the repellent pyrazine odour. The yellowish defensive fluid contains at least two pyrazines (Burdfield-Steel *et al.* under review), which are deterrent to birds (Rojas *et al.* 2017). Pyrazine odour has been shown to reveal hidden, unlearned aversion in avian predators (*Gallus gallus domesticus*) towards conspicuously coloured prey (Lindström *et al.* 2001b) and in particular red and yellow colours (Rowe and Guilford 1996). Pyrazines are commonly found in aposematic moths and butterflies, and may act in concert with the warning colour, facilitating memorization (Rothschild *et al.* 1984, Marples *et al.* 1994). Based on observations during experiments without visual cues (IV), the great tits seemed to generalize their experience with one morph to the other based on odour, in the absence of visual cues, as they often left the second wood tiger moth cup immediately after opening the parafilm seal.

If the two morphs are using different lines of defence, they could have different interactions with their co-prey. The combination of black-and-yellow is common among aposematic prey, and birds have indeed been shown to hesitate longer to attack the yellow than the white morph (Nokelainen *et al.* 2012). *Arichanna melanaria* was found to be a potential model for the yellow morph (IV), showing that the yellow morph can gain further protection by signal sharing with other defended prey. There is mixed evidence for the efficacy of a black-and-white warning colour, however. White Pieridae butterflies have been suggested to form a Müllerian mimicry ring with white coloration acting as a warning signal (Marsh and Rothschild 1974). However, wild pied flycatchers (*Ficedula hypoleuca*) attacked live white *Anthocharis cardamines* and *Pieris napi* equally to brown *Lasiommata maera* and orange *Boloria euphrosyne* (Lyytinen *et al.* 1999). In the same study, great tits also ate *A. cardamines*, *P. napi* and *P. brassicae* without showing disgust behaviours. The white hindwings of the white morph reflect UV, which has been shown to attract rather than repel predators (Lyytinen *et al.* 2001, 2004). White colour as such seems not to be a warning signal. White and cream-white moths and butterflies are common in Finland and Estonia (II), where the white morph is frequent. Thus, I suggest, that the white morph gains protection from an enhanced surprise effect upon a predator effect, caused by conflicting cues of prey palatability – a coloration common among palatable prey combined with the pyrazine odour and bad taste. A somewhat similar effect was termed "type II satyric mimicry" by Howse and Allen (1994), who used it as an alternative explanation to the abundance of apparently imperfect hoverfly wasp mimics. According to Howse and Allen (1994), the hoverflies gain protection by signaling simultaneously cues of palatability (i.e. hoverfly appearance) and cues of unpalatability (i.e. wasp coloration), which confuses the predator when it is making it's decision to attack, thus offering more time for the "satyric mimic" to escape. In the case of the wood tiger moth, I would assume that the predator's confusion together with the surprise effect, will increase the probability, that the bird releases the morph. Satyric mimicry might explain also, why the attack latencies of great tits towards white *A. plantaginis* were not significantly reduced after experience with more palatable black-and-white moths *R. hastata* and *L. marginata* compared to the attack latency without previous trials (Kruskall-Wallis chi-squared = 3.45, df = 2, p-value = 0.178), although the sample size for this comparison was small (IV), and thus the result must be dealt with caution.

Interestingly, the yellow and white morphs have different flight behaviour (Rojas *et al*. 2015). The white morph flies more, whereas the yellow morph flies mostly during female luring peak, and when the yellow morphs were more frequent, the flight activity of both was lower. The authors suggest, that the white morph has presumably less costly defenses, allowing for more investment in flight (Rojas *et al*. 2015). If it is true, however, that the different morphs benefit from different type of mimicry, and that the presence of non-mimetic alternative prey increases attacks towards the yellow morph and reduces attacks towards the white, it would be logical, that the white morph can afford to fly more freely than the yellow morph when alternative prey are common. In addition, if the yellow morph is protected only prior to attack, and is rather palatable to the birds (after they get over the first surprise), the presence of the yellow morph may actually reduce protection towards the white morph, thus making it less profitable for the white morph to fly when the alternative prey community consists mainly of yellow conspecifics. If birds are the main predators of wood tiger moths, and they are most attacked when on the move, it also makes sense to fly primarily during early afternoon and at dusk, when the birds are least active. This is also when it is warmest in the cool summers of higher latitudes, and thus easy to heat up for flight.

Hegna *et al*. (2013) showed a selective trade-off between heat-absorbing melanized area and warning coloured area on moth wings, indicating that thermoregulation can be important for the species. The size of orange warning signal on female larvae is genetically correlated with a less red (i.e. more orange) wing coloration as an adult, but also with a higher reproductive output (Lindstedt *et al*. 2016). On the other hand, more melanic (i.e. more orange) larvae are better adapted to cool environments (Lindstedt *et al*. 2009), but the production of melanin has been suggested to be costly in *A. plantaginis*, especially under limited resources in their diet (Ojala *et al*. 2007). Thus, Lindstedt *et al*. (2016) suggest that the more and less orange ends of the larval color variation continuum represent two different strategies, one adapted to invest in warning signal efficacy as a larva and higher reproduction as an adult, and the other to shorter development time as a larva and better heat absorption and warning signal as an adult in colder climates, where the season progresses quickly and ambient temperatures are lower. The regulatory mechanisms of pigment synthesis in the larvae and adult *A. plantaginis* are not currently known, but if they are similar in the two stages, the red hindwing colour of adult moths could be a by-product of selection for melanistic larvae. The findings of Lindstedt *et al*. (2016) were based on a laboratory stock originating from Finland, and thus nothing is known of potential genetic correlations between larval and adult coloration in the red Georgian males. Adult male coloration may also be affected by trade-offs in immunological response against parasites at the larval stage (Nokelainen *et al*. 2013), and their immunological defences against both parasitism and pathogens may also be related to the level of melanism in the larvae (Friman *et al*. 2009).

Unexpectedly, sexual selection in the wood tiger moths is also positively frequency-dependent, thus selecting for signal monomorphism (Gordon *et al*. 2015). However, geographic variation in the direction of sexual selection coupled with gene flow can allow polymorphism to be maintained (Gordon *et al*. 2015), and this effect combined with the geographic variation in natural selection by local predators is surely causing multiple selective pressures to act on the wood tiger moth coloration simultaneously. If each morph represents an adaptive peak on an adaptive landscape, where different selective pressures represent different dimensions, one of the morphs, e.g. the red morph may be most salient (III), whereas e.g. the yellow morph may gain benefit from mimicking another defended species (IV), and the white gains most matings under stressful conditions (Nokelainen *et al*. 2012). If there is enough variation in the adaptive value of each of these qualities, so that the peak heights are approximately even in the long run, multiple morphs could be maintained.

## 3.4 The spread of a new morph

Finally, the moth hindwing colour, as any trait of any organism, is a product of multiple selective pressures in its past and present habitats. Therefore, it may not be perfectly adapted to current selective pressures. In the case of the wood tiger moth, the colour polymorphism seems to be a relatively recent development (I), and thus the occurrence of locally polymorphic populations may be a transitional stage, that is not evolutionarily stable in the long term. The evolutionary pathway to polymorphism in Batesian and Müllerian mimicry is often associated with supergenes (Joron *et al*. 2011), because without tight genetic linkage the multiple distinct colour forms could not be maintained. More work needs to be done to figure out the inheritance mechanisms of *A. plantaginis* hindwing coloration. Thus far it seems, that the white and yellow male morphs are determined by a single locus with at least three alleles – a dominant white allele, a recessive white allele and a yellow allele intermediate in dominance compared to the white alleles (Galarza unpublished). The genetic dominance of the white allele over the yellow together with high levels of gene flow and spatially and temporally variable level of selection might explain how the white morph spread across the Holarctic. As a single wood tiger moth female can produce hundreds of offspring, it might only take one mated female immigrating to a new location to found a new population. If avian predators are wary of any novel types of aposematic prey (Coppinger 1970), with wariness lasting up to weeks or months in wild birds (Marples and Kelly 1999), or the new white morph is protected by satyric mimicry (see discussion above), predation may have little effect at the early stages of establishing a new moth population.

Another potential mechanism facilitating polymorphism in aposematic organisms could be lateral gene exchange between species (Dasmahapatra *et al*. 2012, Kozak *et al*. 2015). Schmidt and Sperling (2008) suggested that mtDNA introgression is common in *Apantesis* (formerly *Grammia*, see I for classification) tiger moth species, Dasmahapatra *et al*. (2012) found hybrid exchange of genes between mimetic *Heliconius* butterfly species, and Medina *et al*. (2013) found hybridization in aposematic *Oophaga* poison frogs. Many tiger moths are aposematic, share similar chemical defences, and also similar pheromones (Conner 2008). These features may allow interspecies genetic mixing, contributing to the evolution of new aposematic species or spread of new colour variants, if the resulting phenotype (of the hybrid offspring) is not predated upon, i.e. happens to occupy a new adaptive peak. New variants may gain protection from widely generalizing predators, or due to a peak shift phenomena, where the change happens in a stimulus dimension that elicits a stronger or equal avoidance reaction in the predators than the already learned stimulus due to predator's cognitive biases (Gamberale and Tullberg 1996, Gamberale-Stille and Tullberg 1999, Lynn 2005). The short branches and thus poorly resolved branching order within *Arctia* (I) is suggestive of rapid adaptive radiation, which may be facilitated by mimicry and hybridization events.

# 4 CONCLUSIONS

We found locally purifying selection on the warning colour of wood tiger moths by local avian predators, as predicted by the theory of aposematism (II). Selection was dependent on local morph frequency across countries, and not on morph colour (II), and no population-level bias was found towards any of the colours by wild-caught predators in a polymorphic population (III). Birds were also able to discriminate between the wood tiger moth morphs based on hindwing colour only (III). The prevalence of red and yellow hindwing colour in the close relatives of the wood tiger moth (I) hints that the ancestral state of the wood tiger moth hindwing colour has been yellow or red. We also found that birds learned to avoid the red morph quicker compared to white and yellow, corroborating the line of evidence suggesting that red is a particularly salient warning colour (III). The original question of why are not all wood tiger moths red remains puzzling, but several lines of research have already indicated possible mechanisms contributing to the maintenance of polymorphism in some populations.

In this thesis, no selection, or very low levels of selection, was found on hindwing warning colour in the more polymorphic populations in Estonia and Finland (II, Table 1), and wild-caught blue tits in Finland showed no initial bias on hindwing colour (III). This suggests, that natural selection may not always be strong, and could be overruled or counterbalanced by other selective processes (see also Briolat *et al.* in review). The main evolutionary function of adult holometabolous insects is to breed. Sexual selection is therefore expected to play an important role in determining wood tiger moth adult coloration. Contrary to expectations, Gordon *et al*. (2015) found sexual selection to be positively frequency-dependent, which is expected to lead to signal monomorphism. It is thus likely, that both sexual selection by conspecifics and survival selection by predators are affecting the adult coloration of wood tiger moths. It is however very unlikely to find a situation where these mechanisms would create opposing selection that is perfectly balanced, and thus could maintain polymorphism. Even a slight difference in the strength of opposing selection pressures leads to directional, albeit weak, selection that should eventually lead to monomorphism. The maintenance of polymorphism could thus be more easily explained with spatio-temporal changes in selection (see also Nokelainen *et al*. 2014, Gordon *et al*. 2015).

Natural selection is variable for many possible reasons. 1) Differences in prey palatability. The wood tiger moths are distasteful, but not very toxic – some predators can handle them and feed on them. Here we showed variation in both the palatability of wood tiger moths with and without visual cues as well as in relation to alternative prey sharing a similar warning coloration (IV). Recent experience with other warningly coloured prey changed bird reactions towards the yellow wood tiger moth, indicating that the co-occurrence of prey of even vague resemblance can alter selection towards the different morphs. 2) Changes in the abundance of non-mimetic or palatable alternative prey may well cause changes in the direction of selection (II), as predators are more likely to attack defended prey when food recourses are scarce. 3) The bird predators vary in their responses towards the morphs at individual (III), interspecies (IV) and community levels (II, Nokelainen *et al*. 2014). This means, that selection may vary at several different spatial levels, ranging from individual bird territories to different habitats and climates. Depending on the size of wood tiger moth populations and the level of gene flow between them, the spatial variation in the direction of selection can promote polymorphism. In addition, 4) seasonality causes temporal changes in predator and prey communities at the high latitudes. The relative contributions of spatial and temporal variation in selection towards warningly coloured prey are not well known.

I conclude that predation by local avian predators is indeed an important selective force driving the evolution of wood tiger moth warning colour. Colour seems to have a very important role in predator-prey interactions, and it seems that birds are more likely to generalize between similarly coloured prey (IV) than between other cues (III), but it is not the only cue predators use in prey discrimination. Earlier experiences of the predator and its abilities to handle defended prey are likely important in determining whether or not it will attack a wood tiger moth. The co-prey community (II, IV), and possibly also other predators, can thus affect predators' attack decisions. Local predator communities are composed of different types of predators (II), which vary in the way they search for food, and select and handle their prey. Because a proportion of the insectivorous birds are migratory or juvenile, which may not have had many opportunities to learn about the wood tiger moths, neophobia and dietary conservatism can also play a role in reducing the attack risk towards the different morphs. Furthermore, if the wood tiger moth defense is mainly based on a surprise effect, it is likely most effective against the less experienced predators. Taken together, these observations indicate a causal relationship between local predator community composition and geographic differences in the strength and direction of selection on wood tiger moth warning colour, but this remains to be tested further to exclude other possibilities, such as changes in lighting conditions, which could affect both predator community structure and signal salience (II, Nokelainen *et al*. 2014).

In order to figure out the proximate reasons affecting selection on warning colour, and thus the possible mechanisms allowing for polymorphism, more detailed information on predator's attack decisions is needed. For example, it is not known how widely different predators will generalize based on colour (Ham *et al*. 2006), or if they use simple categorization when learning about aposematic prey (Chittka and Osorio 2007). It is also unknown, how the different warning signal components interact and which of them are most important for generalization between prey. The salience of the different signal components is also context-dependent. Predatory decisions are affected by not only how they estimate the nutrient/toxin trade-off in consuming defended prey, but also by how they estimate the likelihood of finding profitable food in the future (Skelhorn *et al*. 2016). Therefore, to investigate the ultimate reasons in how polymorphism is originated and maintained, a more comprehensive approach taking into account the ecological context in which predators make their decisions is needed. This requires studying both frequency- and density dependent interactions between local predators and prey.

## *Postscript: So, why are not all wood tiger moths red and does it matter?*

In nature, all organisms face many selective pressures simultaneously. In order to study causal relationships and theories of how a particular selective pressure works in natural systems, it is necessary to make simplifications of the actual system. In modelling approaches, this is shown as the amount of parameters included in the model built to describe a particular mechanism and the assumptions made on how they affect each other. For experimental approaches, however, we make simple, testable predictions and plan the experiments controlling for other sources of variation as well as possible. The need for controlled circumstances means that experiments are often done in unnatural laboratory conditions, with few organisms, lacking the ecological context. As a result of these approaches, we have a) many simplified models of predation and aposematic selection, which often fail to perfectly explain what is observed in the field, and b) a lot of very detailed information on predator behaviour under certain circumstances towards certain qualities of aposematic prey, but no comprehensive picture of what is relevant in real predator-prey interactions in the wild.

The recent development of genetic (e.g. Mazo-Vargas *et al*. 2017), computational (e.g. Nascimento *et al*. 2017), image analysis and animal vision modeling (e.g. Renoult *et al*. 2017) methods enable new type of questions to be asked, and contribute to a detailed understanding of the evolutionary mechanisms by which selection operates. In other words, the developing toolbox for evolutionary biologists is refining our understanding of the "toolbox of evolution", i.e. the proximate reasons behind it. However, also experimental approaches both in the laboratory and in the field have still a justified role. The cognitive processes in predators' decision making upon predatory events are not well known (Skelhorn *et al*. 2016). Thus, it is not enough to know e.g. what a predator is capable of seeing, but also how it perceives the visual stimuli, how it processes this information, and what kind of information it uses for decision-making. The range of variation in cognitive capabilities of non-human animals is not yet fully understood (see e.g. Edelman and Seth 2009 for a suggestion of how to study animal consciousness). Predator-prey interactions are thus best examined using relevant predators and prey.

In addition to predator specificity, predatory decisions are also affected by the ecological context, i.e. by other predators and prey in the community. It is widely acknowledged that predator-prey interactions and ecological communities are complex and variable, and that repetitive studies using different systems are needed in order to draw generalizable conclusions of evolution, and study the ultimate causes for the origins and maintenance of biodiversity and natural selection. Fieldwork is however not easy, as it is impossible to effectively control for variation e.g. in climate, weather, anthropogenic influence or animal behaviour. The most successful eco-evolutionary study systems for field studies are often naturally simple, such as islands (Hanski 1998, Grant and Grant 2002), small ponds (Ebert 1994) or streams (Endler 1980), but even in these cases, long-term studies are needed in order to detect causes for evolutionary change.

The complementary approaches of fieldwork, modeling and laboratory experiments all contribute to a more detailed understanding of the evolution of signal diversity. Observations made in the field can be tested in controlled laboratory conditions to study selective mechanisms in detail. Experimental results can then be used to refine models of selection to make more accurate predictions of evolutionary processes. Model predictions can again be tested in the field, repeatedly in different systems, to test their generalizability. In this thesis, I have used a wide variety of methods to contribute to the big picture of how warning colour is selected for in a particular system. The wood tiger moth system has become a well-known model system in evolutionary biology, as more and more studies using different approaches and studying different evolutionary mechanisms and processes explaining the warning colour variation observed in this system built up. Such comprehensive knowledge will help understanding the relative contributions of each selective pressure in maintaining and creating biodiversity. These types of more complex and counterintuitive systems will thus offer valuable insights to studies at the interphase of evolutionary and ecological research.

Eventually, we might even also figure out why the wood tiger moths are not all red. If I had to make a wild guess of how the polymorphism originated – based on ideas discussed during these four years – I would look for evidence of lateral gene exchange between the wood tiger moth and e.g. *Ocnogyna boeticum*, a more distantly related, but very similarly patterned tiger moth with black-and-white hindwings (and sexually dimorphic, wingless females), or *Arctia virginalis* (Fig. 2), which is surprisingly similar to the wood tiger moth in COI barcode region. However, as interesting as it might be to know how the sexual dimorphism and colour polymorphism originated, it has been equally rewarding to study the selective mechanisms that maintain the observed variation. In science, asking interesting questions is often more valuable than providing exhaustive answers, as new, and even more fascinating discoveries can be made on the way.

