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Ossi Nokelainen

Many Forms of the Wood Tiger Moth (*Parasemia plantaginis*)

Selective Heterogeneity Favours Polymorphic Warning Signals

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"I was born not knowing and have had only a little time to change that here and there."
Richard Feynman,
American physicist 1918-1988

ABSTRACT

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Diss.

Maintenance of colour polymorphism, whereby individuals with distinct coloration co-exist within a population, is generally problematic in nature. As selection should lead to a fixation of the fittest phenotype evolution of polymorphism should be hampered. This is true especially in aposematism, whereby warning signals of prey are expected to evolve towards monomorphism due to a shared cost of predator education. Therefore, factors favouring polymorphism in aposematic species are most likely powerful selective mechanisms, and understanding those helps us to understand the existence of biological diversity also in a broad sense. This PhD thesis addresses why colour polymorphism evolved, and how it persists in aposematic species. I focus on the male colour polymorphism of the wood tiger moth (*Parasemia plantaginis*), which occurs widely in the northern latitudes. The males display white or yellow wing pigmentation with a varying degree of melanised patterning. I show that selective heterogeneity maintains this polymorphism: Selection favours one of the morphs at a given time and space, depending on local selection pressures. Better warning signal efficacy of the yellow morph comes at the cost of mating success, which was better for the white morph. The white morph was also better at encapsulating foreign intrusions, whereas the yellow morph had higher haemolymphic anti-microbial activity. I also show that the abundance of similar-looking alternative preys affects the survival of both morphs, and that their warning signal efficacy depends on predator community composition, which suggests that small-scale habitat mosaics may develop. Furthermore, populations are connected by gene flow that can maintain genetic variation between local populations. In conclusion, the results suggest that polymorphism in aposematic species may be maintained by differences in warning signal efficacy and mating success as well as spatially variable enemy communities combined with gene flow.

Keywords: Aposematism; colour polymorphism; predation; predator-prey interactions; sexual selection.

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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 am the first author of papers I-III, and I carried out a large part of the planning, writing and data collecting for each:

I Nokelainen, O., Hegna, R.H., Reudler, J.H., Lindstedt, C. & Mappes, J. 2012. Trade-off between warning signal efficacy and mating success in the wood tiger moth. Proceedings of the Royal Society B: Biological Sciences. 279: 257-265.

II Nokelainen, O., Lindstedt C. & Mappes, J. 2013. Environment mediated morph-linked immune and life-history responses in the aposematic wood tiger moth. Journal of Animal Ecology. In Press.

III Nokelainen, O., Valkonen, J., Lindstedt, C. & Mappes J. Prey and predator community composition promotes polymorphic warning signals. Submitted manuscript.

IV Galarza, J., Nokelainen, O., Hegna, R.H., Ashrafi, R. & Mappes, J. Spatio-temporal variation in the warning signal of the wood tiger moth. Manuscript.

The table shows the contributions to the original papers. Smaller contributions are stated in the acknowledgements of the original papers. ON = Ossi Nokelainen, JM = Johanna Mappes, CL = Carita Lindstedt, RH = Robert Hegna, JV = Janne Valkonen, JG = Juan Galarza, JR = Joanneke Reudler Talsma, RA = Rogaeih Ashrafi

	I	II	III	IV
Original idea	JM, CL, ON	ON, CL, JM	ON, JM, CL	JG, JM
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Analyses	ON, CL, JM	ON, CL	ON, JV, JM	JG
Writing	ON, CL, JM	ON, CL, JM	ON, JV, CL, JM	JG, ON, RH, JM

1 INTRODUCTION

1.1 Prelude – On the animal coloration

The beauty and privilege of evolutionary biology is to seek answers to why there is such a wide array of biological variation surrounding us, how it arises, prevails, and perishes. By definition evolution is any change across successive generations in the inherited characteristics of biological populations (Ridley 2004). This is simple: If individuals differ even slightly from each other, and if these differences are heritable, then individuals that are better adapted to their environment are more likely to survive and leave more descendants. As a consequence, selection tends to favour phenotypes that harbour the fittest set of alleles leading to degradation of genetic variability leaving less fit genotypes to perish (Bell 2008). How can the ample biological variation then be explained?

Coloration has inspired scientists to study evolution in action early on (e.g. Fisher et al. 1947, Cain et al. 1954, Kettlewell 1956), and perhaps no other visible animal trait has caught as much attention as coloration (Poulton 1890, Cott 1940, Caro 2005). Rightfully so, because as a key trait coloration has many adaptive functions including camouflage (Stevens et al. 2009), sexual signalling (Andersson et al. 1996), thermoregulation (Majerus et al. 1998), and warning signalling (Ruxton et al. 2004). Therefore, research on coloration is of a great importance in determining fitness in almost any organism, and hence in understanding evolution.

1.2 Colour polymorphism and its persistence

Colour polymorphism is a type of genetic variation in which individuals with distinct coloration co-exists in a population (Ford 1965), and their frequencies are such that the rarest form is too common to be a result of recurrent mutations (Huxley 1955). This phenomenon is fairly widespread in nature, and examples of colour polymorphism are ample in a variety of taxa including insects

(Brakefield 1985), gastropoda (Cain et al. 1954), fish (Seehausen et al. 2008), frogs (Maan et al. 2008), snakes (Forsman 1995), lizards (Sinervo et al. 1996), and birds (Roulin 2004). For example, in the side-blotched lizard (*Uta stansburiana*), the throat colour polymorphism is an index of male reproductive strategy. Aggressive orange-throated-males are strong competitors occupying large territories. Subdominant blue-throated-males defend and occupy smaller territories that they are able to persist. Yellow-throated-males resemble females, do not keep territories, and sneak to mate when territorial males leave a territory unguarded (Sinervo et al. 1996). In Gouldian finches (*Erythura gouldiae*), an aggressive red-headed morph is a strong competitor, but expresses high stress responses to changes in social environment (Pryke et al. 2007). The less aggressive black-headed morph is the more passive competitor, but invests more in parental care than aggression (Pryke et al. 2009). Therefore, in all cases colour morphs can have different qualities depending on their phenotype.

Colour polymorphism is problematic in the evolutionary sense, however, because selection as explained above should favour traits maximizing fitness (Bell 2008), which should prevent the evolution of polymorphisms in the first place. Maintenance of colour polymorphism therefore should require similar net benefits between colour morphs in the long term (Maynard Smith 1982, Gray et al. 2007). If this were not the case, sooner or later, morphs with lower fitness would go extinct (Stearns 1992, McKinnon et al. 2010). For example among prey species, one mechanism to achieve equal net benefits is negative frequency-dependent selection caused by predation (i.e. apostatic selection) (Clarke 1962), in which relative fitness is inversely dependent on the frequency of individuals sharing a similar trait (Fisher 1930, Ayala et al. 1974, Allen et al. 1988, Endler 1988, Merilaita 2006). Visually hunting predators tend to form a 'search image' (Tinbergen 1960, Plaisted et al. 1995), which target the most common form of profitable prey (Bond et al. 1998, Bond 2007). As the frequency of the common morph drops too low for predators to encounter it frequent enough to form a search image, the relationship is reversed, and predators form new search image on the morph that had become the most common morph, which facilitates the existence of colour morphs with fluctuating frequencies. However, maintenance of colour polymorphism can be more complicated especially when warning coloration and secondary defences come into play as these will have a different bearing to the expected evolutionary scenarios.

1.3 The problem of 'aposematic colour polymorphism'

Aposematism is an anti-predator strategy (Poulton 1890, Cott 1940, Edmunds 1974), in which warning signals are used by prey to advertise secondary defences (e.g. aggressiveness, spines, or toxins) to potential predators (Poulton 1890, Cott 1940, Guilford 1990, Mappes et al. 2005). Various signals advertise unprofitability including colours (Poulton 1890), odours (Rowe et al. 1999, Lindström et al. 2001a), sounds (Dunning et al. 1995, Rowe 2002), and even

bioluminescence (De Cock et al. 1999), or electric signals (Guilford 1990). Further, signal efficacy can be enhanced exhibiting different signals simultaneously (Rowe 1999). As attacks are costly in terms of mortality, injury or time to both defended prey and their predators both parties benefit from mutual avoidance (Müller 1879, but note Bates 1862, reviewed in Ruxton et al. 2004). Selection should therefore favour the most efficient warning signal by matching the signal to the cognitive capabilities of the receiver (Speed 2000).

Polymorphism in aposematic species is puzzling to understand. This is because the benefits of warning signals are almost always dependent on a sufficient density and frequency of individuals displaying a similar signal (Sword et al. 2000, Lindström et al. 2001b, Endler et al. 2004), because predator education is based on "strength in numbers" of similar appearance (Müller 1879, Rowland et al. 2007, Rowland et al. 2010). Warning colour patterns are thus expected to be under positive frequency-dependent selection (Speed 2001, Sherratt 2002, Mappes et al. 2005), which should effectively hamper the evolution of multiple colour morphs. In spite of this there are several examples of colour polymorphism also in aposematic species (Brakefield et al. 1985, Siddiqi et al. 2004, Williams 2007). Given this, factors maintaining polymorphism in aposematic species are likely to be powerful selective mechanisms. To understand maintenance of genetic variation, an ultimate goal of evolutionary biology, it is important to know those factors. Herein, I focus on understanding colour polymorphism in aposematic organisms. Evolution is typically modification by selection and many different selective factors could cause polymorphism in aposematic organisms. Hence, it is necessary to look into few of the most likely important ones: Fitness trade-offs, frequency-dependent selection, and the role of environmental heterogeneity.

1.4 Selective mechanisms favouring colour polymorphism

1.4.1 Fitness trade-offs

A trade-off occurs when an increase in the expression of one trait is coupled with decreasing the expression of another trait (Roff 2002). It is plausible to assume that trade-offs expose colour morphs to opposing selection pressures depending on inherent qualities of different colorations. For example in European vipers (*Vipera berus*), the regular zig-zag pattern functions as a warning signal to potential predators (Forsman 1995) whereas the dark melanic morph gains thermoregulatory benefits by its coloration (Clusella Trullas et al. 2007). Moreover, the melanic morph tends to grow larger, which is a key for winning male-male competitions over females (Andrén et al. 1981). In *Heliconius* butterflies (*H. cydno, H. pachinus*) both sexes are polymorphic with combination of white, yellow, orange and black patches on wings. Males show assortative mating towards females similar to them (Kronforst et al. 2006), but predators select for similarity to the population mean (Kapan 2001). Hence, sexual and

viability selection may be in conflict. Similarly, in the polymorphic poison frog system in Central America (Summers et al. 2001), females show assortative mating preferences towards males with an appearance similar to their own (Summers et al. 1999, Maan et al. 2008), yet predators exert purifying selection on novel colour morphs in respective population (Noonan et al. 2009). There is also evidence for a trade-off between conspicuousness and toxicity in this system (Darst et al. 2006, but see Maan et al. 2012).

Trade-offs can also arise through correlational selection whereby selection targets an underlying trait of the phenotype (Blows et al. 2003). This can facilitate polymorphism if colour morphs are bound to produce some trait which expression is limiting expression of some other trait (Roff 2002). For instance, defence against pathogens is a fundamental trait shaping the fitness of organisms (Wilson & Cotter 2008). As maintaining immune defence is considerably costly (Schmid-Hempel 2005), it is possible that individuals need to allocate to different components of the immune system. In side-blotched lizards colour polymorphism in females is linked to differences in immunological defence (Svensson et al. 2001). Survival and antibody responsiveness is positively correlated in yellow morphs, but negatively correlated in orange morphs, which suggests production of orange pigmentation to be limiting an effective antibody response. Hence, if resource allocation of morphs depends on their phenotype (Roff 2002), it could result in a physiological trade-off in colour morphs (Svensson et al. 2001, Pryke et al. 2007). Since it is plausible to assume that all immune defences cannot be concurrently up-regulated because of their costliness (Cotter et al. 2004, Rantala et al. 2005), allocating more to one immune response can expose morphs to different selection pressures depending on the natural pathogen community, therefore facilitating polymorphism.

1.4.2 Frequency-dependent selection

Trade-offs may not be sufficient to maintain polymorphism in the long term, and thus additional evolutionary forces are required to explain the persistence of polymorphism. This is because if morphs (two or more) have equal net benefits at time, all else being equal, anything upsetting the balance should break down the polymorphism due to purifying selection (i.e. the purging of deleterious variations) (Maynard Smith 1982, Gray et al. 2007). Negative frequency-dependent selection is a powerful mechanism preserving genetic variation (Ayala et al. 1974, Allen et al. 1988, Endler 1988) and colour polymorphism in general (Sinervo et al. 2001, Svensson et al. 2005, Merilaita 2006, Calsbeek et al. 2010). However, negative frequency-dependent selection maintaining polymorphism is problematic in aposematic organisms, because as explained above the conventional expectation for prey warning signals is that selection should be positive frequency-dependent due to shared cost of predator education (Müller 1879, Rowland et al. 2007, Rowland et al. 2010).

One possibility allowing some variation in colour morph frequencies can be that frequency-dependent selection may vary geographically depending on

the frequency of signal in a local population (Borer et al. 2010). For example, in Alpine leaf beetles (*Oreina gloriosa*) positive frequency-dependent selection works via predators that have learned to avoid two colour morphs differently in separate locations (Borer et al. 2010). Nonetheless, this should lead to selection towards local optima and result in fixation in small spatial scale (Mallet et al. 1999, Mallet 2010), which should hamper the occurrence of less adaptive colour morphs within a population. Thus, some evolutionary force must allow less adapted phenotypes to persist in low frequencies in the population (O'Hara 2005). Note worthily, sharing the cost of predator education concerns similar appearance in prey community, but not necessarily the absolute frequency of conspecifics (Müller 1879, Kapan 2001, Beatty et al. 2004, Mallet 2010). Hence, other sympatric prey sharing similar appearance could lead selection to maintain polymorphism in locations where numbers of alternative prey are sufficient to promote local polymorphism.

1.4.3 Environmental heterogeneity

Environmental heterogeneity may favour colour polymorphism in nature for example due to constraints involved in producing warning coloration. In a resource-limited environment individuals are compelled to allocate nutrient components among life-history traits (Stearns 1992, Roff 1996, Roff 2002). In insects, the production of cuticular pigmentation relies mainly on food nitrogen uptake, which is an important prerequisite component of amino acids needed to produce coloration (Talloen et al. 2004). Limited food resources may constrain signal expression by constraining the production of pigments (Grill et al. 1998, Ojala et al. 2007, Lindstedt et al. 2010b), especially if there is a cost of producing an effective warning coloration (Darst et al. 2006, Blount et al. 2009, Blount et al. 2012). Moreover, if resources are limited in one place but not in another, it is possible that such limitations cause reduced investment also in secondary defences such as defensive toxins, especially, if individuals are deriving those from food resources (Weller et al. 1999). Weaker secondary defences can have further impact on how well predators associate prey unprofitability with appearance (Skelhorn et al. 2007, Skelhorn et al. 2009, Skelhorn et al. 2010), because predators may not learn to avoid signal. It is possible that some morphs are able to resist the costs of environmental constraints better than others and succeed in respective environment (Karell et al. 2011). Therefore, colour polymorphism may be promoted, when individuals face suboptimal conditions forcing them to use available nutrients differently.

It has been speculated that one benefit of being an aposematic organism is to be released from the constrained use of a particular environment (which limits many camouflaged animals), which allows broader use of habitats and enhanced resource seeking (Joron 2005, Merilaita et al. 2005, Speed et al. 2010). Nonetheless, warning signals can work better in particular habitats (i.e. visual environment effect) (Endler 1991, Endler 1992, Archard et al. 2009). If an aposematic organism's environment consists of a mosaic of different habitats, morphs may occupy different niches (i.e. small scale microhabitats differing in

lighting, vegetation etc.), depending on where their coloration best serves its signalling function (Endler 1991, Endler 1992, Endler et al. 1998).

It is likely that variation in the predator community causes further heterogeneity in selective regimes (Endler et al. 2004), because predators differ in what they consider as unprofitable prey based on their behavioural cautiousness (Marples & Mappes 2011), or because predators perceive (and associate) signals differently (e.g. Endler 1992, Hart et al. 2000, Cuthill 2006). Such conditions can arise particularly when the predator population consists of naïve, uneducated individuals, which have not yet learnt to associate the signal with unprofitability (Marples & Mappes 2011). Additionally, conspicuous warning signals may turn out disadvantageous in populations where different predators, whether it be caused by specific predators or community composition, do not avoid the signal (Endler et al. 2004, Valkonen et al. 2012). Considering these environmental heterogeneity is capable of promoting variation, but pin-pointing the most important factors involved is often complicated especially if heterogeneity facilitates colour polymorphism coupled with other mechanism such as trade-offs.

1.5 Random factors generating colour polymorphism

Colour polymorphism can also be maintained by genetic drift and gene flow. If migration between locally adapted populations occurs, then polymorphism could be explained by gene flow between those populations (Slatkin 1987, Petit et al. 2009, Mochida 2011). The effect of gene flow is less expected among aposematic organisms, because warning signals locally experience high levels of phenotypic selection (Mallet et al. 1999, Mallet 2010). However, at least in the conspicuous sea star species (*Pisaster ochraceus*) colour polymorphism (orange or purple) has an underlying genetic component, and regional colour morph variation is ecologically controlled with considerable effect of gene flow among populations (Harley et al. 2006).

The variation in colour morph (or allele) frequencies can also be due to random change, at least for those genes (or traits), which possess neutral selective value (Masel 2011). It could allow some variation in warning signal components that are not under strong selection. Although it has been suggested that genetic drift maintains colour polymorphism in candy-stripe spider (*Enoplognatha ovata*) (Oxford 2005), and contributes to variation in colour morph frequencies in scarlet tiger moth (*Callimorpha dominula*) (O'Hara 2005), drift is often expected to have a minor role in the maintenance of polymorphism in the long run, and empirical evidence is lacking particularly among aposematic species. In sum, it is likely that the role of genetic drift and gene flow in the maintenance of colour polymorphism requires conjunction of other evolutionary forces such as environmental heterogeneity (Gray et al. 2007).

In order for colour polymorphism to persist in an aposematic species, most likely, several different selection pressures operate in concert to generate

an environment that is favourable for the existence of colour polymorphism. Therefore, like with any other complex problem, to find out what maintains polymorphism in aposematic species, each of the possible alternatives must be approached separately.

1.6 Aims of the study

This thesis aims to address the question why colour polymorphism evolves and persists in aposematic species. My study species, the wood tiger moth (*Parasemia plantaginis*), is a polymorphic species (Hübner 1820), it sequesters defensive chemicals from its diet (Ojala et al. 2005, Lindstedt et al. 2010a), and occurs widely in the northern latitudes (Leraut 2006). Males express white or yellow pigmentation with a varying degree of melanised patterning on their wings (Leraut 2006). I focus on colour polymorphism in males (Figure 1), since females express continuous variation in coloration. The sections of this PhD thesis specifically address: (I) Sexual and survival selection; (II) Environment-mediated fitness responses; (III) Frequency dependency and the role of prey and predator community to attack risk; and (IV) The role of population structure and gene flow in maintaining colour polymorphism.

