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Geographic Variation in the Warning Signals of the Wood Tiger Moth
(*Parasemia plantaginis*; Arctiidae)

JYVÄSKYLÄN YLIOPISTO

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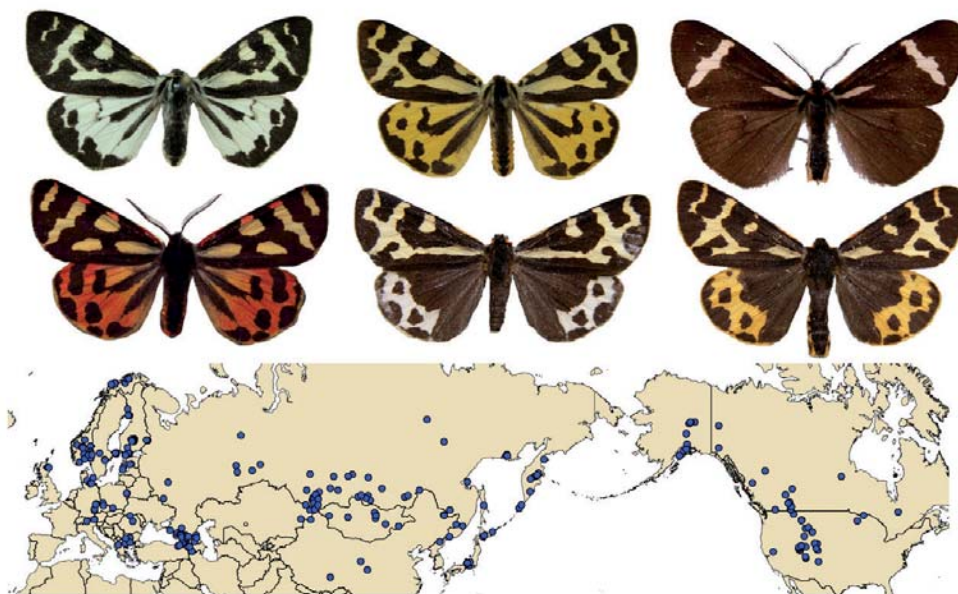
UNIVERSITY OF JYVÄSKYLÄ

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Cover picture by Robert Hegna

Of Research

"Keep your eyes on the stars, and your feet on the ground."
--Theodore Roosevelt

"There's nothing more dangerous than an idea if it is
the only one you have"
--Mark Twain

"If a cluttered desk is a sign of a cluttered mind,
of what, then, is an empty desk a sign?"
--Albert Einstein

"Energy and persistence conquers all things"
--Benjamin Franklin

"There will be plenty of time to sleep
once you are dead"
--Benjamin Franklin

ABSTRACT

Warning signals are not expected to be diverse because they are under stabilizing selection. This dissertation aims to study historic and contemporary selection mechanisms underlying the geographic warning signal diversity in the wood tiger moth (*Parasemia plantaginis*). In the first study, I explored the variation in hindwing warning color frequencies across the distribution of *P. plantaginis* and the phylogeography of the species. Males have polymorphic hindwing color (yellow or white) across much of their distribution but turn reddish in the Caucasus and mostly white in Japan, which coincide with mitogenetic divergence. Females vary continuously in hindwing warning color between yellow and red, becoming yellow east of the Ural mountain range, but does not correspond to mitogenetic divergence. Post-glacial isolation may be one contributing factor to the genetic divergence and the hindwing warning signal differences in the Caucasus region. In the second study, I found that thermoregulation benefits of melanin can trade-off with a larger more effective warning signal, which contributes to variation in melanization within Europe. In the third study, I found that forewing patterns function as warning signals and that generalization by predators might play an important role in warning signal diversity along with frequency dependent selection. In the fourth chapter, I found three populations in Japan differing in warning signal traits with some gene flow suggesting both selection and isolation may be underlying causes. Overall, my results suggest that historical and abiotic factors contribute to the observed warning signal diversity in *P. plantaginis*, rather than predation alone. This adds to our understanding of how historical factors along with environmental heterogeneity in biotic and abiotic factors can promote warning signal diversity. My results highlight the need to take an integrated approach to studying geographic diversity in warning signals.

Keywords: aposematism; color polymorphism; geographic variation; Holarctic; opposing selection; *Parasemia plantaginis*; phylogeography.

Robert H. Hegna, University of Jyväskylä, Department of Biological and Environmental Science, P.O. Box 35, FI-40014 University of Jyväskylä, Finland

Author's address   Robert H. Hegna
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland
robert.hegna@jyu.fi

Supervisors   Professor Johanna Mappes
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland

Dr. Juan A. Galarza
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland

Reviewers   Dr. Mathieu Joron
CNRS Group Leader
Muséum National d'Histoire Naturelle
Departement Systématique et Evolution
CNRS UMR 7205, Case postale 50
45, rue Buffon
75005 Paris
France

Assistant Professor Maren Wellenreuther
Lund University, Box 117
SE-221 00 Lund
Sweden

Opponent   Professor Kyle Summers
East Carolina University
Department of Biology
Howell Science Complex N314
Greenville, NC 27858
USA

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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   Hegna, R.H., Galarza, J.A., & Mappes, J. 2013. Global phylogeography and geographic variation in hindwing warning color of the aposematic wood tiger moth (Arctiidae) with implications for the evolution of color polymorphism and sexual dimorphism. Manuscript.

II   Hegna, R.H., Nokelainen, O., Hegna, J.R., & Mappes, J. 2013. To quiver or to shiver: increased melanization benefits thermoregulation, but reduces warning signal efficacy in the wood tiger moth. *Proceedings of the Royal Society B* 280: 1755.

III   Hegna, R.H., & Mappes, J. 2013. Influences of geographic differentiation in the forewing warning signal of the wood tiger moth in Alaska.
Submitted Manuscript.

IV   Hegna, R.H., Honma, A., Galarza, J.A., & Mappes, J. 2013. Genetic and phenotypic divergence among aposematic wood tiger moth populations in Japan. Manuscript.

The table shows author contributions for the original papers. Smaller contributions are stated in the acknowledgements of the original papers.

	I	II	III	IV
Original Idea	JM, RH, JAG	RH, JM	RH, JM	RH, JM, AH
Data	RH	RH, JH, ON, JM	RH	AH, RH
Analyses	RH, JAG	RH, JM	RH, JM	RH, JAG, AH
Writing	RH, JAG, JM	RH, JM, ON, JH	RH, JM	RH, AH, JAG

RH= Robert H. Hegna, JM= Johanna Mappes, JAG= Juan A. Galarza, ON= Ossi Nokelainen, JH= Jonathan R. Hegna, AH= Atsushi Honma.

1 INTRODUCTION

1.1 Biological significance of coloration

Studies of animal coloration have laid important groundwork for studying natural selection (Wallace 1879, Poulton 1890, Cott 1940, Endler 1980, Majerus 1998, True 2003, Gray & McKinnon 2007, Cook et al. 2012). Coloration is usually closely tied to fitness because it plays important roles in mate choice (Graham et al. 1980, Endler & Houde 1995, Summers et al. 1999, Maan et al. 2004, Maan & Cummings 2008, Estrada & Jiggins 2008), thermoregulation (Tsuji et al. 1986, Guppy 1986b, Davis et al. 2012), and protection from predators (Wallace 1879, Endler 1983, Ruxton et al. 2004). Therefore, the study of color and how animals use coloration is necessary to fully understand the natural history, ecology, and evolution of any organism (Fogden & Fogden 1974).

Butterflies and moths (Lepidoptera) provide an ideal taxonomic group for integrative research on evolution and selective pressures in part because of the variation in coloration between and within species (Brakefield & French 1999). Diversity in the wing patterns and color of lepidopterans has inspired research for over 150 years (Nijhout 1991, McMillan et al. 2002). In the Lepidoptera color patterns appear to be strongly shaped by selective forces because they are a prominent feature (McMillan et al. 2002). Research exploring developmental aspects of lepidopteran coloration has revealed flexibility in color pattern production that provides 'astonishing raw material' that selective forces can act on (Nijhout 1991, McMillan et al. 2002).

1.2 Geographic variation in aposematic species

1.2.1 Geographic variation for different prey

One of the most important long-standing debates in evolutionary biology concerns the relative importance of selection pressures and isolation at different

spatial scales (Endler 1977, Saccheri & Hanski 2006, Conover et al. 2006, Astrid et al. 2009). Genetic variation between populations is often manifested through local adaptations, clines, and has the potential to ultimately lead to speciation. For these reasons, species differing across their distributions in various traits were referred to as, "the most important to us," by Charles Darwin (Darwin 1859).

One of the most notable ways in which animals can vary over geographic space is in color, which is closely tied to fitness as previously described. Visual environment often changes over the distribution of an organism and brings challenges when trying to avoid predation by blending into the background because a change in background can potentially make it easier to be detected by predators. Prey that hide from predators by matching their backgrounds are termed cryptic (Edmunds 1974, Endler 1988, Stevens & Merilaita 2009). Thus, it is not surprising that cryptic organisms can be seen to vary in appearance over their distributions in ways that help them hide from predators (Kettlewell 1961, Forsman & Shine 1995, Cook et al. 2012, Linnen et al. 2013). However, some organisms are protected by defenses that make them unprofitable and advertise their unprofitability to predators with conspicuous warning signals. These organisms are termed 'aposematic' (Wallace 1878, Poulton 1890, Ruxton et al. 2004, Mappes et al. 2005) and often use conspicuous colors in their warning signals (e.g., yellow, orange, red combined with black) because such colors are easier for potential predators to learn and remember (Gittleman & Harvey 1980, Roper & Redston 1987, Lindström et al. 1999b). This leaves us asking whether it is reasonable to expect that visual warning signals of aposematic organisms should differ in the same way as cryptic prey over geographic space.

Warning signals of aposematic species have been of interest since the earliest days of evolutionary biology (Darwin 1859, Bates 1862, Belt 1874, Wallace 1878, Müller 1878, Wallace 1879, Poulton 1890). Effectiveness of warning signals is based on frequency and density because the more common a signal is, the easier it is for predators to learn to avoid (Lindström et al. 2001, Despland & Simpson 2005). Warning signal uniformity is also expected because predators learn to avoid and remember to avoid uniform signals better than variable warning signals (Lindström et al. 2001, Speed 2001). The benefit of signal uniformity should induce positive frequency-dependent selection that acts against novel or rare signals (Joron & Mallet 1998, Ruxton et al. 2004, Beatty & Beirinckx 2004, Rowland et al. 2010). Therefore, we are left with two predictions for the variation of warning signals over geographic space (assuming no environmental heterogeneity).

The first prediction is that within the same spatial proximity we should find warning signal homogeneity in the simplest of scenarios because positive frequency-dependent selection should act against foreign signals (Joron & Iwasa 2005, Sherratt 2006). The second expectation is that it should be difficult for new signals to arise in a population of warningly colored organisms because novel signals should be selected against (Mallet & Singer 1987, but see Alatalo & Mappes 1996). When the warning signal of an aposematic organism is variable across its geographic range or within the same population, then, questions are

raised concerning how the variant evolved and what currently maintains the differentiation.

Contrary to the predictions for warning signal homogeneity, many organisms possess variable warning signals (Endler & Mappes 2004). Some of the species found to have geographically varying warning signals include Neotropical *Heliconius* butterflies (Brown & Benson 1974, Brower 1996, Joron et al. 1999, Mallet 2010), ladybird beetles (Creed 1966, Brakefield 1985, Dolenská et al. 2009, Blount et al. 2012), monarch butterflies (Brower 1958, Davis et al. 2005, Davis et al. 2012), newts (Mochida 2009, Mochida 2011), poison frogs (Daly & Myers 1967, Savage 1968, Summers et al. 2003, Wang & Summers 2010, Wang 2011, Rudh et al. 2011, Maan & Cummings 2012, Willink et al. 2013, Richards-Zawacki et al. 2013, Meuche et al. 2013, Hegna et al. 2013b), velvet ants (Wilson et al. 2012), alpine leaf beetles (Borer et al. 2010), and bumble bees (Plowright & Owen 1980). Interestingly, some aposematic species appear to switch between aposematic and cryptic strategies across their distributions. Such geographic variation in warning signals has been documented in the strawberry poison frog (Wang & Shaffer 2008, Brown et al. 2010, Rudh 2013) and in a closely related sister species the granulated poison frog (Wang 2011, Willink et al. 2013, Brusa et al. 2013). Many of the best known examples of aposematic species exhibiting geographic variation in warning signals are those involved in mimicry complexes, such as burnet moths (Bovey 1941, Turner 1970, Sbordoni et al. 1979) and *Heliconius* butterflies (Turner 1970, Benson 1972, Brown et al. 1974, Brown & Benson 1974, Brower 1996). The term 'variation' has statistical connotations that can provide an additional layer of confusion when trying to describe warning signal differences over geographic space. Therefore, I use the phrase, "warning signal diversity," as a broad umbrella term and attempt to restrict 'variation' to be used in a statistical sense or to refer to characters that vary gradually.