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# YHTEENVETO (RÉSUMÉ IN FINNISH)

## Peto-saalissuhteet ja paikallisen värimuuntelun evoluutio aposemaattisen täpläsiilikkään (*Arctia plantaginis*) populaatioissa

Luonnon monimuotoisuus on seurausta evoluutiosta, jossa luonnon- ja seksuaalivalinnan seurauksena pisimpään elävät tai parhaan kumppanin saavat organismit tuottavat eniten jälkeläisiä. Mikäli valinnan suosimat ominaisuudet ovat perinnöllisiä, yleistyvät ne seuraavassa sukupolvessa, minkä seurauksena keskenään lisääntyvä populaatio sukupolvi sukupolvelta sopeutuu elinympäristönsä valintapaineisiin. Petojen ja niiden saalislajien välisten vuorovaikutussuhteiden seurauksena saalislajeille on kehittynyt erilaisia selviytymistrategioita. Aposemaattinen organismi varoittaa mahdollisia petoja pahanmakuisuudestaan tai muusta puolustuksesta varoitussignaalein, jotka ovat usein huomiota herättäviä värejä. Peto oppii signaalin merkityksen maistamalla tai kokeilemalla saalista, ja yleistää oppimansa välttämiskäyttäytymisen muihin samankaltaisiin signaaleihin.

Mikäli pedot oppivat kokeilemalla, yksittäiseen saalisyksilöön kohdistuva saalistuspaine on sitä pienempi, mitä useampi yksilö jakaa samanlaiset signaalin. Saalistuksen aiheuttama luonnonvalinta suosii siten signaalin yhdenmukaisuutta. Kuitenkin värivaihtelu on yleistä myös aposemaattisten eliöiden keskuudessa. Hyvä esimerkki tällaisesta lajista on kemiallisesti puolustautunut täpläsiilikäs (*Arctia plantaginis*), jonka etusiivet ovat mustavalkokirjaillut ja takasiivet joko mustat, mustavalkoiset, mustakeltaiset tai mustakeltaiset kera vaihtelevan punaisen pigmentin. Värimuotojen yleisyys vaihtelee niin populaatioiden välillä kuin populaatioiden sisällä. Väitöskirjassani tarkastelin, mitkä evolutiiviset valintapaineet voivat selittää täpläsiilikkään varoitusvärien monimuotoisuutta. Erityisesti tutkin, millainen rooli täpläsiilikkäiden todennäköisimmillä pedoilla eli hyönteissyöjälinnuilla sekä vaihtoehtoisella saaliilla eli muilla päiväaktiivisilla perhosilla on varoitusvärien evoluutiossa. Väitöskirjani koostuu neljästä osatyöstä (I-IV), joissa tutkin:

I) Onko täpläsiilikkään vaihteleva väritys sen sukulaisille tyypillinen ominaisuus, ja siten todennäköisesti evoluutiohistorian jäänne, vai
II) onko se seurausta lintujen saalistuksen aiheuttamasta luonnonvalinnasta?
III) Yleistävätkö pedot oppimansa välttämiskäyttäytymisen täpläsiilikkään eri värimuotojen välillä mahdollistaen näin värivaihtelun?
IV) Mahdollistuuko eri värimorfien olemassaolo sillä, että ne jakavat varoitusvärin muiden aposemaattisten lajien kanssa, saaden näiltä suojaa saalistukselta?

Ensimmäisessä osatyössä kerättiin maailmanlaajuisesti DNA-näytteitä täpläsiilikkään mahdollisista sukulaisista, mistä saadun aineiston perusteella täpläsiilikäs sijoitettiin Arctiina–alaheimoon. Sekä maximum likelihood- että bayeslainen analyysi muodostivat samankaltaisen ja hyvin tuetun sukupuun, jonka perusteella ehdotettiin yhteensä 33 siilikässuvun yhdistämistä neljään sukuun. Täpläsiilikäs, *Parasemia plantaginis*, kuten moni sukulaisensa, oli kuvattu sukunsa ainoana lajina, mutta kuuluu fylogeneettisen analyysin mukaan sukuun *Arctia* comb. nov. (*combinatio nova*, uusi nimiyhdistelmä). Täpläsiilikkään lähimmät sukulaiset ovat väritykseltään puna-keltaisia, mikä yhdessä aiempien tutkimustulosten kanssa viittaisi täpläsiilikkään värivaihtelun olevan uudempaa kehityslinjaa. Lajin muuntelevuus tarjoaa mahdollisuuden havainnoida siihen vaikuttavia valintapaineita ja tutkia, mitkä evolutiiviset mekanismit ylläpitävät ja edistävät biologista monimuotoisuutta.

Toisessa osatyössä mitattiin väritykseen kohdistuvaa saalistuspainetta asettamalla valkoista, keltaista ja punaista värimuotoa vastaavia keinotekoisia perhosmalleja täpläsiilikkään luontaisiin elinympäristöihin. Tutkimuskohteiksi valittiin neljä populaatiota neljästä eri maasta, joissa esiintyi luontaisesti joko yhtä tai useampaa eri täpläsiilikkään värimuotoa. Lintujen hyökkäykset havainnoitiin mallien muovailuvahasta tehtyihin ruumiisiin jääneiden nokanjälkien perusteella. Paikalliset pedot hyökkäsivät vähiten paikallisesti yleisiin täpläsiilikkään värimuotoihin, eli luonnonvalinta riippui positiivisesti värimuodon yleisyydestä. Valinta ei ollut riippuvaista väristä sinänsä, sillä pedot välttivät eri värimuotoja eri alueilla. Niissä populaatioissa, joissa useampi värimuoto oli yleinen, ei havaittu eroa eriväristen mallien riskissä tulla hyökätyksi.

Kokeen aikana laskettiin myös tutkimusalueella esiintyneiden erilaisten hyönteissyöjälintujen ja päiväaktiivisten perhosten määrät. Lintuyhteisön koostumus korreloi täpläsiilikkään eri värimuotoihin kohdistuvan saalistuspaineen kanssa eri tavoin eri maissa. Saalistuspaineen on aiemmin havaittu lisääntyvän keltaista muotoa kohtaan yhteisöissä joissa rautiaiset (Prunellidae) ovat edustettuina, verrattuna yhteisöihin joissa tiaiset ovat yleisiä. Aiempi tutkimusaineisto oli rajattu Suomeen ja Viroon niiden lintulajiston samankaltaisuuden perusteella. Nyt havaittiin aiempien tulosten mukaisesti, että Suomessa runsas hyökkäysmäärä keltaiseen muotoon assosioituu rautiaisten läsnäoloon lintuyhteisössä, kun taas Skotlannissa tilanne oli päinvastainen. Vastaavia populaatiotason eroja lintujen saalistus-käyttäytymisessä on aiemmin todettu talitiaisilla.

Viron lintuyhteisön koostumus poikkesi vertailumaista (Suomi, Skotlanti ja Georgia), mikä saattaa selittää sen, miksi malleihin hyökättiin Virossa erittäin vähän. Alueellisesti vaihteleva luonnonvalinta paikallisten lintujen toimesta onkin siten todennäköinen syy sille, miksi täpläsiilikkään varoitusväreissä esiintyy maantieteellistä vaihtelua. Vähäinen saalistuspaine voi mahdollistaa varoitusvärien vaihtelua, koska varoitusfunktion ollessa vähäinen muut valintapaineet voivat vaikuttaa perhosten väritykseen. Varoitusvärien avulla perhonen viestittää mahdollisille saalistajille olevansa puolustautunut, mutta väritys voi vaikuttaa myös mm. perhosten lämmönkeruukykyyn, immuunipuolustukseen sekä lajinsisäiseen viestintään esimerkiksi parinvalintaan liittyen. Saalistuspaine varoitusvärisiä saaliita kohtaan riippuu esimerkiksi petojen motivaatiosta ja tarpeesta hankkia ravintoa sekä siitä, kuinka laajalti se yleistää oppimaansa eri signaalien välillä.

Kolmannessa osatyössä testattiin yleistävätkö pedot yhtä täpläsiilikkään värimuotoa kohtaan oppimansa välttämiskäyttäytymisen toisiin täpläsiilikkään värimuotoihin, mikä johtaisi valinnan heikkenemiseen värityksen suhteen. Petoina käytin sinitiaisia, jotka ovat perhosten luontaisia petoja Suomessa, jossa myös täpläsiilikkään valkoinen, keltainen ja punainen värimuoto esiintyvät yhtä aikaa. Linnut oppivat välttämään punaista värimuotoa nopeammin kuin keltaista tai valkoista. Sinitiaiset eivät kuitenkaan yleistäneet eri muotojen välillä, sillä opittuaan välttämään yhtä värimuotoa valtaosa linnuista kuitenkin hyökkäsi kahteen muuhun, ilmeisesti pitäen näitä erilaisina saaliina yksinomaan takasiipien värin perusteella.

Koska väri on linnuille tärkeä saaliin tunnistamisperuste, neljännessä osatyössä testattiin, voisiko usean värimuodon samanaikainen esiintyminen selittyä sillä, että linnut yleistävät oppimansa välttämiskäyttäytymisen samanväristen puolustautuneiden lajien välillä. Tällöin kukin täpläsiilikkään värimuoto matkisi eri lajia, kuten trooppinen aposemaattinen perhonen *Heliconius numata*, jonka eri värimuodot matkivat useita samalla alueella esiintyviä myrkyllisiä mallilajeja. Kokeeseen valittiin kaksi keltamustaa ja kaksi valkomustaa täpläsiilikkään kanssa yhtä aikaa esiintyvää perhosta. Koska lajien keskinäisestä syömäkelpoisuudesta ei ollut aiempaa tietoa, niiden maistuvuutta talitiaisille testattiin ensin ilman värejä. Varoitusvärin vaikutusta lintujen reaktioihin testattiin tarjoamalla tali- ja sinitiaisille kuolleita yksilöitä ensin yhdestä ja sitten toisesta lajista. Havaintojemme perusteella suomittari, *Arichanna melanaria*, jakaa mahdollisesti puolustuksensa täpläsiilikkään keltaisen värimuodon kanssa.

Testatut mustavalkoiset lajit eivät olleet linnuille yhtä pahanmakuisia kuin valkoinen täpläsiilikäs. Valkoinen värimuoto saattaa kuitenkin hyötyä muutoin vaihtoehtoisesta saaliista. Maastokokeissa havaitsin nimittäin, että lisääntyvä muun kuin täpläsiilikkään värisen saaliin määrä vähensi hyökkäyksiä valkoista värimuotoa kohtaan, mutta lisäsi hyökkäyksiä keltaiseen muotoon. Täpläsiilikkään värisiksi luokittelin mustavalkoiset, mustakeltaiset ja mustapunaiset lajit ja muunvärisiksi muut. Koska iso osa muunväriseksi luokitelluista perhosista on yleissävyltään vaaleita, ja perhosten ollessa liikkeellä niiden kuvioinnit sulautuvat yhteen, on mahdollista että linnut erehtyvät luulemaan valkoista täpläsiilikästä syötäväksi saaliiksi. Kun lintu nappaa täpläsiilikkään nokkaansa syödäkseen sen, täpläsiilikäs vapauttaa erityisistä pään sivuilla sijaitsevista rauhasista kitkerälle maistuvaa ja haisevaa puolustusnestettä. Jos peto yllättyy tästä ja hellittää otteensa, saattaa se lisätä täpläsiilikkään mahdollisuuksia selvitä hyökkäyksestä vahingoittumattomana. Täpläsiilikkään eri värimuodot saattavatkin erikoistua erilaisiin puolustusstrategioihin – keltainen muoto saa suojaa lintujen epäröinnistä ennen hyökkäystä ja valkoinen lintujen epäröinnistä hyökkäyksen aikana.

Tulosteni perusteella voidaan päätellä, että a) paikallisten hyönteissyöjälintujen saalistuskäyttäytymisellä on tärkeä merkitys täpläsiilikkään varoitusvärien evoluutiossa, b) sekä lintujen että vaihtoehtoisen saaliin ominaisuuksien vaihtelu eri tasoilla (yksilöistä yhteisöihin) vaikuttaa valinnan suuntaan, mikä voi edesauttaa värimuotojen (polymorfismin) säilymistä ja c) valinnan voimakkuus voi vaihdella sekä paikallisesti että ajallisesti, jolloin muut evolutiiviset prosessit voivat tasapainottaa tai kumota luonnonvalinnan vaikutuksen edistäen varoitusvärien muuntelevuutta.

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# ORIGINAL PAPERS

# I

# PUTTING *PARASEMIA* IN ITS PHYLOGENETIC PLACE: A MOLECULAR ANALYSIS OF THE SUBTRIBE ARCTIINA (LEPIDOPTERA)

by

Katja Rönkä, Johanna Mappes, Lauri Kaila & Niklas Wahlberg 2016

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# Putting *Parasemia* in its phylogenetic place: a molecular analysis of the subtribe Arctiina (Lepidoptera)

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**Abstract.** Despite being popular among amateur and professional lepidopterologists and posing great opportunities for evolutionary research, the phylogenetic relationships of tiger moths (Erebidae: Arctiinae) are not well resolved. Here we provide the first phylogenetic hypothesis for the subtribe Arctiina with the basic aim of clarifying the phylogenetic position of the Wood Tiger Moth *Parasemia plantaginis* Hübner, a model species in evolutionary ecology. We sampled 89 species in 52 genera within Arctiina s.l., 11 species of Callimorphina and two outgroup species. We sequenced up to seven nuclear genes (CAD, GAPDH, IDH, MDH, Ef1$\alpha$, RpS5, Wingless) and one mitochondrial gene (COI) including the barcode region (a total of 5915 bp). Both maximum likelihood and Bayesian inference resulted in a well-resolved phylogenetic hypothesis, consisting of four clades within Arctiina s.s. and a clade comprising spilosomine species in addition to Callimorphina and outgroups. Based on our results, we present a new classification, where we consider the *Diacrisia* clade, *Chelis* clade, *Apantesis* clade, *Micrarctia* Seitz and *Arctia* clade as valid genera within Arctiina s.s., whereas *Rhyparia* Hübner **syn.n.** and *Rhyparioides* Butler **syn.n.** are synonymized with *Diacrisia* Hübner; *Neoarctia* Neumoegen & Dyar **syn.n.**, *Tancrea* Püngeler **syn.n.**, *Hyperborea* Grum-Grshimailo **syn.n.**, *Palearctia* Ferguson **syn.n.**, *Holoarctia* Ferguson **syn.n.**, *Sibirarctia* Dubatolov **syn.n.** and *Centrarcti*a Dubatolov **syn.n.** are synonymized with *Chelis* Rambur; *Grammia* Rambur **syn.n.**, *Orodemnias* Wallengren **syn.n.**, *Mimarctia* Neumoegen & Dyar **syn.n.**, *Notarctia* Smith **syn.n.** and *Holarctia* Smith **syn.n.** are synonymized with *Apantesis* Walker; and *Epicallia* Hübner **syn.n.**, *Eucharia* Hübner **syn.n.**, *Hyphoraia* Hübner **syn.n.**, *Parasemia* Hübner **syn.n.**, *Pericallia* Hübner **syn.n.**, *Nemeophila* Stephens **syn.n.**, *Ammobiota* Wallengren **syn.n.**, *Platarctia* Packard **syn.n.**, *Chionophila* Guenée **syn.n.**, *Eupsychoma* Grote **syn.n.**, *Gonerda* Moore **syn.n.**, *Platyprepia* Dyar **syn.n.**, *Preparctia* Hampson **syn.n.**, *Oroncus* Seitz **syn.n.**, *Acerbia* Sotavalta **syn.n.**, *Pararctia* Sotavalta **syn.n.**, *Borearctia* Dubatolov **syn.n.**, *Sinoarctia* Dubatolov **syn.n.** and *Atlantarctia* Dubatolov **syn.n.** are synonymized with *Arctia* Schrank, leading to 33 new genus-level synonymies. Our focal species *Arctia plantaginis* **comb.n.** is placed as sister to *Arctia festiva* **comb.n.**, another widespread aposematic species showing wing pattern variation. Our molecular hypothesis can be used as a basis when adding more species to the tree and tackling interesting evolutionary questions, such as the evolution of warning signalling and mimicry in tiger moths.

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## Introduction

Tiger moths are a highly diverse group consisting of about 11 000 species worldwide. Of these, approximately 4000 species in 113 genera belong to the subtribe Arctiina (Erebidae: Arctiinae: Arctiini: Arctiina s.l.) (see Weller *et al.*, 2009 and references therein). Their visual appearance and diverse ecology have made them popular among amateur lepidopterists and some species are studied intensively [e.g. the Ornate Moth *Utetheisa ornatrix* (Linnaeus), Garden Tiger Moth *Arctia caja* (Linnaeus) and the Wood Tiger Moth *Parasemia plantaginis* (Linnaeus)], but in general our knowledge of their diversity and phylogenetic relationships is surprisingly limited (Bendib & Minet, 1998; Conner, 2009). New species are still found, perhaps because many are relatively rare, difficult to observe, or may occur in small numbers in remote places (e.g. *Micrarctia kautti* Saldaitis & Pekarsky, 2015). The present classification of Arctiina s.l. is based mainly on detailed studies based on morphological characters (Dubatolov & de Vos, 2010; Lafontaine & Schmidt, 2010; Fibiger *et al.*, 2011; Vincent & Laguerre, 2014). However, these data have not been subjected to rigorous phylogenetic analyses.

Most tiger moths are chemically defended, advertise their unpalatability with spectacular warning colours and take part in several Müllerian mimicry rings (Conner, 2009). High morphological variability in Arctiinae means that it is difficult to determine unequivocal synapomorphies [shared derived characters that support monophyletic groups (clades)], which makes it challenging to trace the evolutionary relationships within the group (Schmidt, 2007; Weller *et al.*, 2009). As mimicry is very likely to occur within Arctiinae, another phenomenon that can potentially obscure our understanding of the systematics of this group is incomplete lineage sorting. This is likely to be common in many systems, such as mimetic butterflies, resulting from rapid radiation or adaptive introgression facilitated by strong selection on adaptive loci (Kozak *et al.*, 2015). In addition, the tendency of researchers to describe each colourful and uniquely patterned species in its own genus has led to a less informative classification, in which many tiger moth genera are species-poor, monotypic and, in some cases, probably paraphyletic (Weller *et al.*, 2009).

*Parasemia plantaginis* is the only species in its nominal genus *Parasemia* Hübner. The species occurs in the Holarctic, forming two distinct clades, one of which corresponds to *P. plantaginis* ssp. *caucasica* (Ménétries), with both male and female moths expressing 'interrupted' forewing pattern (Hegna & Mappes, 2014; Honma *et al.*, 2015) and hindwing coloration varying from yellow to red (Fig. 1D). The other clade comprises all other forms of *P. plantaginis* with various patterns and polymorphic hindwing coloration (Fig. 1A–C; Hegna *et al.*, 2015). The effects of variation in both larval and adult coloration of *P. plantaginis* on their predation risk and other fitness measures, as well as population genetics, have been intensively studied (e.g. Ojala *et al.*, 2005, 2007; Lindstedt *et al.*, 2011; Nokelainen *et al.*, 2011; Hegna *et al.*, 2013 & Galarza *et al.*, 2014) and the species has great potential for becoming a model system in the study of the evolution of warning coloration (Stevens & Ruxton, 2012) and colour polymorphism.

Thus, to further investigate interesting evolutionary questions in this system, such as the evolution of warning signal polymorphism or convergent evolution in mimicry rings, a well-resolved phylogeny of Arctiina is crucially needed (Simmons, 2009; Hegna *et al.*, 2015). With a phylogenetic hypothesis available, it will be possible to determine when colour polymorphisms have evolved in the group and to study the occurrence of mimetic patterns in detail (Simmons, 2009).