In paper I, I test a potential trade-off between survival and sexual selection. First, the efficacy of warning signal and whether there is a difference in it between morphs is evaluated in laboratory behavioural assays with blue tits (*Cyanistes caeruleus*) as predators. This is complemented by a predation study with natural ranging predators in field conditions, where the conspicuousness of moths against respective backgrounds is taken into account as the efficacy of the warning signal is usually associated with conspicuousness and distinctiveness (e.g. Merilaita et al. 2007).

In paper I, I also test if morphs have different mating success, because sexual selection often plays a role in male colour polymorphism (Sinervo et al. 1996, Seehausen et al. 2004). If the fitness of either of the morphs is increased by survival selection, then sexual selection against it could indicate a trade-off between warning signal efficacy and mating success providing one plausible mechanism that maintain polymorphism.

In paper II, I test if morphs have different competence when exposed to stressful conditions, which can cause differential selection for colour morphs (Lande et al. 1983, Via et al. 1985, Blows et al. 2003). I examine the performance of the colour morphs by rearing larvae solitarily (favourable conditions) or in aggregations (challenged conditions) until adult stage. I test whether growth density affects the males' survival, life-history traits and adult melanin pigment expression. If challenged conditions are energetically costly compared to favourable conditions (Roff 2002, Zuk et al. 2002, Rantala et al. 2005), then larvae in aggregations are expected to gain less weight, have shorter developmental time and experience high mortality (Goulson et al. 1995, Sheldon et al. 1996). I also test whether larval growth density triggers

immunological responses (Goulson et al. 1995). If cuticular pigmentation is an indication of active immune system in insects as is often suggested (Aso et al. 1985, Wilson et al. 1998, Barnes et al. 2000), an increase in the area of wing melanisation is predicted alongside up-regulated immune responses in high rearing density. I test whether larval growing conditions affect any of the measured traits differently depending on the colour morph and whether conditions could thus explain why one morph may sometimes be favoured.

In paper III, I test whether predation pressure on colour morphs differ in separate populations with different colour morph frequencies as well as different prey and predator community composition. First I test the role of conspecific-based frequency-dependent selection to find out whether it explains the predation risk of colour morphs. If a locally dominant colour morph is attacked less than a rare one, its lower predation risk would indicate it benefits from positive frequency-dependent selection by local predators (Müller 1879, Pinheiro 2003). Positive frequency-dependent selection can be reinforced if there are alternative prey species in the community with a similar appearance to the dominant morph (Bates 1862, Müller 1879, Fisher 1930, Leimar et al. 1986). I also study the local predator community structures because heterogeneity in local predators can cause differential selective regimes for colour morphs (*rationale* Endler et al. 2004). Thus, the effect of predator community composition (Passerine bird species) on predation pressure is examined to see if it causes differential selection on colour morphs.

Finally, in paper IV, I study the possibility of neutral selection and connectivity of populations, since one explanation for the regional variation in colour morph frequencies could be that gene flow maintains the occurrence of both morphs within population (Slatkin 1987, Petit et al. 2009). To study genetic structure and gene flow of populations, nuclear microsatellite markers are used to characterize and compare the genetic structure of local populations from both morphs for over three consecutive years. Spatio-temporal patterns of several warning signal components are compared to a neutrally evolving genetic marker to detect potential non-neutral mode of evolution, because some warning signal components could possess a neutral selective value.

2 MATERIAL AND METHODS

2.1 Study species

The wood tiger moth (*Parasemia plantaginis*) is a widely distributed, fairly common moth species of the Arctiidae family (Leraut 2006). The species, and its colour polymorphisms was first described in the 18th century (von Linné 1758), and again in 19th century (Hübner 1820). Although coloration of this species vary considerably through its northern circumpolar distribution range, in Europe, only two male colour morphs are common (Leraut 2006). Adult *P. plantaginis* males have either yellow or white wing colour, which together with a striped melanisation pattern result in its conspicuous appearance (Figure 1). The antennae of males are feathered, and flanks of abdomen and legs are often yellowish. Females exhibit more continuous colour variation (yellow-orange-red, Figure 1). The melanisation pattern is fairly similar in female and male forewings, but often more pronounced in female hind wings in comparison with males. The antennae of females are mildly clubbed, and flanks of a plump abdomen are usually reddish. Here however, as explained previously, I focus on understanding colour polymorphism in males.

Like many other Arctiid moths (Weller et al. 1999, Conner 2009), *P. plantaginis* larvae are able to sequester defensive chemicals from their host plants (Ojala et al. 2005, Lindstedt et al. 2010a). Larvae are often called 'woolly bears', because of their tufted appearance with black and orange hairs (Conner 2009). The orange patch of the larvae can act as a warning signal for visual predators (Lindstedt et al. 2008), whereas dark hairs bring fitness benefits in terms of thermoregulation (Lindstedt et al. 2009). As a food generalist this species does not rely on a particular host plant during its larval stage (Waring et al. 2003), and being a capital breeder (Tammaru et al. 1996), it is not restricted by nutritional sources as an adult either. Perhaps owing to the generalist nature of the larvae this species occurs in a various habitats, and although traditionally one would expect to find this species in moist, open meadows, as well as the

edges of moors and bogs (Waring et al. 2003), dispersing males can be found in virtually any kind of habitat (Nokelainen et al. unpublished).

The life cycle of the *P. plantaginis* typically yields one generation (i.e. univoltinism) in a year (Ojala et al. 2005, Lindstedt et al. 2010a). Flight season occurs approximately concurrently throughout the moth's distribution range. However, this is weather-dependent and in colder areas (such as higher altitudes) flight usually occurs later than in warmer locations. Adult males usually emerge slightly earlier than females to patrol potential mates before they emerge in June or early July. Females attract males by producing pheromones and after a preferable male has been found, mating occurs. The female gains a nutrient-rich spermatophore from the male containing sperm that fertilize the eggs (Chargé et al. unpublished), and the female will lay a clutch shortly thereafter. About one week later the eggs will hatch. Emerged larvae will feed until autumn then prepare for diapause (typically in September). Diapause ends when spring temperatures are adequate (typically in April). Then larvae activate, start rehydrating, and gain body mass by foraging. After the larvae have reached the final instar (in May or in early June), they pupate. A month later this a new adult generation emerges.

FIGURE 1 The colour variation of the European wood tiger moth. Top row: Male colour morphs of the wood tiger moth. The figure shows both the distinct variation in wing colour and the variation in melanisation (black patterning), which is usually more continuous in nature. From left; less melanised white (f. bicolor), regular coloured white (f. hospita), regular coloured yellow (f. plantaginis) and less melanised yellow (f. luteaobsoleta). Bottom row: Female colour variation of the wood tiger moth. Notice that the hind wing pigmentation varies continuously from yellowish to reddish. Also melanisation varies in the wild. © Samuel Waldron

2.1.1 Laboratory stock

All *P. plantaginis* individuals used in the experiments (see below and I, II, III, IV) originated in a laboratory stock, which had been maintained in greenhouse conditions (25°C) since year 2003 at the University of Jyväskylä, Central Finland (62°N, 26°E). At the beginning of the experiments in 2008 11 laboratory generations had been reared. However, in 2008 the lab stock was almost

completely renewed and that lab stock originates in 50 randomly mated wild pairs from Åland, Southern-, Central-, and Northern Finland. Experimental individuals were derived from divergent selection lines for larval coloration (small or large orange pattern, see Lindstedt et al. 2009); henceforth 'selection lines' (small and large lines). Although *P. plantaginis* produces one generation per year in the wild, it is possible to yield up to two, even three generations per year in the laboratory.

The rearing protocol covers the full life cycle of *P. plantaginis*. During the larval period, food (dandelion, Taraxacum sp.) was offered ad libitum (for more details, see Lindstedt et al. 2009, Lindstedt et al. 2010b). Larval maintenance was carried out daily until pupation, after which pupal weight was measured on a laboratory scale to the closest milligram. After eclosion individuals were labelled (on their individual containers), sexed and morph-identified. To produce the next generation a random subset of females were mated with males in a mating container by letting the couples mate freely. After oviposition the eggs were left to hatch, after which the larvae were reared to approximately third instar. The larvae were prepared for diapause by offering willow leaves (Salix sp.) as additional food. Larvae were diapaused at +0°C. After diapause the larvae were reared to adults as above. For more details of rearing protocol see (Lindstedt 2008).

2.1.2 Field sites and sampling

Wild *P. plantaginis* abundances and relative frequencies of the male colour morphs were monitored since 2008 in various geographical locations (Figure 2). This was done in several sampling areas (Table 1), namely, in Estonia (Pärnu surroundings 58°N, 25°E), Åland (60°N, 20°E), Southern Finland (Hanko surroundings 60°N, 23°E), Central Finland (Jyväskylä surroundings 62°N, 26°E), and Northern Finland (Tornio surroundings 66°N, 24°E). Monitoring was also done in Scotland near Portknockie (57°N, 2°W) in 2009 and the Alps near borderline of Austria and Switzerland (46°N, 10°E) in 2009 and 2010. Nets and pheromone traps were used to capture wild males in various semi-open habitats (Figure 3). Pheromone traps are commonly loaded with artificial pheromone, but I used females from the laboratory stock to attract males into the trap. Traps are active as long as the female is alive, and therefore, the females were changed every second day. Naturally, the method selectively captures males only. Field trapping was aimed to cover the whole flight season from June to July (depending on the location).

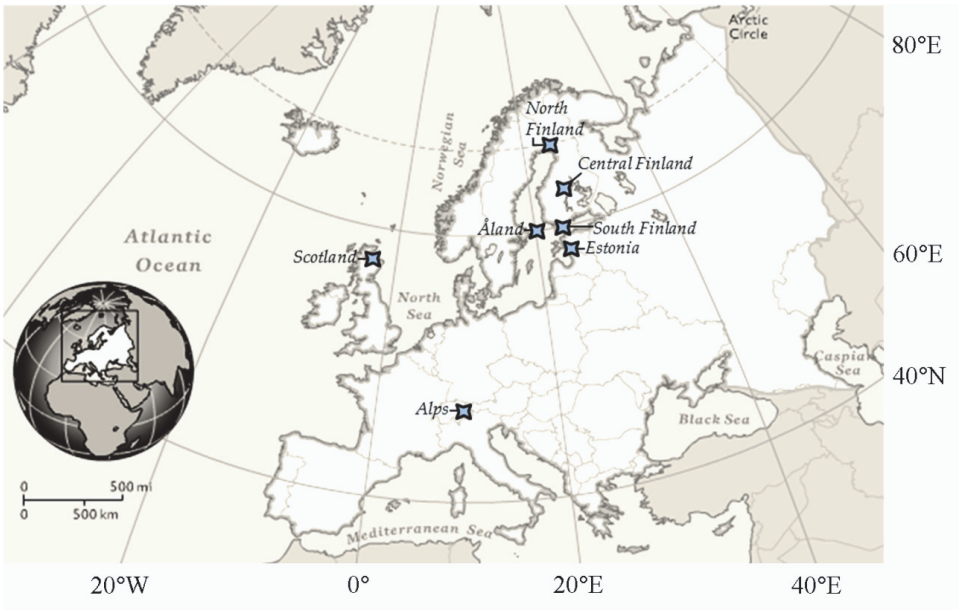

FIGURE 2 Field sites to study colour variation of the European wood tiger moth. Sampling sites are indicated with blue markers. Axis numbers indicate latitudes (°N) and longitudes (°E). Map courtesy National Geographic Education. National Geographic does not review or endorse content added to this background by others.

TABLE 1 Yearly variation of male wood tiger moth colour morph frequencies (%) in sampling sites. Sample sizes are given in brackets. Sampling was not conducted in Alps in 2011-2012 as well as not in the Scotland. Na stands for *not assigned* values (i.e. missing data point).

Site	Morph	2009	2010	2011	2012	Total
Alps	White	37.9 (22)	32.1 (17)	Na	Na	35.1 (39)
	Yellow	62.1 (36)	67.9 (36)	Na	Na	64.9 (72)
Central	White	78.5 (62)	80.0 (8)	81.8 (36)	73.5 (136)	76.1 (242)
Finland	Yellow	21.5 (17)	20.0 (2)	18.2 (8)	26.5 (49)	23.9 (76)
Estonia	White	92.3 (36)	100.0 (17)	97.7 (43)	97.0 (163)	96.6 (259)
	Yellow	7.7 (3)	0.0 (0)	2.3 (1)	3.0 (5)	3.4 (9)
Northern	White	55.2 (32)	100.0 (2)	61.5 (24)	57.1 (28)	58.1 (86)
Finland	Yellow	44.8 (26)	0.0 (0)	38.5 (15)	42.9 (21)	41.9 (62)
Scotland	White	0.0 (0)	Na	Na	Na	0.0 (0)
	Yellow	100.0 (60)	Na	Na	Na	100.0 (60)
Southern	White	60.5 (26)	34.4 (45)	56.8 (21)	32.9 (51)	39.1 (143)
Finland	Yellow	39.5 (17)	65.6 (86)	43.2 (16)	67.1 (104)	60.9 (223)
Åland	White	22.7 (15)	48.3 (14)	42.1 (8)	44.4 (8)	34.1 (45)
	Yellow	77.3 (51)	51.7 (15)	57.9 (11)	55.6 (10)	65.9 (87)

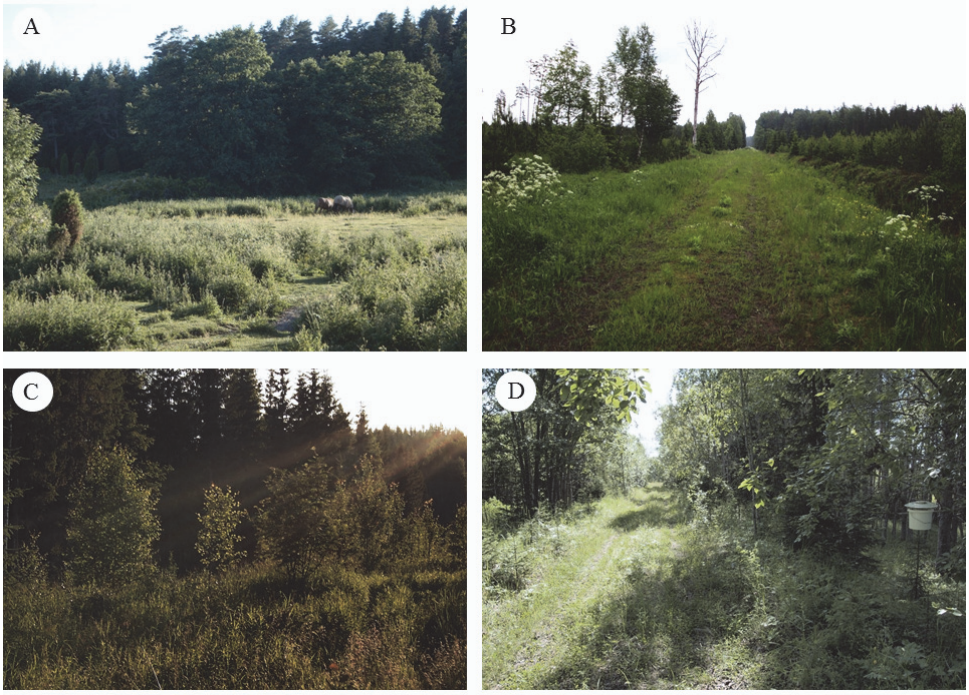

FIGURE 3 An example of field sites used in the wood tiger moth surveys: A) Åland, B) Estonia, C) Central Finland, and D) Northern Finland. This species is commonly found in various semi-open habitats. Notice that in figure D there is a white pheromone trap used in the field surveys hanging from a tree.

Field sites for separate studies (I, II, III, IV) were used as follows. Field experiment of the first predation study to assess warning signal efficacy of colour morphs (I) was conducted in Åland, whereas predators for the behavioural assay originated in Central Finland (I). The extended predation experiment addressing frequency-dependent selection and environmental heterogeneity (habitat, prey and predator community) was conducted simultaneously in Estonia, Åland, Southern and Central Finland as well as Scotland (III). The population colour morph frequencies were followed and individuals were sampled for genotype and phenotype determination from Estonia, Åland, Southern, Central and Northern Finland and the Alps to address spatio-temporal variation in the warning signal of *P. plantaginis* (IV).

2.2 Coloration and visual contrast

I categorised adult *P. plantaginis* males to white or yellow colour morph by eye in all experiments. The colour difference between colour morphs was also confirmed by spectrophotometer in paper I (Nokelainen et al. 2012) allowing using the same visual categorization by eye later on.

I also used digital photographing in the further experiments (II, III, IV). Moths were photographed with an ultraviolet (UV) sensitive Fuji Film Finepix S3 Pro UVIR digital camera under a light bulb emitting both visible and UV wavelengths, because UV may play important role as many Passerine species are capable of sensing low wavelengths making them potentially important in predator perception (Cuthill 2006). Two photographs were taken of each object: (1) a 'human visible light' photo using a UV-blocking filter and (2) a UV photo using as UV transmitting filter. Standard grey reflecting 50% of all light was included in each picture. Using a custom Matlab programme, the photographs were linearized, and further converted into reflectance images (utilising the known reflectance of the standard) where the values of the red (R), green (G) and blue (B) channels of the camera correspond to reflectance in the long (LW), medium (MW) and short (SW) wavelength bands in the 'human visible' photos. UV reflectance was measured from the UV photos using the red channel since it was the most sensitive (Stevens et al. 2007, Lindstedt et al. 2010b). As a measure of brightness (i.e. achromatic intensity) for the colour patterns of interest the mean reflectance over the whole spectrum was used ((LW+MW+SW+UV)/4). As a measure of hue each wavelength area was standardized into a proportion of total reflectance (e.g. LW/(LW+MW+SW+UV)).

The coverage of melanisation on the hind wings was measured from the digital photographs with Paint.Net – software by dividing the area of melanised pattern by the total area of the wings (II, III, IV). Morphological measures of the wings; area, length, and width were measured with Image J software. To analyse pattern composition of fore wings (IV), organization of dichotomous melanin patterns was measured (Todd et al. 2005): all wing images were adjusted into the same position and a grid of 35 x 35 cells was laid over the images using Microsoft PowerPoint software. All the cells comprising black melanin pattern were given the value '1'. Remaining cells without melanin were scored as '0', after which the data were re-arranged for further pattern analysis.