1.2.2 Influence of local predators on warning signals

Across geographic space in nature predators will undoubtedly vary in abundance and density. Predator community variation is thought to have an effect on warning signal appearance (Endler 1988, Mappes et al. 2005). The signals themselves should be evolved to match the sensory ecology of specific predator(s) (Speed 2000). However, warning signals are likely influenced by more than a single predator, which can make a difference in the net selection acting on a warning signal (Lindstedt et al. 2011). Furthermore, predators likely differ within and between species in how distasteful they may find various aposematic prey defenses or abilities to detect signals (Thompson 1984a, Endler 1988, Exnerová et al. 2003, Endler & Mappes 2004, Mappes et al. 2005).

Studying how predator differences may influence warning signal diversity, though, is not straightforward because predation is rare and knowledge of which specific predators attack specific aposematic prey is limited. In natural conditions it is thought that predator changes over the geographic distribution of the poison frog *Oophaga pumilio* may partly explain intra-specific color diversity of morphs (Reynolds & Fitzpatrick 2007, Hegna et al. 2013b). At least two studies have

found some possible taxon-specific influences. The presence of common buzzards is also shown to be associated with increased attacks on aposematic patterns of European vipers (Valkonen et al. 2012). Another study by Nokelainen (2013) found that male wood tiger moths with yellow hindwings were attacked less than those with white hindwings when the predator community was dominated by avian predators in the Paridae family. However, the opposite was true when the predator community composition shifted towards being dominated by species of the family Prunellidae. Future work on the ways intra-specific predator variation and predator community changes influence will be key to understanding geographic structuring of warning signals.

1.2.3 Genetic drift, neutral forces, and Wright's shifting balance

The initial evolution of novel signals can also occur through processes other than selection, such as genetic drift and isolation (Mallet & Joron 1999). Genetic drift is simply a process that causes allele frequencies to change due to random chance so that not all alleles within a population are successfully passed from one generation to the next (Lande 1976, Hartl & Clark 1997). The effects and importance of genetic drift is most apparent in small populations where it is easier for random fluctuations in alleles to lead to fixation of certain alleles by chance (Hartl & Clark 1997). In the case of either genetic drift or isolation, gene flow with other populations is restricted, which can result from past geologic events that change the suitability of climate and limit dispersal.

Investigations into the phylogeography of several aposematic species have revealed that current or historic barriers to gene flow may impact warning signal diversity (e.g., Brown et al. 1974, Owen et al. 1994, Turner & Mallet 1996, Noonan & Gaucher 2006, Albuquerque de Moura et al. 2011, Hines et al. 2011, Gehara et al. 2013, Hill et al. 2013). Over the course of the late Holocene glacial cycles influenced the biota of northern and tropical regions. Expanding and contracting ice sheets have left genetic imprints on species whose distributions were influenced by those patterns (Schmitt & Hewitt 2004, Hewitt 2004, Provan & Bennett 2008) and diversity in general (Dynesius & Jansson 2000, Mittelbach et al. 2007). It has even been suggested that these climate oscillations influenced the evolution of dispersal ability and range sizes, selecting for greater dispersal ability in some species (Dynesius & Jansson 2000). During cycles of glacial expansion populations that were previously split became reunited when habitats and climate became more suitable for dispersal. Secondary contact between separated lineages has been thought to potentially be responsible for some observed polymorphisms in aposematic organisms (Owen et al. 1994, Noonan & Gaucher 2006). Interestingly, work with *Heliconius heurippa* has shown that contact between two species that differ in warning signal appearance can potentially result in speciation by the creation of a novel color pattern (Mavárez et al. 2006, Melo et al. 2009). Changing environmental conditions, such as through habitat modification by humans or changing climate, may also have the potential to cause warning signal differences by enhancing (Owen et al. 1994) or restricting gene flow (Legrand et al. 2011, Albuquerque de Moura et al. 2011).

One of the ways gene flow and drift can influence warning signal diversity is when natural selection begins to act in favor of a trait that evolved while a population was isolated. Selective forces acting on warning signals are likely never to be constant across the entire distribution of any species. Wright (1932) viewed the spatial variation in natural selection as a form of 'adaptive landscape,' across which the fitness of an individual varies. The landscape was filled with fitness peaks and valleys in accordance to an individual's genetic make-up. Wright referred to the idea as 'shifting balance' (Wright 1932, Wright 1978), which he described in three distinct phases. In the first phase, random local processes could allow a new trait to evolve, followed by a second phase where natural selection takes a population to the new adaptive peak, and the third phase when interdemic selection spreads the trait to other populations. Drift or fluctuating selection are two processes thought to allow populations to evolve toward new local fitness peaks in the first phase of Wright's shifting balance. However, Wright's idea of shifting balance has received some criticism (Coyne et al. 1997, Gavrilets 1997), with the first and third phases being viewed as the most controversial. Despite the criticism, the idea of an adaptive landscape has gained traction as a hypothesis for explaining initial evolution of warning signal diversity in Neotropical *Heliconius* butterflies (Mallet & Singer 1987, Mallet 2010) and at least one species of poison frog (Chouteau & Angers 2012) and other cases (Gavrilets 2004).

1.2.4 Frequency-dependent selection

Once a warning signal has been established within a geographic area, positive frequency-dependent selection (FDS) is thought to be responsible to maintain any geographic signal differences (Endler 1988, Mallet & Joron 1999, Sherratt 2006). Once predator-driven positive FDS begins to occur within an area, foreign warning signals should be selected against. This should result in active prevention of gene flow. Predator-driven positive FDS has been investigated theoretically (Joron & Iwasa 2005, Sherratt 2006), empirically under controlled conditions (Lindström et al. 2001), and supported by results from field experiments showing foreign signals are attacked more than local signals (see also Benson 1972, Smith 1979, Mallet & Barton 1989, Noonan & Comeault 2009, Chouteau & Angers 2012). When several different areas of a distribution differ in the predominant warning signal a geographic warning signal mosaic emerges with various locally adapted signals (Sherratt 2006). Such landscapes of locally adapted warning signals fit in with theoretical expectations for how warning signals might vary, but still raise the historical question over what may have caused the initial divergence.

Investigations into mimicry complexes of *Heliconius* butterflies in Central and South America are well-known examples of warning signal differences maintained by positive FDS. Studies in the field (Mallet & Barton 1989, Kapan 2001) and in the lab with local predators (Langham 2004) have found that predators from areas with local predominant morphs are more likely to attack foreign than local morphs. Indeed, even at a broader genomic level

differentiation across hybrid zones is greater at loci directly responsible for coloration than at neutral loci (Baxter et al. 2010).

Poison frogs represent another broad group that positive FDS has been explored as a mechanism maintaining warning signal differences in three species. At least three studies have found evidence that areas differing in predominant warning signal types show selection against foreign signal types in *Dendrobates tinctorius* (Noonan & Comeault 2009, Comeault & Noonan 2011) and *Ranitomya imitator* (Chouteau & Angers 2011, Chouteau & Angers 2012). However, not all studies conducted on poison frogs have found evidence for positive FDS. Two recent studies of predation on polytypic and polymorphic populations of *Oophaga pumilio* did not find evidence that positive FDS was involved in maintaining color differences in two populations (Hegna et al. 2013b, Richards-Zawacki et al. 2013). However, additional investigation would be needed across the geographic distribution of *Oophaga pumilio* where nearly 20 polytypic morphs occur to fully understand if positive FDS maintains differences in some locations (Hegna et al. 2013b). One additional explanation for why positive FDS may not always be found is that predators may generalize their avoidance of warning signals more broadly. Generalized avoidance of warning signals should result in weakened positive FDS (Ihalainen et al. 2012). A recent on *Oophaga histrionica* found some evidence to suggest predators generalized their avoidance to warning signals that differed in color and pattern of foreign warning signals (Amézquita et al. 2013).

Much of the work looking at whether predators avoid foreign warning signals and exert positive FDS that maintains warning signal differences has been done in tropical systems. Less is known about whether predators drive positive FDS in more northern locations that exhibit greater seasonality. The potential that the many migratory species of birds that visit northerly areas widely generalize their avoidance of aposematic taxa by general cues also exists (i.e., avoiding anything with bright conspicuous colors or patterns). Some work on alpine borer beetles in Europe has shown evidence of positive FDS (Borer et al. 2010), but work on the wood tiger moth has not (Nokelainen 2013, Hegna et al. 2013a).

1.2.5 Trait trade-offs and interactions

Conspicuous warning signals are thought to be better because they are more easily learnt and remembered by potential predators trying to avoid aposematic prey (Gittleman & Harvey 1980, Roper & Redston 1987, Lindström et al. 1999b, Ruxton et al. 2004). However, some predators can be unaffected by defenses (Exnerová et al. 2003) or be driven by hunger to overcome defenses (Sherratt et al. 2004, Barnett et al. 2007) leading aposematic organisms to balance warning signal efficacy with detectability to such predators (Bohlin et al. 2008). Therefore, warning signal conspicuousness alone is not always optimized.

Predation is also not the only selective force affecting warning signal design. Warning signal components may serve more than one function and be involved in fitness related trade-offs. Trade-offs occur when the expression of

one trait is linked to a decrease in the expression of another trait (Roff 2002). If the nature of a relationship between warning signal components and physiological requirements changes across the geographic distribution of an organism, then it is possible that a shift in warning signal appearance will also ensue (Thompson 1984b, Brakefield 1985, Hegna et al. 2013a). Two forces other than predation that are thought to be influential on visual warning signal traits through trade-offs are the need for thermoregulation (Speed & Ruxton 2007, Lindstedt et al. 2009) and sexual selection (Nokelainen et al. 2012, Cummings & Crothers 2013).

Thermoregulation in many ectothermic species is important to fitness and benefitted by melanin (Pereboom & Biesmeijer 2003, Trullas et al. 2007, Punzalan et al. 2008). Melanin is a dark compound found in the coloration of many animals and is thought and shown to trade-off with other fitness-related traits (Talloen et al. 2004, Lindstedt et al. 2009). Development may be hindered by the need to produce greater amounts of melanin for thermoregulation that requires more nitrogen (Talloen et al. 2004, Ojala et al. 2007), which is often a limited resource (Mattson 1980). Although not a warning signal trade-off, sex-specific color change in alpine grasshoppers (*Kosciuscola tristis*) demonstrates a signaling trade-off between intraspecific signaling to mates and thermoregulation (Stuart-Fox & Moussalli 2009). Female alpine grasshoppers prefer bright blue males. Above 25°C most males turn bright blue, but below 15°C most males turn highly melanized because melanin seems to benefit thermoregulation (Key & Day 1954). Thus, thermoregulation appears to constrain the ability of males to display more appealing signals to prospective mates in lower temperatures (Key & Day 1954, Stuart-Fox & Moussalli 2009). In a similar way, melanization may limit the amount of other pigments important in the warning signal of aposematic species, thereby setting the stage for trade-offs between signaling and thermoregulation (Speed & Ruxton 2007, Lindstedt et al. 2009).

In lepidopterans, indications of links between melanization and thermoregulation are seen in geographic variation in melanization through elevational (Guppy 1986b, Ellers & Boggs 2003) and latitudinal (Guppy 1986b, Tuomaala et al. 2012) gradients (see Trullas et al. 2007 for more examples). Faster warm-up times (Watt 1968) and increased escape ability (Kingsolver 1987) are two benefits that greater melanization may bestow on lepidopterans prior to flight. However, thermoregulation can also be very important in flight where the primary source of heat gain is solar radiation (Tsuji et al. 1986). More melanization in lepidopteran wings can increase flight duration (Roland 1982, Van Dyck & Matthysen 1998) and improve movement across cooler habitat patches (Guppy 1986a, Van Dyck & Matthysen 1998). Such increased flight mobility can be important for fitness-related activities such as finding mates and locations to lay eggs (Roland 1982). Melanization can also decrease egg maturation time in *Colias* (Ellers & Boggs 2004).