The higher classification of tiger moths (Lepidoptera: Erebidae: Arctiinae) was recently studied with molecular methods by Zaspel *et al.* (2014), but this study had sparse sampling of the species-rich subtribe Arctiina. Zaspel *et al.* (2014) sampled only *Arctia caja* from the diverse *Arctia* group and did not include *Parasemia*. *Parasemia* is thought to be closely related to *Arctia*, with some evidence that it may, in fact, be within the genus (Fibiger *et al.*, 2011). Schmidt's (2007) tree, with combined evidence from barcode and morphology, placed *Parasemia* in the same clade with *Arctia*, *Platyprepia*, *Platarctia* and *Pararctia*. With the broadest sampling of related genera so far, Dubatolov (2008) placed *Parasemia* closest to *Hyphoraia*, which consists of three species [*Hyphoraia aulica* (Linnaeus), *H. dejeani* (Godart) and *H. testudinaria* (Geoffroy)], and *Epicallia* (=*Arctia*) *villica* (Linnaeus), a monotypic genus, based on morphological characters.

In this study, we infer a molecular hypothesis of the phylogenetic relationships of species in the subtribe Arctiina, aiming to clarify the position of *Parasemia* within the subtribe. Based on our results, we revise the classification of Arctiina s.s. By doing this we contribute to establishing the relationships among many monotypic genera, stated by Weller *et al.* (2009) as the next big challenge in arctiine systematics.

## Material and methods

### *Sampling*

Many Palearctic Arctiina species are rare and/or occur in areas that are not easily accessible to collectors. However, with the aid of several amateur lepidopterologists and fellow scientists (see the Acknowledgements) we were able to sample many of the species in the subtribe putatively related to *Parasemia*. The selection of taxa was based on previous studies (Jacobson & Weller, 2002; Schmidt, 2007; Dubatolov, 2008, 2009; Zaspel *et al.*, 2014) and available checklists relevant to our taxon sampling (Dubatolov & de Vos, 2010; Lafontaine & Schmidt, 2010; Fibiger *et al.*, 2011; Vincent & Laguerre, 2014). Within the tribe Arctiini we sampled 11 species representing nine genera of the subtribe Callimorphina and 89 species representing 52 genera of the subtribe Arctiina, but excluded the mostly tropical subtribes Pericopina, Ctenuchina, Euchromiina and Phaegopterina. As outgroups we used *Setina sp.* (Erebidae: Arctiinae: Lithosiini) and *Amata sp.* (Arctiinae: Syntomini), which are closely related to Arctiini according to Zaspel *et al.* (2014).

Our focal study species, *P. plantaginis*, is placed in Arctiini: Arctiina. To our knowledge, *Parasemia* together with other genera putatively related to *Arctia* belong to Arctiina s.s., and,

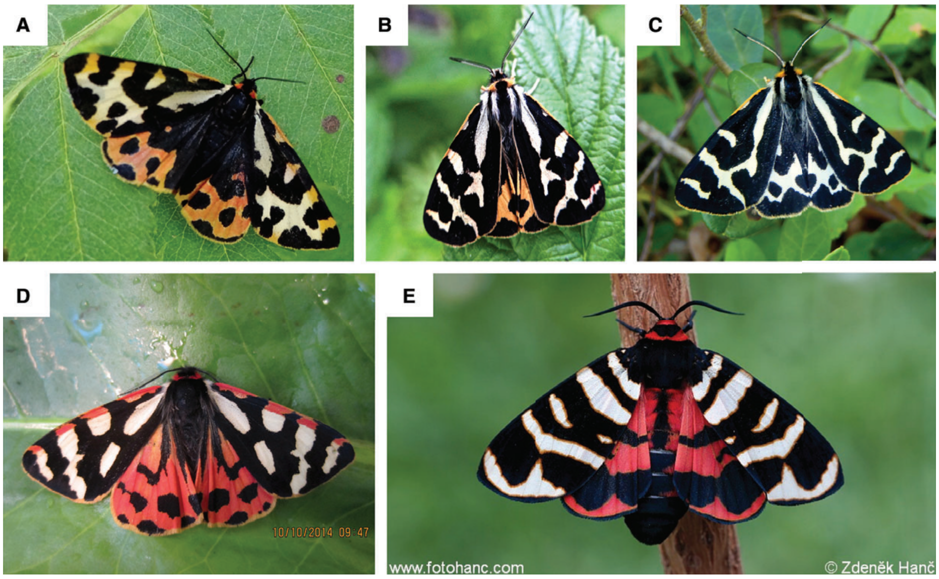

**Fig. 1.** (A–E) Female *Arctia plantaginis* (A), male *A. plantaginis* colour variants (B, C), male *A. plantaginis* ssp. *caucasica* (D) and female of the sister species *Arctia festiva* (E) on natural backgrounds. [Photographs were taken by Bibiana Rojas (A–C), KR (D) and Zdenek Hanc (E).]

within that, to a lineage that has a Holarctic distribution (Weller *et al.*, 2009). Sampling within Arctiini was thus limited to the Holarctic region, with most species having a Palaearctic distribution, although eight species occurring only in the Nearctic were also included. For species with a wide distribution range we aimed to sample at least two individuals representing different populations to avoid possible bias caused by local adaptive evolution. As we focused our sampling to Arctiina s.s. in the hope of finding the closest relatives of *Parasemia*, the so-called spilosomine genera and other mainly tropical lineages of Arctiini were left more sparsely sampled. However, including the sequences of Arctiina used by Zaspel *et al.* (2014) in our analysis broadened our coverage to tropical regions for the spilosomine genera.

We used samples that were as fresh as possible, with the oldest ones sampled successfully being up to 10 years old, stored dry, in alcohol or frozen at −20°C. For DNA extraction we used either one to two legs of adult specimen or a small piece of tissue (e.g. anal prolegs) from larvae. The barcode (COI) sequences of our samples were cross-checked in the Barcode of Life Data System (Ratnasingham & Hebert, 2007) for those species that already had a reference barcode provided. All our sampled taxa, genes and GenBank accession numbers are provided in Appendix S1.

### DNA markers and laboratory protocols

The eight genetic markers used in this study have proven useful in resolving evolutionary relationships between species above and below the family level (e.g. Wahlberg & Wheat, 2008; Zahiri *et al.*, 2011, 2012; Zaspel *et al.*, 2014). We amplified the mitochondrial cytochrome oxidase (COI), including the barcode region, as well as the nuclear gene regions carbamoylphosphate synthase domain protein (CAD), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), isocitrate dehydrogenase (IDH), cytosolic malate dehydrogenase (MDH), elongation factor 1-a protein (Ef1$\alpha$), ribosomal protein subunit S5 (RpS5) and wingless (WGS).

DNA extraction was conducted using the DNeasy Blood + Tissue extraction kit (Qiagen, Hilden, Germany) both in Turku and Jyväskylä according to the manufacturer's protocols, but assisted by a robot (Kingfisher, Waltham, MA, U.S.A.) in Jyväskylä. Washing and eluting DNA in Jyväskylä was thus done using MagAttract tubes and the KingFisher robot with the programme Qiagen Blood. For polymerase chain reaction (PCR) and primer pairs we followed the laboratory protocols of Wahlberg & Wheat (2008). However, for some older samples processed in Jyväskylä, in cases where we did not obtain enough product to be visualized and purified from agarose gel during the first PCR, we did a second PCR using the first PCR product as a template with the same primers. PCR products were sent to Macrogen Europe in the Netherlands for sequencing, except for part of the barcode region (the 5′ half of COI) samples, which were sequenced in Jyväskylä with Big-Dye terminator v3.1, Cycle Sequencing kit (Applied Biosystems, Carlsbad, CA, U.S.A.) and run on an ABI 3130xl Genetic Analyzer (Applied Biosystems). Finally, we aligned DNA sequences manually using MEGA version 5.2.2 (Tamura *et al.*, 2011) or BIOEDIT (Hall, 1999) and stored them on the web-based VOSEQ database software (Peña & Malm, 2012).

### *Phylogenetic analysis and checking for errors*

To check for erroneous sequences, we performed neighbour-joining and Bayesian analyses on single-gene alignments. These analyses were compared with the combined analysis of all genes, and if the species were placed in a radically different relationship between these two, the original sequence data for the differing gene were examined, and, in cases of possible contamination or low-quality sequence, omitted from further analysis.

We performed both maximum likelihood (ML) and Bayesian inference (BI) analyses on the combined dataset of a minimum of two successfully sequenced gene regions (min. of approximately 1000 bp). The Bayesian information criterion using PARTITION FINDER v. 1.1.1 (Lanfear *et al.*, 2012) was used to determine the best-fit partitioning scheme and evolutionary model for the dataset, which was partitioned into each codon position for each gene region. For ML analysis we used RAXML-HPC2 (Stamatakis, 2014) on XSEDE (Towns *et al.*, 2014) and ran 1000 replicates of bootstrapping to calculate support for ML nodes using the Cipres science gateway (Miller *et al.*, 2010). The BI analyses were carried out using MRBAYES v3.2.3 (Ronquist *et al.*, 2012) on the Cipres science gateway. We performed 10 million generations, with sampling every 1000 generations and four chains, one cold and three heated, in two independent runs. The parameters and models of evolution were unlinked across character partitions and the mixed evolutionary model was used. The convergence of the two runs was ascertained by visual inspection of the log-likelihoods stationary distribution, discarding the first 25% of sampled trees, as well as by checking that the final average standard deviation of split frequencies was below 0.05 and that the potential scale reduction factor for each parameter was close to 1. Resulting trees for both ML (Fig. 2) and BI analyses (Appendix S2) were visualized using FIGTREE v.1.4.2. (Rambaut, 2014).

## Results

The most optimal partitioning scheme found by PARTITION FINDER had 16 partitions (out of a total of 24). Most codon positions of each gene were kept in their own partition, except for the following, which were combined: position 3 of CAD and position 3 of MDH; position 2 of CAD and position 2 of IDH; position 3 of GAPDH, position 3 of IDH and position 3 of WGS; position 2 of GAPDH, position 2 of MDH; and position 1 of MDH, position 1 of RpS5 and position 1 and 2 of WGS.

Both ML and BI analyses resulted in well-resolved topologies with nearly identical branching patterns (Fig. 2, Appendix S2). The topologies are rooted with Lithosiini (*Setina* sp.) and the sample representing Syntomini (*Amata* sp.) is positioned as sister to all other clades [bootstrap (BS) = 100, Bayesian posterior probability (BP) = 1.0]. Our 11 species formally placed in Callimorphina are divided into two strongly supported clades, eight species forming Callimorphina (BS = 99, BP = 1.0) and three species of *Nyctemera* + *Secusio* forming another clade (BS = 100, BP = 1.0). The latter is sister to Arctiina with strong support. Within Arctiina s.l., we find strong support for the monophyletic group of spilosomine genera (BS = 100, BP = 1.0) separate from Arctiina s.s. (BS = 49, BP = 0.94).

Within Arctiina s.s., several clades are formed, but the relationships between and within some of these groups are not clear. The first clade comprises *Diacrisia*, *Rhyparia* and *Rhyparioides* (the *Diacrisia*-clade), which form a strongly supported monophyletic group (BS = 100, BP = 1.0). *Hyperborea*, *Sibirarctia*, *Chelis* and *Neoarctia* + *Holoarctia* + *Palearctia* + *Tancrea* + *Centrarctia* also form a clade with strong support (the *Chelis* clade; BS = 99, BP = 1.0) as do *Holarctia*, *Grammia*, *Apantesis* and *Notarctia* (the *Apantesis* clade; BS = 99, BP = 1.0).

*Micrarctia trigona* (Leech) is placed alone as a sister to the monophyletic grouping of '*Arctia*' species (the *Arctia* clade; BS = 86, BP = 0.99), which is divided in two subclades, which we term the 'Northern *Arctia*' (BS = 98, BP = 1.0) and the 'Mediterranean *Arctia*' (BS = 100, BP = 1.0). Six species of *Arctia* form a monophyletic '*Arctia caja* group', of which *A. intercalaris* (Eversmann) + *A. thibetica* Felder are placed as sister to *A. caja* + *A. martinhoneyi* Dubatolov & Gurko + *A. brachyptera* Troubridge & Lafontaine + *A. opulenta* (H. Edwards), which show very little difference in the molecular data. We consider the '*Arctia caja* group' as part of the sister 'Northern *Arctia*' subclade, where *Platyprepia* and *Oroncus* form the most basally arising branches, with some support for a monophyletic grouping of *Preparctia* [including *Sinoarctia sieversi* (Grum-Grshimailo)] + *Gonerda* + *Platarctia souliei* (Oberthür) placed as sister to *Orontobia secreta* (Draudt) + *Acerbia seitzi* (Bang-Haas) + *Arctia rueckbeili* Püngeler and a grouping of *Pararctia* + *Acerbia alpina* (Quensel), *Platarctia parthenos* (Harris), *Pericallia matronula* (Linnaeus), *Borearctia menetriesii* (Eversmann) and *Arctia flavia* (Fuessly) with a non-resolved branching structure. The other subclade of the monophyletic group of '*Arctia*' is the 'Mediterranean *Arctia*', which comprises our focal study species *P. plantaginis* placed as sister to *Eucharia (=Ammobiota/Arctia) festiva* (Hufnagel) (BS = 94, BP = 1.0), next to all three *Hyphoraia* species, which in turn form the sister clade of *Atlantarctia ungemachi* (Le Cerf), *Atlantarctia (=Arctia) tigrina* (Villers) and *Epicallia (=Arctia) villica* (Linnaeus).

## Discussion

### *A molecular hypothesis of Arctiina phylogenetic relationships*

We were able to sample a wide range of Arctiina species throughout their distribution ranges in the Holarctic, while aiming to find all the potential relatives of *Parasemia*. Our sampling is the most comprehensive to date of the subtribe Arctiina and brings many species that have been difficult to place in a phylogenetic context for the first time. The resolution of our hypothesis could well be further improved by adding samples

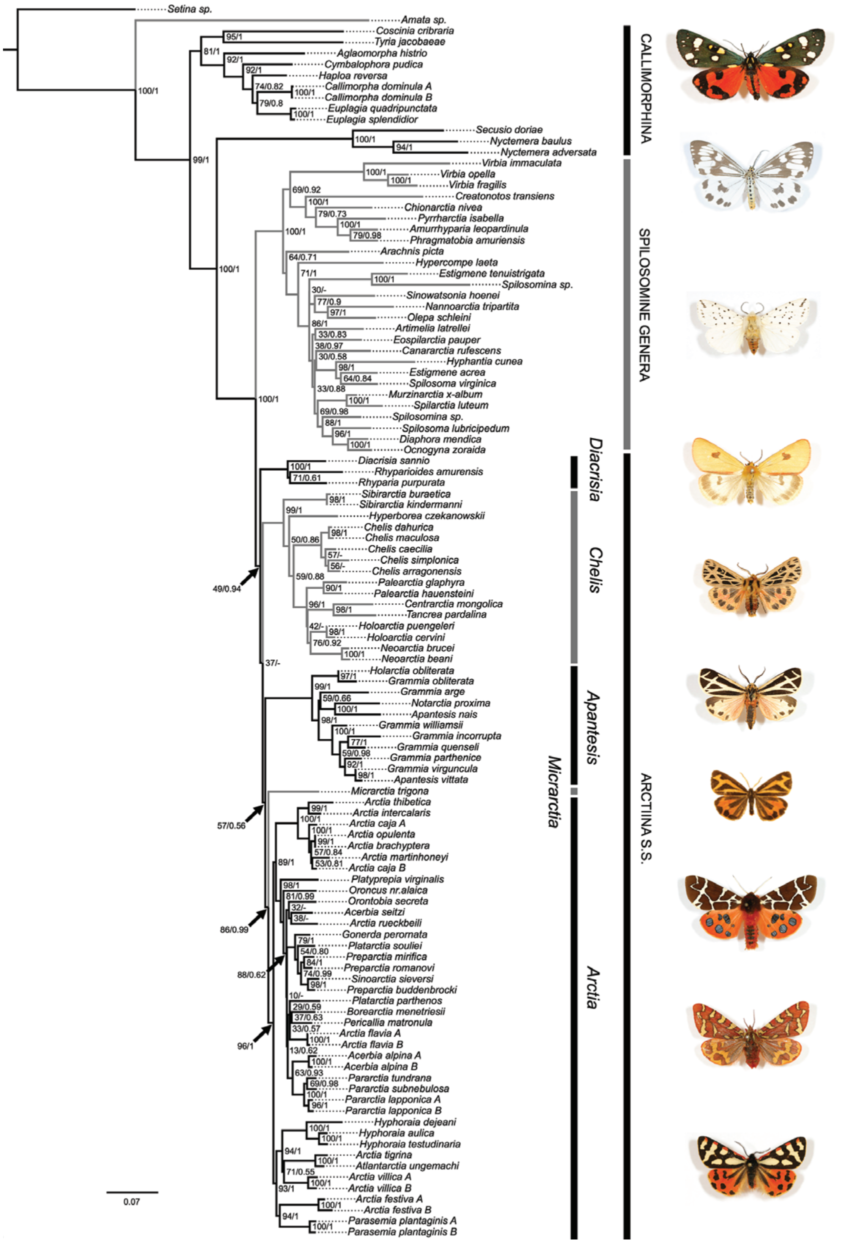

**Fig. 2.** Phylogram of the potentially closest relatives of *Arctia plantaginis*. Bootstrap/Bayesian posterior probability support values are given next to the nodes. Lines on the right delimit the revised genera and other monophyletic groupings formed. Tiger moths illustrated in the pictures from top down are *Callimorpha dominula*, *Nyctemera adversata*, *Spilosoma lubricipedum*, *Diacrisia sannio*, *Chelis dahurica*, *Apantesis vittata*, *Micrarctia trigona*, *Arctia caja*, *Arctia lapponica* **comb.n.** and *Arctia plantaginis* ssp. *caucasica* **comb.n.**

**Table 1.** Formal generic revision of Arctiina s.s.

| Valid genera | Synonymized genera |
|---|---|
| ***Apantesis*** Walker, 1855 | *Grammia* Rambur, 1866 **syn.n.** |
| | *Orodemnias* Wallengren, 1885 **syn.n.** |
| | *Mimarctia* Neumoegen & Dyar, 1894 **syn.n.** |
| | *Notarctia* Smith, 1938 **syn.n.** |
| | *Holarctia* Smith, 1938 **syn.n.** |
| ***Chelis*** Rambur, 1866 | *Neoarctia* Neumoegen & Dyar, 1893 **syn.n.** |
| | *Tancrea* Püngeler, 1898 **syn.n.** |
| | *Hyperborea* Grum-Grshimailo, 1900 **syn.n.** |
| | *Palearctia* Ferguson, 1984 **syn.n.** |
| | *Holoarctia* Ferguson, 1984 **syn.n.** |
| | *Sibirarctia* Dubatolov, 1987 **syn.n.** |
| | *Centrarctia* Dubatolov, 1992 **syn.n.** |
| ***Diacrisia*** Hübner, [1819] 1816 | *Rhyparia* Hübner [1820] 1816 **syn.n.** |
| | *Rhyparioides* Butler, 1877 **syn.n.** |
| ***Micrarctia*** Seitz, 1910 | |
| ***Arctia*** Schrank, 1802 | *Eyprepia* Ochsenheimer, 1810 **junior objective synonym of *Arctia* Schranck, 1802.** |
| | *Epicallia* Hübner, [1820] 1816 **syn.n.** |
| | *Eucharia* Hübner, [1820] 1816 **syn.n.** |
| | *Hyphoraia* Hübner, [1820] 1816 **syn.n.** |
| | *Parasemia* Hübner, [1820] 1816 **syn.n.** |
| | *Zoote* Hübner, [1820] 1816 **junior objective synonym of *Arctia* Schranck, 1802.** |
| | *Pericallia* Hübner [1820] 1816 **syn.n.** |
| | *Nemeophila* Stephens, 1828 **syn.n.** |
| | *Ammobiota* Wallengren, 1855 **syn.n.** |
| | *Callarctia* Packard, 1864 **junior objective synonym of *Arctia* Schranck, 1802.** |
| | *Platarctia* Packard, 1864 **syn.n.** |
| | *Chionophila* Guenée 1865 **syn.n.** |
| | *Eupsychoma* Grote, 1865 **syn.n.** |
| | *Gonerda* Moore, 1879 **syn.n.** |
| | *Platyprepia* Dyar, 1897 **syn.n.** |
| | *Preparctia* Hampson, 1901 **syn.n.** |
| | *Oroncus* Seitz, 1910 **syn.n.** |
| | *Acerbia* Sotavalta, 1963 **syn.n.** |
| | *Pararctia* Sotavalta, 1965 **syn.n.** |
| | *Borearctia* Dubatolov, 1984 **syn.n.** |
| | *Sinoarctia* Dubatolov, 1987 **syn.n.** |
| | *Atlantarctia* Dubatolov, 1990 **syn.n.** |

of the rarer species, e.g. from the small genera *Atlantarctia* Dubatolov, *Divarctia* Dubatolov, *Ebertarctia* Dubatolov, *Leptarctia* Stretch, *Ocnogyna* Lederer, *Oroncus* Seitz, *Orontobia* de Freina, *Palerontobia* Dubatolov, *Sonorarctia* Ferguson*, Allanwatsonia* Ferguson and *Pseudalus* Schaus. However, many of the missing species are described from only a few specimens, or from the type series only, and fresh samples are thus extremely difficult to obtain.