To take into account how the visibility of the colour morphs on different backgrounds affects their attack risk in the field (I), reflectance spectra of tree trunks were obtained using spectrophotometer to back up data for pinning experiment. In paper I, this was done by taking measurements from three individuals of each tree species as contrasts can be depended on the respective background, hence affecting how well predators are able to detect prey coloration. Also ten measurements of both morphs from the hind wings were taken using spectophotometer. An avian vision model was then constructed to estimate visual contrast between the moths and the backgrounds in the eyes of a potential predator (Vorobyev et al. 1998, Vorobyev et al. 2001). The contrasts were further incorporated into the survival analysis to compare attack risk of morphs in the field (I). In paper II, I used photographs to get data of adult wings (and melanisation). In paper III, I also constructed the avian vision model to control for visibility of prey. The same (spectrophotometer) measuring protocol was used, but morph wings were paper-made (see below) and background was green cardboard (III). In paper IV, different warning signal components were measured using digital photographing.

2.3 Predation experiments

2.3.1 Warning signal efficacy (I)

Since predation is one of the most important factors causing mortality in the nature I studied if predation pressure between morphs differ which could help to explain colour polymorphism. I test if bird predators consider *P. plantaginis* colour morphs as unprofitable, if this is the case in both morphs and whether this holds in field. To test whether predators treat yellow and/or white morphs as unprofitable an experiment was conducted in the Konnevesi Research Station, Central Finland (62°N, 26°E). Blue tits (*Cyanistes caeruleus*) were used as predators since they are generalists and found in the same areas as *P. plantaginis*. Birds (n= 36) were captured from feeding sites between March and April, and between September and October in 2009 using a peanut-filled trap (a box 13 x 17 x 40 cm) (Ham et al. 2005, Lindstedt et al. 2008).

A small plywood box was used as an experimental arena (50 x 65 x 45 cm). Temperature inside the arena was approximately 20 °C and the box was lit with a light bulb emitting the entire visible daylight spectrum (including UV). The arena contained a water bowl, a perch for the bird, a hatch from which food was offered, and a visual barrier allowing one to determine precisely when the bird detected the prey. Birds were familiarized with the arena for approximately one hour before the experiment, and they were trained to seek food (i.e. sunflower seeds) from behind the visual barrier. The birds' feeding motivation was controlled by food-depriving them for one hour before the experiment and by offering them a live mealworm (*Tenebrio molitor*) larva as a control at the start to confirm that bird is motivated to attack on insect food.

In the experiment a single live moth (either white or yellow) was offered to the predator (Figure 4). Predator attack latency (i.e. time between detecting the prey and the moment of an attack) was measured in seconds as well as the handling time of the prey. The proportion of the moth eaten was also measured. Prey behaviour under attack was scored as no response, startle, defence fluid, escape attempt, feigning death (I). After the experiment bird's hunger was measured from the consumption of mealworms *ad libitum*, which was measured as mass to the closest milligram. Birds were used only once in the experiment, ringed for further identification, and released to the feeding site after the experiment. None of the birds died or were injured during captivity. The experiment was done under permission from Central Finland Regional Environmental Centre (permission number: KSU-2007-09311/YM-23) and licenced from Experimental Animal Committee (ESLH-2007-L-687/254).

FIGURE 4 Predation experiment in action at the Konnevesi Research Station. The predator, a blue tit, has made an attack decision after evaluating the prey. Notice the visual barrier on the left, which allows the experimenter to evaluate attack latency. © Samuel Waldron

2.3.2 Predation pressure in the field (I, III)

Here I test predation pressure on colour morphs in the field using pinned prey items: First, to test whether the results obtained from warning signal efficacy experiments (see above) are similar in the nature (I); and second to test how local selection pressures affects risk of predation in different locations (III). In the latter, I also test how environmental heterogeneity, habitat, prey and predator community composition, affects the predation pressure in field.

To test whether predation risk of the yellow and white morphs differs in nature (i.e. whether their warning signal efficacy differs in the wild), the first field experiment was conducted in Åland, South-Western Finland in the summer of 2008 (I). The area is characterized by small meadows and deciduous forest patches, which are natural habitats for *P. plantaginis*. Predation risk was measured by pinning dead males on tree trunks and recording bird attacks from them. Seven 1000 meter transects were set in different open forest locations, and alternating yellow and white specimens were pinned in 25 meter intervals (20 white and 20 yellow individuals per transect). Altogether 280 targets (140 white: 140 yellow) were used in the experiment. Moths were pinned randomly on different backgrounds due to variable tree species distribution, and on visible spots on the southern side of tree trunks 1.5 meters from the ground with forewings spread into 45° position with tweezers.

The second field study consisted of a set of pinning experiments, which were conducted in five geographic areas in 2010 (III): Estonia, Åland, Scotland, Central Finland and Southern Finland. The locations represent both polymorphic and monomorphic populations allowing one to study the role of frequency-dependence (see details for morph frequencies above). Attack risk was measured by pinning artificial moths on standard green backgrounds (to control for potential differences in visibility between morphs on natural

backgrounds) and by recording bird attacks from them (Figure 5). The artificial moths had plasticine bodies and paper wings, which were designed to resemble the real *P. plantaginis* male colour morphs, were used as prey. The resemblance of the artificial preys to real moths was confirmed *a priori* by combining digital photography, spectrophotometric measurements, and avian vision modelling. Altogether, four types of prey were formed resembling natural variation (and including melanisation variation) of *P. plantaginis* males. Additionally, control prey not resembling any known species (i.e. no potential for learned avoidance by predators) was used. A total of 15 transects were set in each location (except in Åland there were 16 transects) resulting in 76 transects. On 67 transects 50 prey items (10 of each morph) were pinned in 20 meter intervals (white, yellow and control alternating). For 9 transects (all in Central Finland) controls were not used (40 moths / transect). In total 3710 prey items were used.

In both experiments a moth was determined as attacked, if a clear damage on the body was observed. Bird attacks can be identified by v-shaped markings on the body or wings (Cuthill et al. 2005). Other cases such as missing individuals, moths that were heavily nibbled by ants or beetles, moths covered with slime tracks (snails) and hollow exoskeletons (spiders) were censored from the analyses. The experiments lasted for 5 days, and the survival of the prey specimens was checked every 24 hours. Attacked prey were replaced with the new ones to keep the frequencies of available prey constant but only the first attacks on the original prey were included in the analyses.

FIGURE 5 Field predation experiment. The artificial prey is presented on a standard green background, and attacks by birds can be detected from V-shaped beak marks on the plasticine body.

2.3.3 Assessing selective heterogeneity (III)

To test whether environmental heterogeneity alters the attack risk of the colour morphs I categorized the habitat (open, bush, forest) in which the artificial prey

were presented and recorded the alternative prey community surrounding the local population as well as the avian predator community. The obtained variables were used in the analysis of the bird attack data from five populations to if study local selection pressures affect the attack risk (III).

Other Lepidopterans in the field sites were recorded using the line transect counting method (Sutherland 2006). The observed Lepidopterans were classified into crude visual groups based on their coloration. The groups were: Black, white, grey, brown, green, blue, red, orange, and others. Groups were further divided into warning coloured (black, red, orange, others) and non-warning coloured (white, grey, brown, green, and blue) (Exnerová et al. 2006, Ham et al. 2006). Butterflies were counted from all populations except Scotland (n = 61 transects, mean = 29 butterflies on transect, SD = 30.797).

To examine whether predator community promotes selective heterogeneity, birds alongside the experimental transects were identified and counted using the line transect counting method (Sutherland 2006). This was important because warning signal efficacy can depend on what types of predators are around. Monitoring was done in good weather conditions during mornings (04:00-12:00), when birds were active. All birds within a 50 meter-wide 'research stripe' adjacent to transects were counted. Birds observed to be further than 25 meters from the observer were excluded, to make sure that the birds represented the most potential predators in close vicinity of the experimental transects. To describe the predator community, observed Passeriformes were grouped into family level, which conveniently represented functional groups of bird behaviour and foraging tactics. Only relevant insectivore families were considered as potential predators resulting in 9 genera, which were used in a principal component analysis (PCA) yielding three principal components (PCs) as a measure of predator community composition (III).

2.4 Life-history experiments

2.4.1 Mating experiment (I)

To study whether life-history traits can cause differential selection for colour morphs and affect occurrence of colour morphs, I test different life-history traits of colour morphs in the absence of predation. Firstly, I test what is the mating success of the morphs. A mating experiment was conducted to test whether mating success and fecundity of male *P. plantaginis* was affected by their coloration and condition. The experiment was conducted in the greenhouse at the University of Jyväskylä in Central Finland during June and July 2009. To test the mating success of the male morphs in two different conditions, white and yellow males were divided into two treatment groups: 1) controls; and 2) manipulated, where males were forced to produce abdominal defensive fluid. Males were forced to produce the defensive fluid once by gently lifting the

moth from its wings with tweezers. The fluid produced was sucked into a capillary tube for volume measurement. The males' ability to produce defensive fluid varies and the amount fluid produced decreased when repeatedly harassed (Suisto et al. unpublished) suggesting its general costliness. Since the defensive fluid has a distinct odour and males produce it under threat, it likely serves an anti-predator function (Weller et al. 1999, Conner 2009).

One virgin male (i.e. excluding male-male competition) was offered to a randomly chosen virgin female. In total 42 white males (18 from the small and 24 from the large selection line) and 43 yellow males (20 from the small and 23 from the large selection line) were used. Pairs were placed in a mating container (10 x 13 x 12 cm) in the evening before the sunset between 20:00 - 21:00, since mating (*mean* = 01:08 *hrs*, *s.d.* = 02:41 *hrs*) typically occurred during the night. The pairs were observed continuously for 10 hours from 21:00 to 07:00, and after mating had ceased, males were removed from the boxes and females were allowed to lay eggs for four days. Altogether, male mating success (whether the male mated or not), delay until the beginning of mating, duration of mating, egg yield and offspring hatching success were recorded.

2.4.2 Growth environment and fitness responses (II)

To study correlated fitness traits in *P. plantaginis* male colour morphs, I test if morphs have different competence when exposed to stressful conditions in a common garden experiment with manipulated growth densities. Study was conducted at the University of Jyväskylä, Central Finland from July to October 2008. Food (dandelion, *Taraxacum* sp.) was offered *ad libitum* during the larval period (for details, see also Lindstedt et al. 2009, Lindstedt et al. 2010b). Larvae were checked and fed, and their rearing containers were cleaned daily until they reached adulthood. Until their third instar the larvae were reared together in family groups consisting on average of 143 (SD ± 101) individuals (total number of families = 50) under greenhouse conditions. Next the larvae were divided into two densities: solitary or aggregated. Solitary larvae were reared individually on petri dishes (diameter 90 mm). Aggregated larvae were reared in groups of 40 individuals in plastic rearing containers (100 x 130 x 120 mm). Initially 1517 individuals were divided into the treatments. After division all experimental larvae were reared in controlled environmental chambers (for details, see paper II). Mortality of larvae, larval developmental time to pupa, pupa weight, and were recorded. I also counted the amount of eclosed adult males, determined the heritability (h^2) for colour and measured the melanisation variation of the wings (II).

I used a sub-sample of individuals from the density treatments for immune assays to measure encapsulation and humoral response. Encapsulation assessment is a commonly used method to simulate an animal's response against foreign intrusions (e.g. eggs of parasitoids, spores or fungi) (Carton et al. 1997, Schmid-Hempel 2005, Siva-Jothy et al. 2005). Encapsulation assessment was conducted using the fifth instar larvae, which were anaesthetized with CO^2, after which a nylon implant (length = c. 6mm) was inserted between the

second and the third body segments (Ojala et al. 2005, Friman et al. 2009). The immune system was allowed to react for 5 hours before taking the implant out. The implant was dried and photographed under a microscope with 10 x magnification using a Panasonic wv-CL702 video recorder. From the images I took three measurements of grey value of the first 1mm of the end of implant that was inside the larva with ImagePro Plus 4.0 software (Media Cybernetics). The grey value of the background was subtracted from the grey value of the implant to correct for variation in lighting conditions. I used the mean of those three measurements for the analyses. High grey values (darker implant) indicated a strong encapsulation response.

The lytic activity of haemolymph was measured using a lytic zone assay. This is a type of humoral immune response, where quick production of multiple small antimicrobial peptides (Khush et al. 2000, McKean et al. 2001), is targeted against microbial pathogens (Morishima et al. 1995, da Silva et al. 2000, Shelley 2004). A haemolymph sample was taken from the larva at the insertion site of the nylon implant (see above), by puncturing a sterile needle between the second and third segment of the larva, and letting the haemolymph to form a droplet. 10 µl of haemolymph was withdrawn with a pipette and ejected on a filter paper disc on an agar plate. Petri dishes were kept at room temperature (+25°C) for three days, after which plates were photographed and the area of the lytic zone was measured with Image J software. I also recorded whether the samples expressed a rapid melanisation reaction within five minutes after the sample was ejected on petri dish.

2.5 Connectivity of populations and neutral selection

2.5.1 Genetic structure and temporal stability of populations (IV)

Because variation in allele frequencies that govern colour morph frequencies in nature can also be altered due to gene flow, it was important to determine the genetic structure of the populations and its temporal stability. The level of genetic diversity in the COI indicated by the number of haplotypes (h), number of segregating sites (S), nucleotide (pi) and haplotype diversity (H), per sampling site/year was calculated using dnaSP v. 5.10 software (Librado et al. 2009) (See Paper IV). Haplotype relationships were analysed by constructing a network with the software TCS version 1.21 (Clement et al. 2000). To examine the possible association between colour and haplotypes, an analysis of molecular variance (AMOVA) was conducted using Arlequin v.3.5.1.3 software (Excoffier et al. 2010). For this analysis only the sampling sites containing samples from both morphs over consecutive years were included. Hierarchal comparisons were performed among years, among morphs within years, and within years. Statistical significance was attained by means of 10100 random permutations. Tajima's D test was used by dnaSP v. 5.10 (Librado et al. 2009) to infer if observed haplotypes conforming to a neutral model of evolution (IV).

To study spatio-temporal population structure within the morphs, allele frequency variation at 10 species-specific microsatellite loci was analysed (Galarza et al. 2011). The samples were first subdivided according to region (Finland, Åland, Estonia, and the Alps). Deviations from Hardy-Weinberg expectations (HWE) and linkage disequilibrium within regions and year were estimated according to the level of significance determined by means of 10,000 MCMC iterations using GENEPOP v.3.4 software (Raymond et al. 1995). Bonferroni corrections were applied for multiple comparisons setting a significance level of 0.05 (Rice 1989). Genetic diversity was characterized by calculating the number of alleles per locus (Na), the effective number of alleles (independently of sample size, Ar), the fixation index (FIS), as well as the observed (HO) and expected heterozygosities (HE) computed by FSTAT software (Goudet 2001) and Arlequin v.2.0 (Schneider et al. 2000). Finally, to test for temporal stability in population structure, pairwise F_{ST} comparisons between sampling locations was performed using GENETIX v.4.05 software (Belkhir et al. 2000). Pairwise F_{ST}s and their estimated probabilities were calculated by 10,000 permutations according to Weir et al. (1984). The alternative hypothesis of significant genetic differentiation was accepted if the probability was equal or less than 0.05.

2.5.2 Spatio-temporal variation in warning signal components (IV)

Lastly, I compare spatio-temporal patterns of warning signal components to a neutrally evolving genetic marker to detect non-neutral mode of evolution. All of the analyses include only complete datasets (i.e. COI, microsatellite, all signal components), and that have been obtained in the same location for at least two consecutive years. Correlations between several different datasets were analysed using a series of Mantel and partial Mantel tests, which assess the correlation between any two matrices. Distance matrices were compared to each other (for details, see paper IV): (1) Correlation between a genetic distance matrix (COI, microsatellite) and a signal component distance matrix, (2) the distance matrices for the different signal components (i.e. melanin coverage and colour whereby the Euclidean distance using the first principal component values of hue and brightness), and (3) forewing black pattern composition. Then, if the signal component has an effect on the correlation between the black pattern and genetic distance was tested. Significance of partial correlations was calculated by comparing the actual r-statistic to a distribution of r-scores based on 1000 random permutations of the genetic distance matrix.

3 RESULTS AND DISCUSSION

3.1 Warning signal and mating success trade-off (I)

It was important to first address whether the coloration of *P. plantaginis* males has a warning signal function, because signal theory assumes that warning coloration has evolved by visually hunting predators selecting for the signal (Poulton 1890, Cott 1940, Edmunds 1974, Ruxton et al. 2004). In the behavioural assays blue tits hesitated on average 15 seconds and 128 seconds to attack the white and yellow males, respectively. As predators showed nine times longer hesitation time for yellow males in comparison with white males, the results indicate that yellow is a more efficient warning signal. The moths behaved similarly under attack irrespective of morph, handling times did not differ between morphs, nor did the proportion of moths eaten by the birds.

In concordance with the behavioural assays, when real, dead prey specimens were exposed to free ranging predators in the wild, yellow males were approximately three times more likely to survive compared to white males. Of the 140 individuals of each morph, 61 white males (43.6%) and 31 yellow males (22.1%) were attacked, total mortality being 33.3 % for all prey specimens. Intuitively, yellow coloration seems rather conspicuous against natural backgrounds (Poulton 1890, Cott 1940), but what appears generally conspicuous to humans may be effectively concealed to real predators in the natural visual environment (Endler 1983, Endler 1991). For example, the white morph could adopt camouflage (e.g. disruptive coloration) as an anti-predator strategy when resting stationary on tree trunks (Cuthill et al. 2005, Stevens 2007), such as birch that has white bark, which could help to conceal it. However, when visual contrasts of the moths against the substrate types (13 tree species including birch) were analysed based on receptor limits of bird discrimination ability (Vorobyev et al. 1998, Vorobyev et al. 2001), both colour morphs were clearly visible for avian predators. In more detail, yellow had higher chromatic contrast than white but achromatic contrast did not seem to affect the attack risk, which might indicate that chromatic contrast is the

essential feature of a better warning signal in this species. This result suggests that both colour morphs possess warning coloration, although yellow is the more effective one. Note worthily, this does not exclude the possibility that predators may perceive coloration differently when prey is in motion. Apparently predators are selecting against the white morph. There must, however, be something balancing the success of the yellow morph, as otherwise white males should be extinct.

Coloration can have functions other than predator avoidance and often sexual selection plays a role in maintaining colour polymorphism (Sinervo et al. 1996, Seehausen et al. 2004). White males were approximately eight times more likely to mate in comparison to yellow males. White males also released more defensive fluid, which however, appeared to not affect the mating probability. The latency of copulation (i.e. mating delay) did not differ between the colour morphs, but interestingly, copulation took longer if males were forced to give defensive fluid. This could mean it could decrease the quality of the males if releasing the fluid decreases male's condition (Mappes et al. 1996, Rowe et al. 1996, Tomkins et al. 2004). No difference was observed in post-mating life-history traits (i.e. clutch size or offspring number) between colour morphs. As conspicuousness (i.e. warning coloration) and secondary defence (e.g. chemical toxins) can be expensive to produce and/or maintain (Blount et al. 2009, Blount et al. 2012), signal production costs (e.g. costly pigmentation; Freitak et al. 2005, Stoehr 2006) can impact the individuals' condition (Mappes et al. 1996, Rowe et al. 1996, Tomkins et al. 2004). This can also affect mating success, which can offer white males an alternative strategy to balance the deficiency of their visual warning signal. The result that there is a difference in a mating success is very important since differential mate choice can provide a powerful evolutionary force that can significantly improve the fitness of the white morph.