Features that are involved in warning coloration may also be become involved in sexual signaling. When warning signals also serve as sexual signals it can potentially set the stage for diversification. Such a scenario was first suggested for the interspecific diversification of firefly warning signals (Branham

& Wenzel 2003), where selection by predators and mate choice are thought to impact the evolution of firefly flashes (Moosman et al. 2009). Sexual selection is also thought to play a role in the intra-specific diversification of warning signals in the strawberry poison frog (*Oophaga pumilio*) (Rudh et al. 2011, Cummings & Crothers 2013). Although it is well established that females prefer color morphs that are similar to their own coloration (Summers et al. 1999, Maan & Cummings 2008), recent work has shown that predators may not select for local morphs on the basis of color alone (Hegna et al. 2013b, Richards-Zawacki et al. 2013). Instead, base coloration of *O. pumilio* may be related to their toxicity (Maan & Cummings 2012). Therefore, a possible synergistic effect between sexual and warning signaling, and how an element of honest signaling of toxicity may further interact certainly poses many questions for future work (Cummings & Crothers 2013).

In *Parasemia plantaginis,* a trade-off appears to exist between warning signal efficacy and mating success (Nokelainen et al. 2012). Yellow males are better protected from predators, but white males have greater mating success. Currently, though, it is not known if this apparent trade-off influences any broad patterns of geographic diversity in male hindwing coloration observed across the wood tiger moth's distribution. Certainly, the potential of sexual selection to be both an agent of diversification or limitation to defensive signaling is intriguing and worth additional future study in aposematic organisms in general.

1.3   Within population variation

Local (intra-population) warning signal diversity presents a challenge in aposematic species because it should cause increased mortality due to less efficient association by predators between the signal and unpalatability (Joron & Mallet 1998). If the warning signal is variable within the same locality or population, then we might wonder why the more effective warning signal does not predominate. Just as in the case of geographic warning signal diversity, predator community, drift, and trade-offs also have the possibility to influence local warning signal diversity. Local signal diversity can manifest itself in continuous variation, polymorphisms, or combinations of these. Color polymorphism is described as two or more genetically determined morphs within the same population, with the rarest morph occurring above the frequency of being a recent mutation (Huxley 1955). The presence of polymorphic populations of aposematic organisms poses one of the most perplexing situations because of the expectation that there should be strong selection against color polymorphism in aposematic organisms.

Species exhibiting stable color polymorphisms are some of the most interesting to evolutionary biology because they give potential windows into studying speciation mechanisms (Gray & McKinnon 2007). Although negative FDS is thought to help maintain local color polymorphisms (Gray & McKinnon 2007), it is not thought to be a predominant driver of polymorphisms in

aposematic organisms because the expectation is for positive FDS selection to maintain a monomorphic warning signal (Müller 1878, Thompson 1984a, Endler 1988, Greenwood et al. 1989, Joron & Mallet 1998, Lindström et al. 2001). When polymorphisms in color or pattern within populations of aposematic organisms exist it is nearly paradoxical because stabilizing selection should quickly move populations to signal monomorphism (Thompson 1984a, Endler 1988, Joron & Mallet 1998). In aposematic species pockets of polymorphic populations have been found in many contact zones between distinct morphs in *Heliconius* species (Mallet 1986, Mallet et al. 1990). Increases in gene flow can also result in the formation of polymorphic areas (Owen et al. 1994, Twomey et al. 2013). In addition to the influences of migration-related phenomena, trade-offs and dual fitness peaks may be explanations for local polymorphisms (Brakefield 1985). The previously described trade-off between warning signal efficacy and mating success in male wood tiger moth is suggested to be one factor explaining the local color polymorphism (Nokelainen et al. 2012). This trade-off along with environmental heterogeneity is thought to explain the high degree of color polymorphism exhibited in some European populations in general (Nokelainen 2013).

Temporal variation in predator and alternative prey community are two ecological factors that may affect warning signals (Mappes et al. 2005). Indeed, studies of some tropical species of *Heliconius* butterflies have suggested that concurrent temporal and spatial variation in mimetic environment may promote maintenance of polymorphisms in aposematic species (Brown & Benson 1974, Joron et al. 1999). However, many studies are generally only able to investigate single snapshots of selection, phenotype diversity, or genetic diversity. One of the most thorough temporal datasets on genotypic variation inferred from phenotypes is the scarlet tiger moth, which has been studied for over 60 years to investigate the causes of wing color fluctuations (Jones 1989, Jones 2000, O'Hara 2005). At least one recent study of forewing pattern frequencies across the years showed that fluctuations appeared to be due to genetic drift (O'Hara 2005). Similarly the diversity of warning signals in African *Hypolimnas misippus* (mimic of *Danaus chrysippus*) was found to be dependent on temporal fluctuations in population density and other aspects not related to mimicry (Smith 1976). The degree to which temporal stability in coloration and gene flow within populations maintain localized signal diversity represents an area of questions that will be undoubtedly important to answer to fully understand local warning signal diversity.

1.4 Aims of the study

The general aim of this thesis is to explore the evolution and maintenance of warning signal diversity using the wood tiger moth (*Parasemia plantaginis*). The wood tiger moth displays a range of geographic warning signal diversity in the degree of hindwing color polymorphism, gradual variation in hindwing

melanization, and forewing pattern differences across its Holarctic distribution. It is known to be aposematic and sequesters defensive chemicals from its diet and synthesizes others de novo. In my thesis I focused on developing an understanding of how historical factors and predation have influenced the warning signal diversity in *Parasemia plantaginis*. To this end, my thesis consists of four chapters.

In the first chapter (paper I) I investigate the phylogeographic history of *Parasemia plantaginis* and relate it to male and female hindwing warning coloration across the entire Holarctic distribution. I used both mtDNA and microsatellite markers to investigate phylogeography and population structure. This basic understanding of geographic pattern in phenotypic variation and phylogeography is crucial to developing specific questions and hypotheses about the evolution and maintenance of warning coloration.

In the second chapter (paper II) I address the question of whether signal efficacy trades off with thermoregulation at the adult stage of *P. plantaginis*. A trade-off was previously found in the warning-colored larvae of *P. plantaginis*. I also look at whether a possible trade-off could explain geographic patterns of hindwing melanization in Europe. Many times geographic diversity in warning signals is attributed to biologically driven selection mechanisms (mostly predation). Whether abiotic factors could drive signal differences is less studied.

In the third chapter (paper III) I examine geographic differences in the white forewing pattern of *P. plantaginis* in Alaska. Many diurnal (and presumably aposematic) arctiid moths have forewing patterns that bear some similarity to each other (van Bemmelen 1918). However, work with arctiid moths has focused on the often brightly colored hindwings, and it is not known whether forewing patterns have any defensive function. I assess whether the forewing pattern could be a warning signal. I also investigate whether selection by predators against foreign forewing types could explain the differences in forewing pattern over the Alaskan geographic distribution of *P. plantaginis*.

In the fourth chapter (paper IV) I study phenotypic differences across Japan and how they relate to genetic differences. Previous work has debated whether three or four populations exist in Japan based on phenotypic differences. Knowledge about population structure and how connected populations may be is critical to understanding whether drift or selection may maintain warning signal differences. I hypothesized that warning signal traits would differ among the genetic populations.

2 MATERIAL AND METHODS

2.1 Study species

The wood tiger moth (*Parasemia plantaginis*) is in the family Arctiidae and occurs across most of the northern hemisphere in what is referred to as the Holarctic region (Leraut 2006, Powell & Opler 2009). Like many arctiid moths, *P. plantaginis* is unpalatable and warningly colored, both in the larval (Ojala et al. 2007, Lindstedt et al. 2008) and adult stages (Nokelainen et al. 2012). Larvae have a hairy appearance and are black with an orange blotch that varies in size on their dorsum. The defense chemicals of *P. plantaginis* are obtained from a variety of host plants while in the larval stage and synthesized de novo. Owing to its generalist nature, *P. plantaginis* can be found in a variety of habitats. Habitats range from bogs, meadows, alpine habitat, dry shrub meadows, and others. Shifts in host plant use can promote speciation (Nosil et al. 2009). Given the expansive distribution that covers nearly half the globe one might expect such shifts. However, the highly generalized diet (i.e., *Lupinus*, *Taraxicum*, *Plantago*, *Trifolium*, *Betula*, etc.) of *P. plantaginis* likely acts against this possibility.

The wood tiger moth has a life cycle that is univoltine, producing one generation per year (Ojala et al. 2007). The flight season for the moth occurs between the end of June and the end of July across the entire distribution. The time of day during which the moth flies is roughly the same across the distribution as well, starting in mid-afternoon and extending until very late (can be midnight in the northernmost areas where daylight is continuous in the summer). Females are typically calling males with pheromones during this time, which means that the vast majority of individuals observed flying are males. Once males find a female and she accepts to mate, a nutrient rich spermatohphore is deposited by the male during copulation (Chargé et al. unpublished data). In some arctiids this spermatophore contains not only nutrients, but also defensive chemicals (Conner 2008). Larvae emerge from eggs the female deposited a few weeks later and feed until they enter diapause in the Fall. In the spring when diapause ends, larvae forage until they reach their final

instar between May and June and pupate. Following pupation, larvae emerge as new adults.

Across the distribution, male appearance can be extremely diverse (Fig. 1). The diversity has been recognized across the distribution (Coolidge 1910, Inoue 1961, Okano & Katayama 1976, Koçak 1989, Chenglai 2000, Dubatolov & Zahiri 2005, Leraut 2006, Powell & Opler 2009), but no concerted effort to study the variation as a whole has been made. Male hindwing color is polymorphic over much of its distribution (yellow or white) with variable melanized markings that can obscure the color completely in some areas and limit warning signal efficacy (Hegna et al. 2013a). Both yellow and white male hindwing colors function as warning signals (Nokelainen et al. 2012). European and most Russian areas can be comprised of polymorphic areas with yellow and white males occurring together (I). Yellow coloration becomes less frequently observed, though, in the Eastern portion of Russia, Japan, and North America (I). At the same time, the degree of melanization increases in these same areas compared to Europe and other areas in the western distribution. Within the Caucasus region (between Turkey and Azerbaijan), males have hindwing coloration that varies continuously between orange and red with most males displaying a more reddish color (Koçak 1989, De Freina 1993, Dubatolov & Zahiri 2005). Diversity also exists in the different forewing patterns that can be observed across the distribution that appear to be polymorphic, though, their heritability is less well understood compared to hindwing characteristics. In general, forewing diversity increases from Europe through North America. Forewing pattern appears to function as a warning signal as well (III).
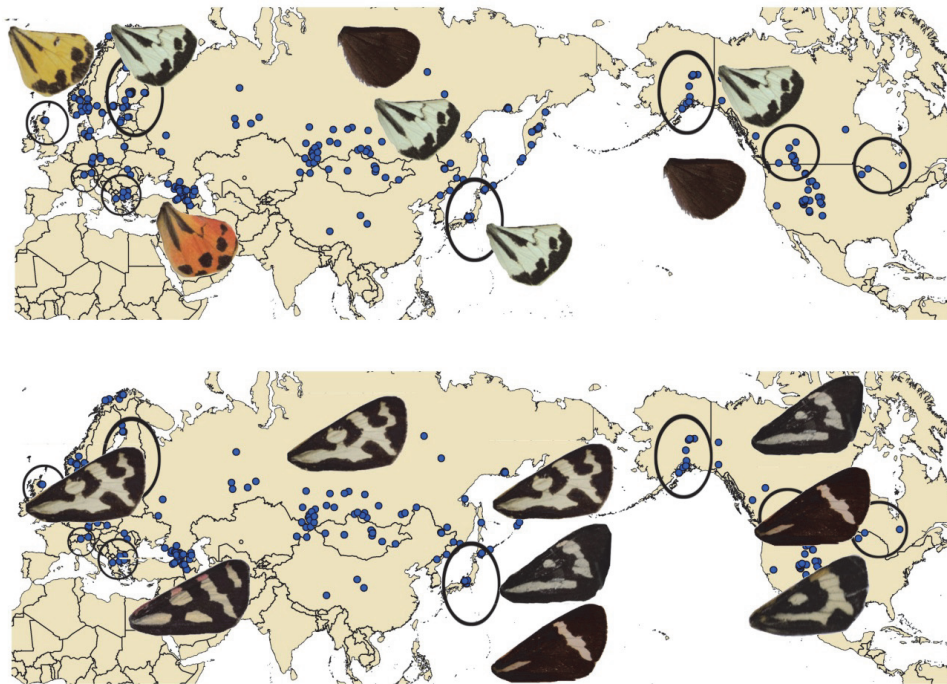
FIGURE 1   Generalized distribution of male hindwing color (top) and forewing pattern
distribution of both males and females (bottom). Blue dots represent sites that
specimens originated from that were used in examining the geographic
distribution of hindwing warning color and in genetic analyses. Areas that are
circled are where myself, or colleagues working in close collaboration,
personally collected specimens.