Both ML and BI analyses resulted in nearly identical topologies. Within Arctiini, the selected 11 species of Callimorphina are segregated into the Callimorphina clade and *Nyctemera + Secusio*, forming a clade sister to Arctiina. Whether reinstating Nyctemerina as a separate subtribe would be necessary, as discussed in Zaspel *et al.* (2014), is beyond the scope of this study. We find strong support for a large monophyletic grouping of the spilosomine genera as separate from Arctiina s.s. Within Arctiina s.s., four well-supported clades are recovered. We find it most informative, and probably also most stable, to consider these clades to represent the generic level within the subtribe. Each clade and the implications of our results on the taxonomy of Arctiina are discussed further in the following. Formal taxonomic revision of the genera is given in Table 1.

In the broad sense, our molecular hypothesis of the evolutionary history of *P. plantaginis* and relatives is in concordance with earlier phylogenies by Ferguson (1985), Schmidt (2007) and Dubatolov (2008, 2009), which were based on morphological characters, as well as the COI barcode region in Schmidt (2007). Dubatolov (2008, 2009) divided the Arctiina s.s. into ‘Micrarctiini’ and ‘Arctiini’. Dubatolov’s (2009) ‘Micrarctiini’ comprises mostly same genera as in our *Diacrisia*, *Chelis* and *Apantesis* clades, but with different hypothesized phylogenetic relationships. All of Dubatolov’s (2008) ‘Arctiini’ are placed in *Arctia* as delimited below. Dubatolov (2008) divided ‘Arctiini’ into two clades, one associated with ‘northern and mountainous areas of Asia and North America’ and the other with ‘plains of moderate altitudes’, which correspond largely to our subclades

'Northern *Arctia*' and 'Mediterranean *Arctia*', but again his tree derived from morphology has a different branching order. Interestingly, *Micrarctia* is placed as sister to our *Arctia*.

### *Spilosomine genera*

The *Spilosoma* group has been considered part of Arctiina s.l. (e.g., Ferguson, 1985) or as a separate tribe or subtribe called Spilosomina (e.g. Schmidt, 2007; Vincent & Laguerre, 2014). Zaspel *et al.* (2014) did not find Spilosomina separate from Arctiina and discussed whether the division has been made in an attempt to categorize moths by similar appearance. In our tree with a larger sampling of Arctiina, the spilosomine genera come out as a well-supported monophyletic group corroborating the preliminary results of Schmidt (2007) – a hypothesis that is also supported by the light wing coloration shared by many species within the group. However, as the spilosomine genera are highly diverse and globally distributed, with hotspots of diversity in the tropical Asia and Africa (Ferguson, 1985), our sampling does not allow substantive interpretation of the interrelationships within the clade. We agree with Fibiger *et al.* (2011) that this species group needs more work and a thorough phylogenetic revision. We thus prefer to retain the spilosomine genera in the subtribe Arctiina s.l. for the time being.

### *Arctiina s.s.:* Diacrisia*,* Chelis *and* Apantesis *clades*

*Diacrisia*, *Rhyparia* and *Rhyparioides* have been suggested to be closely related in several studies (Ferguson, 1985; Koda, 1987; Dubatolov, 2009). Our analyses corroborate these studies as we also find them to form a monophyletic entity. Species in this clade differ in their adult forewing coloration and pattern from other Arctiina by their bright yellow and red hues. This group has the highest species diversity in Asia. As *Diacrisia* is the oldest available genus name for these, we synonymize *Rhyparia* **syn.n.** and *Rhyparioides* **syn.n.** with *Diacrisia*.

The second clade combines the rather large genera *Chelis* and *Palearctia* together with many smaller genera. Ferguson (1985) noted the close relationship of *Neoarctia*, *Holoarctia*, *Palearctia* and *Hyperborea*. The internal relationships of this clade are not well resolved and would benefit from adding more samples of species and genera than are included in our analysis. Due to the well-supported monophyly of this clade, all genera in the *Chelis* clade are here combined into *Chelis*.

The third clade comprises almost solely species assigned to *Grammia*, but also *Notarctia proxima* (Guérin-Méneville), *Apantesis nais* (Drury) and *A. vittata* (Fabricius). The close relationship of *Grammia*, *Notarctia* and *Apantesis* has previously been suggested based on morphological characters (Ferguson, 1985). *Arctia* [later in *Grammia*] *obliterata* Stretch was placed in its own genus *Holarctia* by Smith, based on its more variable morphology and wider distribution than other *Grammia* species. Schmidt (2009) considered the species *obliterata* to be related and probably basal to *Grammia*, a view corroborated by our analysis. Contrary to Schmidt (2009), however, we find the clade consisting of *Grammia* **syn.n.**, *Holarctia* **syn.n.**, *Notarctia* **syn.n.** and *Apantesis* monophyletic with high support, and therefore place all these genera under *Apantesis* (see Table 1). Synonymy of *Holarctia* with *Apantesis* and *Holoarctia* **syn.n.** with *Chelis* will also clarify the confusion caused by the similar orthography of these two genus names (Ferguson, 1985).

### *Micrarctia*

*Micrarctia trigona* is an especially interesting case of Arctiinae tiger moths. The tribe Micrarctiini (originally established by Seitz as Micrarctiinae) was used by Dubatolov (1990, 2009) to host many superficially similar arctiine genera that could not be placed elsewhere. Later, most of these genera were moved to other (sub)tribes, leaving *M. trigona* the only genus and species of Micrarctiini. Recently, a second species was described in *Micrarctia* that is sympatric with *M. trigona* (Saldaitis & Pekarsky, 2015). This species, *M. kautti*, is nocturnal, unlike its sister species, and perhaps this is why it had remained unnoticed for so long. It would be intriguing to include *M. kautti* in an analysis to further elucidate the position of *Micrarctia* and thus potentially help to resolve the branching order of all four clades within Arctiina s.s. As the position of *Micrarctia* is not as strongly supported (BS = 86, BP = 0.99) as the other clades (BS = 99–100, BP = 1.0), we prefer to retain it as a valid genus until further work can ascertain its phylogenetic position.

### *The* Arctia *clade*

The unusually short branching within the *Arctia* clade and low support values for internal nodes suggest rapid radiation. This type of quick speciation leaves little phylogenetic evidence in the nuclear genes to study the species-level branching. '*Arctia*' species (excluding *Micrarctia* at the base of the clade) form a well-supported clade. The superfluous number of monotypic genera that also causes polyphyly of *Arctia* is obviously unwarranted. To render the classification more natural, and also simplify it, we combine all these species under *Arctia* (see Table 1). However, two well-supported subclades can be distinguished – our 'Northern *Arctia*' and 'Mediterranean *Arctia*'.

### *Northern* Arctia *and* A. caja *group*

Many Arctiina species, especially in the 'Northern *Arctia*' clade, are better adapted to cooler environments than most other noctuoid moths (Ferguson, 1985). Adapting to cold environments could be one mechanism behind the apparently rapid diversification that has occurred in this clade. The subclade has been divided into many monotypic genera containing some of the most rarely encountered species with almost mysterious life histories. For example, there was a gap lasting for decades between the observations of the Menetries's Tiger Moth *Borearctia menetriesii* in Finland and the next discovered sites are not only separated by hundreds of kilometres but are also in different habitats (Bolotov *et al.*, 2013).

The species in this subclade are very distinctive, with their conspicuous wing patterns, bright colours and large size. The Garden Tiger Moth *Arctia caja* is no exception, but is in addition very variable in its patterning. Many species, such as *A. intercalaris*, *A. martinhoneyi*, *A. thibetica*, *A. brachyptera* and *A. opulenta*, have been split from *A. caja* based on appearance, but in our molecular hypothesis all these species group together with high support and very little genetic difference. However, as the molecular markers we used in this study are too conservative for inferring interrelationships between very closely related species, other markers should be used to study patterns and levels of differentiation at the species level. We consider the *A. caja* group to be part of the 'Northern *Arctia*' clade.

Dubatolov (2008) arranged his 'Northern mountainous clade' to (*Gonerda* + *Preparctia*) + *Sinoarctia* + (*Borearctia* + (*Pararctia* + *Platarctia*)) + (*Orontobia* + (*Oroncus* + (*Acerbia* + *Platyprepia*))). These genera form our 'Northern *Arctia*' subclade, supplemented with *A. caja* group, *A. flavia*, *A. rueckbeili* and *Pericallia matronula*. There is also some evidence in our dataset (Appendix S1) indicating that *Ebertarctia nordstroemi* (Brandt) could belong to the 'Northern *Arctia*'. According to our hypothesis the Nearctic genus, *Platyprepia* is closer to the base and not at the tip of the subclade and *Sinoarctia sieversi* is nested within *Preparctia.* Based on the short branching, we combine all these genera under *Arctia* (see Table 1). By so doing, we again move away from the uninformative monotypic genera.

Some other monotypic genera, such as *Leptarctia* and *Palerontobia*, that we were not able to sample or to obtain good-quality sequences of, are likely to belong to this subclade, and including them could help to resolve the internal relationships within the subclade. However, we consider it more likely that the low resolution within this subclade results from rapid diversification rather than sparse sampling, as both morphological and molecular data have repeatedly proved indecisive within this subclade (Ferguson, 1985; Dubatolov, 2008, 2009; Weller *et al.*, 2009).

### *Mediterranean* Arctia

This is another subclade consisting of the equally showy and colourful *Atlantarctia ungemachi*, *Arctia* (=*Epicallia*) *villica*, *Arctia* (=*Atlantarctia*) *tigrina*, *Eucharia* (=*Ammobiota*/*Arctia*) *festiva*, *Hyphoraia* spp. and *Parasemia*. As their distribution ranges meet at the Mediterranean, we call this group 'Mediterranean *Arctia*'. This monophyletic group includes only a few species, and several of them are already ascribed to *Arctia*. We combine both this subclade and the 'Northern *Arctia*' subclade under *Arctia* (see Table 1). The species in the two subclades are also morphologically quite similar to each other, and these clades lack reliable synapomorphies.

## Concluding remarks and future applications of the phylogeny of Arctiina

This study stemmed from the need to find the closest relatives of *Arctia plantaginis* to be able to further understand the evolutionary origins of its peculiar polymorphic warning coloration and also tiger moths in general. *Arctia plantaginis* has been suggested to originate in the Caucasus or south-eastern Europe based on COI, ten microsatellite loci haplotypes and species distribution modelling (Hegna *et al.*, 2015). Hegna *et al.* (2015) hypothesized that, as sexually monomorphic hindwing coloration seems to be ancestral in arctiines, the Caucasian form, *A. plantaginis caucasica*, of which hindwing coloration varies continuously from yellow to red in both sexes, would be ancestral to all other *A. plantaginis*. In other populations, female hindwing coloration still varies continuously from yellow to red, but male hindwing coloration is polymorphic and the ground colour can be white, yellow or black (Fig. 1A–D). Based on our results, the closest relatives of *A. plantaginis*, like *Arctia festiva* (Fig. 1E), are indeed sexually monomorphic in their hindwing coloration, although many species continuously vary in forewing pattern. This comparison implies that the polymorphism in *A. plantaginis* male hindwing coloration is a more recent development.

Another obvious application of our phylogenetic hypothesis is in the study of diversification patterns of Arctiina species. Most Arctiina species are diurnal with polyphagous larvae, feeding on, amongst others, dandelion (*Taraxacum* spp.) and plantain (*Plantago* spp.), including in the Nearctic, where these plants are naturalized European species (Conner, 2009). Dubatolov (2008, 2009) suggests that Arctiina most probably originated in Asia, from where they have spread in multiple occasions to the Western Palearctic and Nearctic. It is also possible, however, that there were some refugia during glaciation periods in the Mediterranean region, which enhanced diversification.

In conclusion, we would like to encourage researchers to study below the surface of these popular, colourful and dazzling species, so as to gain information that escapes our eyes. Our work offers long-awaited clarification of the phylogenetic relationships of Arctiina, especially within Arctiina s.s. – a group of spectacular and popular moths that have been much studied, yet proven difficult to classify with traditional methods. It was beyond our scope to provide a complete systematic revision of Arctiina s.l., with a vast majority of the 4000 species occurring in the tropics, and more work needs to be done to solve the evolutionary relationships between and within clades in this highly diverse and specialized group of moths. We hope that our molecular hypothesis for Arctiina will work as a backbone, where many more tiger moth species can find their relatives. With rigorous phylogenetic hypotheses, it will be possible to tackle many interesting evolutionary questions to come.

## Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/syen.12194

**Appendix S1.** Taxon sampling table. Letter A or B after the species name refers to the voucher positioned to the trees in Fig. 2 and Appendix S2. Samples with less than two successfully sequenced gene region (min. of approximately 1000 bp) were not included in the final analysis. Samples marked with an asterisk (*) in collection country are from Zaspel *et al.* (2014).

**Appendix S2.** Bayesian topology for the same dataset as in the maximum likelihood phylogram in Fig. 2.

## Acknowledgements

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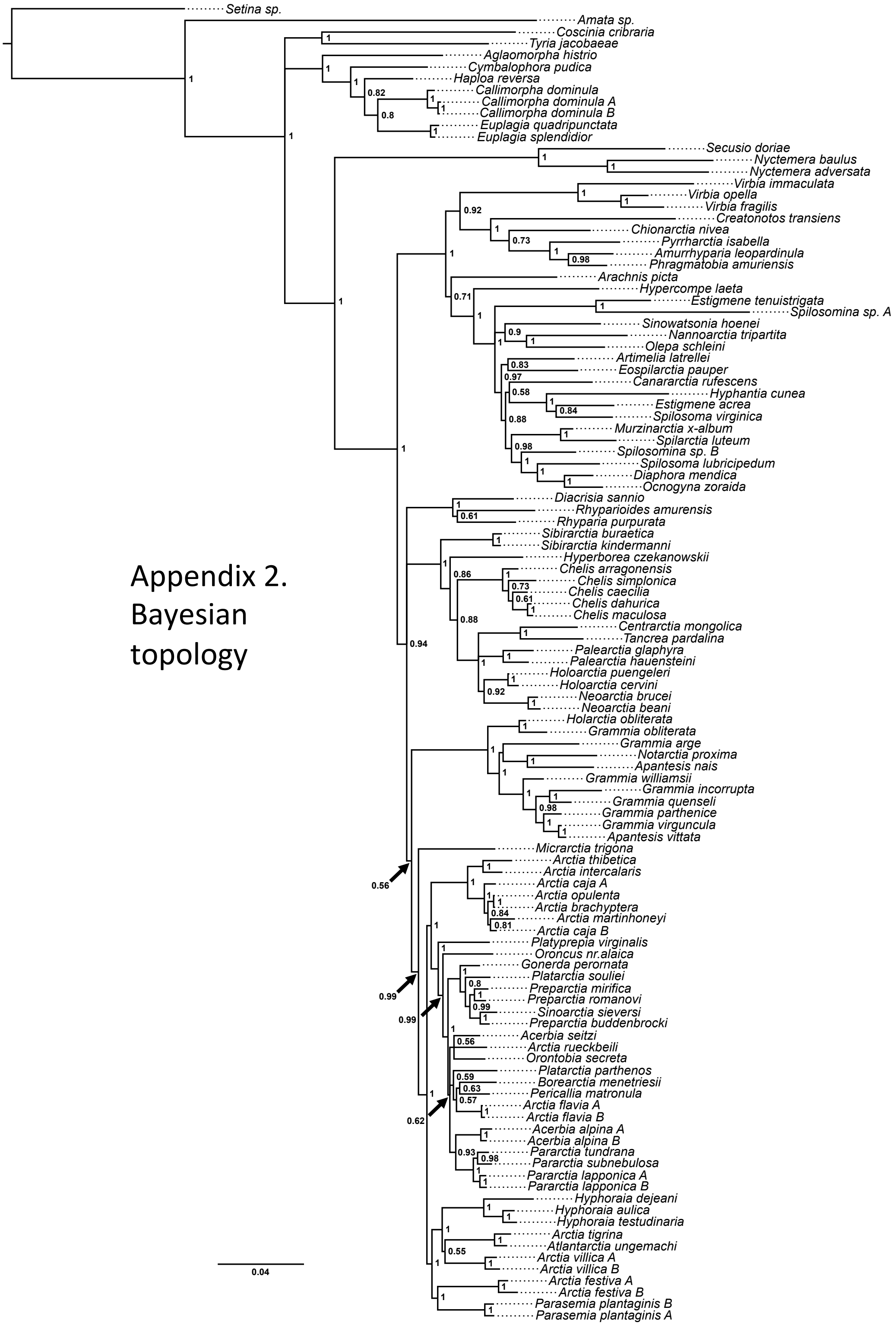
**Taxon sampling table**