3.2 Environment-mediated fitness responses (II)

Heritability analysis indicated that the colour of the father mostly explains yellow and white coloration ($h^2 = 0.42$). This was important to confirm, because like in desert locust (*Schistocerca gregaria*), warning coloration can be a facultative response to the density of conspecifics (Sword 1999, Sword 2002). Hence, polymorphism of *P. plantaginis* appears to be genetically controlled rather than facultative response to its environment.

Density did not even affect the area of melanisation expressed on adult wings. This was surprising as abundant evidence suggests that cuticular melanisation is an indication of investment in immunity (e.g. Reeson et al. 1998, Wilson et al. 2001, Cotter et al. 2008, Friman et al. 2009, but see Jacot et al. 2005, Joop et al. 2006, Karl et al. 2010). The amount of melanin in the larval stage translated into the adult stage (II), where wings of yellow males tended to have larger melanin pattern (60.1%, SE = 0.01) than the wings of white males (54.6%, SE = 0.01). This may be important in nature, because variation in melanisation

pattern can be a subject to selection in terms of thermoregulation (Majerus et al. 1998). In *P. plantaginis*, increased melanin coverage on wings appears to hinder the efficacy of warning signal, but bringing thermoregulatory benefits to its carrier (Hegna et al. 2013). A similar trade-off is also confirmed in the larvae (Lindstedt et al. 2009), which may further be selected for and translate adults to possess more melanin on their wings (Nokelainen et al. 2013). Exposure to cold (Hegna et al. 2013), or humid (Majeurs et al. 1998) environments may thus favour different properties of wings and affect the evolution of warning signals.

Colour polymorphism can be promoted by phenotype differences other than appearance (i.e. correlated characters) (Sinervo et al. 1998, Roulin 2004, Gray et al. 2007, McKinnon et al. 2010). High growth density increased larval mortality: 17.2% of solitary reared larvae (n = 557) died during rearing, whereas 70.8% of larvae grown in aggregations died (n = 960), indicating general costliness of the crowded environment (Goulson et al. 1995, Sheldon et al. 1996). The colour morphs eclosed at the same proportions from the solitary treatment (51.9% white and 48.1% yellow), but more yellow individuals eclosed from the aggregated treatment (40.7% white and 59.3% yellow).

One possible reason for the observed differences in the eclosion frequencies could be differential allocation to immune response. In aggregations larvae which later eclosed as white males had higher encapsulating response than individuals that eclosed as yellow males, which suggested a competent parasitoid resistance (Rantala et al. 2005, Siva-Jothy et al. 2005, Wilson et al. 2008). In comparison, the lytic zone assay indicated that yellow males were better than white males at in mounting their lytic activity of haemolymph in aggregations, which in turn suggests increased defence against bacterial pathogens (Morishima et al. 1995, da Silva et al. 2000, Shelley A. 2004). Yellow males may have eclosed more abundantly than white males from aggregations, because they are better defended against bacterial pathogens, which could prevail especially in dense rearing conditions in a laboratory where parasitoid attacks are excluded.

3.3 Frequency-dependency and heterogeneity of selection (III)

As stated previously the benefits of warning signals depend on a sufficient density and frequency of individuals displaying the signal (e.g. Sword et al. 2000, Lindström et al. 2001b, Endler et al. 2004). Therefore, it was necessary to study whether the colour morphs' survival differs in populations with differing colour morph frequencies. Conventional view sets an expectation that predators avoid warning signals depending positively on the local dominant colour morph (e.g. Müller 1879, Pinheiro 2003). However, the predation risk of the colour morphs (i.e. artificial prey) was not associated with the natural colour morph frequencies of male *P. plantaginis* in the respective populations. Overall white artificial moths were attacked significantly more than yellow ones. Out of a total of 3710 artificial prey individuals 477 (12.9%) were attacked.

Prey fauna can benefit of shared appearance even across species (Bates 1862, Müller 1879, Fisher 1930, Leimar et al. 1986). This can lead to synergistic selection for common signal components in the alternative prey community (Rosenberg 1991, Leimar et al. 1997). Results show that the abundance of butterflies sharing the white coloration increased the attack risk on white artificial moths and decreased attack risk of the yellow ones, whereas abundance of butterflies sharing the yellow coloration lowered attack risk on both colour morphs. Yellow interspecifics can lower the attack risk of both colour morphs because some predators may show innate avoidance of yellow (Marples et al. 1998, Ham et al. 2006). White males might benefit from the sympatric yellow interspecifics if predators generalised their avoidance from yellow to white (Lindström et al. 2004, ten Cate et al. 2007, Skelhorn et al. 2008). In contrast, presence of white butterflies increased the attack risk of both colour morphs. If white butterflies in the experimental locations were mostly palatable species, such as most geometer moths, their abundance could have been the reason why the birds had not learned to avoid white (and through generalisation, yellow) warning signals (Speed 2000, Sherratt & Beatty 2003, Barnett 2007). Speculatively, even though the exact mechanism is not known, the surrounding butterfly community can alter predation risk and induce synergistic selection on organisms with similar appearance (Rosenberg 1991, Leimar et al. 1997, Mappes et al. 1997). Through attack risk, the surrounding community can have consequences for the predation pressure and within-population morph ratio in the long term.

One possible mechanism for maintaining polymorphism could be heterogeneity of local predators, which could cause differential selective regimes for the colour morphs (Endler et al. 2004). Hence, the effect of predator community composition on predation risk was examined. Artificial moths tended to get more attacks overall in bush habitats, which probably stems from predators like Paridae species preferring those habitats. According to earlier experiments the yellow morph possesses the more efficient warning signal than the white morph (I). In this same respect yellows were attacked less than whites when the predator community was characterized by Paridae species. However, as the community composition shifted towards Prunellidae, yellows were attacked more than whites. Prunellidae do not necessarily cause this alone, as it is possible that a change in species composition causes a change in competition dynamics. Either way, variation in predator community composition can have a realistic contribution to the maintenance of colour polymorphism in aposematic prey. This is a rather novel finding since although it has been suggested that the role of predator community is important (Endler et al. 2004), only one empirical experiment has found that the presence of a specific predator in the community is important for maintaining variation in warning signals (Valkonen et al. 2012).

3.4 Gene flow and temporal variation (IV)

Colour polymorphism can be fuelled by continuous gene flow from surrounding populations (Gray et al. 2007, McKinnon et al. 2010). For this reason the genetic structure and connectivity of populations was investigated using mitochondrial DNA and nuclear microsatellite data. When examining the mitochondrial DNA results, the level of genetic diversity observed at the COI gene fragment was overall low. The haplotype network identified one main haplotype, which was shared by all sampling sites, years and colour morphs. A second major haplotype was shared between samples of both morphs from Southern and Central Finland from across all years. A third haplotype was shared interestingly between yellow morphs from Lapland and white morphs from Estonia belonging to two different years suggesting that these populations are, or at least were once connected, and hints of the species good dispersal ability. The analyses of molecular variance (AMOVA) indicated that only 5.4 per cent of the variation is explained by differences among the years, whereas 99.01 are explained within the years. Thus, the observed variation in colour morph frequencies must be due to selection on local phenotypes rather than the genetic structure of the populations. Accordingly, negative variance components (-4.45) were found among colours within years suggesting an absence of temporal structure (Weir et al. 1984, Weir 1990).

When testing genetic differences by microsatellites (IV), no evidence of linkage disequilibrium was found between any locus pair. Significant deviations from Hardy-Weinberg equilibrium were observed within regions suggesting that the genetic control of colour polymorphism is not due to one locus two alleles inheritance (i.e. classic Mendelian inheritance). None of the F_{ST} between-morphs comparisons showed statistical differences within years, but there were significant differences within morphs between years for all sampling locations. When spatio-temporal variation in the warning signal components of colour morphs were analysed, low or negative correlation values in the Mantel and partial Mantel tests performed for both the nuclear and mitochondrial data indicate that most of the signal components do not evolve in a neutral fashion (IV), but are rather under selection and cannot be explained only by genetic structure. These results are in concordance with findings from earlier results (I, II, III) highlighting the importance of selection favouring either of the morphs under certain circumstances at given time in local population. Taken together, these results suggest that although populations are connected by gene flow temporal differences in the colour morph frequencies are mostly driven by selection, and not by the inherent genetic structure of the population.

4 CONCLUSIONS

Based on these combined results colour polymorphism persists in the aposematic wood tiger moth males due to selective heterogeneity. Selection favours either of the morphs at a given time and space, depending on the local conditions. Better warning signal efficacy of the yellow morphs comes at the cost of mating success, in which the white morphs performs better. White morphs were better at encapsulating foreign intrusions whereas yellow morphs were better at mounting anti-microbial activity of the haemolymph. This in turn suggests that both morphs can be maintained due to their differential investment in immune defences in a heterogeneous environment (since the risk of diseases and parasites varies spatially and temporally in the wild). The selective regime is further altered by the abundance of other butterfly species of a similar appearance, as this seems to affect predation risk. In addition, the efficacy of the warning signal depends on predator community composition, which promotes the development of a small-scale habitat mosaic. Furthermore, different geographical populations are connected by gene flow fuelling genetic variation between local populations, although results indicate selection on local phenotypes rather than the genetic structure of the populations. Thus, the evidence suggests that the existence of polymorphism in the *P. plantaginis* is maintained by differences in warning signal efficacy and mating success as well as spatially variable enemy community combined with gene flow.

Conclusively, as Ruxton et al. phrased: "The reasons for these different instances of polymorphism are likely to be complex and varied, and indeed there may be no single most important explanation" (Ruxton et al. 2004). To extend our comprehension of colour polymorphism in aposematic species, these results support the role of spatial variation generating the heterogeneous selective regime. Given that the colour morphs are more or less adapted into their local environment, and available genetic variation is an outcome of selection in successive generations, there is there a wide array of outcomes of morph frequencies in different populations. This results in persistence of polymorphic warning signals, and explains how biological variation surrounding us stems.

This thesis addressed why colour polymorphism evolved and what maintained it in aposematic species. The results presented here touch some of the important parts where the big gaps in understanding polymorphism in aposematic species are, which makes findings of this thesis timely ones. As emphasized in the beginning, as an easily intelligible trait research on coloration has fascinated people for many years and offered early possibilities to study biological diversity and evolution in action. Recently, in science there has been a resurgence of interest to study colour polymorphism as this day methodology allows taking new perspectives to quantify phenotypic traits. Consequently study of coloration continues to be an important field of evolutionary biology allowing tracing causes and consequences of evolution.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

**Täpläsiilikkään (*Parasemia plantaginis*) monet muodot –
Valintapaineiden epäyhtenäisyys suosii varoitussignaalien polymorfiaa**

Väripolymorfiaksi kutsutaan vaihtelua, jossa kaksi tai useampia geneettisesti erilaista lajin värimuotoa esiintyy samassa populaatiossa. Yleisesti ottaen tämänkaltainen vaihtelu on ongelmallista luonnossa, koska luonnonvalinnan tulisi johtaa parhaimman fenotyypin yleistymiseen populaatiossa, ja siten estää sekä polymorfian syntymistä että sen säilymistä luonnossa. Näin on eritoten aposemaattisilla eläimillä, jotka viestittävät pedoille puolustuskyvystään varoitussignaalien (kuten värityksen) avulla. Varoitussignaalit toimivat siten, että pedot oppivat yhdistämään varoitussignaalin saaliin puolustuskykyyn (esimerkiksi kemialliseen, morfologiseen, käyttäytymiseen), ja siten välttämään niitä. Koska saalistajien on helpompaa oppia tunnistamaan yksi varoitussignaali useamman sijasta, tehokkaimman varoitusvärin (yhdenmukainen ja helposti tunnistettava) tulisi yleistyä populaatiossa, ja tämän vuoksi varoitussignaloinnissa voidaan olettaa esiintyvän vain vähän vaihtelua. Näin ollen tekijät, jotka ylläpitävät polymorfiaa aposemaattisilla lajeilla, ovat todennäköisesti voimakkaita valintapaineita, jotka ylläpitävät lajien geneettistä monimuotoisuutta. Lisäksi niiden tutkiminen auttaa ymmärtämään lajien potentiaalia sopeutua vaihteleviin ympäristöoloihin.

Tämän väitöskirjatutkimuksen tavoitteena oli selvittää, miksi väripolymorfia on kehittynyt ja mitkä valintapaineet luonnossa ylläpitävät sitä aposemaattisilla lajeilla. Tutkimuslaji, täpläsiilikäs (*Parasemia plantaginis*), on siilikkäiden heimoon kuuluva päiväaktiivinen perhonen, joka esiintyy pohjoisella pallonpuoliskolla. Lajin koiraat ovat siipien väritykseltään polymorfisia; keltaisia tai valkoisia, minkä lisäksi perhosen melanisaatiokuvio vaihtelee. Toukat ottavat puolustuskemikaaleja ravintokasveistaan, ja osa näistä kemikaaleista siirtyy aikuisvaiheeseen asti. Tässä tutkimuksessa osoitan, mitä etuja tehokas varoitusväritys tuo mukanaan, mutta myös niitä kustannuksia, jotka aiheuttavat vastakkaisia valintapaineita. Tulosten mukaan keltaisten koiraiden varoitussignaali on tehokkaampi kuin valkoisten, jotka puolestaan ovat naaraiden suosiossa. Aikuisiksi kehittyvät valkoiset koiraat ovat myös parempia enkapsuloimaan taudinaiheuttajia kehostaan toukkina, kun taas keltaisiksi kehittyvillä koirailla on parempi hemolymfan antimikrobinen aktiivisuus. Todennäköisesti valkoiset yksilöt ovat parempia loisrikkaassa ympäristössä, kun keltaiset ovat parempia bakteeripatogeenejä vastaan. Lisäksi osoitan, että vaihtoehtoisten saaliiden määrä populaatiossa vaikuttaa luonnossa täpläsiilikkäiden saaliiksi joutumisen riskiin, kuten myös lintusaalistajayhteisön rakenne. Populaatiot ovat myös geenivirran avulla yhteydessä toisiinsa, mikä ylläpitää geneettistä vaihtelua populaatioissa.

Tämän tutkimuksen tulosten mukaan valintapaineiden epäyhtenäisyys voi suosia varoitussignaalien polymorfiaa. Väripolymorfiaa voi aiheuttaa muun muassa erot varoitussignaalien tehokkuudessa ja parittelumenestyksessä, tai niiden kytkeytymisessä erilaiseen panostukseen immuunipuolustuksessa. Li-

säksi valintapaineet voivat vaihdella niin ajallisesti kuin paikallisestikin, aivan kuin paikallinen saalistajayhteisö. Lopuksi, populaatioiden ollessa toisiinsa yhteydessä riittävä geenivirta voi edesauttaa väripolymorfian säilymistä ja geneettisen vaihtelun ylläpitoa sekä sopeutumispotentiaalia populaatioissa.

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ORIGINAL PAPERS

I

TRADE-OFF BETWEEN WARNING SIGNAL EFFICACY AND MATING SUCCESS IN THE WOOD TIGER MOTH

by

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Trade-off between warning signal efficacy and mating success in the wood tiger moth

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The coloration of species can have multiple functions, such as predator avoidance and sexual signalling, that directly affect fitness. As selection should favour traits that positively affect fitness, the genes underlying the trait should reach fixation, thereby preventing the evolution of polymorphisms. This is particularly true for aposematic species that rely on coloration as a warning signal to advertise their unprofitability to predators. Nonetheless, there are numerous examples of aposematic species showing remarkable colour polymorphisms. We examined whether colour polymorphism in the wood tiger moth is maintained by trade-offs between different functions of coloration. In Finland, males of this species have two distinct colour morphs: white and yellow. The efficacy of the warning signal of these morphs was tested by offering them to blue tits in the laboratory. Birds hesitated significantly longer to attack yellow than white males. In a field experiment, the survival of the yellow males was also higher than white males. However, mating experiments in the laboratory revealed that yellow males had lower mating success than white males. Our results offer an explanation for the maintenance of polymorphism via trade-off between survival selection and mating success.

Keywords: avian vision model; colour polymorphism; *Parasemia plantaginis*; predation; sexual selection; warning signalling

1. INTRODUCTION

Colour polymorphism provides a classic opportunity to understand the process of natural selection in maintaining biodiversity [1]. Processes involved in maintenance of colour polymorphism are of particular interest because they may be important precursors to mechanisms that cause speciation [2]. Several hypotheses have been proposed to explain the maintenance of multiple colour morphs, though none are mutually exclusive; frequency-dependent selection via predation or sexual selection being the most prominent ones [3,4].

In cryptic species, negative frequency-dependent selection often leads to polymorphism of prey [5], but selection is expected to be opposite when prey is aposematic. Aposematic prey often advertises unprofitability (i.e. spines, toxins, noxious chemicals, etc.) to predators via conspicuous coloration [6,7]. Aposematism confers protection to individuals carrying the warning signal, but the benefits are often dependent on a sufficient density of individuals displaying the signal [8–10]. Colour polymorphism is therefore *not* expected in aposematic organisms, like Müllerian mimics, because predator education is based on 'strength in numbers' of similar phenotypes [11–13] and selection is positively frequency-dependent (i.e. anti-apostatic selection) [9]. Despite the expected adaptive function of signal monomorphism in aposematic organisms, colour polymorphism has been reported in many aposematic species (e.g. [14,15]).

Conflicts among the selective pressures acting on coloration can contribute to the maintenance of colour polymorphism in aposematic species. In the strawberry poison frog (*Oophaga pumilio*), the aposematic coloration influences the behaviour of male conspecifics [16] and female preference [17]. Females prefer males based on familiar dorsal coloration, but female tolerance for unfamiliar colour patterns may facilitate the establishment of novel phenotypes that could be favoured further by predator bias [17]. Thus, the combined effects of sexual selection and predation may facilitate colour polymorphism [16–18].

The wood tiger moth (*Parasemia plantaginis*) offers a particularly attractive possibility to investigate selective forces favouring colour polymorphism. Males of this species show extensive colour polymorphism both locally and on broader geographical scales. *Parasemia plantaginis* is widely distributed over the Northern Hemisphere and inhabits a variety of habitats [19], but rarely occurs in high densities. The genetic morphs of males have visually distinct hind wing colours ([20]; figures 1*a* and 2*b*); the most typical in Europe are yellow and white with various degrees of melanization. *Parasemia plantaginis* larvae and females are shown to be aposematic [21,22], and the defence chemicals (e.g. iridoid glycosides) larvae sequester are transferred to the adult females and males [20].