Females vary primarily in their hindwing coloration (Fig. 2), and in forewing
pattern in a similar way as males. Hindwings of females are generally highly
melanized (between 70-90%). Female hindwing color in Europe varies
continuously from yellow to deep red and functions as a warning signal
(Lindstedt et al. 2011), but appears to vary less across the entire geographic
distribution than males (I). Females in the Caucasus region have hindwings that
vary to some degree in their redness. East of Europe females appear to lose red
coloration and have hindwings that are yellow. In North America, it is even
possible to find females with hindwings that are completely melanized or with
white hindwings (RHH personal observation). However, accurate estimates of
global female phenotypic variation are not currently as reliable in a quantitative
sense as males because they are harder to find in nature. Therefore, I focus herein
on male warning signal diversity and describe female coloration in the
phylogeographic study of the species.

FIGURE 2   The range of female hindwing coloration also showing some typical melanization patterns.

2.2 Study sites and sampling

To study geographic diversity in the warning signal of *Parasemia plantaginis* I used many museum samples. However, I also conducted field work in many locations throughout North America and the European Alps to collect recent samples and carry out field experiments. I worked with colleagues in Finland, Bulgaria, and Japan to collect additional fresh samples from other locations. Male and females were captured using butterfly nets. I was also able to use pheromone traps baited with adult female moths to locate areas with *P. plantaginis* and catch males. What I found is that *P. plantaginis* occurs in a variety of habitats (Fig. 3). In the Alps, Japan, and Canada, the moth could be found at relatively high elevations (up to 2000m-3000m). In these areas the moth occupied alpine tundra and small meadow areas that were interspersed in forested areas over a variety of elevations. In the Alps I did not find the moth at lower elevations, but I also found myself unable to go lower than 1000m in many areas and extensive farming or development often existed below 1400m. In Finland the moth could be found occasionally around bogs, but in Canada I occasionally found the moth in very dry areas where only ephemeral pools of water could occasionally be seen.

FIGURE 3   Habitats where the moth lives from Canada with a moth trap hanging from the trees that was used to capture males (top), the European Alps (middle), and Alaska (bottom).

2.3   Coloration measures (I, II, III, IV)

Description of male hindwing coloration across the geographic distribution of *Parasemia plantaginis* was categorical (I, II), owing to the populations I dealt with were polymorphic in terms of hindwing color (males had either yellow or white

hindwings). Within the Caucasus region males can vary more continuously in the degree of redness expressed in the hindwings. However, because this variation exists only in one region I used a simplified 'red' category for all males bearing reddish hindwings (I).

Design of artificial moth model wing colors (II, III) required greater accuracy than using by-sight comparisons to real moths because avian vision can differ and it is important to take predator vision into account (Endler 1978, Endler & Mielke 2005, Osorio & Vorobyev 2005). Therefore, I used avian vision modeling to help construct accurate replicas of real moths that would be visible against the standard green backgrounds used in both predation experiments (II, III). To run the avian vision models I measured the reflectance spectra of the black and white/yellow areas of the real and artificial moth wings along with the green background using an AvaSpec-2048-SPU (Avantes, USA) spectrophotometer illuminated by a AvaLight DHS Deuterium-Halogen light. Five individuals of each hindwing color type were used to generate an average reflectance reading for comparing real individuals to the background. The spectra were then used to model excitation values for Blue Tits' single and double cones (Hart et al. 2000) with a standard D65 irradiance spectrum. I used the Blue Tit's photon catch values in particular because it is a known predator that occurs in both locations where the experiment was performed, and because its retinal sensitivity is known. Avian color vision is comprised of four single cone types (Cuthill 2006), while luminance discrimination is based on the double cones (Osorio & Vorobyev 2005). Therefore I used the four single cones to model chromatic vision and the double cone values to model achromatic vision (Siddiqi et al. 2004). I 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 (long wave = 1.00, medium wave = 0.99, short wave = 0.71, and ultraviolet sensitive = 0.37). The contrasts are reported as just noticeable difference values (or 'JNDs') (Vorobyev et al. 1998, Vorobyev & Osorio 1998). The model predicts that values of < 1 are indistinguishable, values between 1- 3 are hard to distinguish unless under optimal conditions, and values > 5 are easy to tell apart under most conditions (D. Osorio personal communication; see also Vorobyev et al. 1998).

In addition to describing color, I also measured the amount of melanization present in the hindwings of specimens (II, IV). Melanization in *Parasemia plantaginis* is not mixed with other aspects of the coloration, as to change the luminance of the base color (yellow, white, red). Instead, the amount of wing surface area covered by black melanized pigment changes. To measure melanization, then, I used the digital photographs to obtain the percentage of hindwing covered by melanized markings. I used the threshold selection tool (also called the 'magic wand' tool) in the program PAINT.net to obtain the number of pixels containing the black pigment and the area of the hindwing.

2.4   Pattern analyses (IV)

In two of the studies I analyzed forewing patterns in greater detail to use it in conjunction with genetic data (IV). This allowed me to look at relationships between the neutral genetic markers and forewing pattern (IV) and also taking geographic distance into account (IV). To analyze forewing patterns I followed methods developed by Todd et al. (2005). The method analyzes the position of pattern elements through constructing distance matrices that compare pattern similarities in a pairwise manner between each individual. This approach permits a holistic quantitative comparison of pattern similarity.

Specimens were photographed using a Fujifilm S3 Pro camera against a background of 0.5cm graph paper as a size standard. The forewing was then copied and pasted onto a Microsoft PowerPoint ® slide where it was adjusted and enlarged to be in the same position as all other forewings. Within PowerPoint a grid measuring 35x35 cells was overlaid on each image. Cells containing the pattern element were then marked with a '1'. A macro was created within Microsoft Excel ® to fill in all other empty cells with a '0' and then re-arranged the square grid into a single column of data. Each moth's grid then comprised a single column and was labeled with an alphanumeric code denoting population membership and other factors of interest, so that samples could easily be assigned the appropriate factors later.

The resulting pattern data was analyzed in PRIMER (version 6.1.B) (Clarke & Gorley 2006). The Excel file containing all specimens of interest (one specimen per column) was imported and columns were classified as being samples while rows were classified as variables. A resemblance matrix was calculated using the adjusted Bray-Curtis similarity. The adjusted Bray-Curtis similarity index is capable of dealing with the large number of zero's present in each sample better than the Pearson correlation (Clarke et al. 2006). From the similarity matrix a one-way analysis of similarity (ANOSIM) was used to determine if pattern differences existed between the two regions (Clarke 1993). I used an non-metric multidimensional scaling (nMDS) plot to visually depict the differences and similarities in pattern design among populations (Clarke 1993).

2.5   Molecular Methods (I, III, IV)

2.5.1 Phylogeographic analyses and population structure from mtDNA (I, IV)

I amplified a 630bp fragment of the cytochrome oxidase I (COI) gene to look at relationships among populations (I, III) with the universal primer pairs reported in Folmer et al. (1994) LCO (fwd) G GTC AAC AAA TCA TAA AGA TAT TGG and HCO (rev) T AAA CTT CAG GGT GAC CAA AAA ATC A. Amplifications were performed with a total reaction volume of 20μL. Included were ~50ng of DNA, 0.5μM of forward and reverse primer, 2μL of 10x buffer with 20 mU MgCl,

and 5U/µl DreamTaq buffer. PCR amplification consisted of an initial denaturation step at 95ᵒC for 3 min followed by 30 cycles of 30s at 95 ᵒC, 30s at an annealing temperature of 50 ᵒC, and 1.5min at 72 ᵒC; samples then underwent 5 min at 72 ᵒC. Sequencing reactions were conducted with the BigDye® Terminator v3.1, Cycle Sequencing Kit (Applied Biosciences) using the M13 primers connected to the COI primers and were analyzed on an ABI 3130xl Genetic Analyzer (Applied Biosystems). The M13 primer consisted of a forward 5'-TTGTAAAACGACGGCCAGT-3' or reverse 5'-CAGGAAACAGCTATGACC-3'. Sequences were edited in Seqscape v2.6 (Applied Biosystems) against a template sequence and no gaps were observed.

Haplotype networks were constructed to examine relationships among haplotypes and the populations that potentially shared haplotypes (I, IV). I did this using TCS 1.2.1 with a 95% connection limit (Clement et al. 2000). To investigate relationships among haplotypes and populations with different haplotypes (I) I took a Bayesian approach using the software MRBAYES v3.2.1 (Ronquist & Huelsenbeck 2003, Ronquist et al. 2012).

I was interested to infer information about the demographic history of *Parasemia plantaginis* (I) to consider how the demographic history might compare to any patterns in warning signal diversification in general. I used dnaSP v 5.10 (Librado & Rozas 2009) to calculate nucleotide diversity (π), haplotype diversity (h), Tajima's D, Fu's Fs, and average nucleotide differences. Universal molecular clocks for mitochondrial sequences do not exist for lepidopterans or for *P. plantaginis* (Ho & Lo 2013; Niklas Wahlberg personal communication). Therefore, I avoided using molecular clocks in our analyses. I estimated general demographic trends through time without explicitly incorporating a mutation rate in Bayesian skyline plots (Ho & Shapiro 2011). I used BEAST v1.7.2 (Drummond et al. 2012) to implement a coalescent-based Bayesian Skyline Plot model (BSP; Drummond et al. 2005) for the main five geographic groups shown in the Bayesian analysis of relationships among haplotypes (Caucasus, Europe, Russia, Japan, and North America). In cases where no molecular clock is used the x-axis of the BSP is read as the substitutions per site, rather than substitutions per site, per unit of time (Ho & Shapiro 2011; see also BEAST user manual). The y-axis represents log transformed values of population size (Ne) multiplied by the mutation rate (µ). Datasets for the BSP reconstruction included all samples for the Caucasus Region (n=37), North America (n=190), Europe (n=229), Russia (n=52), and Japan (n=79). I used the HKY+G substitution model and fixed the strict molecular clock rate to 1.0 with the number of groups in the BSP analyses set to five. I used a uniform prior for estimating BSP population sizes. Two simulations were run for each of the five regional groups that included 20 million generations for the Caucasus, Russia, and Japan, sampling every 2000 generations. The Europe and North America group required 200 million generations, sampling every 10,000 generations. I checked the convergence using the program TRACER (Rambaut & Drummond 2003) to make sure that effective sample size (ESS) values were greater than 200, indicating convergence.

2.5.2 Microsatellite marker amplification (I, III, IV)

To investigate current population dynamics and structure in a more thorough way I amplified a total of 10 di- and tri- microsatellite loci (I, III, IV) as identified by Galarza et al. (2010; Ppla107, Ppla109, Ppla279, Ppla313, Ppla317, Ppla323, Ppla363, Ppla382, Ppla414, Ppla439). Each microsatellite locus was amplified separately by PCR in 20μL total volume, which included ~50ng of DNA, 2mM of $MgCl_2$, 0.5 μM of reverse primer, 0.45 μM of forward primer, 0.05 μM of labeled forward primer, 200 μM of dNTP's, 10X DreamTaq Buffer (Fermentas), and 0.75 units of DreamTaq DNA polymerase (Fermentas). Following amplification products were initially multiplexed into two plates and genotyped by assessing allele size on an ABI 3130xl Genetic Analyzer (Applied Biosystems) using fluorescently labeled forward primers (6-FAM, NED, PET, and VIC; Applied Biosystems). Scoring of alleles was carried out using GENEMAPPER V4.0 software (Applied Biosystems). I used MICRO-CHECKER v2.2.3 (Van Oosterhout et al. 2004) to check for scoring errors arising from stuttering and large allele drop out. Possible departure from Hardy-Weinberg and the existence of linkage disequilibrium among loci was tested with GENEPOP (Guillot et al. 2008).

Defining what constitutes a population is not always intuitive or straightforward. Throughout this thesis I used two principle methods to determine genetic clusters that were considered populations. The first method I employed was STRUCTURE 2.3.3 (Pritchard et al. 2000), which examines only the genetic data across multiple tentative numbers of populations to arrive at a probable population number (known as K) (I, III, IV). It is then possible to examine the specimens after the analysis to determine which specimens are assigned to each population. To help infer the number of populations (K) I used the ΔK statistic (Evanno et al. 2005) calculated with the program STRUCTURE HARVESTER (Earl & vonHoldt 2012).