| Voucher code | Subfamily | Tribe | Genus | Species | Country | CAD | COI | EF1A | GAPDH | IDH | MDH | RPS5 | WINGLESS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CNLEP113677 | Arctiinae | Arctiini | *Acerbia* | *alpina A* | Canada | KX050167 | KX050200 | KX050290 | KX050363 | KX050408 | KX050452 | KX050536 | KX050611 |
| MM23468 | Arctiinae | Arctiini | *Acerbia* | *alpina B* | Finland | KX050168 | KX050201 | KX050291 | KX050364 | KX050409 | KX050453 | KX050537 | KX050612 |
| KN00860 | Arctiinae | Arctiini | *Acerbia* | *seitzi* | Kyrgyzstan | - | KX050202 | KX050292 | - | - | KX050454 | KX050538 | KX050613 |
| NSGJZ017 | Arctiinae | Arctiini | *Aglaomorpha* | *histrio* | * | - | KF533450 | KF533522 | - | - | - | KF533644 | KF533578 |
| AMSP13GE1 | Arctiinae | Syntomini | *Amata* | *sp.* | Georgia | - | KX050203 | KX050293 | KX050365 | KX050410 | KX050455 | KX050539 | KX050614 |
| KN00881 | Arctiinae | Arctiini | *Amurrhyparia* | *leopardinula* | China | KX050169 | KX050204 | KX050294 | - | KX050411 | KX050456 | KX050540 | - |
| CNLEP113667 | Arctiinae | Arctiini | *Apantesis* | *nais* | Canada | - | KX050205 | KX050295 | KX050366 | - | KX050457 | - | - |
| SCDNA83 | Arctiinae | Arctiini | *Apantesis* | *vittata* | * | - | KF533442 | - | - | - | - | KF533636 | - |
| SCDNA169 | Arctiinae | Arctiini | *Arachnis* | *picta* | * | - | KF533443 | KF533515 | - | - | - | KF533637 | KF533571 |
| CNLEP81969 | Arctiinae | Arctiini | *Arctia* | *brachyptera* | Canada | - | KX050206 | KX050296 | KX050367 | - | KX050458 | KX050541 | KX050615 |
| ACAJ13RUS | Arctiinae | Arctiini | *Arctia* | *caja A* | Russia | KX050170 | KX050207 | KX050297 | KX050368 | KX050412 | KX050459 | KX050542 | KX050616 |
| W15 | Arctiinae | Arctiini | *Arctia* | *caja B* | * | - | KF533444 | KF533516 | - | - | - | KF533638 | KF533572 |
| AFES10RUS | Arctiinae | Arctiini | *Arctia* | *festiva A* | Russia | KX050171 | KX050208 | KX050298 | - | - | KX050460 | KX050543 | KX050617 |
| AFESIRAN | Arctiinae | Arctiini | *Arctia* | *festiva B* | Iran | KX050172 | KX050209 | KX050299 | - | - | KX050461 | KX050544 | KX050618 |
| AFLA11RUS | Arctiinae | Arctiini | *Arctia* | *flavia A* | Russia | - | KX050210 | KX050300 | KX050369 | KX050413 | KX050462 | KX050545 | KX050619 |
| AFLA14AUS | Arctiinae | Arctiini | *Arctia* | *flavia B* | Austria | KX050173 | KX050211 | KX050301 | KX050370 | KX050414 | KX050463 | KX050546 | KX050620 |
| AINT13TAJ | Arctiinae | Arctiini | *Arctia* | *intercalaris* | Tajikistan | KX050174 | KX050212 | KX050302 | KX050371 | KX050415 | KX050464 | KX050547 | KX050621 |
| KN00873 | Arctiinae | Arctiini | *Arctia* | *martinhoneyi* | Pakistan | - | KX050213 | KX050303 | - | - | KX050465 | KX050548 | - |
| CNLEP113664 | Arctiinae | Arctiini | *Arctia* | *opulenta* | Canada | KX050175 | KX050214 | KX050304 | KX050372 | KX050416 | KX050466 | KX050549 | KX050622 |
| KN00874 | Arctiinae | Arctiini | *Arctia* | *rueckbeili* | Kyrgyzstan | - | KX050215 | - | - | - | KX050467 | - | - |
| KN00875 | Arctiinae | Arctiini | *Arctia* | *thibetica* | Pakistan | - | KX050216 | - | - | - | - | KX050550 | - |
| KN00862 | Arctiinae | Arctiini | *Arctia* | *tigrina* | Spain | - | KX050217 | KX050305 | - | KX050417 | KX050468 | KX050551 | KX050623 |
| AVIKRUSK | Arctiinae | Arctiini | *Arctia* | *villica B* | Italy | KX050176 | KX050218 | KX050306 | KX050373 | KX050418 | KX050469 | KX050552 | KX050624 |
| AVIL12SPA | Arctiinae | Arctiini | *Arctia* | *villica A* | Spain | KX050177 | KX050219 | KX050307 | KX050374 | - | KX050470 | KX050553 | KX050625 |
| ALAT15KK | Arctiinae | Arctiini | *Artimelia* | *latrellei* | Nz. Israel | - | KX050220 | KX050308 | KX050375 | - | KX050471 | KX050554 | KX050626 |
| AUNG10MOR | Arctiinae | Arctiini | *Atlantarctia* | *ungemachi* | Morocco | KX050178 | KX050221 | KX050309 | KX050376 | KX050419 | KX050472 | KX050555 | KX050627 |
| AUNG15MOR | Arctiinae | Arctiini | *Atlantarctia* | *ungemachi* | Morocco | - | KX050222 | - | - | - | - | - | - |
| BMEN11RU2 | Arctiinae | Arctiini | *Borearctia* | *menetriesii* | Russia | KX050179 | KX050223 | KX050310 | KX050377 | KX050420 | KX050473 | KX050556 | KX050628 |
| CDOM14GE1 | Arctiinae | Arctiini | *Callimorpha* | *dominula B* | Georgia | - | KX050224 | KX050311 | KX050378 | KX050421 | KX050474 | KX050557 | KX050629 |
| RZ136 | Arctiinae | Arctiini | *Callimorpha* | *dominula A* | * | HQ006965 | HQ006169 | HQ006266 | HQ006444 | HQ006514 | HQ006594 | HQ006685 | HQ006778 |
| CRUF15SPA | Arctiinae | Arctiini | *Canararctia* | *rufescens* | Spain | - | KX050225 | KX050312 | - | - | KX050475 | KX050558 | KX050630 |
| KN00888 | Arctiinae | Arctiini | *Centrarctia* | *mongolica* | Mongolia | - | KX050226 | - | KX050379 | - | KX050476 | - | KX050631 |
| CARR13SPA | Arctiinae | Arctiini | *Chelis* | *arragonensis* | Spain | KX050180 | KX050227 | KX050313 | KX050380 | KX050422 | KX050477 | KX050559 | - |
| CCAE11GRE | Arctiinae | Arctiini | *Chelis* | *caecilia* | Greece | KX050181 | KX050228 | KX050314 | KX050381 | - | KX050478 | KX050560 | KX050632 |
| CDAH10RU1 | Arctiinae | Arctiini | *Chelis* | *dahurica* | Russia | - | KX050229 | KX050315 | KX050382 | KX050423 | KX050479 | KX050561 | KX050633 |
| CMAC10RUS | Arctiinae | Arctiini | *Chelis* | *maculosa* | Russia | - | KX050230 | KX050316 | - | - | - | - | - |
| KN00904 | Arctiinae | Arctiini | *Chelis* | *simplonica* | Italy | - | KX050231 | - | - | - | KX050480 | KX050562 | - |
| NSGJZ009 | Arctiinae | Arctiini | *Chionarctia* | *nivea* | * | - | KF533502 | KF533560 | - | - | - | KF533686 | KF533624 |
| MM05671 | Arctiinae | Arctiini | *Coscinia* | *cribraria* | Finland | HQ006949 | HQ006149 | HQ006247 | - | HQ006499 | KJ723677 | HQ006666 | HQ006758 |
| RZ30 | Arctiinae | Arctiini | *Creatonotos* | *transiens* | China | HQ006991 | HQ006198 | HQ006294 | - | HQ006537 | HQ006619 | HQ006711 | HQ006806 |
| CPUD13SPA | Arctiinae | Arctiini | *Cymbalophora* | *pudica* | Spain | KX050182 | KX050232 | KX050317 | - | KX050424 | KX050481 | KX050563 | KX050634 |
| MM12598 | Arctiinae | Arctiini | *Diacrisia* | *sannio* | Finland | KX050183 | KX050233 | KX050318 | KX050383 | - | KX050482 | KX050564 | KX050635 |
| MM07688 | Arctiinae | Arctiini | *Diaphora* | *mendica* | Finland | - | KX050234 | KX050319 | KX050384 | KX050425 | KX050483 | KX050565 | KX050636 |
| KN00876 | Arctiinae | Arctiini | *Ebertarctia* | *noerdstroemi* | Iran | - | - | - | - | - | - | KX050566 | - |
| KN00901 | Arctiinae | Arctiini | *Eospilarctia* | *pauper* | China | - | KX050235 | KX050320 | - | - | KX050484 | KX050567 | - |
| W13 | Arctiinae | Arctiini | *Estigmene* | *acrea* | * | - | KF533462 | KF533532 | - | - | - | KF533654 | KF533590 |
| T1 | Arctiinae | Arctiini | *Estigmene* | *tenuistrigata* | Zambia | - | KP081793 | KP082336 | KP082339 | - | KP082464 | KP082590 | KP082726 |
| NSGJZ003 | Arctiinae | Arctiini | *Euplagia* | *quadripunctata* | * | - | KF533468 | KF533536 | - | - | - | KF533657 | KF533594 |
| KN00905 | Arctiinae | Arctiini | *Euplagia* | *splendidior* | Armenia | - | KX050236 | - | KX050385 | - | KX050485 | KX050568 | - |
| KN00863 | Arctiinae | Arctiini | *Gonerda* | *perornata* | Pakistan | - | KX050237 | - | - | - | KX050486 | - | KX050637 |
| CNLEP113672 | Arctiinae | Arctiini | *Grammia* | *arge* | Canada | - | KX050238 | KX050321 | KX050386 | KX050426 | KX050487 | KX050569 | KX050638 |
| CNLEP113678 | Arctiinae | Arctiini | *Grammia* | *incorrupta* | USA | KX050184 | KX050239 | KX050322 | - | - | KX050488 | KX050570 | KX050639 |
| KN00884 | Arctiinae | Arctiini | *Grammia* | *obliterata* | Russia | - | - | - | - | - | KX050489 | - | KX050640 |
| CNLEP113671 | Arctiinae | Arctiini | *Grammia* | *parthenice* | Canada | - | KX050240 | - | - | - | KX050490 | KX050571 | - |
| MM10596 | Arctiinae | Arctiini | *Grammia* | *quenseli* | Finland | KX050185 | KX050241 | KX050323 | - | KX050427 | KX050491 | KX050572 | KX050641 |
| CNLEP113665 | Arctiinae | Arctiini | *Grammia* | *virguncula* | Canada | KX050186 | KX050242 | KX050324 | - | KX050428 | KX050492 | KX050573 | KX050642 |
| CNLEP113670 | Arctiinae | Arctiini | *Grammia* | *williamsii* | Canada | - | KX050243 | KX050325 | KX050387 | KX050429 | KX050493 | KX050574 | KX050643 |
| JMZI001 | Arctiinae | Arctiini | *Haploa* | *reversa* | * | - | KF533471 | - | - | - | - | KF533660 | KF533597 |
| CNLEP113669 | Arctiinae | Arctiini | *Holarctia* | *obliterata* | Canada | - | KX050244 | KX050326 | KX050388 | KX050430 | KX050494 | KX050575 | KX050644 |
| KN00878 | Arctiinae | Arctiini | *Holoarctia* | *cervini* | Austria | KX050187 | KX050245 | KX050327 | - | - | KX050495 | KX050576 | - |
| HPUE12SUE | Arctiinae | Arctiini | *Holoarctia* | *puengeleri* | Sweden | KX050188 | KX050246 | KX050328 | KX050389 | KX050431 | KX050496 | KX050577 | KX050645 |
| KN00882 | Arctiinae | Arctiini | *Hyperborea* | *czekanowskii* | Russia | - | KX050247 | KX050329 | - | KX050432 | KX050497 | KX050578 | - |
| CR055 | Arctiinae | Arctiini | *Hypercompe* | *laeta* | * | - | JQ562561 | KF533540 | - | - | - | - | KF533599 |
| W72 | Arctiinae | Arctiini | *Hyphantria* | *cunea* | * | - | KF533474 | KF533541 | - | - | - | KF533662 | KF533600 |
| MM08779 | Arctiinae | Arctiini | *Hyphoraia* | *aulica* | Finland | KX050189 | KX050248 | KX050330 | KX050390 | KX050433 | KX050498 | KX050579 | KX050646 |
| HDEJ14SP2 | Arctiinae | Arctiini | *Hyphoraia* | *dejeani* | Spain | - | KX050249 | - | KX050391 | - | KX050499 | - | - |
| HTES14ITA | Arctiinae | Arctiini | *Hyphoraia* | *testudinaria* | Italy | KX050190 | KX050250 | KX050331 | KX050392 | KX050434 | KX050500 | KX050580 | KX050647 |
| KN00903 | Arctiinae | Arctiini | *Maurica* | *breveti* | Morocco | - | KX050251 | - | - | - | - | - | - |
| KN00880 | Arctiinae | Arctiini | *Micrarctia* | *trigona* | China | - | KX050252 | KX050332 | - | KX050435 | KX050501 | KX050581 | KX050648 |
| KN00899 | Arctiinae | Arctiini | *Murzinarctia* | *x-album* | China | - | KX050253 | - | - | - | KX050502 | KX050582 | - |
| KN00902 | Arctiinae | Arctiini | *Nannoarctia* | *tripartita* | Thailand | - | KX050254 | KX050333 | - | - | KX050503 | KX050583 | KX050649 |
| CNLEP113661 | Arctiinae | Arctiini | *Neoarctia* | *beani* | Canada | - | KX050255 | KX050334 | - | KX050436 | KX050504 | KX050584 | KX050650 |
| CNLEP113660 | Arctiinae | Arctiini | *Neoarctia* | *brucei* | USA | - | KX050256 | - | - | - | KX050505 | - | - |
| CNLEP113679 | Arctiinae | Arctiini | *Notarctia* | *proxima* | USA | - | KX050257 | KX050335 | KX050393 | KX050437 | KX050506 | KX050585 | KX050651 |
| NSGJZ008 | Arctiinae | Arctiini | *Nyctemera* | *adversata* | * | - | KF533485 | - | - | - | - | KF533673 | KF533610 |
| RZ387 | Arctiinae | Arctiini | *Nyctemera* | *baulus* | * | - | JN401287 | JN401402 | JN401611 | JN401714 | JN401818 | JN401909 | JN400969 |
| OZOR13SPA | Arctiinae | Arctiini | *Ocnogyna* | *zoraida* | Spain | - | KX050258 | KX050336 | KX050394 | KX050438 | KX050507 | KX050586 | KX050652 |
| OSCHLEINI14 | Arctiinae | Arctiini | *Olepa* | *schleini* | Nz. Israel | - | KX050259 | KX050337 | KX050395 | KX050439 | KX050508 | KX050587 | KX050653 |
| KN00868 | Arctiinae | Arctiini | *Oroncus* | *gurkoi* | Pakistan | - | KX050260 | - | - | - | - | - | - |
| MM23400 | Arctiinae | Arctiini | *Oroncus* | *nr.alaica* | RUSSIA | KX050191 | KX050261 | KX050338 | KX050396 | KX050440 | KX050509 | KX050588 | KX050654 |
| KN00869 | Arctiinae | Arctiini | *Orontobia* | *secreta* | China | - | KX050262 | - | - | - | KX050510 | - | - |
| KN00896 | Arctiinae | Arctiini | *Palearctia* | *erschoffi* | Kyrgyzstan | - | KX050263 | - | - | - | - | - | - |
| KN00889 | Arctiinae | Arctiini | *Palearctia* | *glaphyra* | China | - | KX050264 | KX050339 | - | - | KX050511 | - | - |
| KN00897 | Arctiinae | Arctiini | *Palearctia* | *gracilis* | Kyrgyzstan | - | KX050265 | - | - | - | - | - | - |
| KN00898 | Arctiinae | Arctiini | *Palearctia* | *hauensteini* | China | - | KX050266 | - | - | - | KX050512 | KX050589 | - |
| CNLEP113675 | Arctiinae | Arctiini | *Pararctia* | *lapponica A* | USA | - | KX050267 | KX050340 | KX050397 | - | KX050513 | KX050590 | KX050655 |
| MM23469 | Arctiinae | Arctiini | *Pararctia* | *lapponica B* | Finland | KX050192 | KX050268 | KX050341 | - | KX050441 | KX050514 | KX050591 | KX050656 |
| CNLEP113676 | Arctiinae | Arctiini | *Pararctia* | *subnebulosa* | Canada | KX050193 | KX050269 | KX050342 | KX050398 | KX050442 | KX050515 | KX050592 | KX050657 |
| KN00865 | Arctiinae | Arctiini | *Pararctia* | *tundrana* | Russia | - | KX050270 | KX050343 | - | - | KX050516 | KX050593 | - |
| BULDOR001 | Arctiinae | Arctiini | *Parasemia* | *plantaginis A* | Bulgaria | KX050194 | KX050271 | KX050344 | KX050399 | KX050443 | KX050517 | KX050594 | KX050658 |
| GEO13-2.2 | Arctiinae | Arctiini | *Parasemia* | *plantaginis B* | Georgia | KX050195 | KX050272 | KX050345 | KX050400 | KX050444 | KX050518 | KX050595 | KX050659 |
| PMAT11RUS | Arctiinae | Arctiini | *Pericallia* | *matronula* | Russia | KX050196 | KX050273 | KX050346 | KX050401 | KX050445 | KX050519 | KX050596 | KX050660 |
| NSGJZ011 | Arctiinae | Arctiini | *Phragmatobia* | *amuriensis* | * | - | KF533492 | KF533556 | - | - | - | KF533679 | KF533616 |
| CNLEP113673 | Arctiinae | Arctiini | *Platarctia* | *parthenos* | Canada | KX050197 | KX050274 | KX050347 | - | - | KX050520 | KX050597 | - |
| KN00867 | Arctiinae | Arctiini | *Platarctia* | *souliei* | China | - | KX050275 | KX050348 | - | KX050446 | KX050521 | KX050598 | KX050661 |
| CNLEP78241 | Arctiinae | Arctiini | *Platyprepia* | *virginalis* | USA | - | KX050276 | KX050349 | - | - | KX050522 | KX050599 | KX050662 |
| KN00872 | Arctiinae | Arctiini | *Preparctia* | *buddenbrocki* | China | - | KX050277 | KX050350 | - | - | KX050523 | KX050600 | - |
| KN00870 | Arctiinae | Arctiini | *Preparctia* | *mirifica* | China | - | KX050278 | KX050351 | - | - | KX050524 | KX050601 | KX050663 |
| KN00871 | Arctiinae | Arctiini | *Preparctia* | *romanovi* | China | - | KX050279 | KX050352 | KX050402 | KX050447 | KX050525 | KX050602 | KX050664 |
| CNLEP113666 | Arctiinae | Arctiini | *Pyrrharctia* | *isabella* | Canada | - | KX050280 | KX050353 | KX050403 | - | KX050526 | KX050603 | KX050665 |
| MM08356 | Arctiinae | Arctiini | *Rhyparia* | *purpurata* | Finland | KX050198 | KX050281 | KX050354 | KX050404 | KX050448 | KX050527 | KX050604 | KX050666 |
| NSGJZ014 | Arctiinae | Arctiini | *Rhyparioides* | *amurensis* | * | - | KF533489 | KF533554 | - | - | - | KF533676 | KF533613 |
| NSGJZ021 | Arctiinae | Arctiini | *Secusio* | *doriae* | * | - | KF533500 | - | - | - | - | KF533684 | KF533622 |
| SIR14GEO | Arctiinae | Lithosiini | *Setina* | *sp.* | Georgia | KX050199 | KX050282 | KX050355 | KX050405 | - | KX050528 | KX050605 | KX050667 |
| KN00887 | Arctiinae | Arctiini | *Sibirarctia* | *buraetica* | Russia | - | - | - | - | - | KX050529 | - | KX050668 |
| KN00885 | Arctiinae | Arctiini | *Sibirarctia* | *kindermanni* | Russia | - | KX050283 | - | - | - | KX050530 | - | - |
| KN00864 | Arctiinae | Arctiini | *Sinoarctia* | *sieversi* | China | - | KX050284 | KX050356 | - | KX050449 | KX050531 | KX050606 | - |
| KN00900 | Arctiinae | Arctiini | *Sinowatsonia* | *hoenei* | China | - | KX050285 | KX050357 | - | - | KX050532 | - | - |
| MM07409 | Arctiinae | Arctiini | *Spilarctia* | *luteum* | Finland | - | KX050286 | KX050358 | KX050406 | KX050450 | KX050533 | KX050607 | KX050669 |
| MM01038 | Arctiinae | Arctiini | *Spilosoma* | *lubricipedum* | Finland | - | KX050287 | KX050359 | KX050407 | KX050451 | - | KX050608 | KX050670 |
| SCDNA81 | Arctiinae | Arctiini | *Spilosoma* | *virginica* | * | - | KF533503 | KF533561 | - | - | - | KF533687 | KF533625 |
| M5 | Arctiinae | Arctiini | *Spilosomina* | *sp. A* | Ghana | - | KP081794 | KX050360 | KP082340 | - | KP082465 | KP082591 | KP082727 |
| TTAM15MOR | Arctiinae | Arctiini | *Spilosomina* | *sp. B* | Morocco | - | KX050288 | KX050361 | - | - | KX050534 | KX050609 | - |
| KN00877 | Arctiinae | Arctiini | *Tancrea* | *pardalina* | Kazakhstan | - | KX050289 | KX050362 | - | - | KX050535 | KX050610 | KX050671 |
| DNA1252 | Arctiinae | Arctiini | *Tyria* | *jacobaeae* | * | - | KF533506 | KF533564 | - | - | - | KF533690 | KF533628 |
| JZ000 | Arctiinae | Arctiini | *Virbia* | *fragilis* | * | - | KF533508 | KF533565 | - | - | - | KF533693 | - |
| KC33 | Arctiinae | Arctiini | *Virbia* | *immaculata* | * | - | KF533509 | - | - | - | - | KF533694 | KF533631 |
| KC11 | Arctiinae | Arctiini | *Virbia* | *opella* | * | - | KF533511 | - | - | - | - | KF533696 | KF533633 |