In the first part of this study, we investigated the strength and direction of selection by predators on male coloration. We determined whether predators found male morphs aversive by offering white and yellow

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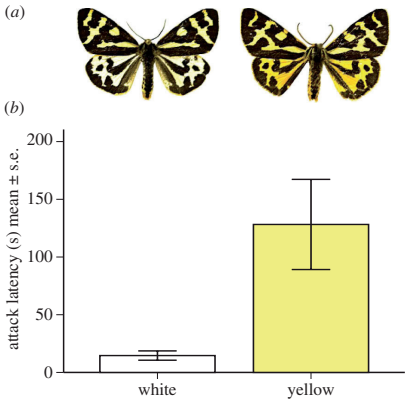

Figure 1. (*a*) The white (left) and yellow (right) male morph of wood tiger moth. (*b*) Predators' hesitation (in seconds) to attack against colour morph.

P. plantaginis males for blue tits under laboratory conditions. The more conspicuous morph (yellow) should have a selective advantage compared with a less conspicuous morph (white) [21,23–25] and therefore, we predicted that birds should hesitate longer and attack yellow morphs less than white morphs.

Next, we compared the survival of dead white and yellow *P. plantaginis* moths pinned to various different tree trunks in the field. Conspicuousness of the colour pattern depends on both receiver perception and visual background [26–28]. If an environment is heterogeneous in substrate type, the potential exists for several different colour morphs to persist, as each morph has an advantage within a given visual microhabitat [5,29]. Therefore, it is important to address the survival of different morphs in the wild where light conditions, predator community and visual environment vary. In addition, we used avian vision modelling [30] to analyse whether the conspicuousness of white and yellow morphs against different backgrounds predicted their survival. We again assumed that the more conspicuous yellow morph should be attacked less than the white morph if conspicuousness is beneficial against predators. Alternatively, the less conspicuous morph may benefit from a lower detection risk by predators (e.g. white morph on a white birch trunk). It is also possible that colour variation is neutral in terms of predation [31] and colour polymorphism can be maintained in the population.

In the second part of this study, we examined mating success and reproductive output of white and yellow males since sexual selection often plays a role in male colour polymorphism [4,32]. We hypothesized that if the conspicuous morph is favoured by increased survival against predation, then either better mating success of the less conspicuous form or signal production costs (e.g. costly pigmentation; [33,34]) could provide a plausible explanation for the observed male colour polymorphism. As mating success is shown to be dependent on an individual's condition [35–37], we forced part of the yellow and white males to excrete a defensive fluid before mating to see if males of different colour morphs are able to bear costs differently in benign and stressful conditions.

2. MATERIAL AND METHODS

(a) *Rearing of* Parasemia plantaginis

Individuals for the experiments came from a laboratory stock reared under constant temperature and density. During the larval stage, food (dandelion, *Taraxacum* sp.) was offered ad libitum (more detailed description in Lindstedt *et al.* [38,39]). Adults do not feed. Males used in the assays originated from divergent selection lines for larval coloration (small and large orange patch, see Lindstedt *et al.* [38]. However, larval coloration does not affect male colour [20]. Both white and yellow morph are visually distinctive and easy to distinguish by the human eye (figure 1), which allowed easy categorization of individuals for the experiments. The colour classification was also confirmed by spectrophotometer measurements, which showed clear differences between the two groups based on chromatic contrast values (figure 2*b*).

(b) *Warning signal efficacy of white and yellow morph*

We conducted behavioural assays with blue tits (*Parus caeruleus*) in an aviary, where we tested whether predators treat yellow or white morphs as unprofitable prey by measuring the hesitation to attack and the handling time. The experiment was conducted in Konnevesi Research Station, Central Finland. Blue tits are generalist predators distributed in the same areas as *P. plantaginis*. Birds were captured from feeding sites between March–April and September–October 2009. A peanut-filled pre-baited trap (a box $13 \times 17 \times 40$ cm), which could be manually operated, was used to catch the birds [21]. Captured birds ($n = 36$) were used only once in the experiment, ringed for the identification and immediately released back to the feeding site after the experiment.

We used a small plywood box as an experimental arena ($50 \times 65 \times 45$ cm). The temperature inside the arena was approximately 20°C. The light bulb used emitted the entire visible daylight spectrum (including UV; Litetronics, made in Germany, SPE CE 20 W, spiral-lite, 220–240 V, 50/60 Hz, 5700 K, Cape 27). Bird behaviour was observed through a small mesh-covered window, and all trials were done in a dark room to prevent disturbance to the birds. The experimental box contained a water bowl and a perch for the bird. In the opposite wall from the perch, there was a hatch from which food was offered. Between the perch and the feeding platform was a visual barrier, allowing us to determine when the bird detected the object for the first time. Prior to the experiment, the birds were familiarized with the experimental boxes for approximately 1 h, and trained to seek food from behind the visual barrier by giving them sunflower seeds. To confirm the birds' feeding motivation, they were food-deprived for 1 h before the experiment and at the beginning of the experiment a living mealworm (*Tenebrio molitor*) larva (weight: 0.10–0.15 mg) was offered.

In the experiment, only one living moth was offered to each bird for every trial. The moth was placed against a green background (*Epiprenum pinnatum* leaf), because *P. plantaginis* is often found resting on a green background in the wild (O.N. 2008 personal observation). In total, we

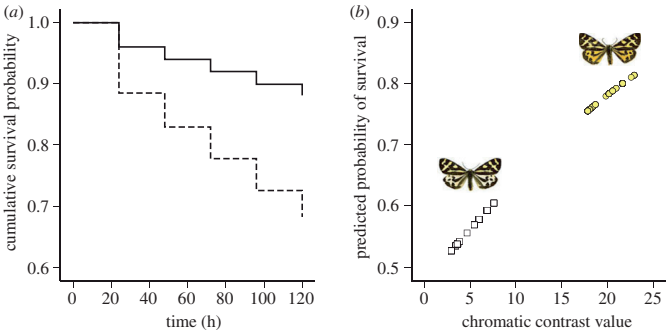

Figure 2. (*a*) Survival plot of the yellow (solid line) and white (dashed line) colour morph survival in the field. The lines are the probability of surviving avian predation as a function of time (hours) based on Cox regression estimates to account for censored data during the 5 day experiment. (*b*) Chromatic contrast values of hind wing colour compared against different backgrounds and their relation to predicted probability of survival. Squares represent white males and circles represent yellow males.

used 18 white males (four from small and 14 from large orange patch size selection line of larval colour [38], and 18 yellow males (four from small and 14 from large orange patch size selection line of larval colour) of equal size (ANOVA for size: colour morph: $F_{1,30} = 0.884$, $p = 0.355$; line: $F_{1,30} = 0.355$, $p = 0.556$).

Latency to attack (i.e. hesitation) was measured in seconds from the first moment a bird detected the prey to the moment of the attack. The maximum time allowed for a bird to make an attack decision was 10 min. Handling time (in seconds) was measured from the first moment the bird attacked the prey to the time when the bird ceased handling the prey. Moth behaviour during the bird attack was scored into five categories: (i) no response (the moth was standing on the background without moving), (ii) startle (the moth flashed its forewings actively revealing its hind wings), (iii) defensive fluid (moth released an aversive defensive fluid from abdomen), (iv) escape attempt (individuals trying to fly away), and (v) feign death (when the moth relaxed its legs and remained motionless). The proportion of moth eaten was scored into four categories: 0 per cent (no observable damage), 10 per cent (head taken), 50 per cent (abdomen taken) and 100 per cent (fully eaten). The categories refer only to the proportion of the body consumed because the birds always pulled the wings off before they ate the moth (O.N. 2009 personal observation).

After the experiment, birds' hunger level was measured by offering them 12 mealworms. The weight of the mealworms consumed within 5 min was used to estimate the bird's satiation level. Birds were fed with peanuts and sunflower seeds before they were released. None of the birds were injured or died during temporary captivity.

We used analysis of variance (ANOVA) to test whether the attack latency (initial avoidance) or handling time by blue tits differed between the two male colour morphs (white and yellow). We also included larval selection line as a fixed factor into the model. Hunger level of the bird and size of the moth (pupa weight) were set as covariates in both models. We log-transformed 'the latency of attack' and 'the handling time', as neither were normally distributed to fit the assumptions of tests. The defence behaviour of the moth and proportion of the moth eaten was tested with Fisher's exact test.

(**c**) ***Predation on white and yellow morphs in the field***

To study potential differences in predation on the colour morphs in natural conditions, we pinned dead adult *P. plantaginis* males on tree trunks to estimate avian predation pressure. Moths were kept in the freezer and thawed approximately 1 h before the experiment. The field experiment was conducted in the Åland Archipelago, southwestern Finland in summer 2008. The area is characterized by small meadows and deciduous forest patches, which are natural habitats for *P. plantaginis*. The avian predator community was observed once with the line transect method [40] in the early morning between 04.00 and 10.00 h, when the birds are most active. Only *Passeriformes* were included in the analysis, as most of the known and potential predators of *P. plantaginis* are within this group.

A total of seven transects were set in different open forest locations and morphs were pinned in 25 m intervals, making a total count of 40 moths (20 white : 20 yellow), and length of one transect 1000 m. Altogether 280 targets (140 white : 140 yellow) were used in the experiment. Moths were placed in visible locations enabling birds to identify them easily. Moths were always pinned on the southern side of tree trunks 1.5 m from the ground with forewings spread to 45° position. Tweezers were used to simulate this natural resting posture. Moths were pinned randomly on 13 different backgrounds, because of varying tree species composition between study sites. The majority of moths were pinned on *Pinus sylvestris* (36%), *Betula pubescens* (25%), *Picea abies* (13%) and *Fraxinus excelsior* (9%). Additional tree species comprised less than 5 per cent of study sites, respectively (see list in the electronic supplementary material, appendix table S2).

The experiment was lasted 5 days and the survival of prey specimens was checked every 24 h. A moth was determined to be attacked if we observed clear damage to the body or wings. Other cases were counted as censored values in the survival analysis to ensure that we did not inflate the predation estimate. Attacked moths were replaced with the new ones in order to keep the frequencies of available moths constant, but only the first attacks of individuals were taken into account in the analysis. We identified avian attacks by v-shaped rips and beak marks left in the body or wings [41]. We excluded missing individuals, moths which were

heavily damaged by ants and beetles, covered with slime tracks (snails), and hollow exoskeletons (consumed by spiders) from the analysis [41]. However, results remained the same if we included the insect attacks and/or missing targets into the analysis.

To analyse the overall survival of white and yellow morphs in the field, we ran Cox regression analysis. We included time as the dependent variable and avian attacks as status variable, along with male colour, study site and number of predator species and their interactions as covariates. To test if vulnerability to predation differs among tree species, we constructed a separate binary logistic regression model with male colour and tree species and their interaction as a covariate. Finally, we tested whether the conspicuousness against different backgrounds predicts survival of yellow and white morphs. Chromatic contrast and achromatic contrast values of moth (see further) against the background are correlated properties, thus their effect on survival has to be analysed in two separate models. In the first model, we had survival as a binomial-dependent variable and the colour (chromatic) contrast value as a covariate. In the second model, we had survival as a binomial-dependent variable and luminosity (achromatic) contrast value as covariate. For all the results, expected beta coefficients are reported as odds ratios (OR) to describe the effect size. A value 1.00 indicates that two treatments have identical survival probabilities (i.e. event is equally likely to happen in both groups).

(d) *Variation in conspicuousness and predation risk*

The conspicuousness of the moths on different backgrounds was estimated with an avian vision model [42,43]. We measured the reflectance spectra of tree trunks by taking measurements from three individuals of each tree species along with the 10 measurements of both morphs. We then constructed an avian vision model to analyse contrasts between moths and backgrounds.

We used discrimination threshold modelling to predict whether the bird (blue tit) can discriminate between the colour and luminance of the white and yellow hind wing colours against several different backgrounds used in the field predation experiment. The discrimination threshold model used assumes that noise in the receptors limits discrimination ability [42,43]. The model uses information about the visual system, such as the sensitivity and relative abundance of different receptor types, and estimates of noise that arise in the photoreceptors.

We first took five measures per individual from 10 white males and 10 yellow males with an Ocean Optics (Dunedin, FL, USA) USB4000 spectrometer held at 45° to normal, with illumination by a PX-2 pulsed xenon lamp, recorded from 300 to 750 nm, expressed relative to a SpectralonTM 99 per cent white reflectance standard (Labsphere, Congleton, UK). Data were reduced to 1 nm intervals prior to analysis by selecting the first value of each nanometre. Colour of the various backgrounds (see list in the electronic supplementary material, appendix table S2) was measured similarly except with AvaSpec-2048-SPU (Avantes, USA) spectrometer with illumination by AvaLight DHS Deuterium–Halogen light source. Average spectra were taken for each stimulus type, followed by modelling of a blue tit's photon catch values for the single and double cones [44] with a standard D65 irradiance spectrum. Colour vision in birds stems from the four single cone types [45], whereas luminance discrimination apparently stems from the double cones [30]. For the colour model, we therefore used the four single cones, whereas the luminance model was based on the double cones [15]. For the discrimination model, we used a Weber fraction of 0.05 for the most abundant cone type, and the relative proportions of cone types in the blue tit retina (longwave = 1.00, mediumwave = 0.99, shortwave = 0.71 and ultraviolet sensitive = 0.37). For the results of the discrimination model, values (just noticeable differences, or 'JNDs') of less than 1 are indistinguishable, values between 1 and 3 are hard to distinguish unless under optimal conditions and values more than 5 are easy to tell apart under most conditions [46]. Finally, we incorporated obtained colour contrast and luminosity contrast values as covariates in the survival analysis of the field experiment data (described above).

(e) *Reproductive output of white and yellow morphs*

To determine whether mating success and reproductive output of males was affected by coloration, we performed a mating experiment. The experiment was conducted in a greenhouse at the University of Jyväskylä in Central Finland (62° N, 26° E) during June and July 2009. The temperature in the greenhouse varied between 20°C and 30°C during the day (approx. 20 h) and during the night (approx. 4 h) the temperature decreased to 15°C–20°C. In the northern latitudes (greater than 62° N), the flying and mating period of *P. plantaginis* males occurs during the midsummer when the days are very long (22 h of light). Thus, the males' colours should be visible to females, and could potentially be a target of sexual selection.

In the experiment, one virgin male was offered to a virgin female. Each 'couple' was placed within a separate container to control for the possibility of male–male competition. Variables measured to determine mating success included latency of copulation (the time between introduction and copulation), the duration of mating, egg production and offspring hatching success. In total, we had 42 white males (18 from small and 24 from large orange patch size selection line for larval coloration) and 43 yellow males (20 from small and 23 from large orange patch size line). Males were mated with a randomly chosen female to control any potential bias in experimenter pairing choices. Pairing was done in the evening (a mating box $10 \times 13 \times 12$ cm) before sunset between 20.00 and 21.00. After pairing the males and females, individuals were observed continuously for 10 h from 21.00 to 07.00 (when mating naturally occurs), and their mating success (whether the male mated or not) was recorded. If males had not mated during this time they were scored as 'unsuccessful'. After mating, males were removed from the boxes and females were allowed to lay eggs for 4 days and on the fifth day the eggs were counted. Larvae were counted on a second day after hatching to confirm that all the larvae had hatched.

In order to be able to disentangle the mating success of male morphs in benign and stressful conditions, males were divided into two treatment groups; (i) controls and (ii) manipulated, where males were forced to produce defensive fluid (e.g. [47]). The defensive fluid has a distinct odour and we have observed that males produce it when threatened and thus, it most likely has an anti-predatory function in Arctiid moths (see also [48,49]). As capital breeders, production of defence fluid must be restricted for adult *P. plantaginis* individuals making it costly trait to produce repetitively both in terms of resources (e.g. water) and energy (e.g. metabolic processes to synthesize and expel the fluid). In addition,

Table 1. ANOVA-table of hesitation and handling times by blue tits.

source of variation	d.f.	MS	*F*	*p*
hesitation to attack (s)				
morph	1	5.347	9.469	0.005*
signal line	1	0.438	0.777	0.386
morph × signal line	1	0.708	1.254	0.273
male weight	1	0.050	0.089	0.767
satiation	1	1.035	1.834	0.187
error	27	0.565		
handling time (s)				
morph	1	0.140	0.792	0.382
signal line	1	0.668	3.791	0.062
morph × signal line	1	0.026	0.150	0.701
male weight	1	0.074	0.417	0.524
satiation	1	0.352	2.001	0.169
error	26	0.176		

*Significant at 5%.

releasing frequency of the defence fluid also varies among individuals under predation (table 2) and preliminary tests indicate a negative relationship between the releasing frequency and the amount of fluid exuded supporting its costliness for males (K. Suisto and C. Lindstedt, 2009 personal observation). Fluid was released from the males before the mating on the same day of the experiment. Males were forced to produce the defensive fluid only once by gently lifting the moth with tweezers. The defensive fluid produced was then drawn into a capillary tube and its volume was measured.

To determine whether male colour influenced mating success we used binary logistic regression. Mating success (mated or not mated) was set as the dependent variable and male colour, selection line for larval colour, defensive fluid treatment along with their possible interactions were set as covariates. We tested whether the amount of defensive fluid produced varies between morphs by setting the amount as a dependent variable, morph and selection line as fixed factors, and male weight as covariate in ANOVA. The defensive fluid volume was transformed to a logarithmic scale to fit the assumptions of ANOVA. The mating delay (in minutes) and duration of copulation (in minutes) were tested with an analysis of variance by setting the time as a dependent variable with morph, selection line for larval colour, and defensive fluid volume as fixed factors. Male weight was included as a covariate in the ANOVA. To determine if fecundity differed between the male colour morphs we also used an ANOVA. Fecundity measure (number of eggs, number of offspring) was set as a dependent variable. Colour of the male, selection line and defensive fluid treatment were set as fixed factors. Female weight was set as a covariate. Non-significant parameters were omitted from the final analyses (general protocol; $p > 0.05$, smallest omitted significance was $p = 0.488$).

3. RESULTS

(a) *Warning signal efficacy of white and yellow morph*

Blue tits hesitated significantly longer to attack yellow males compared with white males (figure 1 and table 1). The average hesitation time for yellow males (mean = 128 s, s.d. = 166) was nine times longer than for white males (mean = 15 s, s.d. = 17). The handling time was not different between two morphs (table 1), even though birds' handling time for the yellow males (mean = 161 s, s.d. = 190), was approximately two times longer than for the white males (mean = 82 s, s.d. = 50). The proportion of moths eaten by blue tits did not differ between colour morphs, and both colour morphs behaved similarly when threatened by predators (table 2).