A second program used in examining populations in Japan (JAPAN MS) was GENELAND 4.0.2 (Guillot et al. 2005b, Guillot et al. 2005a). GENELAND incorporates explicit information about spatial relationships among samples and defines spatial genetic units (IV). Because the number of populations within Japan has been somewhat debated I used both GENELAND and STRUCTURE to independently examine population structure (IV). GENELAND also includes a useful model that is able to adjust estimates of population structure for the influence of null alleles. However, a recent modeling and simulation study indicated that STRUCTURE and other population delimitation programs are robust to the effects of null alleles (Carlsson 2008). In that study loci with as much as 98% null allele frequencies influenced results by less than two percent.

In addition to being interested in understanding more about what the population structure among various samples was, I wanted to know relationships among populations that were found (I). I analyzed relationships among populations by constructing a neighbor-joining tree with 1000 bootstrap replicates using the D_A measure of genetic distance (Nei et al. 1983) in the program POPTREE2 (Takezaki et al. 2010).

Analysis of molecular variance allows one another way to examine how differences are partitioned among and within groups. Therefore, I conducted AMOVA analyses using GENALEX in all three chapters containing genetic data (I, III, IV). In the first chapter, I conducted a hierarchical AMOVA analysis that included regions based on broader groupings of populations as seen in the population level tree of relationships among populations.

I also calculated F_{ST} values (Weir et al. 1984; Weir 1996) to further investigate population structure and differences among populations (I, III, IV, V). Values of F_{ST} are based on comparing heterozygosity estimates to expected values. However in recent years much conversation has emerged about how good such comparisons are at describing population differentiation (Jost 2008, Heller & Siegismund 2009). To that extent some have suggested alternatives to the traditional F_{ST} including the R_{ST}, G_{ST}, and D_{EST} measures. At least one recent comparison of these suggested D_{EST} to represent the best measure of population differentiation (Gerlach et al. 2010). Therefore, I also calculate D_{EST} to compliment F_{ST} calculations (I, IV). Because null alleles can inflate F_{ST} values I used FREENA to calculate adjusted F_{ST} estimates (Weir 1996) when null alleles were present to compare to F_{ST} values calculated without taking null alleles into account (I, III, IV).

Measures of differentiation have been historically used to infer the amount of gene flow between populations. Advances in computational techniques, though, have resulted in analyses that can give better estimates. One such technique makes use of Bayesian MCMC resampling methods to infer directional gene flow occurring within the past 3-4 generations implemented in the program BAYESASS v3.0 (Wilson & Rannala 2003). A confidence interval can be easily calculated to allow inference of which asymmetrical migration rates are informative. I used BAYESASS to examine gene flow among populations in all three chapters incorporating genetic data (I, III, IV). The program TRACER v1.5 (Rambaut & Drummond 2003) was used to check that analyses reached convergence.

In one chapter (IV) I was interested to explore whether warning signal characteristics could be evolving non-neutrally with a series of Mantel and partial Mantel tests using ZT v1.0 (Bonnet & Van de Peer 2002). A growing recognition of the problems associated with such tests is acknowledged (see Guillot & Rousset 2013), and here I use these analyses as one tool to get an idea of how traits may be evolving and not as 'proof' for selection in and of themselves. For morphology measurements (body length, forewing length, hindwing length) I used euclidian distance matrices and Bray Curtis dissimilarity matrices were calculated for melanization (forewing and hindwing) and forewing pattern matrices using PRIMER v6. In the Mantel tests I looked for associations (or lack of association) between phenotypic characters, geographic distance (km), and genetic distance inferred from the microsatellite markers, presumed to be neutral. In partial Mantel tests I set genetic distance to be a covariate in order to look at associations between phenotypic and geographic differences, while attempting to control for neutral genetic divergence (IV). This way, I was able to take neutral genetic differentiation into account to examine the possible influence

of selection on warning signal differences more clearly (Antoniazza et al. 2010, Kawakami et al. 2011).

2.6 Species distribution modeling (I)

To further develop an understanding of the phylogeography (I) of *Parasemia plantaginis* I employed species distribution modeling. Combining climate-based distribution modeling with phylogeographic data allows for better developed spatially explicit hypotheses of past distribution patterns (Waltari et al. 2007). To perform the climatic species distribution model (SDM) of *P. plantaginis* I used MAXENT 3.3.3 (Phillips et al. 2006, Phillips & Dudík 2008). I used 211 locality records of *P. plantaginis* from my genetic data and from museum samples. Modeling was replicated 20 times to explore the reliability and robustness of the results using 75% of the 211 records for model training and 25% were omitted for model testing. The model followed the logistic approach. Testing was assessed by the strength of the predictive relationship using the area under the receiver operating characteristic curve (AUC).

I obtained climate data for the present day and for the Last Glacial Maximum (LGM) from the WorldClim 1.4 database (Hijmans et al. 2005). I used the data at a grid resolution of 2.5 arcmin. Simulations used to create the climate data for the LGM used the Community Climate System Model (CCSM). Of the 19 BIOCLIM variables used for species distribution models (Busby 1991), I used 13 known to have an influence on lepidopteran biology (Habel et al. 2010): annual mean temperature (BIO1), mean monthly temperature range (BIO2), isothermality (BIO3), maximum temperature of the warmest month (BIO5), minimum temperature of the coldest month (BIO6), annual temperature range (BIO7), mean temperature of the wettest quarter (BIO9), annual precipitation (BIO12), precipitation seasonality (BIO15), precipitation of the driest quarter (BIO17), precipitation of the warmest quarter (BIO18), and precipitation of the coldest quarter (BIO19).

2.7 Thermoregulation (II)

Because thermoregulation is important to ectotherms, I was interested to see if greater amounts of melanization resulted in being able to warm up more quickly (II). To determine whether more melanized individuals warm up faster, I measured the temperature gained under a heat source over a fixed time across males with varying amounts of melanin and of different colors (yellow or white). Fifty-five dead individuals were used originating from the Alpine Region of Europe (n=13), the non-alpine Central Europe (n=11), and Finland (n=31). Specimens were all undamaged and in a body position with their wings open. The open winged position is relevant because small lepidopterans loose heat

during flight (Tsuji et al. 1986, Heinrich 1986, Heinrich 1993). By using dead moths we were able to control for any differences in thermoregulation-related behavior, which might exist among differently melanized individuals. At least two previous studies have also used either dry (Guppy 1986a) or freshly killed (Punzalan et al. 2008) individuals for the same reason to look how coloration relates to thermoregulation.

Measurements were taken with a thermal imaging camera (InfraCAM by FLIR Systems) in a controlled environment (mean=15.6ºC, s.d.=1.27), representing the lowest temperature at which I observed flying individuals in the field (personal observation). The environment was created using a plastic container filled with approximately 3cm of ice. I fixed a piece of dry foam (1cm x 1cm x 4cm) in the center to provide a platform on which the specimen was mounted using an insect pin. To insure that all measurements started in similar conditions I checked the initial temperature of the moth with the camera, and the temperature of the environment with both the camera and a sensor mounted at the same level as the moth. Initial temperature was included as a covariate in the analysis as well. The heat source for the temperature gain measurements was a 60 Watt incandescent heat lamp mounted 52cm above the specimen. I took temperature readings once after 45 seconds. Measurements were taken from the thorax of each specimen between the attachment points of both forewings, being careful to not take readings of the pin. I systematically alternated among colors and relative amounts of melanin to prevent any sample order effects confounding the results.

2.8 Predation experiments (II, III)

Here, I studied how predators in the wild view the warning signals of *Parasemia plantaginis* in two experiments (II, III). Previous studies of predation in lepidopterans have occasionally been done by looking at damage to the wings of specimens collected in the wild, but attacks can be rare and difficulties often exist in re-capturing specimens (Bowers et al. 1985). Studying actual predation in the wild is even more difficult when prey are aposematic because the rate of predation is usually low. Therefore, I used clay models that record attacks as marks left on the clay surface to study predation on different forms of *P. plantaginis*. The use of clay models is somewhat common in studies of predation (Madsen 1987, Brodie 1993, Kuchta 2005, Saporito et al. 2007, Nokelainen et al. 2012) and even frugivory (Arruda et al. 2008) in birds. In both experiments where fake model moths were used the same methodology was employed in designing wings that accurately represented the real moths and ensured visibility by using avian vision modeling. Models were designed in both experiments to best reflect their real counterparts and to make sure different morphs contrasted well against the standard background using an avian vision model as described above. Although the models themselves differed between the two studies, methods for carrying out each study were the same.

FIGURE 4   A picture showing a model moth from the study in chapter III against a standard green background.

Models were constructed and placed against a standard green background that ensured all model types used were visible to avian predators (Fig. 4) (Hegna et al. 2013a, Hegna & Mappes. in prep). The backgrounds were constructed from corkboard that was painted green and placed atop a wooden stake approximately 0.3-0.4m in length. The models were placed every 15m down a linear transects that varied in length depending on the experiment. Models were counted as being attacked if they had imprints on the clay surface in the shape of a 'V' or other marks consistent with avian attacks (Fig. 5). Missing models or those bearing signs of arthropod bites were excluded from analyses. In both experiments (II, III), logistic regression was used to analyze the odds of being attacked for different artificial moth model types.
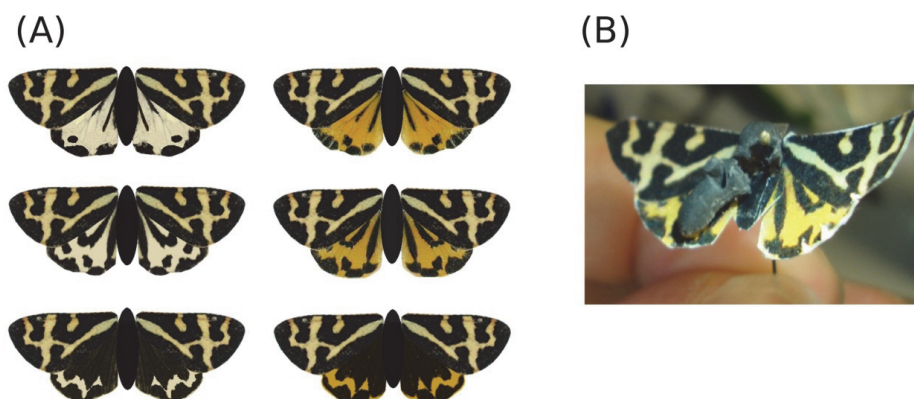

FIGURE 5   Picture showing the model types used in the first experiment (A) and an attacked model from experiment II with a low amount of melanization present on the hindwing (B). This figure is reprinted from work published in Proceedings of the Royal Society with permission.

In the first experiment (II) I investigated whether the increased amounts of melanin covering the hindwing influenced probability of being attacked. Because increasing amount of melanin reduces the amount of hindwing color (yellow or

white), higher amounts of melanization could potentially reduce signal size and decrease its effectiveness. Models were designed with three different levels of melanization for each male color (yellow and white; Fig. 5). Additionally, the forewing pattern was made to be either whitish or yellowish to represent natural variation in forewing color. Therefore, a total of 12 model types were used. This experiment was carried out in Central Finland and in the Alpine Region of Europe in June and July of 2010. The two geographically distinct areas were used because male hindwing melanization is different between the two areas, which allowed us to see if any evidence for a more 'universal' trend existed in how predators treated highly melanized hindwings. Models were constructed depicting the moths as they would be seen moving through the vegetation or just before flight with forewings cracked open to expose the hindwing coloration. In the first experiment looking at melanization in the hindwings I used 36 transects (350m) in the Alpine region (864 models) and 15 longer transects (1km) in Central Finland (840 models). In the Alpine region models were left for 3 days and in Central Finland models were left for 5 days, but checked each day so that a combined analysis with only 3 days of predation could be done.