# Appendix 2. Bayesian topology



# II

# WARNING SIGNAL POLYMORPHISM DESPITE POSITIVE FREQUENCY-DEPENDENT SELECTION

by

Katja Rönkä, Janne Valkonen, Ossi Nokelainen, Bibiana Rojas, Swanne Gordon,
Emily Burdfield-Steel, Tönis Tasane & Johanna Mappes 2017

Manuscript.

# III

# COLOUR ALONE MATTERS: NO PREDATOR GENERALIZATION AMONG MORPHS OF AND APOSEMATIC MOTH.

by

Katja Rönkä, Chiara De Pasqual, Johanna Mappes, Swanne Gordon & Bibiana Rojas 2017

Animal Behaviour, In press.

# Colour alone matters: no predator generalization among morphs of an aposematic moth

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## Keywords

learning, polymorphism, predator generalization, predator–prey interactions, warning signals, wood tiger moth

## Abstract

Local warning colour polymorphism, frequently observed in aposematic organisms, is evolutionarily puzzling. This is because variation in aposematic signals is expected to be selected against due to predators' difficulties associating several signals with a given unprofitable prey. One possible explanation for the existence of such variation is predator generalization, which occurs when predators learn to avoid one form and consequently avoid other sufficiently similar forms, relaxing selection for monomorphic signals. We tested this hypothesis by exposing the three different colour morphs of the aposematic wood tiger moth, *Arctia plantaginis*, existing in Finland to local wild-caught predators (blue tits, *Cyanistes caeruleus*). We designed artificial moths that varied only in their hindwing coloration (white, yellow and red) keeping other traits (e.g. wing pattern and size) constant. Thus, if the birds transferred their aversion of one morph to the other two we could infer that their visual appearances are sufficiently similar for predator generalization to take place. We found that, surprisingly, birds showed no preference or aversion for any of the three morphs presented. During the avoidance learning trials, birds learned to avoid the red morph considerably faster than the white or yellow morphs, confirming previous findings on the efficacy of red as a warning signal that facilitates predator learning. Birds did not generalize their learned avoidance of one colour morph to the other two morphs, suggesting that they pay more attention to conspicuous wing coloration than other traits. Our results are in accordance with previous findings that coloration plays a key role during avoidance learning and generalization, which has important implications for the evolution of mimicry. We conclude that, in the case of wood tiger moths, predator generalization is unlikely to explain the unexpected coexistence of different morphs.

## Introduction

Aposematic organisms display warning signals that predators learn to associate with their unprofitability (Poulton, 1890). The survival of such prey is thus highly dependent on a predator's ability to learn, remember and generalize their learned avoidance to other individuals sharing the same warning signal (reviewed in Ruxton, Sherratt, & Speed, 2004). Signal sharing among aposematic prey benefits both the prey and their potential predators: (1) a given individual has a lower risk of predation when more individuals share the same warning signal, and (2) predators benefit from not having to sample as many unprofitable or toxic prey and can more easily remember one and not multiple signals (Ghirlanda & Enquist, 2003; Guilford & Dawkins, 1991; Müller, 1878; Rowland, Ihalainen, Lindström, Mappes, & Speed, 2007; ten Cate & Rowe, 2007). Therefore, local polymorphism in warning coloration is expected to be selected against (Chouteau, Arias & Joron 2016; Endler 1991; Joron & Mallet, 1998; Lindström, Alatalo, Lyytinen, & Mappes, 2001; Mallet & Barton, 1989; Mallet & Joron, 1999; but see also Ihalainen, Lindström, & Mappes, 2007 who found no evidence for slower avoidance learning of single versus multiple signals).

Despite the predicted disadvantages, warning signal polymorphisms are present in several aposematic taxa, such as frogs (Amézquita, Castro, Arias, González, & Esquivel, 2013; Rojas & Endler, 2013), ladybirds (O'Donald & Majerus, 1984; Průchová et al. 2014) and butterflies (Jiggins & McMillan, 1997). In fact, they seem to be more common than expected considering that warning signals are predicted to be under positive frequency-dependent selection (Müller, 1878; Ruxton, Sherratt, & Speed, 2004). One possible explanation for the co-occurrence of several warning signal forms within the same population is predator generalization. This refers to a predator's ability to transfer its learned avoidance of a particular signal to other signal(s) that share common characteristics (Gamberale-Stille & Tullberg, 1999; Lindström, Alatalo, Mappes, Riipi, & Vertainen et al. 1999b; Guilford & Dawkins, 1991; Mappes & Alatalo 1997). Generalization can be symmetric, meaning that once one colour is learned it is equally possible to transfer the learned aversion to other similar colours, or asymmetric, implying that transferring a learned avoidance from one colour to other(s) depends on the signal salience (Aronsson & Gamberale-Stille, 2008; Exnerová et al., 2006; Gamberale & Tullberg, 1996; Gamberale-Stille & Tullberg, 1999; Ham, Ihalainen, Lindström & Mappes, 2006; Ruxton, Franks, Balogh, & Leimar, 2008; Waldron et al., 2017).

Predator learning involves different cognitive processes that establish the association between warning coloration and unprofitability, and aid the memorization of this association once established. This learning process may vary between predators even at intraspecific levels (e.g. Adamová-Ježová et al., 2016; Endler & Mappes, 2004; Exnerová et al., 2010; 2015; Karlíková et al., 2016; Lindström, Alatalo, & Mappes, 1999a; Sherratt & Macdougall, 1995; Skelhorn, Halpin, & Rowe, 2016). Predators may also vary in their ability to cope with defended prey, due for example to dietary conservatism (Marples & Kelly, 1999; Mettke-Hofmann et al., 2002; Webster & Lefebvre, 2000; Turini, Veselý & Fuchs, 2016). Therefore, investigating how predators learn to associate the appearance of prey with the noxious effects of their unprofitability is crucial to understanding how signal variation can be maintained within a population. During the learning process predators acquire information about the nutrient and toxin content of aposematic prey. Thus, individual predators are expected to make different decisions on how to use the information gathered from an encounter with aposematic prey (Exnerová et al., 2003; Exnerová et al., 2007; Halpin, Skelhorn, & Rowe, 2014; Lynn, 2005; Skelhorn et al., 2016; Trimmer et al., 2011), and modify their ingestion of toxic prey according to their toxic burden (Skelhorn & Rowe, 2007).

Generalized avoidance should be broad and persist for a relatively long time to offer protection to different warningly coloured prey morphs. On the other hand, naïve predators can also avoid warningly coloured prey due to innate wariness, neophobia or dietary conservatism (Exnerová et al., 2007; Lindström, Alatalo & Mappes, 1999; Marples & Kelly, 1999; Marples & Mappes 2011), which could be further reinforced by the short-term effects of negative experience with other aposematic prey. It has been suggested that multiple modalities of warning signals can help predators discriminate between palatable and unpalatable prey (Siddall & Marples, 2008, Kazemi, Gamberale-Stille & Leimar, 2015). However, generalized avoidance of aposematic prey can also be based on cues of different sensory modalities, such as odour, sound, colour or pattern or combinations of these. Depending on the cognitive processes of predators, they could also associate their negative experience with certain stimuli to any other stimuli encountered simultaneously (Mackintosh, 1975; Pavlov, 1927). These results emphasize the importance of studying how multiple cues and separate signal components influence a predator's decision to attack prey (Kikuchi, Mappes, Sherratt & Valkonen, 2016; Rowe & Halpin, 2013).

Here, we tested the hypothesis that the hindwing colour polymorphism of an aposematic moth is enabled by predator generalization, and investigated whether or not that generalization is symmetric. We exposed paper models of the different hindwing colour morphs of the wood tiger moth, *Arctia plantaginis* (formerly known as *Parasemia plantaginis*, Rönkä, Mappes, Kaila, & Wahlberg, 2016) to natural predators (blue tits, *Cyanistes caeruleus*), and examined whether, once they learned to avoid one of the colour morphs, they would generalize this aversion to the two unlearned colour morphs, which would allow multiple morphs to coexist. A lack of generalization among colour morphs would mean that birds pay more attention to colours than to other cues of the moth wings.

## Methods

The wood tiger moth is an aposematic diurnal moth with a Holarctic distribution (Hegna, Galarza, & Mappes, 2015). They have two different chemical defences, one of which is secreted from the prothoracic glands. Although the chemical composition is not fully known, these fluids contain two types of methoxypyrazines, which are produced de novo (Burdfield-Steel, Pakkanen, Rojas, Galarza & Mappes 2016) and make them a deterrent to birds. Experiments with bird predators suggest that the fluids of yellow males have a more repulsive odour (Rojas et al. 2017), while those of white males taste worse (Rojas, Burdfield-Steel & Mappes 2015). Individuals vary in the degree of melanization and black patterning of the wings, as well as in levels of chemical defence, but the most striking feature of the wood tiger moth is its local hindwing colour polymorphism (Hegna et al., 2015). In Europe, its forewings present a black and white pattern in both males and females, whereas the hindwing colour combined with black pattern differs between the sexes (e.g. Galarza, Nokelainen, Ashrafi, Hegna, & Mappes, 2014; Hegna & Mappes, 2014). The distinct white and yellow male morphs are genetically determined by one autosomal locus and at least three alleles, dominant white, recessive white and intermediate yellow (Galarza, Nokelainen & Mappes 2016), while female hindwing coloration varies continuously from yellow to red (Lindstedt et al. 2017; Fig. 1). In Finland, for example, yellow and white males may occur within one population (Nokelainen, Valkonen, Lindstedt, & Mappes, 2014) whereas female hindwing coloration is mostly red (Hegna et al., 2015).

To study the reaction of bird predators (see below for details on procedure) to the different hindwing colour morphs, we used artificial moth models. The usage of artificial prey allows for the controlled manipulation of one or more warning signal components at a time, while accounting for how predators (i.e. birds) would see them (Endler & Mielke 2005). In this way, other components can be kept constant and independent of prey qualities, such as the variation in the level of chemical defence or behaviour (Karlíková et al., 2016; Lindström et al., 1999a; Veselý & Fuchs 2009). Here, our artificial moth models eliminated individual variation in moth size, shape, degree of melanization, wing pattern, wing posture, behaviour, smell or taste. Model wings were constructed with the software GIMP (2.8.16; http://www.gimp.org/) from pictures of a real male wood tiger moth specimen collected in Finland. Pictures of one forewing and one hindwing of a typical white moth were duplicated to obtain a symmetric pattern for the whole model. The melanization pattern of the moths used was a representative sample of a wing pattern in Finland (Fig. 1). To control for the amount and shape of melanized (mainly black) pattern of the wings, yellow and red models were created from the same wing picture, changing the hue of the white parts of the hindwing towards yellow or red. Finished models were printed double sided (HP Color LaserJet CP2025) on waterproof (Rite In The Rain, Tacoma, WA, U.S.A.) paper. To ensure that the model colours resembled the real wood tiger moth morphs, colour reflectance was measured with an Ocean Optics Maya2000 Pro spectrometer and average reflectance curves from three spots in the model hindwing coloration were compared to average reflectance curves of white, yellow and red moth hindwings (Fig. 1). Models were then cut out from the paper and completed with a body made of rolled pastry, composed of two parts of lard, six parts of coarse wheat flour and one part of water to make them edible. The total body weight was 0.04 ± 0.005 g. Bodies were dyed on top with black food colouring, to make models resemble the real moths as accurately as possible. Finally, bodies were glued on the paper models with nontoxic glue (UHU stick).

### *Bird predators*

Blue tits were chosen as predators for several reasons: (1) they are visual foragers and their visual capabilities are well known (Hart, Partridge, Cuthill, & Bennett, 2000; Hart & Vorobyev, 2005), ensuring that they are able to distinguish all of the wood tiger moth's colour morphs; (2) they have been used in several experiments on coloration (e.g. Dimitrova & Merilaita, 2010; Exnerová et al., 2007; Kikuchi et al., 2016) and wood tiger moths (Nokelainen, Hegna, Reudler, Lindstedt, & Mappes, 2012), and also with similar moth models (Rojas, Burdfield-Steel & Mappes 2015); (3) tits are likely to be important natural predators of wood tiger moths in Finland

(Nokelainen, Valkonen, Lindstedt, & Mappes, 2014); and (4) blue tits are common in central Finland, and easy to capture and keep in captivity for a short period of time.

The birds used for the experiment were caught from Konnevesi Research Station and City of Jyväskylä (central Finland), maintained individually in plywood cages with a perch, water bowl and food ad libitum, and kept on a 12:12 h light:dark cycle. Each bird was weighed before and after the experiment, ringed, and its sex and age were determined before being released to the same place of capture. Birds were used with permission from the Central Finland Centre for Economic Development, Transport and Environment and licensed from the National Animal Experiment Board (ESAVI/9114/04.10.07/2014) and the Central Finland Regional Environment Centre (VARELY/294/2015). All experimental birds were used according to the ASAB/ABS Guidelines for the treatment of animals in behavioural research and teaching.

### *Experimental procedures*

The experiment consisted of three phases: a preference test, a learning test and a generalization test (see details below). Each bird was tested individually and only once for each part of the experiment. The experiment was conducted between November 2015 and March 2016 at Konnevesi Research Station, in central Finland and lasted, on average, 3 days for each individual, depending on how long the bird took to complete the different tests.

Trials took place in experimental custom-built plywood cages (50x50 cm and 70 cm high) illuminated with a daylight lamp (Exo Terra Repti Glo 10.0 UVB, http://exo-terra.com/). Each aviary had a perch and a water bowl (access ad libitum). Birds were observed through a small mesh-covered window situated on the front of the cage, and filmed with a Canon Powershot S120 camera. The experiment took place in a dark room to minimize observers disturbing the birds.

Food and experimental models were offered on a green platform through a moveable tray behind a visual barrier, allowing us to estimate the exact time when the bird first saw the model and thus started the trial (see details in Nokelainen et al. 2012). A standard green background was used, because wood tiger moths rest on green leaves in nature (Hegna et al. 2013, Nokelainen et al., 2012). All colours used in the moth models are easily distinguished from the background by birds: Hegna et al. (2013) reported just noticeable difference (JND) values in colour contrast ranging from 8.6 to 11.6 for white and yellow artificial moth models and real wood tiger moths against the green background used also in this experiment, and Lindstedt et al. (2011) calculated JND values above 27.27 for orange and red females on natural green leaves of *Alnus incana*.

During pretraining, birds were allowed to habituate to the experimental cages and learned to eat three sunflower seeds from the green platform. To motivate the birds to attack the moth models during the experiment (see below), they were food deprived for 2 h before the preference test, 1 h before the learning test and 1 h before the generalization test. After food deprivation, bird motivation was tested with a sunflower seed; if eaten, the bird was considered ready to begin the test.

### *Phase 1: Preference test*

A preference test was included in the experimental protocol for two reasons. As we used wild-caught birds, we first tested whether they had any pre-existing biases towards white, yellow or red moth morphs. Second, by offering palatable morphs several times to birds we ensured that any potential unlearned or learned biases disappeared, allowing us to test the effect of the coloration on learning and generalization (Ghirlanda & Enquist, 2003).

All three morphs (white, yellow and red) were offered simultaneously on the green platform for 5 min, starting from when the bird first saw them. If the bird did not attack (i.e. grab or peck) any of the edible model pastry bodies during the 5 min, the models were taken away and presented again after a break. Once the first attack was made, the models were kept in the cage until the bird finished eating all the pastry bodies. To ensure that all birds had an equally rewarding experience with all the colours, we let the birds finish eating the pastry bodies of all models in three consecutive trials during the preference test. Between the trials, the presentation (order) of the models on the platform was always changed (Fig. 2).

As birds were hesitant to attack the moth models for the first time (hesitation times varying from 17 s to 2 h consisting of 5 min presentations), we did not use time to attack in analyses. Instead, we recorded the order in which the models were attacked and eaten during the three consecutive trials. We compared the order of attacks between the first and the last preference test to be sure that all the birds got rid of any potential bias in preferences before the learning phase. Preference test presentations were continued for a maximum of 2 days. Eight of 53 birds did not attack or finish eating the artificial moth models offered during the preference test and were, therefore, excluded from further tests.