(b) *Predation on white and yellow males in the field*

Yellow males were nearly three times more likely to survive in the field compared with white males (Wald = 3.937, d.f. = 1, $p = 0.047$, OR = 2.727; figure 2). Total avian predation in the experiment was 33.3 per cent of all prey specimens. Of the 140 individuals of each morph, 61 white males (43.6%) and 31 yellow males (22.1%) were attacked. The survival rate of white and yellow males did not depend on the site (Wald = 0.121, d.f. = 1, $p = 0.728$, OR = 1.196) nor did it vary among the study sites (study site × colour morph: Wald = 0.042, d.f. = 1, $p = 0.839$, OR = 1.026). In addition, the amount of predator species (Wald = 0.161, d.f. = 1, $p = 0.688$, OR = 1.045), or their interaction with site (Wald = 0.875, d.f. = 1, $p = 0.350$, OR = 0.979) did not impact the survival rate of the pinned moths. When we tested whether the tree species, against which the moth was pinned, predicted survival we found that only male colour was a significant predictor (Wald = 14.153, d.f. = 1, $p < 0.001$, OR = 0.368). Neither the tree species (Wald = 6.534, d.f. = 12, $p = 0.886$) nor their interaction with male colour (Wald = 2.273, d.f. = 8, $p = 0.971$) predicted predation.

Both colour morphs were clearly conspicuous for avian predators against all backgrounds, though yellow males were generally more conspicuous in terms of colour (all JND values for colour contrasts varied for white males from 2.95 to 7.59 and for yellow males from 17.83 to 22.90). For luminance, JND values varied from 0.48 to 19.27 and 0.22 to 16.54 for white males and yellow males, respectively. Conspicuousness was beneficial for moths, because survival probability increased with increasing colour (chromatic) contrast against the background (Wald = 15.440, d.f. = 1, $p < 0.001$, OR = 1.071; figure 2). However, luminosity contrast alone did not predict predation (Wald = 0.824, d.f. = 1, $p = 0.364$, OR = 1.018).

(c) *Reproductive output of white and yellow morphs*

White males released more defensive fluid than yellow males (figure 3 and table 3). White males were also more than eight times more likely to mate than yellow males (Wald = 8.339, d.f. = 1, $p = 0.004$, OR = 8.212; figure 3). Extracting defensive fluid before the mating did not affect the mating probability (Wald = 1.490, d.f. = 1, $p = 0.222$, OR = 2.880), nor was there an interaction between the defensive fluid treatment and male colour (Wald = 1.177, d.f. = 1, $p = 0.278$, OR = 0.309). Neither the selection line (Wald = 0.097, d.f. = 1, $p = 0.756$, OR = 1.241) nor its interaction with the defensive fluid treatment (Wald = 0.136, d.f. = 1,

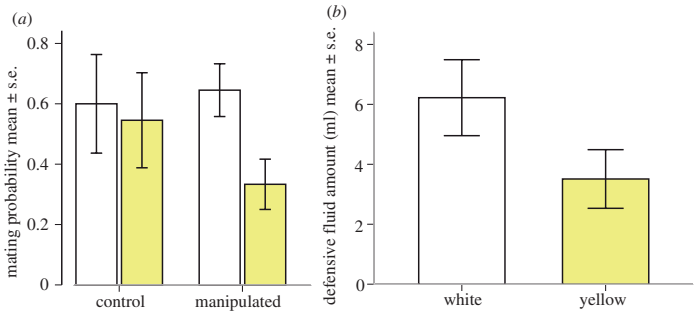

Figure 3. (*a*) The mean mating probability of wood tiger moth males. On the *x*-axis, the 'control' represents individuals, which were not in the defensive fluid treatment, and 'manipulated', stands for the defensive fluid (i.e. droplet) treatment group. Left bars within the group (white) stands for the white males, and right bars (yellow) represent yellow males. On the *y*-axis is the mating probability of males, when females have no alternative choice. (*b*) The mean volume (mm^3) of defensive fluid between two male colour morphs of wood tiger moth.

Table 2. Observed behaviour and damage of moths under predation threat in the aviary.

variable	observation	white	yellow	total	Fisher's exact
behaviour	no response	11	8	19	0.505
	startle	0	2	2	1.000
	defensive fluid	2	6	8	0.228
	escape attempt	2	1	3	1.000
	feign death	3	1	4	0.603
proportion eaten	0% no damage	3	6	9	0.443
	10% head taken	4	5	9	1.000
	50% abdomen eaten[a]	2	1	3	1.000
	100% fully eaten[a]	9	6	15	0.500

[a]Notice that wings were never eaten.

$p = 0.713$, OR = 0.673) had an effect on mating probability. There was also no interaction between male colour and larval selection line (Wald = 3.035, d.f. = 1, $p = 0.081$, OR = 0.194) affecting mating probability. Male weight did not differ significantly among the treatment groups (colour morph: $F_{1,79} = 1.887$, $p = 0.173$; line: $F_{1,79} = 0.032$, $p = 0.858$; defensive fluid treatment: $F_{1,79} = 0.008$, $p = 0.928$), thus variation in male size does not explain the differences in mating success.

The latency (i.e. mating delay) of copulation did not differ between the colour morphs (table 3). Larval coloration lines, defensive fluid treatments and their interactions did not affect mating delay (table 3). However, if we include only the males that mated, the male colour did not significantly affect the mating delay (time to start copulation). Interestingly, copulation took longer if males were forced to give defensive fluid (table 3). This could mean that the fluid may be a part of courtship signals delivered to females or it could decrease the quality of the males in other ways, prolonging the latency of copulation. Male weight also affected copulation time (table 3), which was shorter for heavier males ($r = -0.330$, $p = 0.031$).

No difference was observed in post-mating fitness differences between yellow and white males who successfully mated. We found that male colour did not affect the number of eggs or number of offspring produced (table 3). However, heavier females produced more offspring (table 3).

4. DISCUSSION

Genetic polymorphism can be maintained if different morphs have equal mean fitness (e.g. [3,4,32]). In the present study, we demonstrate a trade-off between warning signal efficacy and mating success between yellow and white *P. plantaginis* males. The more conspicuous yellow males were better defended against predators, as they hesitated longer and were less likely to attack yellow males compared with white males. However, white males had better mating success in comparison with yellow males, which would lead to a higher number of egg clutches sired by white males. This trade-off can partly explain the sympatric occurrence of white and yellow male morphs.

Predators are expected to select for conspicuous [21,23,24,50,51] and convergent [11–13,52,53] warning signals in aposematic prey. In concordance, our results showed that increased colour contrast was beneficial for *P. plantaginis* males, with yellow males benefitting the most because of their greater conspicuousness. In addition to colour contrast, greater prey luminance contrast may increase the detection of prey, but also facilitate predator aversion [54] and memory retention

Table 3. ANOVA-table from reproductive output experiment.

source of variation	d.f.	MS	*F*	*p*
volume of defensive fluid				
morph	1	6.467	5.291	0.025*
signal line	1	0.0000424	<0.001	0.995
morph × signal line	1	2.473	2.024	0.160
male weight	1	0.030	0.024	0.877
error	57	1.222		
mating delay (min)				
morph	1	0.00000001567	1.783	0.190
signal line	1	0.000000579	0.659	0.422
defensive fluid	1	0.00000001372	1.561	0.220
morph × signal line	1	0.0000001519	0.173	0.680
signal line × defensive fluid	1	0.0000008921	1.015	0.320
morph × defensive fluid	1	0.0000007827	0.891	0.352
error	36	0.0000008789		
duration of copulation (min)				
morph	1	24 296.056	0.930	0.342
signal line	1	8344.096	0.319	0.576
defensive fluid	1	188 563.120	7.220	0.011*
morph × signal line	1	4247.928	0.163	0.689
morph × defensive fluid	1	70 372.151	2.695	0.110
signal line × defensive fluid	1	50 370.910	1.929	0.174
male weight	1	145 233.020	5.561	0.024*
signal line × defensive fluid × morph	1	105 179.770	4.027	0.053
error	33	26 116.467		
egg number				
morph	1	7.645	0.001	0.973
signal line	1	468.966	0.070	0.794
defensive fluid	1	3209.485	0.479	0.495
morph × signal line	1	3095.068	0.462	0.503
morph × defensive fluid	1	3137.048	0.469	0.500
signal line × defensive fluid	1	1207.520	0.180	0.675
female weight	1	24 581.420	3.672	0.067
error	24	6694.44		
offspring number				
morph	1	170.262	0.029	0.866
signal line	1	22.905	0.004	0.951
defensive fluid	1	14 866.183	2.524	0.126
morph × signal line	1	4368.392	0.742	0.398
morph × defensive fluid	1	38.909	0.007	0.936
signal line × defensive fluid	1	5228.009	0.887	0.356
female weight	1	27 921.408	4.740	0.040*
error	23	5890.859		

*Significant at 5%.

of an invertebrate predator [55]. Our data showed that despite white *P. plantaginis* males having stronger luminosity contrast compared with the yellow males, it did not increase the survival of white males. Thus, we did not find any support for the hypothesis that the variation in predator perception and/or background coloration could maintain colour polymorphism in this system.

Selection for conspicuous morphs by predators could be relaxed if toxicity and conspicuousness are expensive to produce and maintain, but an increase in either of the components may offer equally good protection against predators [56,57]. The present study did not provide clear support for this assumption as birds were handling and consuming white and yellow males similarly. In addition, tendency to produce defensive fluid under attack did not differ among morphs, although white males excreted higher quantities of bitter smelling defensive fluid before the mating experiment. However, more detailed investigations would be needed to determine whether white and yellow males have different defence strategies before definitive conclusions can be made.

Allocation of resources to conspicuous warning coloration may have an impact on other fitness-related traits, such as reproduction [57,58]. Although we do not have any direct evidence of production or maintenance costs of male coloration, we suggest that one cost of effective warning signal expression is impaired reproductive output because the mating probability of white males was higher when compared with yellow males. Based on the current data, we cannot offer a clear reason underlying the observed female preference. Females may gain indirect benefits of mating with attractive males and having attractive sons. According to our results, male

quality did not differ between colour morphs in terms of number of eggs or offspring produced per female. However, we did not measure the quality of the offspring further, thus it is possible that offspring of more attractive white males would have enhanced performance or viability. This will be a topic for future study.

In Arctiid moths, female mate choice can have more direct benefits as males leave spermatophores after mating, termed 'nuptial gifts', containing nutrients, water and defence chemicals [48]. Therefore, it is possible that white males could offer larger and better quality nuptial gifts. The additional nutrients may be particularly valuable for females, considering that *P. plantaginis* adults do not feed. Thus, one can assume that by mating with the males that can offer extra nutrients, females would gain direct benefits which could extend their individual lifespan and reproductive output. This may allow females to seek suitable egg-laying habitats longer, and thus evaluate the most suitable site for laying a clutch or to spread its clutch on several host plants (i.e. bet-hedging). Defensive chemicals transferred to females in the nuptial gifts can be used by the female for herself or for the defence of the eggs [48,59]. We did not find significant differences in the mating duration of the white and yellow males. However, larger males copulated for a shorter amount of time and individuals that were forced to produce defensive fluid mated longer, offering indirect support for the costs of producing defensive fluid. In order to test the possible differences in the spermatophore size between males of different condition and colour, further experiments are needed. In addition, tests where females are able to choose between the males are needed to see whether the results are in concordance with the current experiment when male–male competition is allowed.

Factors that contribute to the maintenance of colour polymorphisms continue to be a central focus in evolutionary research. We found that white male *P. plantaginis* were better at mating, but possessed a less effective warning signal. Although the mechanism behind the mate choice remains partly speculative, our results offer evidence that female mate choice along with selection by predators contributes to the maintenance of colour polymorphism in this species.

The experiment was carried out with permission from the Central Finland Regional Environmental Centre (permission no.: KSU-2007-09311/YM-23) and licence from the Experimental Animal Committee of the University of Jyväskylä (ESLH-2007-L-687/254).

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Electronic appendix table 1: Tree species distributions according to site and number of pinned moths on specific tree species.

species	site 1	site 2	site 3	site 4	site 5	site 6	site 7	total
Pinus sylvestris	25	15	7	9	38	2	0	96
Betula pubescens	9	14	11	3	1	20	9	67
Fraxinus excelsior	0	0	0	3	0	10	20	33
Picea abies	4	4	5	17	1	0	0	31
Populus tremula	0	6	2	0	0	5	0	13
Betula pendula	0	0	5	6	0	0	0	11
Alnus glutinosa	2	0	7	1	0	0	1	11
Corylus avellana	0	0	0	1	0	0	8	1
Acer platanoides	0	0	1	0	0	1	2	4
Alnus incana	0	1	1	0	0	0	0	2
Prunus padus	0	0	0	0	0	1	0	1
Tilia cordata	0	0	1	0	0	0	0	1
Electric pole	0	0	0	0	0	1	0	1
	40	40	40	40	40	40	40	280

Electronic appendix table 2: Observed *Passeriformes* - species in sites and the total amount of passerine bird species per sites as presence/absence (0 = absent, 1 = present). Table is presented in systematic order. Birds are count with line transect method.

species	site 1	site 2	site 3	site 4	site 5	site 6	site7	total
Lullula arborea	0	0	0	0	1	0	0	1
Alauda arvensis	0	0	0	0	1	1	0	2
Hirundo rustica	0	0	1	1	0	0	0	2
Anthus trivialis	1	1	1	1	1	0	0	5
Troglodytes troglodytes	0	0	1	1	0	0	0	2
Prunella modularis	1	0	0	1	0	0	0	2
Erithacus rubecula	1	1	1	1	1	1	1	7
Luscinia luscinia	0	0	0	1	0	0	0	1
Turdus merula	1	1	1	1	1	1	1	7
Turdus pilaris	1	1	1	1	0	0	0	4
Turdus philomelos	1	1	1	1	0	1	1	6
Turdus iliacus	1	0	0	0	0	0	0	1
Turdus viscivorus	0	0	0	0	0	0	1	1
Hippolais icterina	0	0	0	0	0	0	1	1
Sylvia atricapilla	1	1	1	0	1	1	0	5
Sylvia borin	1	1	1	1	0	1	1	6
Sylvia curruca	1	1	1	1	0	0	0	4
Sylvia communis	0	1	1	1	0	1	1	5
Phylloscopus sibiltraxis	1	1	1	1	0	0	0	4
Phylloscopus collybita	0	0	1	1	1	0	0	3
Phylloscopus trochilus	1	1	1	1	1	1	1	7
Regulus regulus	1	1	1	1	1	1	0	6
Muscicapa striata	1	1	1	1	1	1	1	7
Ficedula parva	0	0	1	0	0	0	1	2
Ficedula hypoleuca	1	1	0	0	0	0	0	2

Aegithalos caudatus	0	1	1	0	0	0	0	2
Parus montanus	0	0	0	0	1	0	0	1
Parus cristatus	1	0	0	0	1	0	0	2
Parus ater	0	1	1	1	1	0	0	4
Parus caeruleus	0	1	1	1	1	1	0	5
Parus major	1	1	1	1	1	1	1	7
Certhia familiaris	1	0	1	1	1	0	0	4
Lanius collurio	0	0	0	0	0	1	0	1
Pica pica	0	1	0	1	0	0	0	2
Nucifraga caryocatactes	0	1	1	1	0	0	0	3
Corvus corone cornix	1	1	1	1	1	1	0	6
Corvus corax	0	0	0	0	0	1	0	1
Sturnus vulgaris	0	0	0	1	0	0	0	1
Fringilla coelebs	1	1	1	1	1	1	1	7
Carduelis chloris	1	1	1	1	0	0	0	4
Carduelis spinus	1	1	1	1	1	0	1	6
Carduelis cannabina	1	0	1	0	0	0	0	2
Loxia curvirostra	1	1	1	1	0	1	0	5
Loxia pytyopsittacus	0	1	0	1	0	0	0	2
Carduelis erythrinus	0	0	1	0	0	0	0	1
Emberiza citrinella	1	1	1	0	1	1	1	6
total	25	27	31	30	20	18	14	species total 46

II

ENVIRONMENT-MEDIATED MORPH-LINKED IMMUNE AND LIFE-HISTORY RESPONSES IN THE APOSEMATIC WOOD TIGER MOTH

by

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Environment-mediated morph-linked immune and life-history responses in the aposematic wood tiger moth

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Summary

1. Warning signals are expected to evolve towards conspicuousness and monomorphism, and thereby hamper the evolution of multiple colour morphs. Here, we test fitness responses to different rearing densities to explain colour polymorphism in aposematic wood tiger moth (*Parasemia plantaginis*) males.

2. We used larval lines sired by white or yellow adult males selected for small or large melanization patterns of coloration. We reared these selected lines either solitarily (favourable conditions) or in aggregations (challenged conditions), and followed their performance to adult stage. We tested whether differences in larval density affected life-history traits, adult melanin expression, adult morph (white or yellow) survival and immunological responses.

3. We found that the aggregated environment increased mortality of larvae, but decreased larval developmental time and pupa weight. Adult wing melanin pigmentation was dependent on larval melanin expression but not rearing density. We also confirmed that adult wing coloration had a genetic basis (h^2 = 0·42) and was not influenced by larval growth density. Adult yellow males survived better from aggregations in comparison with white males, which may be related to differences in immune defence. White males had better encapsulation ability, whereas yellow males had increased lytic activity of haemolymph in the aggregations.

4. Our main results highlight, that morph-linked immune responses mediated by differential growth density may facilitate the maintenance of colour polymorphism in aposematic species. In nature, risk of diseases and parasites vary spatially and temporally. Therefore, both yellow and white adult morphs can be maintained due to their differential investment in immune defence in heterogeneous environments.

Key-words: aggregation, aposematism, colour polymorphism, immune defence, *Parasemia plantaginis*

Introduction

Elaborate colour signals represent some of the most well-documented examples of discrete phenotypic variation both in vertebrates (Seehausen & Alphen 1998; Roulin 2004) and invertebrates (Sandoval & Nosil 2005; Svensson & Abbott 2005). The maintenance of different genetic morphs within the population implies similar net benefits for all morphs (Ford 1965; Maynard Smith 1982), and therefore, different colour morphs should have equally rewarding strategies in the long term (Gross 1996; Sinervo & Lively 1996). Otherwise, morphs with lower fitness would decrease leading to fixation of the fittest morph (Fisher 1930; Stearns 1992). Although some polymorphisms are maintained by selective heterogeneity in predation pressure (Olendorf *et al.* 2006), polymorphisms can also be maintained by intraspecific competition (Calsbeek & Cox 2010), female preference (Maan & Cummings 2008) via frequency-dependent selection or differential thermoregulation benefits of morphs (Forsman 1995; Williams 2007). Alternative colour morphs can also differ in features other than colour (i.e. correlated characters) (Sinervo & Svensson 1998; Roulin 2004; Gray & McKinnon 2007; McKinnon & Pierotti 2010). For example, in side-blotched lizards (*Uta stansburiana*) hormone levels of different female morphs have been shown to respond to their social environment (Comendant *et al.* 2003).