In the second experiment (III) I set out to determine if evidence supported the forewing patterns acting as warning signals, or potentially being involved in disruptive coloration. Patterns similar to the forewing of *Parasemia plantaginis* can sometimes act in a disruptive manner by preventing predators from detecting the outline of a prey item. I carried out the experiment between June and July of 2011 in two regions of Alaska where local moths differ in the locally dominant forewing pattern (Fig. 6). The first region was the area around Fairbanks, Alaska. The predominant forewing pattern in this region is known as the 'hash' forewing pattern that is also very melanistic. The second region is the Anchorage region where the forewing pattern is the 'cross' pattern observed also in Europe.
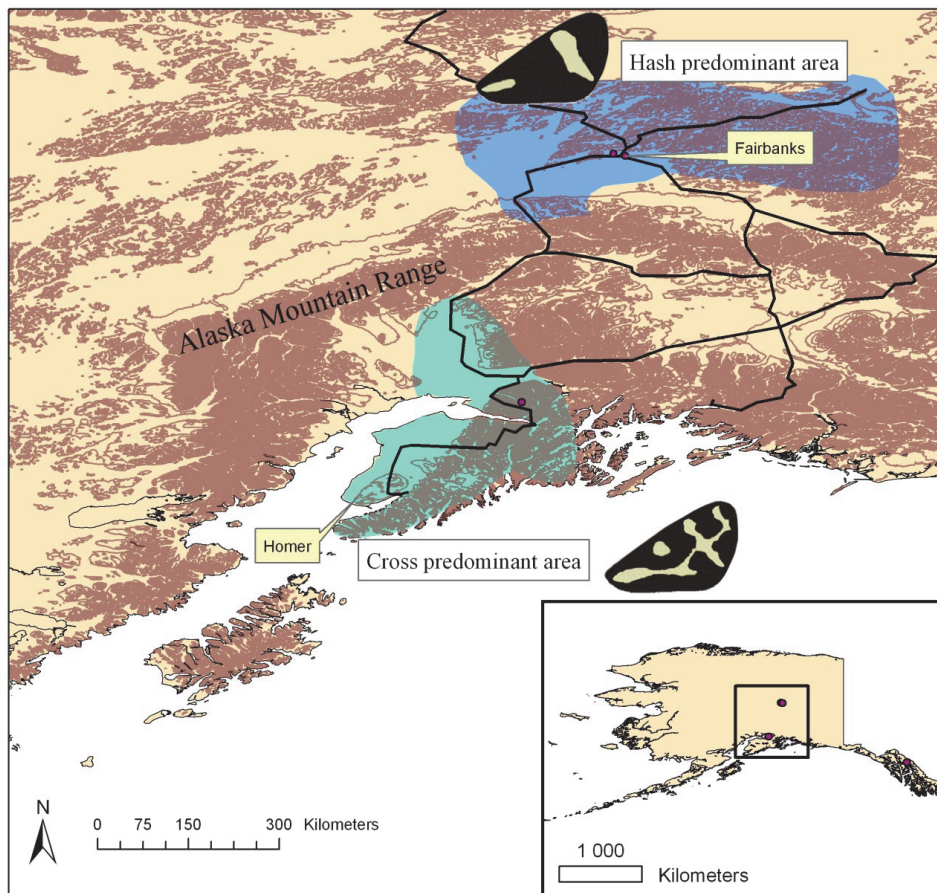
FIGURE 6   A map showing the distribution of the two areas where the forewing pattern
shifts from being dominated by the 'hash' form to being dominated by the
'cross' form.

I designed artificial moth models in the second experiment with different
forewing patterns to determine if the foreign forewing pattern suffered increased
predation (Fig. 7), as would be expected if the pattern functions as a warning
signal. Unlike the first experiment, models displayed the forewings in the closed
position that would prevent predators from seeing the hindwing color. The two
different forewing patterns were designed to have the same amount of white
patterning, so that differences in attacks could be attributed to pattern and not
amount of white (i.e., controlling for different signal sizes). I also modified the
two patterns, such that the white patterns would either touch or not touch the
edge of the forewing (forming a border around the outside). By touching and not
touching the edge of the wing I increased and decreased, respectively, the level
of disruptiveness in the pattern. Decreased disruptiveness from the presence of a
border has increased detectability to predators in all previous work on disruptive
patterns in moth wings. In warning signals, one might predict that signal

recognition and conspicuousness would overcome any disadvantage of being more conspicuous from the presence of a border outlining the aposematic organism. Therefore, this manipulation allowed a second way of looking if evidence supported a warning function for the forewing pattern. I also included a patternless black forewing in the experiment as a way to compare how birds treated the same forewing shape without the presence of any pattern. If the forewing pattern functions as a warning signal, I also expected the black forewing to potentially receive more attacks than either pattern. Five model types in total were used. Along each transect, six replicates of each model type were used. In Alaska I used 40 transects (450m long) divided between the two locations where the local forewing type differed (1200 models total).
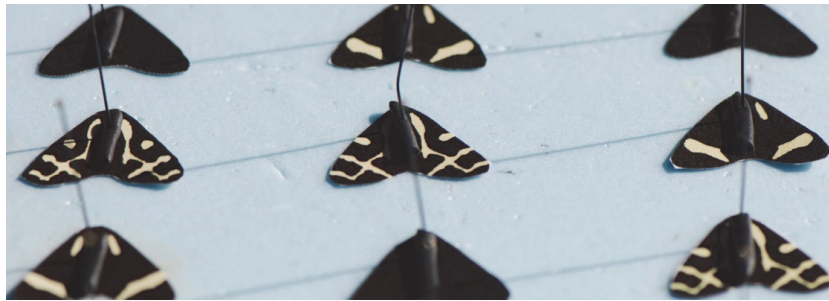

FIGURE 7   Models that were used in Alaska pinned against a Styrofoam board before being placed on the platforms with the green backgrounds.

3 RESULTS AND DISCUSSION

3.1 Phylogeography of the wood tiger moth and geographic variation in its hindwing warning signal (I)

To fully understand warning signals and their diversity it is necessary to develop a historical framework in which to gain perspective on their evolution (Härlin & Härlin 2003). Developing such a framework requires the understanding of how phenotypes are distributed across geographic space and melding that knowledge with a phylogenetic hypothesis. The phylogeographic reconstruction indicated a deep mitogenetic divergence between Caucasus haplotypes and those from other areas across the Holarctic, but overall mitogenetic structure was surprisingly shallow. One specimen from the Caucasus, though, had a haplotype in common with Central Russian specimens. Japanese specimens also largely showed some divergence in their haplotypes from other specimens in the Palearctic, but one specimen did have a European haplotype. Divergence in male hindwing warning color paralleled mitogenetic structure in the Caucasus and in Japan. However, divergence in male warning color in the Caucasus was not complete because yellow males can still be seen there and while most male Japanese specimens are white there are some yellow males there as well. Our finding of males with hindwing color not matching the local color matches previous reports for both the Caucasus (De Freina 1993, Dubatolov & Zahiri 2005) and Japan (Okano & Katayama 1976, Kishida 2011). A shift in female hindwing warning color from being red and orange in the Caucasus and the Western Palearctic to being yellow in the Eastern Palearctic and Nearctic did not parallel any mitogenetic structure.

Despite not forming a well-supported group in the phylogenetic analyses, the haplotype network showed evidence that North American specimens did not have any haplotypes in common with Palearctic specimens. One predominant European haplotype was spread across the Palearctic region, including one specimen in Japan. The species distribution models suggest past population connectivity during the Last Glacial Maximum, rather than excessive isolation. Furthermore, demographic reconstruction with Bayesian Skyline Plots shows

evidence of demographic stability and only evidence of demographic expansion in the most glaciated areas (Europe and North America). Values from Tajima's D and Fu's Fs showed similar results. Therefore, glacial refugia likely did not play a large role in forming the observed mitogenetic structure. This stands in contrast to many studies that have shown a greater impact of glacial cycles on mitogenetic structure (Wahlberg & Saccheri 2007, Gratton et al. 2008, Von Reumont et al. 2012). Shallow mitogenetic structure in other species of lepidopterans (Forister et al. 2004, Todisco et al. 2012) and other species (Crease et al. 2012, Porretta et al. 2013) has been interpreted in a similar way and is thought to be linked to increased dispersal capabilities.

Current population structure inferred from microsatellites was stronger than indicated from the mitochondrial marker, suggesting that populations are currently more differentiated. The AMOVA analysis indicated that 29% of molecular variance was accounted for by differences among regions (i.e., Europe, Caucasus, Russia, Japan, Alaska, lower North America; df=5, SS=1072.4, MS=214.5, p<0.001) and an additional 10% was accounted for by differences among populations within those regions (df=11, SS=275.9, MS=25.1, p<0.001). When comparing one population to another individually, extremely high degrees of population differentiation were only seen when comparing the most geographically separated populations (i.e., Caucasus-North American populations F_{ST}=0.5-0.7). Less differentiation existed among adjacent populations (F_{ST} typically ranged between 0.05-0.25 in such comparisons). Although not the strongest degree of current population structure observed, the Caucasus populations were differentiated from adjacent Central European and Finnish populations (F_{ST}=0.12-0.19). Caucasus males are also the most diverged in hindwing color. Past work demonstrates that hindwing color can influence mating success (Nokelainen et al. 2012), but whether it has become a factor contributing to pre-zygotic isolation of Caucasus populations is unknown. I only detected evidence of recent migration with BAYESASS between the two Caucasus populations and between two populations in Japan. Pheromones are one aspect of lepidopteran mating biology that can give clues to whether speciation is ongoing (Lumley & Sperling 2011) and observations from using Finnish females to attract males in Japan and North America indicate no obvious mating hesitancy there (Atsushi Honma and RHH, personal observation). More detailed studies of sexual selection in the laboratory and field along with better sampling in the Caucasus will be needed to understand how diverged some populations are.

Analyses of the COI gene fragment suggest the origin of *Parasemia plantaginis* to be either Southeast European or Caucasian based, which fits with a previous hypothesis by Dubatolov (2008). The hypothesized origin also fits with the results of the distribution model reconstructed for the Last Glacial Maximum showing southern Europe and the Caucasus region to be well connected by suitable climate conditions. Analysis of the phenotypic data reveals that the exact origin poses interesting questions concerning the evolution of coloration characteristics. European populations have males that are polymorphic in their hindwing coloration (yellow/white) and females vary continuously between

yellow and red. On the other hand, Caucasus populations have males with red hindwings that vary in the degree of redness in a similar fashion to females.

3.2 Thermoregulation and its geographic effect (II)

Warning signal efficiency appeared to be constrained by thermoregulation needs via melanin present on the hindwing in *Parasemia plantaginis*. I found that greater amounts of melanin in both white and yellow males resulted in an increased ability to absorb radiation ($F_{1,55}$=28.4, p<0.001). Interestingly, I also found a significant interaction between color and hindwing melanin ($F_{1,55}$=12.17, p<0.001) suggesting males with yellow hindwings were better able to warm up with less melanin than white males. More melanization also resulted in an increased likelihood of attack by avian predators. The results of the predation experiment suggested that every 10% increase in melanin increased the odds of attack by 16% in the overall analysis (Wald=13.4 df=1, p<0.001). One mechanistic explanation for the increased predation risk of more melanized individuals is that the amount of color (yellow or white) decreases as the proportion of the hindwing covered by melanin increases. Nokelainen et al. (2012) showed that both yellow and white pigments in the hindwings of male *P. plantaginis* serve as warning signals, even though yellow males were avoided more by avian predators than white males. Larger warning signals increase detection by predators (Bohlin et al. 2008, Lindstedt et al. 2008), which should enhance signal recognition and avoidance by predators (Lindström et al. 1999a, Ruxton et al. 2004). Therefore, the effectiveness of the signal can be diminished by reduced signal size (Gamberale & Tullberg 1996, Lindstedt et al. 2008), as I found in the results.

In addition to showing evidence that signal efficacy appeared to trade off with thermoregulation benefits of melanin, my results offer an explanation for the geographic difference in the hindwing warning signal between Central Finland and the Alpine Region. Temperatures at elevations where *P. plantaginis* occur in the Alps are generally cooler with fewer days reaching above the observed flight threshold of 15°C. The cooler climate should theoretically make melanization an important fitness-related trait in the Alps because it should benefit mate finding opportunities in the Alps more compared to Central Finland. The lower odds of being attacked in the Alps in general (Wald=14.2, df=1, df=1, p<0.001) most likely made it easier for males to possess higher levels of melanization compared to Central Finland as well. Although I did not directly link warm-up ability to fitness in this study, previous work on lepidopterans has shown fitness benefits of increased melanization (Rawlins 1980, Roland 1982, Guppy 1986a, Van Dyck & Matthysen 1998). Therefore, cool temperatures at high elevations in the Alps likely make the benefits of additional melanin outweigh its predation costs and tip selection in favor of highly melanized phenotypes. Of course, it is entirely possible as well that melanization could benefit an

individual moth's ability to escape from predators (Kingsolver 1987), along with other behaviors not tested by this experiment.