### *Phase 2: Learning test*

In the second phase of the experiment we tested whether blue tits learn to avoid white, yellow and red models differently, and established learned avoidance towards one of the colour morphs before the following generalization test. Birds that completed the preference test were divided into three groups for avoidance learning: 15 birds were offered white models, 15 yellow models and 16 red models as unpalatable. Groups of birds were selected as similar as possible (i.e. similar sex, age and size distribution) and birds from all groups were tested simultaneously. All models were made unpalatable by replacing the water in pastry bodies with 15% chloroquine diphosphate solution (Sigma Life Science, St Louis, MO, U.S.A.). As the pastry bodies were coloured with black dye on top, we also added 15% chloroquine diphosphate solution on top of the bodies and let it dry before the following trials. Chloroquine solution was used because it is odourless (Hong 1976) and thus all qualities other than palatability (i.e. taste) of the prey items remained the same throughout the experiment.

During the learning test, unpalatable models were presented individually in consecutive trials alternating with sunflower seeds (Fig. 2). Sunflower seeds were offered to monitor the birds' motivation to forage and avoid unnecessary starvation. If the bird did not attack the sunflower seed, it got a 10 min break without food and was then offered a sunflower seed again. If the bird attacked the sunflower seed, the next unpalatable model was offered 2 min after the bird finished eating. As long as the bird attacked the models, trials were continued alternating with sunflower seeds. If the bird did not attack the unpalatable model, but ate the sunflower seed, it was considered to reject the model. After a bird did not attack the moth model the second time in a row, a small live mealworm (< 20 mm *Tenebrio molitor* larva) was offered instead of the sunflower seed to test the bird's motivation to attack insect prey and increase its motivation to forage. If the bird now attacked the unpalatable model offered after the mealworm, trials were continued again alternating with sunflower seeds, but if it rejected the unpalatable model, it got another mealworm (Fig. 2). We considered the bird to have learned to avoid the unpalatable models when it refused to attack three models in a row, but consumed the sunflower seeds and mealworms offered in between and after the rejected models.

Presentation time was set to 5 min from when the bird first saw the model for the first three trials to make sure that each bird had the opportunity to attack and taste the model. To keep the overall duration of the generalization experiment within the permitted 4-day limit, a maximum of 30 presentations divided into 2 days was set for the avoidance learning. Furthermore, we reduced the presentation time to 2 min from the fourth to the sixth trial, and to 1 min for the rest of the trials. Based on our observations during a pilot experiment with six birds and fixed durations of trials, birds were unlikely to attack the model and did not consume it within 5 min if they did not attack within the first minute. Sunflower seeds were usually attacked quickly, and hesitation time declined to a few seconds as the trials proceeded, implying that 1 min was sufficient to test the bird's willingness to attack the models. Two of the 46 birds did not stop attacking (white and red) models within 30 presentations and were therefore excluded from the following generalization test.

### Phase 3: Generalization test

In the third phase, we tested whether the 44 birds that had learned one of the colour morphs as unpalatable would avoid attacking the other two colour morphs. When birds had completed the learning test, half of them had a break of at least 2 h with food and 1 h of food deprivation before the last phase of the experiment, and half were tested the following day. The generalization test started after the bird had consumed a sunflower seed offered to test its motivation to attack. Birds were tested for the generalization with the colours that they did not learn as unpalatable: yellow and red for those that learned white as unpalatable, white and red for those that learned yellow, and white and yellow for those that learned red (Fig. 2). The two colours tested were presented simultaneously on the green platform in alternating positions for three trials lasting 5 min each. This allowed us to test the repeatability of bird behaviour. The trials were interspersed with sunflower seed presentations to make sure that birds were not attacking the models due to lack of motivation. Moreover, offering alternative food ensured that birds were not forced to eat the models simply because of hunger. Models used for the generalization test were palatable.

*Statistical analysis*

*Phase 1: Preference test*

The potential colour bias of blue tits was analysed separately for the first trial (Fig. 2) and all three trials pooled. Colour biases are most likely to be detected reliably by checking the order of attacks on the white, yellow and red models in the first trial ($N$=53), when the birds first encountered the models. The number of moths of each colour taken first, as well as left last, were compared by means of a chi-square test. Additionally, all three trials were pooled in another analysis to find out whether the potential biases disappeared as the birds learned to eat all the models. In the pooled data, each colour was scored based on the order of choice by the bird in each trial; the colour chosen first was scored 1, that chosen second was scored 2 and the colour chosen last was scored 3. Thereby, the minimum score expected for a preferred colour was 3 (i.e. always chosen first), and the maximum score expected for an avoided colour was 9 (i.e. always chosen last). To study the population level bias to all colours, the scores of each colour in each of the three trials were summed and compared to an even distribution by means of a chi-square test. The potential influence of the first colour chosen on the subsequent choice was checked with a binomial exact test.

*Phase 2: Learning test*

Potential differences in learning rate between the three colour morphs were analysed using a mixed-effect Cox regression model, using the 'coxme' package (version 2.2-5; Therneau, 2015) in RStudio (v. 0.99.902; RStudio, 2015). The response variable was the probability that the presented model was attacked in each trial; time was represented as number of trials. Model colour was added as an explanatory factor and bird individual as a random effect.

*Phase 3: Generalization test*

If birds generalized their learned aversion of a given colour to the two nonlearned colours, we would expect them to refrain from attacking models offered during the generalization test but eat the alternative prey offered between trials. Hence, the probability of attack on palatable models is expected to be significantly lower than random (< 0.5). If, in contrast, birds were unable to generalize their learned avoidance, we would expect the attack probability to be significantly higher than 0.5. High attack probability is expected (in the case of no generalization) since the birds had attacked and eaten similar palatable models in the preference test and did attack the models presented first in the learning test within the 5 min presentation. Thus, to test whether the birds generalized and the attack probability on the models was lower (or higher) than random, we built two generalized linear mixed models (GLMM 1 and 2) with a logit link and binomial distribution, including whether the prey was attacked (1) or not (0) as the dependent variable. Bird ID and bird ID nested within trial in GLMM 2 were added as random factors using package lme4 (Bates et al. 2015) in R. GLMM 1 was used to test for generalization in the first trial only, and GLMM 2 in all three trials.

To test for asymmetric generalization, we divided the birds into six treatment classes by the colours they learned (white, yellow and red) and were offered (yellow and red, white and red, white and yellow, respectively). This classification was then used as the explanatory variable ('colour combination') in two GLMM models separately for the first trial only (Table A1 in the Appendix) and all three trials (Table A2 in the Appendix) of generalization (again with a logit link and binomial distribution, including whether the prey was attacked (1) or not (0) as the dependent variable, and bird ID nested within trial and/or bird ID as random factors). A chi-square test was used to check whether the birds attacked one colour morph first more frequently between the two colour morphs offered, both in the first trial and in the first three trials pooled (Table A3 in the Appendix). Birds tested the same or the following day after avoidance learning were pooled in all analyses, as there were no differences in the number of attacks between birds tested the same or the following day after avoidance learning in the first trial (unpaired two-sample Wilcoxon test: $W$=276, $N$=44, $P$=0.21) or in the three trials pooled ($W$=262, $N$=44, $P$=0.59). We also checked whether the rate of learning correlated with the number of attacks in the generalization test with a Spearman correlation.

## Results

### *Preference test*

At the population level, birds did not show any preferences (Table 1) or aversion (chi-square test: $\chi^2_2$=2.577, $P$=0.28) towards any of the colours (white, yellow or red) during the first trial. Birds chose the second colour to attack with the same probability between the two colours left, irrespective of the first colour chosen (binomial exact test: $P$>0.05 for all comparisons).

At the individual level, 35 birds (85.4%, $N$=41) chose at least one colour in the same order for two different trials (for instance, the same bird chose the yellow morph as last choice in two trials out of three). Two birds showed a strong preference for one of the colour morphs, choosing the same colour (yellow and red, respectively) first for all three trials. Three birds showed avoidance for one colour morph (one for white, two for red), leaving the same colour as last in all the trials. All other birds changed their order of choice during the three trials, showing that they got rid of potential biases towards the colours during training. When we tested the overall scores for each colour morph during the three trials, birds did not show differences between the colour morphs (chi-square test: $\chi^2_2$=0.789, $P$=0.67).

### *Learning test*

Apart from two individuals, all birds ($N$=44) learned to avoid their moth model according to the criterion of no attack over three subsequent trials. The number of trials needed to learn to avoid the unpalatable model varied between 2 and 23 among the birds (mean=7). The Cox regression model (Fig. 3) showed that birds learned to avoid the red colour morph significantly faster than the yellow ($Z$=2.17, $P$=0.03), but showed no significant differences between the yellow and white morphs ($Z$=0.87, $P$=0.38).

### *Generalization test*

Overall, blue tits did not generalize their learned avoidance from one colour morph to the other two, as the attack probabilities were significantly higher than 0.5 in the first trial (GLMM 1: $Z$=4.33, $P$<0.001; Fig. 4) and the three trials pooled (GLMM 2: $Z$=6.42, $P$<0.001). Only three of 44 individuals did not attack any of the palatable models during the generalization test, showing generalized avoidance.

We did not find clear evidence of asymmetric generalization. The estimated attack probabilities did not differ significantly between the combinations of colour learned and colour offered in generalization trials (Tables A1 and A2 in the Appendix), and no differences were found in which colour the birds attacked first during the first trial of the generalization test (chi-square test: $P$>0.05 in all cases; Table A3 in the Appendix). In the first trial, however, birds that learned yellow attacked fewer white models compared to the other colour combinations, and the effect is near the 0.05 significance level (Table A1 in the Appendix). Also, when the three trials were pooled, we found that birds that learned to avoid the white morph attacked the red morph first significantly more often than the yellow one (chi-square test: $\chi^2_1$=5.9, $P$=0.02).

The rate of learning did not correlate significantly with the proportion of attacked models in the first generalization trial (Spearman correlation $r_S$=0.12, $N$=44, $P$=0.45) or the total number of attacks in the three generalization trials (Spearman correlation: $r_S$=0.27, $N$=44, $P$=0.07), thus allowing us to compare the effect of the colour learned on generalization despite different learning rates of red versus the other colours.

## Discussion

### *No generalization based on hindwing colour*

Generalized avoidance by local predators from one warning signal to another has been proposed to contribute to the maintenance of local warning signal polymorphism in aposematic species (Amézquita et al., 2013; Exnerová et al., 2006; Gamberale & Tullberg, 1996; Gamberale-Stille & Tullberg, 1999; Ham et al., 2006; Hegna & Mappes, 2014; Rojas, Rautiala, & Mappes, 2014; Ruxton et al., 2008; Waldron et al., 2017). Here we studied in more detail how bird predators learn and generalize the warning colours of a polymorphic (red, yellow, white) wood tiger moth population using artificial moth models. Attack rates during the generalization test were in general very high. Indeed, the birds did not generalize their learned avoidance among the wood tiger moth morphs, but instead treated them as different prey types based on the differing hindwing colour alone, as the morph models used did not differ in size, shape, pattern, taste or smell.

### *The importance of colour*

Our findings are in line with previous experiments showing that colour is of foremost importance in avian predator learning, contributing especially to the discrimination between palatable and unpalatable prey (Aronsson & Gamberale-Stille, 2008, Kazemi, Gamberale-Stille, Tullberg, & Leimar, 2014). A large body of research has demonstrated birds' ability to learn to avoid conspicuous, unpalatable prey (Aronsson & Gamberale-Stille, 2008; Rowe, Lindström, & Lyytinen, 2004; Svádová et al., 2009). This is because conspicuous warning coloration enhances prey recognition (Guilford, 1986; Sherratt & Beatty, 2003), speed of avoidance learning and memorability (e.g. Roper & Redston, 1987). Different predators may use different components of the warning signal as a primary cue depending on their sensory systems (Aronsson & Gamberale-Stille, 2012; Endler, 1992; Guilford & Dawkins, 1991) and disregard others. Studies done with birds have demonstrated that colour seems to be a more important feature in warning signals than size or pattern (Aronsson & Gamberale-Stille, 2008; Exnerová  et al., 2006; Sillén-Tullberg, 1985; Terhune, 1977).

As predators can associate palatability or unpalatability with several different kinds of prey traits, it is convenient to compare the relative importance of those traits with how much they facilitate associative learning. The expectation is that more salient signals are learned faster (Kazemi et al. 2014). Our results indicate that red was the most salient warning colour: birds learned to avoid the red morph faster than the other morphs. This is in accordance with Lindstedt et al. (2011), who found that the red female morph of the wood tiger moth was better protected against bird predators, suffering fewer attacks than its orange or yellow counterparts. Indeed, red has been shown to be a very efficient warning signal compared to other warning colours such as orange, yellow or white, and other colours such as violet, blue, green and brown, at least for some bird predators (Cibulková, Veselý, & Fuchs, 2014; Exnerová  et al., 2006; Gamberale-Stille & Tullberg, 1999; Lindstedt et al., 2011; Svádová et al., 2009).

In the present study prey items were made to resemble real wood tiger moth morphs as closely as possible, keeping all traits other than hindwing colour constant. This allowed us to compare the effects of warning coloration of hindwings only. Changing the warning colour hue altered not only the internal contrast on model hindwings, but also the contrast between the model and the green background. Although all colours in our experiment were clearly conspicuous to the birds, red had the highest colour contrast against the green background whereas white had the lowest. This might explain why red seems to be the most salient signal. Aronsson and Gamberale-Stille (2009) found similar results using domestic chicks, *Gallus gallus domesticus*, which learned to avoid red prey faster if presented on a contrasting background compared to a background of similar hue. In another experiment, however, red prey colour was found to influence predator avoidance independent of background colour (Sillén-Tullberg, 1985). Thus, it seems that both prey coloration per se and its contrast against the background can contribute to predator avoidance, but it is still relatively unclear which properties of prey coloration, chromatic or achromatic, play the most important role. Previous work with wood tiger moths has shown that the achromatic contrast against a green background is highest for white morphs, which are the most luminous of the three (Lindstedt et al. 2011; Henze, Lind, Mappes, Rojas & Kelber 2017). Luminance has not been found to affect predator responses towards the wood tiger moth, while the chromatic contrast in hue seems to be very important (Nokelainen et al., 2012).

Generalization has been suggested to stabilize selection towards aposematic signals via a peak shift phenomenon (Leimar, Enquist, & Sillen-Tullberg, 1986; Lindström et al. 1999b). The minimum (and maximum) responses of predators (i.e.

peaks of the generalization gradient) have been found to be displaced from the negative (and positive) stimulus (Gamberale & Tullberg, 1996; Hanson, 1959), such as yellow, towards a similar, but more salient novel stimulus, such as red. Overall, we did not find strong evidence of asymmetric generalization, but there were some trends between the colours tested. Birds that learned to avoid red models attacked almost all the white and yellow models in the generalization trials, whereas birds that learned the less salient colours yellow and white generalized more, hinting at a tendency to generalize from the less salient signals towards the more salient signal.

Svádová et al. (2009) found asymmetric generalization using great tits, *Parus major*, which did not generalize from red firebugs, *Pyrrhocoris apterus*, to white or yellow mutants, but did generalize from yellow mutants to red firebugs. Interestingly, in our experiment, four blue tits that learned to avoid the yellow morph (*N*=15) refrained from attacking white models, while only two did so for the red ones. Birds that learned to avoid the white morph attacked both unlearned morphs equally, but chose red models first more often than yellow ones. This indicates that birds tended to generalize more between the white and yellow than between white and red. The yellow morph seems to benefit least from the other colours, since only between 7 and 34% of yellow models were left unattacked (Table 2, Fig. 4).

### *Limitations of testing generalization in the laboratory*

Despite the majority of birds showing no generalization in our experiment, the possibility remains that predators might generalize among morphs of the wood tiger moth under different circumstances. Studying generalization in the wild is practically impossible due to the rareness of predation events on aposematic prey as well as difficulties in observing the choices of individual predators in natural conditions. A previous study aiming to explain the variability in the warning signals of the harlequin poison frog, *Oophaga histrionica*, showed that predators avoided attacking aposematic frog models but not cryptic ones in areas where aposematic frogs occur, exhibiting some generalization among different frog colour morphs in the field. However, the same study found no generalization by naïve chicks tested in the laboratory (Amézquita et al., 2013). This might imply that naïve and experienced predators in the wild can use different generalization strategies (see also Ihalainen, Lindström, Mappes & Puolakkainen, 2008). Birds might also be prone to generalize more or less widely under different circumstances (Aronsson & Gamberale-Stille, 2012), for example under physiological stress during winter months (Barnett, Bateson & Rowe 2007; Chatelain et al. 2013; Veselý et al. 2017), limited food availability (Lindström, Alatalo, Lyytinen & Mappes, 2004; Ihalainen, Rowland, Speed, Ruxton, & Mappes, 2012), limited time to make decisions (Ings & Chittka, 2008), when the prey is dangerously toxic (Lindström, Alatalo, & Mappes, 1997; Sherratt, 2002), when the prey community is complex versus simple (Ihalainen et al., 2007), or when the prey population has palatable Batesian mimics in addition to the unprofitable prey (Plowright & Owen, 1980).

Avoidance learning has been suggested to happen in two steps: first, the birds learn simple rules based on certain cues, and once the basic rules are formed, they then learn in more detail about prey quality (Chittka & Osorio, 2007). Recent studies indicate that birds are able to assess the nutritional benefits of unprofitable prey and use this information in subsequent encounters (Halpin et al., 2014). This ability could have affected not only bird learning rates, but also their decision to attack in the generalization phase of our experiment. As our models' pastry bodies were of high nutritional value and the birds were hungry, it is possible that the birds were willing to take more risks and thus took more trials to learn to avoid the models than it would take them to learn to avoid defended prey in the wild. In addition, the 5 min presentations gave the birds plenty of time to decide whether to attack or not, and to make more sophisticated assessments of prey quality than might be possible in the wild. Birds were given alternative food between the presentations, but not enough for saturation, and would thus have benefited energetically from discriminating between the unprofitable and profitable models. Nevertheless, the cost–benefit relationship was exactly the same for all morphs in our experiment and, thus, we can safely compare the relative differences between morphs in their salience.

The avoidance learning was based on counterconditioning, where the colour signal was first associated with a positive reinforcement (i.e. palatability) and then with a negative reinforcement (i.e. unpalatability). Previous research has shown that in cases of single counterconditioning the associations learned second are forgotten at higher rates than those learned first (Speed, 2000 and references therein). Therefore, it is possible that the birds' experience and learned association with palatability in the preference test exceeded the effect of generalized avoidance among the morphs for most of the birds, which could partly explain the low level of generalization observed. Offering the models as palatable at the beginning of the experiment was necessary to get rid of any pre-existing biases or neophobia prior to learning and testing generalization effects of the birds; this was also necessary to motivate the birds to attack and taste the unpalatable models during the first learning trials.