The maintenance of colour polymorphism becomes puzzling to explain under the context of aposematism (Poulton

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1890; Cott 1940; Edmunds 1974). This is because warning colour patterns are expected to be under positive frequency-dependent selection (Endler 1988; Mallet & Joron 1999; Sherratt 2002), which should prevent the evolution of multiple colour signals (Lindström *et al.* 2001; Rowland *et al.* 2007; Marples & Mappes 2011). In spite of this there are several examples of aposematic polymorphism (Brakefield 1985; Siddiqi *et al.* 2004; Williams 2007), which may stem from a variety of reasons. For example, in a resource-limited environment genetic morphs are bound to allocate nutrient components among necessary life-history traits (Stearns 1992). Thus, if there is a cost to producing an effective warning coloration (Darst, Cummings & Cannatella 2006; Blount *et al.* 2009, 2012), limited resources may constrain signal expression (Grill & Moore 1998; Ojala, Lindström & Mappes 2007; Lindstedt *et al.* 2010b). In addition, if genetic morphs are allocating available resources in a different way (Roff 2002), it could result in a physiological trade-off between competing colour morphs in terms of immune defence (Svensson, Sinervo & Comendant 2001; Pryke *et al.* 2007).

Correlational selection may favour colour polymorphism especially if individuals are bound to produce some traits which expression is excluding some others (Roff 2002). For example, in side-blotched lizards the female throat colour polymorphism is linked to immunological defence (Svensson, Sinervo & Comendant 2001). This appears as positive relationship between survival and antibody responsiveness in the yellow morph, whereas this relationship is negative in the orange morph, suggesting that producing an orange throat colour is limiting an effective antibody response in this species. Indeed, defence against pathogens is one fundamental trait shaping the fitness of organisms (Wilson & Cotter 2008), and it has been shown to result in physiological trade-off between colour morphs in terms of immune responses (Svensson, Sinervo & Comendant 2001; Pryke *et al.* 2007).

The insect immune system provides defence against different pathogens, such as parasitoids, viruses, bacteria and fungi, mainly via humoral and cellular components (Ashida & Brey 1998; Khush & Lemaitre 2000; Rolff & Reynolds 2009). The humoral immune response (i.e. the lytic activity of haemolymph) is mainly targeted against microbial pathogens (Morishima *et al.* 1995; da Silva, Dunphy & Rau 2000; Shelley 2004), and is characterized by fast production of multiple small antimicrobial peptides (Khush & Lemaitre 2000; McKean & Nunney 2001). Cell-mediated immune response (i.e. encapsulation ability), however, is primarily directed against foreign intrusions such as eggs and larvae of parasitoids, spores of fungi and microbes (Carton & Nappi 1997; Schmid-Hempel 2005; Siva-Jothy, Moret & Rolff 2005). Importantly, encapsulation ability is linked to phenoloxidase (PO) cascade through series of micropeptide formation starting from tyrosine being further catalysed by phenoloxidase and eventually leading to melanin (Gotz & Boman 1985; Ashida & Brey 1998; Siva-Jothy, Moret & Rolff 2005). Thereby, expression of melanin-based pigments in phenotype can link to an active immune system (Aso *et al.* 1985; Wilson & Reeson 1998; Barnes & Siva-Jothy 2000), or alternatively, deposition of melanin in the cuticle can serve some other function like warning coloration or thermoregulation (Lindstedt, Lindström & Mappes 2009).

Males of the aposematic wood tiger moth (*Parasemia plantaginis*) show discrete phenotypic variation locally and on a broader geographic scale (Leraut 2006), and therefore provide a suitable system to study the maintenance of colour polymorphism. The most typical colour morphs in Europe are yellow and white with various degrees of melanization (Lindstedt *et al.* 2010a; Nokelainen *et al.* 2012). Larvae possess variable orange-black coloration, and the size of the orange patch functions as a warning signal (Lindstedt, Lindström & Mappes 2009). As a generalist herbivorous capital breeder (Tammaru & Haukioja 1996), this species occurs in a wide variety of habitats without strict dietary preferences (O. Nokelainen, R.H. Hegna, J. Valkonen, C. Lindstedt & J. Mappes unpublished). Both male morphs are aposematic, but the yellow males have a fitness benefit by possessing the more efficient warning signal against visually hunting bird predators, whereas white males seem to benefit from female preference (Nokelainen *et al.* 2012). It also appears that more melanized adult individuals have a fitness benefit of increased thermoregulatory properties at the cost of less efficient warning signalling (R.H. Hegna, O. Nokelainen, J.R. Hegna, & J. Mappes unpublished). A similar trade-off is also confirmed in the larvae of this species (Lindstedt, Lindström & Mappes 2009). Females show continuous variation (yellow-orange-red) in coloration (Lindstedt *et al.* 2010a), for simplicity, however, here we only focus on the maintenance of male-limited colour polymorphism.

Here, we ask whether morph-linked responses to differential larval density can facilitate the co-occurrence of white and yellow male morphs. To do so, we tracked fitness-related traits in both favourable (solitary) and unfavourable (aggregated) environments. We used lines selected for small or large melanization patterns of larval coloration, reared larvae sired by white and yellow males solitarily (favourable conditions) and in aggregations (challenged conditions) and followed their performance to adult stage. First, we estimate the heritability of adult colour pigmentation (i.e. phenotype) with parent–offspring regression to test whether the phenotypic expression of coloration in adults is genetically controlled. Secondly, we examine performance of different colour morphs once the larvae reared under different larval densities reached adulthood. From a life-history point of view a highly competitive environment is expected to be costly (Roff 2002; Zuk & Stoehr 2002; Rantala & Roff 2005). Therefore, compared with favourable conditions, larvae grown in aggregated groups are expected to have shorter developmental time (e.g. escaping the costly environment; Goulson & Cory 1995; Sheldon & Verhulst 1996) and to experience high mortality. Thirdly, as larval aggregations are prone to pathogen infections (Goulson & Cory 1995),

achieving immunity under life-history bound resource costs could allow for differential resource allocation between adult morphs in the challenging environment (Svensson, Sinervo & Comendant 2001; Zuk & Stoehr 2002). If melanin pigmentation is linked to activation of immune system in the wood tiger moth (see also Friman *et al.* 2009), increased disease risk may also constrain warning signal expression by increasing cuticular melanization (Aso *et al.* 1985; Wilson & Reeson 1998; Barnes & Siva-Jothy 2000). Thus, we predict an increase in the area of wing melanization for individuals in the aggregated environment as an indication of investment in immune defence together with up-regulated immune responses. If adult colour morphs also have differential immune responses it could reveal alternative strategies in pathogen resistance.

Materials and methods

REARING OF *PARASEMIA PLANTAGINIS*

Parasemia plantaginis used for the experiment originated from a laboratory stock (12th generation), and experimental individuals derived from 11 generations of two divergent selection lines for larval coloration (small or large melanization pattern, see Lindstedt, Lindström & Mappes 2009); hereafter they are referred to as 'selection lines'. The experiment was conducted at the University of Jyväskylä, Central Finland (62°N, 26°E) from July until October 2008. Food (dandelion, *Taraxacum* sp.) was offered *ad libitum* during the larval period (for more details see Lindstedt *et al.* 2010b; Lindstedt, Lindström & Mappes 2009). Larvae were checked and fed, and rearing containers were cleaned daily until they reached adulthood.

Larvae were divided into treatment groups as follows: Larvae were first reared together in family groups consisting on average of 143 (SD ± 101) individuals (total number of families = 50) until their third instar under regular greenhouse conditions. On the third instar, larvae were divided into two different densities (solitary or aggregations). Solitary larvae were reared individually on petri dishes (diameter 90 mm) representing favourable (i.e. less stressful) growing conditions with typical density for later larvae instars. Aggregated larvae were reared in groups of 40 individuals in plastic rearing containers (100 × 130 × 120 mm). In total we had 1517 individuals divided among each treatment group (Fig. 1). After division, all experimental larvae were then reared in controlled environmental chambers (MLR-351; Sanyo, Etten-Leur, Netherlands, Table S1).

Wood tiger moth eggs are usually laid in egg clusters located in close vicinity, and all together contribution of one female can be up to 400 larvae. Given this, it is possible that in their early development larvae are in even more dense conditions than in our experiment's aggregations, but as larvae grow, they soon start to disperse and larval clusters will be scattered. In this experiment, however, our goal was to seek for correlated fitness characters by manipulating growing conditions of larvae.

ADULT PHENOTYPE DETERMINATION

The visual difference between adult yellow and white male colour morph was confirmed by spectrophotometer measurements

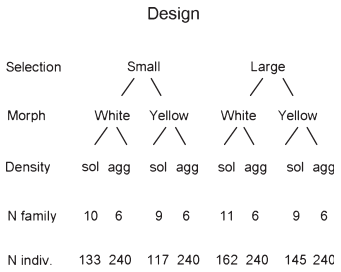

Fig. 1. Schematic illustration of the study design used to examine density-dependent effects on mortality and immune defence in two colour morphs of wood tiger moth males (white, yellow). *Selection* represents the origin of the larvae based on selection lines for more or less melanization (or conversely small or large warning signal patch) deposited on larval coloration. *Morph* represents the colour morph of fathers. *Density* refers to the larval rearing density treatment (solitary treatment (*sol*) larvae were grown individually and aggregated treated (*agg*) larvae were grown in groups of 40 individuals). Next, *n family* indicates number of families in treatments, and *n indiv*. stands for total number of individuals at the start of experiment. Each family consists of on average 14 members. In aggregations we used 24 rearing containers resulting in a total six high-density containers in each treatment. In each of high-density containers we divided members into four different families to control for family-based close relatedness.

(Nokelainen *et al.* 2012), allowing easy classification of colour morphs of eclosed adult moths by eye. We used digital photographing to quantitatively measure the variation in wing melanization pattern in adult males. Photographs were taken of dead specimens with a FujiFilmFinepix S3 Pro UVIR digital camera with standard illumination (Arcadia Reptile D3; Salfords, Redhill, UK). A subsample of adult individuals was used to measure variation in melanization pattern between treatment groups (Table S2). The coverage of wing melanization pattern was measured with Paint.Net – software (dotPDN LLC) – from right fore- and hind wing. The proportion of melanization pattern on the wings was then calculated by dividing the area of melanization pattern by the total area of the wings.

HERITABILITY OF COLOUR PIGMENT

Heritability of wing coloration was estimated using the regression between mean trait values of sire and their singly reared offspring. First, the average colour expression of a family from known sire was calculated. Then, the colour of male progeny (white, yellow) was regressed against the colour morph of the sire in linear regression. The obtained heritability estimate then equals twice the slope of the regression line (Lynch & Walsh 1998). This approach was chosen for categorical trait value, as the colour polymorphism in wood tiger moth is likely the result of two or more linked genes that reside on the sex chromosomes (Honkola *et al.* unpublished).

Relationships between larval density, colour morph of the sire (white, yellow) and selection line (small, large melanin patch) on adult wing colour (white or yellow) of the eclosed moths was analysed in binary logistic regression starting with the full model,

Table 1. Binary logistic model fitting of the eclosion proportions of two male colour morphs of wood tiger moth: relationships between colour morph of the sire, larval density, larval selection line and their interactions. Chi-square statistics describe the difference between log-likelihood function for the current model and initial model; the lower the chi-square statistics the more accurate the model. The degrees of freedom for the model chi-square statistics are equal to the difference between the numbers of parameters estimated in the current model. Difference χ^2 *df* 1 describes the χ^2 improvement to the next step if one term is removed from the model. Underline describes the best model fit

Model	Term removed	χ^2	DF	Difference χ^2 df 1
1. D + S + C + D × S + D × C + C × S + D × C × S		32·178	7	
2. D + S + C + D × S + D × C + C × S	D × C × S	31·691	6	−0·487
3. D + S + C + D × S + D × C	C × S	31·575	5	−0·116
4. D + S + C + D × S	D × C	31·388	4	−0·187
5. D + S + C	D × S	30·928	3	−0·460
6. S + C	D	29·864	2	−1·064
7. C	S	28·413	1	−1·451
8. intercept	C	<0·001	0	−28·413*

D = density, S = larval selection line & C = Colour morph of the sire. + describes the main effects, whereas × stands for interactions. All models incorporate constant, but in the last step it is highlighted. * Sig < 0·001 departure from Chi-square distribution

and omitting non-significant ($P > 0{\cdot}05$) covariates stepwise (Table 1). This test was done using IBM SPSS statistics 19·0 software (Armonk, NY, USA).

REARING DENSITY AND FITNESS

To measure the costs and benefits affecting the fitness of colour morph of the eclosed adult moth, we measured life-history responses from larvae and adult males. The overall larval mortality was calculated from all individuals who died before the adult stage. Developmental time from larva to pupation was measured in number of days. As soon as individuals reached the pupation stage they were weighted to the closest milligram (mg). In addition, we counted amounts of eclosed adult moths and their colour morphs. As colour morphs cannot be distinguished before adult stage, the measure of eclosed adult moths serves as an indirect measure of morph-dependent mortality.

Total larval mortality was analysed using a Chi-square test, as it is frequency-based data. In addition, frequencies of eclosed adult moths were analysed using a Chi-square test. First, frequencies of eclosed adult moths were tested over all treatment groups, and then eclosions were tested on both density groups. Linear mixed-effects (LME) analysis was used to examine the life-history responses of interest using R version 2·12·2 (R Development Core Team 2009). Models were simplified starting from the full model and sequentially removing non-significant interactions ($P > 0{\cdot}05$). All main effects were kept in the final model. To account for the fact that individuals from the same rearing containers may express similar kind of responses, we incorporated rearing container (i.e. the jar effect) as a random effect in the intercept. As there is no within-jar variance for individuals reared solitarily, variance for the low-density individuals was fixed to zero, but allowed to be estimated for the aggregated treatment. We constructed linear mixed-effects models for two response variables: developmental time and pupa weight. The explanatory variables were colour morph of the eclosed adult (*morph*), growth density (*density*) and selection line for larval coloration (*selection*). We incorporated the jar as a random effect in the intercept as indicated above.

IMMUNE DEFENCE AND MELANIN EXPRESSION

For immune defence assays, a subsample of individuals from each treatment was taken to measure the variation in immunological parameters (Table S2). Encapsulation assessment (i.e. induced response) is a commonly used method to stimulate an animal's response against foreign intrusions (e.g. parasitoids) (Schmid-Hempel 2005). Encapsulation assessment was conducted using the fifth instar larvae, which were reared on average 29 days and reaching a mean weight of 208·62 mg (SD ± 39·38) before the immune assays. Larvae were anaesthetized with carbon dioxide (CO^2), after which a sterilized nylon implant (diameter = 0·11 mm, length = c. 6 mm) was inserted inside the larvae between the second and the third segments from the dorsal side of the larva (Ojala *et al.* 2005; Friman *et al.* 2009). The larva was kept still by hand under the microscope while approximately two thirds of the implant was inserted with tweezers into the larva. The immune system of the larvae was allowed to react for 5 h before the implant was removed by pulling on the remaining one third of the implant outside the larva. The resulting encapsulating reaction was seen as a darkening of the implant. Five hours reaction time was selected because it yields optimal darkening of samples for later analysis with image software. Shorter time would produce too pale and longer times completely blackened samples (Ojala *et al.* 2005; Friman *et al.* 2009). The implant was dried and photographed under a microscope with 10× magnification using a Panasonic wv-CL702 (Panasonic, Osaka, Japan) video recorder. The mean grey value of the implant was measured with ImagePro Plus 4·0 (Media Cybernetics, Rockville, MD, USA) on 1 mm of the implant measured from the end implanted inside the larva to avoid measuring melanized tissue formed at the wounding sites. The grey value of the background was subtracted from the grey value of the implant to correct for any variation in lighting during photography. Three measurements were taken from each implant and their average was used. Higher grey values (darker implant) indicated stronger response of encapsulation.

Lytic activity against bacterial pathogens was determined using the area of inhibition (i.e. lytic zone) assay. Unexpectedly, the traditional method testing lytic activity (e.g. Kurtz & Sauer 1999) did not succeed to give a response, and thus we modified the method as follows. Prior to the experiment agar plates were prepared containing a solution of 10 g of Nutrient Broth, 2·5 g of yeast extract, 15 g of agar and 1 L of distilled water after which the solution was autoclaved for 25 min. The bacteria solution (*Micrococcus luteus*, ATCC strain 4698) was grown in the LB medium in 34 degrees Celsius until the lag phase. After this it was kept in the stationary phase to keep the bacterial concentra-

tion stable. Next, we injected 500 μL of the live bacterial solution onto each petri dish (diameter = 90 mm) yielding a dense film of bacteria on top of the plate. To attain a homogenous coverage of the culture across the agar solution we smeared manually with a heat sterilized spreader. Plates were then left to dry for 15 min to attain gelatinous finishing, after which five sterile filter discs (diameter = 5 mm) were placed on the plate in cross formation to designate spots for haemolymph samples.

As a measure of the lytic activity of haemolymph we measured the area of inhibition on the agar plates. 10 μL of sterile water was first added to the centremost filter paper as a negative control, which was used to control that any produced zones of inhibition were due to antimicrobial activity and not a failure in bacterial growth. The haemolymph sample was taken from the larva from the insertion site of the nylon implant by injecting a sterile needle between the second and third segment of the larva, and allowing the haemolymph to form a droplet. This was done before the nylon implant (see above) was inserted, and thereby, lytic activity of haemolymph serves as a measure of constitutive immunity (Schmid-Hempel 2005). We then withdrew 10 μL of haemolymph with a pipette and ejected it on a filter paper disc on the agar plate. Some of the samples expressed a rapid melanization reaction of haemolymph, which is often due to a strong phenoloxidase reaction (PO; Cerenius & Söderhäll 2004), and this was categorized as absent or present based on whether it occurred less than five minutes after the sample was ejected onto the petri dish. All petri dishes were kept at room temperature (+25 °C) for 3 days, after that, the plates were photographed and the area of inhibition was measured using Image J software. We subtracted the maximum area of the inhibition from the area of the filter disc as a data point. Area of inhibition was always visible around the filter disc when it occurred, but some samples never expressed the area of inhibition. Larval weight at immune assays did not differ between colour morphs of the eventually eclosed adult moths ($F_{1,68}$ = 0·047, P = 0·829) or between larval selection lines ($F_{1,68}$ = 0·680, P = 0·413).