3.3 Predator-driven selection on forewing pattern (III)

The effectiveness of warning signals is dependent on their density and frequency (Endler 1988, Greenwood et al. 1989, Lindström et al. 2001, Ruxton et al. 2004), which suggest geographic diversity should be characterized by shifts in dominant signals rather than expecting local diversity. Collection of specimens showed the two areas differed in the local forewing morph during the experiment. The Fairbanks region is dominated by the 'hash' morph while the Anchorage region is dominated by the 'cross' morph.

Results from the predation experiment suggest that the forewing pattern functions as a warning signal. Manipulating the level of disruptiveness in the forewing pattern did not influence attack rates (p>0.20), in contrast with studies finding that less disruptive patterns receive more attacks (Cuthill et al. 2005, Schaefer & Stobbe 2006, Stevens et al. 2006). I also found that the local forewing pattern was attacked less than the foreign pattern, but only in the Fairbanks region (Wald=5.85, df=1, p=0.0157). Selection against foreign warning signals as such is predicted by theory and suggests that positive FDS selection may be at least partially responsible for maintaining local signal differences. Lastly, the black patternless forewing was attacked more than either pattern type in the Anchorage region (Wald=7.39, df=1, p=0.006), potentially suggesting that the presence of a pattern protected models there.

My results from the predation experiment also suggest that predators may have generalized between some model types. In Fairbanks I observed that the black forewing pattern was attacked at a rate equivalent to that of the local melanistic 'hash' morph. Meanwhile, in the area around Anchorage in the south I observed an increase in the attack rate on the black forewing model. Despite findings and theoretical models that predict selection for similarity in local warning signals, evidence also exists suggesting that generalization by predators may allow for some localized warning signal variation (Rowe et al. 2004, Ham et al. 2006, Rowland et al. 2007, Aronsson & Gamberale-Stille 2012).

The relative importance of patterns or colors in warning signals remains a somewhat poorly understood psychological aspect of aposematic strategies (Aronsson & Gamberale-Stille 2008, Hegna et al. 2011, Aronsson & Gamberale-Stille 2012, Stevens & Ruxton 2012). Although many seemingly aposematic arctiid moths possess noticeably similar conspicuous forewing patterns (van Bemmelen 1918), no work has examined whether such patterns could function as warning signals. The experiment gives some evidence to suggest that the patterns can function as a warning signal.

When I analyzed the genetic data for specimens caught throughout the two regions where forewing morphs differed the analyses showed two distinct populations existed. The F_{ST} value obtained when comparing the two

populations was 0.09 (p<0.001). The AMOVA found that 9.4% of molecular variance existed among populations (df=1, SS=10.8, MS=10.7, p<0.001). Furthermore, results looking at gene flow between the two populations suggested little gene flow has occurred within the past 2-4 generations (both estimates included zero in the confidence interval). However, it is important to note that a lack of gene flow does not necessarily indicate migration was not attempted because if frequency dependent selection acts in these two regions, predators should attack migrants with foreign looking forewings. Such patterns of predation are observed in *Heliconius* butterflies (Mallet & Barton 1989, Kapan 2001, Langham 2004) and poison frogs (Noonan & Comeault 2009, Chouteau & Angers 2011, Chouteau & Angers 2012). The effects of selective predation can be seen even at the genomic level where differentiation between *Heliconius melpomene* morphs is greater at loci coding for coloration than neutral loci (Baxter et al. 2010). Overall, forewings appear to be warning signals and the geographic differences seen in Alaska is in part due to predation and likely helped by some restricted gene flow from physical barriers, or both.

3.4   Genetic and warning signal divergence in Japan (IV)

In Japan, phenotypic differences across various areas have spurred debate over whether three or four populations (or even subspecies) exist (Okano & Katayama 1976, Kishida 2011). I used microsatellite markers and mtDNA, along with phenotypic data, to investigate warning signal diversity in Japan.

Microsatellite marker analysis showed the existence of three populations instead of four. One population existed on the northern island of Hokkaido and two populations adjacent to each other were found on the island of Honshu. The AMOVA analysis indicated that 30% was among populations (df=2, SS=58.35, MS=29.18, p<0.001) and 70% of the molecular variation was contained within populations (df=2, SS=195.99, MS=1.10, p<0.001). Values of F_{ST} estimating pairwise population differentiation ranged from 0.26-0.28. Forewing pattern and hindwing melanization are both likely involved in warning predators of the moth's unpalatability and differed among the three populations (Fig. 8). However, other morphological aspects did not differ significantly among all three populations. Interestingly, an area thought to comprise a fourth population was shown to have some unique mitochondrial haplotypes. The unique area could be the product of climatic shifts during the Pleistocene or sex biased dispersal because females appear to have a harder time flying than males (see Baba et al. 2001, Ikeda & Setoguchi 2006).
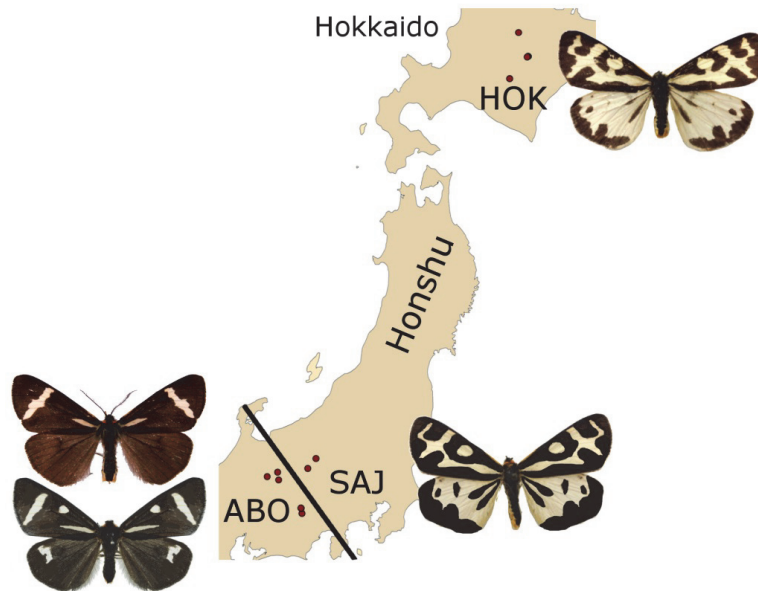
FIGURE 8   A map of Japan showing the phenotypic differences that exist among the three populations (labeled ABO, SAJ, and HOK).

I detected low levels of migration between the two populations on Honshu using the program BAYESASS that also differed the most in appearance. Interestingly, none of the three migrants I detected showed phenotypes that did not match with the local warning signal type in the area they were collected in. The differences in warning signal traits combined with limited migration suggests predator driven selection may play a role in maintaining the warning signal differences between the two populations. When I looked at correlations between phenotypic traits and geographic distance while controlling for genetic distance, results also supported traits evolving in a way that was non-neutral. Hindwing melanization can potentially be beneficial for thermoregulation (Hegna et al. 2013a) and lower temperatures at some of the higher elevation sites in Japan may drive such differences, apart from predators. Differences in elevation between the two populations on Honshu that differ the most in appearance, though, are not that great (200-300m). Only direct experimentation will be able to fully elucidate whether thermoregulation, predation, or even sexual selection could be influencing phenotypic differences.

It is also important to recognize that geographic factors and urbanization are also two influences that could contribute to the limited migration between the two populations on Honshu. Interestingly, previous work shows that mountainous terrain in the European Alps does not appear to impede gene flow (Galarza et al. in prep). I did detect an isolation by distance effect with (r=0.374, p<0.001) and without (r=0.45, p<0.001) including specimens from the northern

island of Hokkaido. Population structure combined with an isolation by distance effect raises the possibility that genetic drift could have originally led to the differences in warning signal features among the three populations or be helping to maintain the differences. Work examining demographic changes and population size more closely would be necessary to investigate drift further.

4 CONCLUSIONS

Diversity in warning signals is complex, and unlikely to be the product of simple processes (discussed in Ruxton et al. 2004). Indeed, complexity of explanations for the evolution and maintenance of warning signal diversity in *Parasemia plantaginis* is one resounding general conclusion from this dissertation. I found that a combination of explanations involving both selection and isolation could be plausible explanations for the evolution of signal differences and/or their maintenance.

The aspect of warning color in *Parasemia plantaginis* most likely related to historical factors was found in the hindwing color of specimens from the Caucasus region where divergent hindwing color coincided the most with mitogenetic divergence (I). One way to interpret a divergence between the Caucasus and neighboring populations is that the Caucasus was a glacial refugia. However, the lack of widely dispersed Caucasus haplotypes and the species distribution model suggests post-glacial isolation caused the genetic divergence. This is important because it suggests that the first differentiation in warning signal appearance in *P. plantaginis* may have faced the obstacle of gene flow. Therefore, although climatic isolation may have ultimately benefitted the evolution or retention of the reddish male hindwing, some form of divergent selection was also likely involved. Had refugia played a greater role in historic population structure, one may hypothesize that different male morphs could have evolved in isolation (i.e., red, yellow, white). However, partitioning of mitochondrial genetic variation among hindwing coloration (Galarza et al. in prep) and visual inspection of a haplotype network showing hindwing coloration (I) suggest this is not the case. Unfortunately, one can only speculate the degree to which climatic isolation or selection influenced the coloration of the Caucasus populations.

While historical factors isolating the Caucasus region may have eventually formed or preserved the reddish morph seen there, selection may be behind the evolution of color polymorphism in males (i.e. the formation of yellow and white morphs). Recent work has shown that white males have greater mating success and yellow males have better protective coloration (Nokelainen et al. 2012),

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suggesting such selection forces may have contributed to the divergence of yellow and white male hindwing color. The influence of sexual selection across the geographic distribution of *P. plantaginis* is not well known, but may shed light in the future on whether it could have been influential in the divergence of coloration between Caucasus and European populations.

I also found that hindwing melanization limited hindwing warning signal efficacy in *P. plantaginis* (II). Geographic differences in the amount of variation in melanization between Finland and the Alps could be due to competing selective forces benefitting thermoregulation or predation. Previous work has suggested thermoregulation may influence the geographic diversity of warning signals (Thompson 1984b, Brakefield 1985, Williams 2007), but empirical examination of such hypothetical scenarios appear to be lacking. Broadly speaking, then, my study adds to general discussion in the field of warning signal biology in showing that an abiotic factor appears to influence geographic diversity.

Forewing patterns are the least well understood aspect of warning signal diversity in *Parasemia plantaginis*, and they are also mostly unexplored as warning signals in other presumably aposematic arctiid moths. My experiment in Alaska showed evidence that forewings function as warning signals and can be under positive frequency-dependent selection (III). Migration between two areas in Japan that differ somewhat dramatically in forewing appearance, with migrants only found to exhibit local warning signal types and not those of their originating population also suggest a warning function for the forewing (IV). Additionally, some indirect evidence from European populations (Galarza et al. in prep) also suggests that selection has acted on this aspect of the signal. The similarity in forewing pattern across many arctiids (van Bemmelen 1918) raises the possibility that they may benefit from predators generalizing their avoidance across the similar forewings, if they all function as warning signals.

My own investigation into the forewing appearance in Alaska (III) suggested that predators may generalize across some variation in forewing patterns. A limited flying season for lepidopteran species likely reduces the time in which tight relationships between predator and prey can develop when predators initially learn to avoid signals, leading to greater generalization by predators. Relationships between predators and prey may be more specialized in the tropics, which could increase the degree of signal homogeneity in a population (discussed in Ihalainen et al. 2012). If predators in northern areas generalize more widely compared to predators in tropical areas, it may permit more intraspecific variation in warning signal appearance for aposematic species occurring at higher latitudes. Nokelainen (2013) found some suggestive evidence that predators may generalize across the color of other local butterflies in his experiments looking at frequency dependent selection on *Parasemia plantaginis*. The presence of other yellow butterflies decreased attacks on both white and yellow morphs of *P. plantaginis*. Meanwhile, the presence of other white lepidopterans resulted in decreased attacks on yellow morphs, but increased attacks on white morphs of *P. plantaginis*. My work in concert with that of Nokelainen (2013) raises the question whether greater predator generalization might permit increased warning signal variation in northern areas, but much

additional behavioral work is needed to probe the question. At the same time, recent evidence in some poison frog systems (Richards-Zawacki et al. 2013, Amézquita et al. 2013) and from examining a number of aposematic taxa (Alice Exnerová and Pavel Štys, personal communication) may lead to a conclusion that generalization occurs to a higher degree in the tropics than previously thought. An empirical finding that any latitudinal behavioral differences in generalization behavior exist would be illuminating in discussions of biogeographic patterns of diversity (see also Ricklefs 2009). In the case of *Parasemia plantaginis*, though, the large amounts of gene flow that seem to occur over hundreds of kilometers (I) may limit the extent to which geographic mosaics can form, regardless of the extent to which predators generalize.