In the preference and generalization tests, simultaneous prey choice was used to decrease the numbers of birds needed to accomplish the experiment. Simultaneous prey choice is also a very powerful set-up to detect any potential predator biases but, obviously, this approach has disadvantages too (Fig. 2). For example, it is possible that long hesitation delays during the first presentation of the preference test were partly due to an aggregation effect, as aggregations of conspicuous prey have been found to be aversive to predators (Gamberale-Stille, 2000; Riipi, Alatalo, Lindstrom, & Mappes, 2001). On the other hand, Nokelainen et al. (2012) presented wood tiger moths singly to birds, several of which also hesitated for a long time before attacking them. Thus, it is difficult to say how much the simultaneous presentation influenced our results, but during the flying season wood tiger moth morphs typically aggregate at the same sites. Males of both morphs are often found near calling females, and thus all morphs can be visible and vulnerable to predators simultaneously.

Lastly, if the wood tiger moths are able to survive bird attacks, the use of artificial models does not necessarily give an accurate estimate of selection. A considerable proportion of attacks in the generalization test were just a single peck, leaving the models uneaten, and thus not necessarily 'killed'. The birds' willingness to attack but reluctance to consume the models could stem from the psychology of birds' decision making (Marples & Kelly, 1999). Adamová-Ježová et al. (2016) showed that for great tits and coal tits, *Periparus ater*, neophobia (i.e. the avoidance of novel prey affecting the decision to attack), but not dietary conservatism (i.e. restriction of diet to certain prey types affecting the decision to consume the prey), was deactivated during pretraining with a palatable prey, but the initial hesitation of blue tits was not affected by earlier experience. Blue tits have been found to show higher general aversion even towards palatable prey than, for example, great tits, probably because of higher dietary conservatism (Veselý et al. 2006, 2013, Prokopová et al. 2010, Turini et al. 2016). This indicates that predators' decisions of whether to attack or not after avoidance learning might be species specific and, thus, not generalizable from blue tits to other predators. In our experiment, 45 of 53 blue tits overcame their initial avoidance during the preference test and attacked and consumed the models readily in the following avoidance trial. As those birds that did not overcome their hesitation during the preference test were not included in the following phases of the experiment, our results describe the generalization tendency of the less hesitant individuals, which are more likely to attack aposematic prey in the wild in the first place. Many of these less hesitant individuals, however, seemed to regain their dietary conservatism after they had learned avoidance, as they no longer consumed the palatable models attacked. In conclusion, whereas no generalization was found regarding the attack probabilities, we did find individual variation in avoidance learning and dietary conservatism, which could affect selection in the wild.

### *The importance of other cues*

Somewhat surprisingly, the blue tits had no initial biases towards any of the hindwing colours. Earlier studies on the wood tiger moth have found differential predation pressure in the field (Lindstedt et al., 2011; Nokelainen et al., 2012; Nokelainen et al., 2014) and different hesitation times by local predators (Lindstedt et al., 2011; Nokelainen et al., 2012) towards the different colour morphs. As the differences in hesitation times were found using living moths (Nokelainen et al., 2012), it is possible that other cues, such as odour, influenced the results. In nature, the wood tiger moth relies on multiple signal components (i.e. odour, taste) in addition to the visual cues when exposed to potential predators (Rojas, Burdfield-Steel & Mappes 2015). Its chemical defence contains pyrazines (Rojas et al. 2017; Burdfield-Steel, Pakkanen, Rojas, Galarza & Mappes 2016), a group of compounds with a characteristic aversive smell, which is effective against birds (Guilford, Nicol, Rothschild, & Moore, 1987; Rowe & Guilford, 1996). In fact, pyrazine is known to trigger hidden aversions to red and yellow colours (Rowe & Guilford, 1996) and conspicuous prey (Lindström, Rowe, & Guilford, 2001), and enhance both learning and memorability of yellow (Siddall & Marples, 2008) or red coloured prey (Barnea, Gvaryahu, & Rothschild, 2004) at least in domestic chicks (Siddall & Marples, 2008). Pyrazine odour has been associated with Müllerian mimicry rings of insects and suggested to function as a warning signal (Rothschild, 1961). On the other hand, pyrazine odour has also been shown to assist in discriminating prey and thus reduce avoidance generalization between differently coloured prey if the odour is present on only some of them (Siddall & Marples, 2008; Rowe & Guilford, 1996). The specific roles of different cues in predator–prey interactions are uncertain. It might be that odour is easy to associate with palatability, but only functions close up, whereas conspicuous colours aid in memorizing which prey to avoid even from a distance. Here we were interested in colour only. However, it has been shown that when colour is kept constant, predators can discriminate prey based on pattern (e.g.

Prokopová et al. 2010; Veselý et al. 2013), and when both colour and pattern are equal, other visual and/or chemical features of the prey are used for prey recognition (Karlíková et al. 2016).

### *Conclusions*

Overall, the colour polymorphism of the wood tiger moth in Finland seems unlikely to be maintained by generalized avoidance based on its warning coloration only. However, predators were hesitant to attack any of the aposematic morphs in the first place, and if they were to encounter them in the wild sharing other warning cues such as pyrazine odour, general aversion seems likely to occur. More knowledge on how predators acquire and use information on prey qualities in different contexts is needed to conclude whether predator generalization contributes to the maintenance of multiple aposematic morphs (see also Skelhorn et al. 2016). The possibility that wild predators can generalize on the basis of the pyrazine odour or the combination of colour and odour requires further investigation. Alternative explanations for the occurrence of local warning signal polymorphism include negative frequency-dependent natural selection, sexual selection, frequency-dependent flight activity (Rojas, Gordon, & Mappes, 2015), signal efficacy trade-offs with other life history traits (Hegna, Nokelainen, Hegna, & Mappes, 2013; Nokelainen et al., 2012), predator species-specific mortality differences between morphs (Nokelainen et al., 2014) or combinations of these mechanisms (Gordon, Kokko, Rojas, Nokelainen, & Mappes, 2015). Colour polymorphism could also be explained by multiple-model mimicry (Edmunds, 2000), if the different morphs share warning colours with other defended prey species, and predators generalize their avoidance from one species to the other based on similar coloration. Thus, generalization of learned avoidance remains as a possible contributor to the maintenance of local polymorphism in wood tiger moth populations. In conclusion, we argue based on our results that although predator generalization could well contribute to the maintenance of different aposematic morphs under certain circumstances, it is unlikely to occur among distinct colour morphs of otherwise similar prey of visually oriented avian predators.

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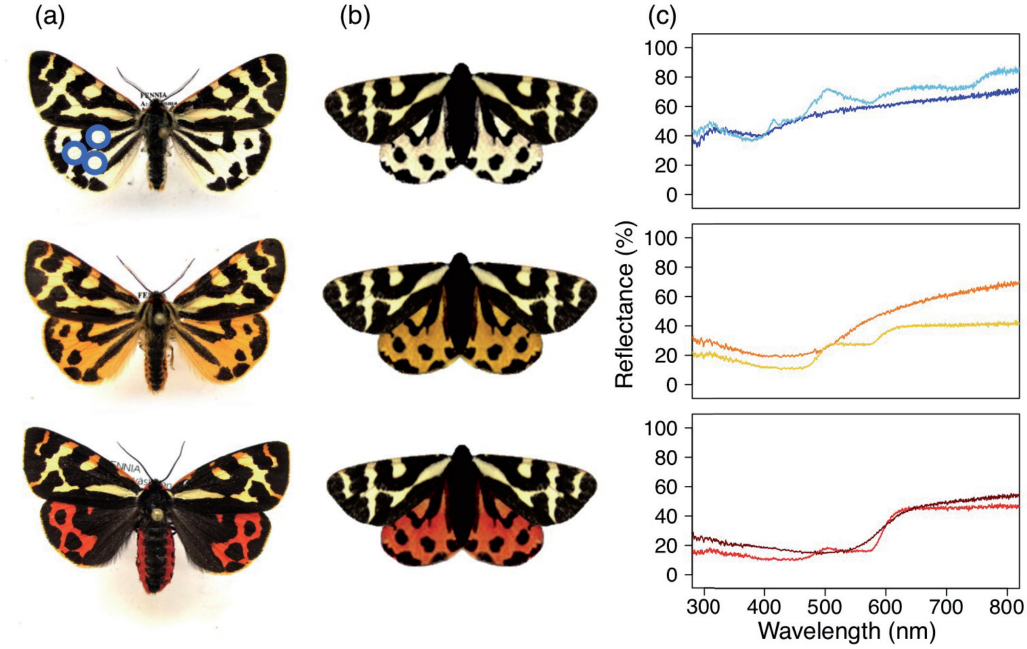

FIGURE 1 (a) Typical wood tiger moth colour morphs from central Finland (Coll. Kari Kulmala), (b) artificial white, yellow and red moth wings used in the experiment and (c) reflectance curves of the white, yellow and red hindwings of real moths (darker colours) compared to reflectance curves from white, yellow and red model hindwings (lighter colours). Spectral measurements were taken from three wild-caught individuals of each colour from the spots marked with blue circles on the white moth. The same spots were used to measure the model hindwing colours. Model wings were set in a more natural posture, less spread than the spread collection samples, but unfolded to show the hindwing colour.

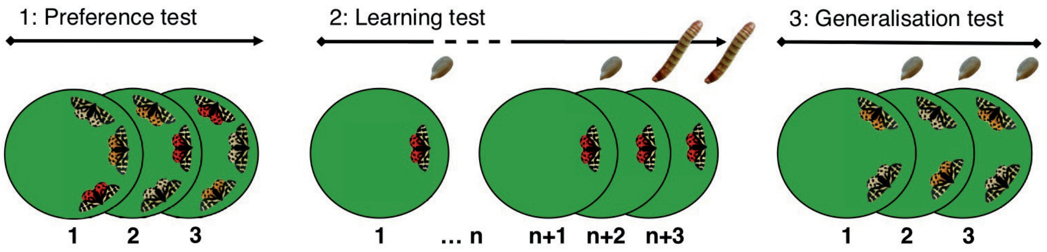

FIGURE 2 Schematic illustration of the experimental design. Each green circle represents a platform presented to a bird in one trial. Moth models as presented to a bird that learned to avoid the red morph are illustrated on the platforms and alternative food offered between the trials is shown above the platforms. For details of the experimental protocol see Methods (Experimental procedures; phases 1-3).

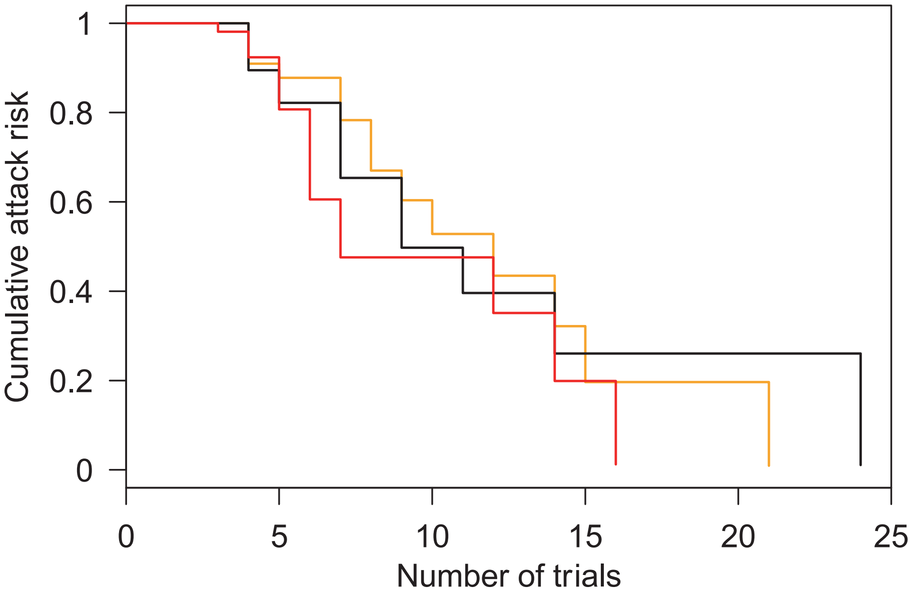

FIGURE 3 Proportion of models attacked during the learning trials for each colour model. The lines represent the cumulative attacks on unpalatable moth models of white (black line), yellow (yellow line) or red (red line) hindwing colour.

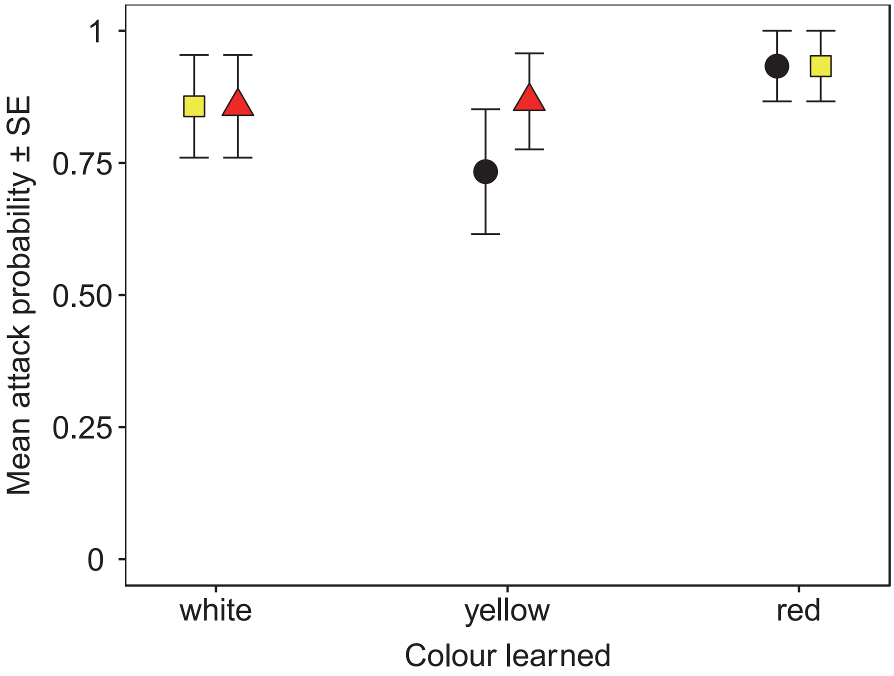

TABLE 1 Distribution of colours chosen first in the preference test trials (phase 1)

| | White first | Yellow first | Red first | *N* | *df* | $\chi^2$ | *P* |
|---|---|---|---|---|---|---|---|
| Trial 1 | 34 | 32 | 34 | 53 | 2 | 0.04 | 0.98 |
| Trial 2 | 44 | 22% | 34 | 41 | 2 | 2.98 | 0.23 |
| Trial 3 | 29 | 27% | 44 | 41 | 2 | 2.10 | 0.35 |

The percentages of birds that chose white, yellow or red models first in the three trials of the preference test, and the corresponding chi-square comparison for preference for each trial, are shown.

TABLE 2 Proportions (± SE) of tested colour morphs attacked in the generalization test trials (phase 3) in relation to the colour learned

| Colour learned | Colour tested | First trial | Second trial | Third trial |
|---|---|---|---|---|
| White | Yellow | 0.85 ± 0.09 | 0.92 ± 0.07 | 0.64 ± 0.13 |
| White | Red | 0.85 ± 0.09 | 0.78 ± 0.11 | 0.78 ± 0.11 |
| Yellow | White | 0.73 ± 0.11 | 0.73 ± 0.11 | 0.73 ± 0.11 |
| Yellow | Red | 0.86 ± 0.09 | 0.73 ± 0.11 | 0.60 ± 0.13 |
| Red | White | 0.93 ± 0.06 | 0.86 ± 0.09 | 0.73 ± 0.11 |
| Red | Yellow | 0.93 ± 0.06 | 0.86 ± 0.09 | 0.86 ± 0.09 |

## Appendix: tables A1-A3

TABLE A1 Test for asymmetric generalization in the first generalization trial

| Model | *df* | LRT | Pr(Chi) | Model AIC |
|---|---|---|---|---|
| **(Intercept) + colour combination** | 5 | 10.70 | 0.058 | 37.4 |
| (Intercept) | | | | 38.1 |

| Random effects | Variance | SD |
|---|---|---|
| Bird ID | 4808 | 69.34 |

| Fixed effects | Estimate | SE | Z | P |
|---|---|---|---|---|
| (Intercept: colour combination: yw) | 12.48 | 3.32 | 3.75 | <0.001 |
| Colour combination: yr | 14.85 | 5.22 | 2.85 | 0.0044 |
| Colour combination: rw | 2.27 | 8.04 | 0.28 | 0.78 |
| Colour combination: ry | 2.27 | 8.19 | 0.28 | 0.78 |
| Colour combination: wr | 1.38 | 6.16 | 0.22 | 0.82 |
| Colour combination: wy | 1.38 | 6.15 | 0.22 | 0.82 |

LRT: likelihood ratio test; y: yellow; w: white; r: red. Model selection was based on model fit, i.e. the model chosen was the one with the lowest Akaike information criterion (AIC) value. The significance level of $\chi^2$ (Chi) indicates a change from the model with colour combination as an explanatory variable to the model below, with intercept only. Estimates of the best-fitting model (in bold) are shown below. Of the colour combinations, the combination of yellow learned and white offered had least attacks, and was thus set to the intercept.

TABLE A2 Test for asymmetric generalization in all three generalization trials

| Model | *df* | LRT | Pr(Chi) | model AIC |
|---|---|---|---|---|
| (Intercept) + colour combination | 5 | 4.32 | 0.50 | 161.9 |
| **(Intercept)** | | | | 156.2 |

| Random effects | Variance | SD |
|---|---|---|
| Trial: Bird ID | 167.82 | 12.96 |
| Bird ID | 58.01 | 7.62 |

| Fixed effects | Estimate | SE | *Z* | *P* |
|---|---|---|---|---|
| (Intercept) | 10.05 | 1.57 | 6.42 | < 0.001 |

LRT: likelihood ratio test. Model selection was based on model fit, i.e. the model chosen was the one with the lowest Akaike information criterion (AIC) value. The significance level of $\chi^2$ (Chi) indicates a change from the model with colour combination as an explanatory variable to the model below, with intercept only. Estimates of the best-fitting model (in bold) are shown below.

TABLE A3 Comparisons of colours attacked first in the generalization trials

| | Colour learned | Colour tested | Attacked first | *df* | $\chi^2$ | *P* |
|---|---|---|---|---|---|---|
| First trial | White | Yellow | 6/14 | 1 | 0 | 1 |
| | | Red | 6/14 | | | |
| | Yellow | White | 6/15 | 1 | 0 | 1 |
| | | Red | 7/15 | | | |
| | Red | White | 8/15 | 1 | 0.13 | 0.71 |
| | | Yellow | 6/15 | | | |
| Three trials pooled | White | Yellow | 12/42 | 1 | 5.89 | **0.02** |
| | | Red | 24/42 | | | |
| | Yellow | White | 20/45 | 1 | 0.75 | 0.39 |
| | | Red | 15/45 | | | |
| | Red | White | 22/45 | 1 | 0.41 | 0.52 |
| | | Yellow | 18/45 | | | |

Chi-square comparisons of how many times each tested colour morph was attacked first in the generalization trials, by the colour morph learned. Models that were not attacked or attacked second are included in the total number of models offered. Bold indicates significant difference.

## IV

# CAN MULTIPLE-MODEL MIMICRY EXPLAIN WARNING SIGNAL POLYMORPHISM IN THE WOOD TIGER MOTH?

by

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