Linear mixed-effects (LME) models with R version 2·12·2 (R Development Core Team 2009) were used to examine encapsulation and lytic activity. All models were simplified starting from the full model and removing non-significant interactions (discard P > 0·05, retain P < 0·05). All main effects were kept in the final model. All models incorporate jar as a random effect in the intercept to control for the similarity within rearing containers. As there is no within-jar variance for individuals reared solitarily, within-jar variance for the low-density individuals was fixed to zero yet allowed to be estimated for the aggregated treatment. LME analyses were carried out to explain both encapsulation response and lytic activity of haemolymph (i.e. area of inhibition) in relation to colour morph of the eclosed adult (*morph*), larval density (*density*) and selection line for larval coloration (*selection*). Generalized linear mixed model (GLMM) with a binomial response variable (absent or present) and a logit link function were used to test rapid melanization of haemolymph (PO) reaction, where colour morph of the eclosed adult (*morph*), larval density (*density*) and selection line for larval coloration (*selection*) were set as fixed factors. The model was fit by Laplace approximation using the lmer function in R package lme4 (Bates & Maechler 2009). Proportional melanization of adult wings was analysed using LME analysis, where wing melanin expression was set as dependent variable, and colour morph of eclosed adult individual (*morph*), larval density (*density*) and selection line for larval coloration (*selection*) were fixed factors. Finally, immunological traits and wing melanization were tested with Spearman correlation coefficients, to examine potential relationships between them. All reported P-values are two-tailed tests.

Results

HERITABILITY OF COLOUR PIGMENT

The heritability of adult colour pigmentation was h^2 = 0·422. This was obtained from the average colour of male progeny, which was regressed against the colour morph of the sire in linear regression ($F_{1,35}$ = 4·462, P = 0·042, B = 0·211, SE = 0·100). Colour pigmentation of eclosed males was best explained by colour morph of the sire (*Wald* = 27·184, DF = 1, P < 0·001, OR = 0·306), which reflects fathers getting higher proportion of sons similar in phenotype (Fig. 2). In spite of this, white males still sired 38·2% of yellows and yellow males sired 33·1% of whites of those individuals that survived to eclosion. Density, larval selection line and possible interactions were non-significant when colour morph of eclosed adult moth was included in the model (Table 1).

REARING DENSITY AND FITNESS

High larval density increased larval mortality (χ^2 = 405·258, DF = 1, P < 0·001): 17·2% of solitary reared larvae (N = 557) died during rearing, whereas 70·8% larvae grown in aggregations died (N = 960). Development time was 5 days (or 8·1%) faster in aggregations (Table 2), but was not dependent on selection line or colour morph of the eclosed adult moth. On average, developmental time for solitarily grown white and yellow males was 54 (SE ± 1·445) and 54 (SE ± 1·537) days, compared to 50 (SE ± 1·942) and 48 (SE ± 1·625) days in aggregations respectively. The average pupa weight of solitary larvae (mean = 207 mg, SE ± 3·031) was 27·4%

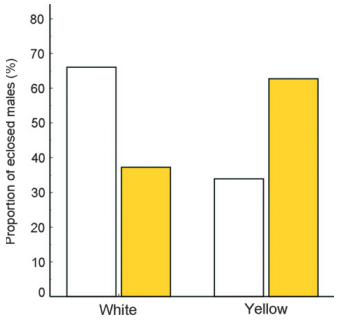

Fig. 2. The proportion of eclosed white and yellow adult morphs. Pooled data accounting both growth environments are used. On the *x*-axis, the colour morph of the sire, and on the *y*-axis the proportion of eclosed colour morphs. White bars represent the white males and yellow bars the yellow males (grey bars in print version).

Table 2. Linear mixed-effects model analysis of examined life-history responses. The model represents the best fit model to explain life-history response (developmental time and pupa weight) in relation to colour morph of the eclosed adult (*morph*), larval density (*density*) and selection line for larval coloration (*selection*). Rearing jar is incorporated as a random effect in the intercept

Response variable	Estimate	SE	T	*P*
Developmental time				
(intercept)[a]	54·933	1·717	31·976	< 0·001*
Morph	−1·158	1·607	−0·720	0·472
Density	−7·702	3·780	−1·859	0·064
Selection	−0·914	1·988	−0·459	0·646
Pupa weight				
(intercept)[a]	205·981	5·084	40·513	< 0·001*
Morph	2·071	4·738	0·437	0·663
Density	−34·487	11·175	−3·085	0·002*
Selection	1·857	5·879	0·315	0·752

*$P < 0·05$.
[a]Intercept includes factor levels: morph [white], density [solitary], selection [less melanized].

heavier than in aggregations (mean = 181 mg, SE ± 3·685, Table 2).

The eclosion of the different adult colour morphs was density-dependent ($\chi^2 = 3·941$, DF = 1, $P = 0·047$). Colour morphs eclosed at the same proportions from solitary treatment ($N_{white} = 112$, $N_{yellow} = 104$, $\chi^2 = 0·296$, DF = 1, $P = 0·586$), but more yellow males eclosed from aggregated treatment ($N_{white} = 50$, $N_{yellow} = 73$, $\chi^2 = 4·301$, DF = 1, $P = 0·038$). In solitary treatment 51·9% of eclosed males were white and 48·1% were yellow, whereas in aggregations 40·7% of eclosed males were white and 59·3% were yellow.

IMMUNE DEFENCE AND MELANIN EXPRESSION

From the eclosed adult moths white males had seemingly higher encapsulating ability levels while larvae in aggregations than yellow males (Fig. 3), and encapsulation was in general increased by density (Table 3). Nevertheless, yellow males had a larger zone of inhibition while larvae than white males in aggregations (Fig. 3, Table 3). The rapid melanization of haemolymph was affected by an interaction of melanin and colour morph of the eclosed adult moth (Table 4), as white males that were less melanized as larvae were less able to produce a more rapid PO reaction than rest of the groups.

The wings of yellow males in general were more melanized (60·1%, SE = 0·01) than white males (54·6%, SE = 0·01). There was also an interaction between colour morph of eclosed adult moth and larval selection line affecting the area of wing melanization (Table 3) because white males that were less melanized as larvae were less melanized in general compared with other groups. In general, however, the more melanin expressed in the larval stage translated to more melanin expressed in the adult stage (Fig. 4, Table 3). The rapid melanization of haemolymph (PO) was correlated with encapsulation ability but not with haemolymph's lytic activity or wing melanization (Table 5).

Discussion

These results demonstrate that wood tiger moth males have differential morph-linked immune responses. First, we confirm heritability of wing colour ($h^2 = 0·42$), which indicates that the colour polymorphism of wood tiger moth is genetically determined, and not favoured by a plastic response of colour expression to differing environments. The eclosion of adult morphs, however, was dependent on growth environment. White adult morphs have increased encapsulation ability when reared in aggregated larval environments, whereas yellow morphs express increased lytic activity of haemolymph.

Immune responses are generally stronger when individuals are grown in challenged conditions (Wilson & Cotter 2008), and thus, immune defence can have consequential trade-offs with life-history traits (Rantala & Roff 2005; but see Freitak *et al.* 2005) or competence (Kraaijeveld & Godfray 1997; Kortet, Rantala & Hedrick 2007). We observed a 53 percentage point higher larval mortality in aggregations than in solitary treatment suggesting that the aggregated environment was costly (Kazimirova 1992; Wilson *et al.* 2002; Cotter *et al.* 2008). Furthermore, there was a trend that aggregated larvae pupated faster than solitary ones, which may suggest a life-history strategy of quickly escaping the highly competitive and potentially contagious environment (Goulson & Cory 1995; Longson

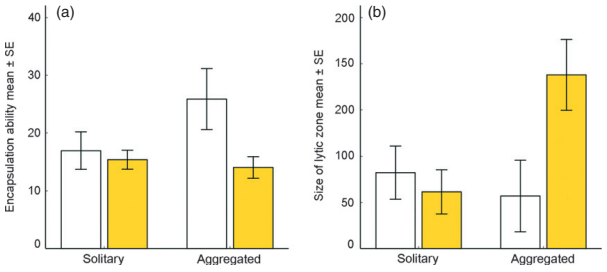

Fig. 3. The immunological responses of eclosed male wood tiger moth colour morphs to density treatment. White bars represent the white males and yellow bars yellow males (grey bars in print version). (a) On the *x*-axis, the larval growth density, and on the *y*-axis the mean encapsulation ability. (b) On the *x*-axis, the larval growth density, and on the *y*-axis the size of lytic zone ability (mm^2).

Table 3. Linear mixed-effects model analysis of examined immunological responses and phenotypic melanin expression of wood tiger moth males to density treatment. The model represents the best model fit to explain assigned responses in relation to colour morph of the eclosed adult (*morph*), larval density (*density*) and selection line for larval coloration (*selection*). Rearing jar is incorporated as a random effect in the intercept

Response variable	Estimate	SE	T	P
Encapsulation ability				
(intercept)[a]	13·747	3·979	3·623	< 0·001*
Morph	1·101	5·242	0·193	0·849
Density	16·726	7·096	2·357	0·022*
Selection	5·886	5·137	1·145	0·257
Morph × density	−15·118	8·049	−1·878	0·084
Morph × selection	−4·434	7·581	−0·572	0·577
Density × selection	−14·340	9·792	−1·464	0·149
Morph × density × selection	13·339	10·780	1·237	0·239
Lytic activity				
(intercept)[a]	71·124	31·729	2·241	0·002*
Morph	−17·030	38·684	−0·440	0·664
Density	−7·863	58·080	−0·135	0·892
Selection	19·916	34·798	0·572	0·569
Morph × density	156·543	66·071	2·369	0·028*
Wing melanin expression				
(intercept)[a]	0·627	0·016	39·013	< 0·001*
Morph	0·012	0·018	0·640	0·525
Density	−0·005	0·017	−0·029	0·772
Selection	−0·103	0·018	−5·568	< 0·001*
Morph × selection	0·050	0·023	2·141	0·037*

*$P < 0·05$.
[a]Intercept includes factor levels: morph [white], density [solitary], selection [less melanized].

Table 4. Generalized linear mixed model analysis of rapid melanization of haemolymph (PO). The model represents the best model fit to explain haemolymph melanization response in relation to colour morph of the eclosed adult (*morph*), larval density (*density*) and selection line for larval coloration (*selection*). Rearing jar is incorporated as a random effect in the intercept

Response variable	Estimate	SE	Z	P
Melanization of haemolymph				
(intercept)[a]	−0·142	0·526	−0·271	0·786
Morph	−0·506	0·659	−0·767	0·442
Density	0·457	0·501	0·913	0·361
Selection	−1·952	0·803	−2·431	0·015*
Morph × selection	2·450	1·024	2·392	0·016*

*$P < 0·05$.
[a]intercept includes factor levels: morph [white], density [solitary], selection [less melanized].

& Joss 2006; Ojala, Lindström & Mappes 2007). This evidence would also explain why pupae in aggregations were 27·4% smaller than solitary ones (Cotter, Kruuk & Wilson 2004; Cotter *et al*. 2004). Although individuals were given food *ad libitum*, solitary larvae likely had more resources *per capita*, as competition was excluded. Interestingly, we observed morph-dependent mortality in aggregations where there was a 18·6 percentage point difference in eclosion frequencies, whereas in solitary treatment there was no difference suggesting divergence in underlying traits in challenging conditions.

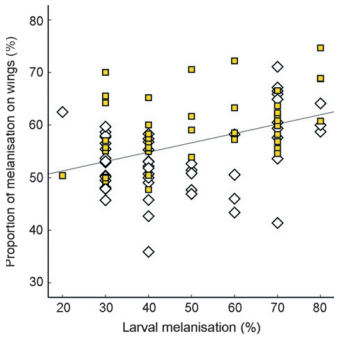

Fig. 4. Relationship between melanization of larvae (*x*-axis) and the proportion of melanization pattern on the adult wings (*y*-axis). White diamonds represent white adult males and yellow squares represent yellow males (grey squares in print version). The linear fit line $R^2 = 0·187$.

The immunological assays revealed a difference between two colour morphs of eclosed adult moths in two immune defences. Larvae that later eclosed as white males had a higher encapsulating response than individuals that eclosed as yellow males when grown in aggregated treatment (see Fig. 3). This hints of competent parasitoid resistance of white males suggesting that while larvae they are well able to encapsulate foreign intrusions (e.g. eggs of parasitoids) from their bodies (Rantala & Roff 2005; Siva-Jothy, Moret & Rolff 2005; Wilson & Cotter 2008). In comparison to encapsulation ability, the lytic zone assay indicated yellow males being better in mounting their lytic activity of haemolymph in aggregations than white males, which in turn suggests an increased defence against bacterial pathogens (Morishima *et al*. 1995; da Silva, Dunphy & Rau 2000; Shelley 2004). This pattern hints of correlational selection on larval stage. It could be that larvae have intrinsic genetic mechanism that regulate their resource-use physiologically depending on which morph individual is going to develop into. Also, morph may be bound to store their resources differently regarding the use of resources later in life history, which can result in differential investment in immune defence.

The encapsulation ability is usually linked to PO cascade via regulated series of micropeptide formation initiating from phenoloxidase proceeding with an oxidation of tyrosine to dopaquinone and further polymerization to melanin (Ashida & Brey 1998; Cerenius & Söderhäll 2004). Hence, low encapsulation of yellow males may be due to early depletion of tyrosine that prevents the completion of the PO cascade (Riley 1997). Furthermore, if yellow pigmentation is more costly to produce (Blount

Table 5. Correlations between immunological traits and wing melanization area

	Encapsulation	Phenoloxidase	Lytic activity	Wing melanization
Encapsulation				
Phenoloxidase	0·295* (74)			
Lytic activity	−0·014 ns (74)	0·040 ns (85)		
Wing melanization	0·055 ns (13)	−0·113 ns (17)	0·421 ns (17)	

*$P < 0{\cdot}05$.
ns = non-significant effect. Sample sizes are indicated inside brackets. All correlations are two-tailed nonparametric Spearman correlation coefficients.

et al. 2009), it can suggest a trade-off between PO activity and cuticular pigmentation (Gotz & Boman 1985; Rolff & Siva-Jothy 2003; Siva-Jothy, Moret & Rolff 2005; Cotter *et al.* 2008). On the other hand, possibly all immune defence traits simply cannot be simultaneously up-regulated (Cotter, Kruuk & Wilson 2004; Rantala & Roff 2005) or there can also be a potential cost of autoimmunity associated with immune responses (e.g. Sadd & Siva-Jothy 2006). To confirm whether encapsulation reaction trade-offs with lytic activity, we would need to perform a more detailed infection experiment to exclude the possibility that measured immunological pathways are merely two ways to obtain equal pathogen resistance.

Immune responses are often found to correlate with other individual traits. Abundant evidence suggests that cuticular melanization is an indication of investment in immunity (e.g. Reeson *et al.* 1998; Wilson *et al.* 2001; Cotter *et al.* 2008; Friman *et al.* 2009; but see Jacot *et al.* 2005; Joop *et al.* 2006; Karl, Hoffmann & Fischer 2010). However, here we did not find evidence that wing melanization would have indicated an investment in immunity, but instead, more melanin expression on the larval stage yielded larger area of wing melanization in the adult stage. Furthermore, white males from the less melanized selection line comprise less melanized wing patterning compared with respective yellow males resulting in a significant interaction. Perhaps yellow males share partly the same metabolic pathway to produce yellow and melanin pigmentation, but this needs further confirmation. One reason why we did not observe the cuticular melanization correlating with immune responses could be that the cuticular pigments are synthesized independently of the immune response. The rapid melanization of haemolymph (PO) had a significant interaction effect between colour morph and selection line because white males from less melanized larval selection lines expressed rapid melanization of haemolymph less often than the rest of the groups. We do not have plausible explanation of this interaction, and future work will be needed to understand if the difference between morphs that eclose from high melanin larvae has any biological significance. Haemolymph's phenoloxidase (PO) activity and encapsulation reaction were the only correlations found between the immune traits. However, as we measured PO reaction as presence–absence trait and not gradual accumulation of phenoloxidase activity, we must be cautious interpreting its role (Ashida & Brey 1998; Cerenius & Söderhäll 2004; Shelley 2004). Also, it is possible that sample sizes of the correlation analysis set limitations to its further interpretation.

There are a wide variety of mechanisms driving the maintenance of colour polymorphism (Gray & McKinnon 2007), and likely many different selection pressures influence to the puzzle of polymorphism in aposematic species. For instance, confirmed warning signal efficacy and mating success trade-off in the wood tiger moth is a plausible explanation to the maintenance of colour polymorphism (Nokelainen *et al.* 2012). Furthermore, the efficacy of warning signal likely depends on heterogeneity in predator community (Endler & Mappes 2004). It is also possible that spatial heterogeneity of the parasitic and disease community can create variable selection on immune responses. As immune responses are morph linked, variable enemy communities can indirectly contribute to colour polymorphism maintenance. It would be interesting to study the role of immune defence directly to see how susceptible colour morphs are against different parasitoids and pathogens, and furthermore, to examine in the wild whether the abundances of parasitoids and pathogens would tie in with the colour morph frequencies of the wood tiger moth. Regardless of these mechanisms our results suggest that genes underlying the phenotypic traits can be selected differently in heterogeneous environment, which may cause selection to favour polymorphism in aposematic species. The maintenance of polymorphism in aposematic species can therefore be contributed by environment through trade-offs, or even alternative strategies that are advantageous in different environmental conditions.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Standardised rearing conditions.

Table S2. Sample sizes of three different data sets: proportional melanisation of wings (*wing melanin*), encapsulation assessment (*encapsulation*), and lytic activity of haemolymph (*lytic activity*).

Table S1. Standardised rearing conditions. Moth larvae were reared in family groups until the third instar in greenhouse conditions. Greenhouse temperature range varied between 20-30°C (day is 18 h), and during the night (6 h) it decreased to 15-20°C. After division to experimental groups larvae were reared under fluctuating temperature and light regimes in controlled environmental chambers (Sanyo, MLR-351). The light/temperature rhythm was set to mimic Finnish summer conditions in a continuous loop. In table; *time* indicates the time of procedure start, *temp* indicates temperature in degrees of Celsius, and *light* stands for the light environment. Before and after bright day light conditions lights were set on dim to mimic sunrise and sunset.

time	00:00	06:00	08:00	11:00	18:00	21:00
temp	16	18	22	25	22	18
light	dark	dim	light	light	light	dim

Table S2. Sample sizes of three different data sets: proportional melanisation of wings (*wing melanin*), encapsulation assessment (*encapsulation*), and lytic activity of haemolymph (*lytic activity*). *Line* is selection line for small or large larval melanisation levels, *morph* is white or yellow for different male morphs, and *density* is solitary (*sol*) or aggregated (*agg*) for larval growth density manipulation.

line	small				large			
morph	white		yellow		white		yellow	
density	sol	agg	sol	agg	sol	agg	sol	agg
wing melanin	8	8	15	9	23	22	13	22
encapsulation	10	5	12	8	11	9	8	10
lytic activity	11	5	12	13	14	9	7	10