Overall, my work begins to fill in gaps in the detailed understanding of aposematism by exploring what causes geographic diversity in warning signals of a Holarctic species. Most of the quantitative work on geographic diversity in warning signals has been done in tropical systems. Ecological differences between lower and higher latitudes are likely important in developing a more comprehensive understanding of warning signal function and evolution. The wood tiger moth has a warning signal influenced by historical, abiotic, and selection factors. We know from previous work with wood tiger moths that biotic environmental heterogeneity, in terms of predator and prey community, influence warning signal diversity in *P. plantaginis* (2013). Therefore, my study extends our knowledge of the influences of warning signal diversity in *P. plantaginis* to include abiotic and plausible historic factors. Many more questions remain to be answered concerning geographic or local warning signal diversity. The large spatial scale of many populations (up to several hundred kilometers in diameter, see chapter I) suggest that gene flow and selection within populations are important to understanding warning signal diversity in this species. How variable coloration became polymorphic, which color represents an ancestral condition, and what ultimately led to the evolution of greater sexual dimorphism in *P. plantaginis* represent three additional evolutionary questions posed by the results that deserve further investigation. Further research beyond the wood tiger moth will be needed on aposematic species in northern areas to understand similarities and differences in underlying causes of warning signal diversity between lower and higher latitudes. Undoubtedly, aposematic species will continue to generate interest as systems in which to study natural selection, color polymorphism, and diversification.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Varoitussignaalin maantieteellinen muuntelu täpläsiilikkäällä, *Parasemia plantaginis* **(Arctiidae)**

Biologisen monimuotoisuuden ymmärtäminen on yksi evoluutiobiologian päätavoitteista. Eliöt, joiden levinneisyysalue kattaa laajoja maantieteellisiä alueita, tarjoavat monimuotoisuuden tutkimiselle erinomaiset edellytykset. Laajalle levinneet lajit elävät hyvin erilaisissa ympäristöissä ja kohtaavat erilaisia valintapaineita, tarjoten erinomaisen mahdollisuuden tutkia valinnan, kuten saalistuksen ja lämpötilan ja sattuman, kuten geneettisen ajautumisen ja geenivirran sekä historiallisten tekijöiden, kuten eristyneisyyden, suhteellisia vaikutusia lajien evoluutioon.

Eläinten väritys antaa erinomaisen mahdollisuuden tutkia monimuotoisuuden syntyä ja säilymistä, koska ne vaikuttavat suoraan eliön kelpoisuuteen ja ovat tutkijalle helppoja havainnoida. Värimuuntelu aposemaattisilla eliöillä, jotka varoittavat saalistajia myrkyllisyydestään tai pahanmakuisuudestaan varoitussignaalein, on erityisen kiinnostavaa, koska niiden monimuotoisuuteen liittyy ilmeinen paradoksi. Luonnonvalinnan pitäisi nimittäin suosia yhdenmukaisia ja näkyviä signaaleja, kuten värikuvioita ja karsia huonot signaalit pois. Yleinen varoitussignaali, jonka kaikki saalistajat helposti tunnistavat on ilmeinen valintaetu puolustatutuville saaliseläimille. Kelta-musta raidoitus on esimerkki erittäin laajalle levinneestä, monien petojen helposti tunnistettavasta värityksestä. Myrkylliset saaliseläimet eivät kuitenkaan aina turvaa yhteen, "parhaaseen" varoitusvärikuvioon. Luonnossa esiintyy populaatioita, joissa värimuuntelu on runsasta eikä maantieteellinen muuntelu saman lajin varoitussignaaleissa myöskään ole tavatonta. Tätä varoitussignaalien vaihtelua ei vielä pystytä selittämään.

Täpläsiilikäs (*Parasemia plantaginis*) on siilikkäisiin (Arctiidae) kuuluva perhonen, joiden toukat ja aikuiset ovat aposemaattisia. Aikuisilla perhosilla on puolustukseen erikoistuneet rauhaset niskassa, joista ne erittävät pahanhajuista ja –makuista ainetta, jota erityisesti linnut pitävät epämiellyttävänä. Täpläsiilikäs varoittaa petoja tästä ominaisuudesta varoitusväreillä, joka on hyvin muunteleva: koirailla on mustavalkoiset etusiivet ja takasiivissä joko kelta-mustat, valko-mustat tai puna-mustat kuviot. Naaraiden etusiipien väritys on samanlainen kuin koirailla, mutta takasiipien väritys vaihtelee oranssi-mustasta puna-mustaan. Lajin levinneisyys kattaa koko pohjoisen pallonpuoliskon; lajia tavataan Pohjois- ja Keski-Euroopassa, Keski-Aasiassa, Venäjällä, Kiinassa, Japanissa sekä uudenmantereen pohjoisosassa.

Väitöskirjassani tutkin vaikuttaako saalistuksen lisäksi esimerkiksi populaatioiden eristyneisyys siihen, minkälaisiksi varoitussignaalit ovat paikallisesti kehittyneet. Hyödynsin väitöskirjassani yliopistojen ja luonnonhistoriallisten museoiden kokoelmia. Valokuvasin ja otin pienen kudosnäytteen DNA-analyysejä varten kokoelmiin säilötyistä yksilöistä. Tutkin myös saalistuksen vaikutusta perhosten ulkoasuun käyttämällä keinoperhosia, joiden vartalo on

muotoiltu muovailuvahasta, jolloin lintujen hyökkäykset niihin on helppo näh-
dä. Hyödynsin viimeisimpiä tietoja lintujen värinäöstä ja käytin niin kutsuttua
linnunsilmämallinnusta suunnitellessani keinoperhosille siivet, jotta ne olisivat
mahdollisimman lähellä oikean perhosen väritystä. Keinoperhoskokeita tein
Keski-Suomessa, Alpeilla ja Alaskassa.

Ensimmäisessä osatyössä kuvailen täpläsiilikään värimuuntelun sen koko
levinneisyysalueella. Koirailla sekä keltainen että vakoinen takasiipien väritys
on yleinen koko Euroopassa. Takasiipien valkoinen väritys on sen sijaan ylei-
sempi itäisillä levinneisyysalueilla. Esimerkiksi Japanista ja Kiinasta keltasiipi-
set muodot olivat jo erittäin harvinaisia. Koiraiden punainen väritys oli rajoit-
tunut ainoastaan Kaukasuksen alueelle. Naarailla punainen siipien väritys oli
selkeästi rajoittunut Eurooppaan. Uralin itäpuolella, Kiinassa, Japanissa ja Poh-
jois-Amerikassa naaraat olivat keltaisempia. Geneettisissä tutkimuksissa selvisi,
että populaatioiden geneettinen rakenne vastasi perhosten väritystä vain osit-
tain. Kaukasuksen punainen populaatio erottuivat selkeästi kaikista muista po-
pulaatioista. Samoin Japanissa erottui kolme selkeästi toisistaan erottuvaa ge-
neettistä populaatiota, jotka ovat myös ulkoasultaan erilaisia. Mitään geneettis-
tä yhteyttä valkoisen ja keltaisen värin suhteen ei löytynyt. Geneettisessä tutki-
muksessa selvisi myös, että laji on levinnyt hyvin nopeasti viimeisen jääkauden
jälkeen koko sen levinneisyysalueelle ja geneettistä erilaistumista on vain vähän
lukuun ottamatta Kaukasuksen aluetta.

Osatöissä kolme ja neljä tutkin petojen aiheuttamaa valintaa perhosten vä-
ritykseen. Hypoteesinani oli, että paikalliset pedot ovat oppineet välttämään
omalla alueella esiintyviä tietynlaisia perhosia, joka selittäisi eri maantieteellisil-
lä alueilla olevien perhosten erilaisen ilmiasun. Tein kokeen kahdella eri alueel-
la Alaskassa (Fairbanks ja Anchorage). Tulokset eivät yksiselitteisesti tukeneet
hypoteesia saalistuksen eriyttävästä merkityksestä: ainoastaan toisessa popu-
laatiossa (Fairbank) linnut saalistivat enemmän vierasta kuin tuttua värityyp-
piä.

Osatyössä kaksi erityinen mielenkiintoni oli paitsi maantieteellinen muun-
telu myös populaation sisäisen muuntelun selittäminen. Värin (valkoinen, kel-
tainen tai punainen) lisäksi täpläsiilikkään siiven värityksessä muuntelee mus-
tan melaniinin määrä. Melaniini auttaa perhosen lämmönsäätelyssä; osoitin
tummempien perhosten lämpiävän nopeammin kuin perhosten, joilla on vä-
hemmän melaniinia siivissä. Siiven melanisaatioasteessa oli selkeä etelä-
pohjois-gradientti: Virossa ja Etelä-Suomessa täpläsiilikkäät olivat vaaleampia
kuin Keski-Suomessa ja Pohjois-Suomessa. Tummimmat perhoset löytyivät Al-
peilta yli 1600 metrin korkeudesta. Koska siipien melanisaatiosta on selkeä hyö-
ty lämmönsäätelyssä, on aiheellista kysyä mikä sitä rajoittaa? Keinoperhosilla
toteutetussa saalistuskokeessa havaitsin, että linnut saalistivat enemmän me-
lanistisia perhosia. Mitä enemmän keltaista tai valkoista pigmenttiä perhosessa
oli suhteessa mustaan, sitä enemmän pedot sitä välttelivät. Tämä johtuu toden-
näköisesti siitä, että lisääntynyt melaniinin määrä siivissä rajoittaa varoitussig-
naalin (valkoinen tai keltainen väri) tehokkuutta. Tulos osoittaa, että melaniinis-
ta aiheutuu kustannuksia, joka todennäköisesti on syy siihen, että sen määrässä

on niin suuri populaation sisäinen ja populaatioiden välinen vaihtelu. Todennäköisesti melaniinista aiheutunut hyöty lämmönsäätelyssä on kuitenkin pohjoisessa ja korkealla vuoristossa kustannuksia suuremmat kuin siitä aiheutuva saalistuskustannus.

Väitöskirjatyöni on ensimmäinen työ, joka pureutuu selittämään varoitusvärien maantieteellistä muuntelua pohjoisella lajilla. Tähänastiset työt ovat lähinnä keskittyneet trooppisiin lajeihin kuten kaposiipi-perhosiin (*Heliconiinae*) *ja* myrkkynuolisammakoihin (*Dendrobatidae*). Väitöskirjatyöni on myös ensimmäinen, jossa tutkitaan lajia, joka on maantieteellisesti näin laajalle levinnyt. Pohjoisuus on todennäköisesti yksi merkittävä syy varoitussignaalin muunteluun tällä lajilla. Pohjoisessa lintusaalistajat ovat harvoin erikoistuneita tiettyyn lajiin tai lajiryhmään vaan yleistävät helpommin oppimansa signaalin riittävän samankaltaisiin signaaleihin. Petojen "suurpiirteisyys" on todennäköisesti yksi selitys varoitussignaalien muuntelevuuteen pohjoisilla leveysasteilla. Toinen syy löytyy lajin historiasta ja geneettisistä tekijöistä. Osa populaatioista, esimerkiksi Japanissa, on ollut melko eristyneitä ja näissä populaatioissa geneettinen ajautuminen näyttäisi olevan suurin yksittäinen tekijä, joka on ajanut populaatioita erilaisiksi. Toisaalta Euroopan Uralin länsipuolisten populaatioiden välillä ei ole käytännössä mitään isolaatiotekijää ja geenivirta populaatioiden välillä on tehokas muuntelua ylläpitävä tekijä.

Väitöskirjatyöni herätti myös paljon lisäkysymyksiä sekä täpläsiilikkään biologiasta että yleisemmin aposemaattisuudesta. Mistä täpläsiilikkäät ovat peräisin? Mitkä valintatekijät ovat johtaneet naaraiden ja koiraiden seksuaalisen dimorfiaan? Vaikka varoitusvärien tutkimus on pysynyt evoluutiobiologien mielenkiinnon keskiössä jo 150 vuotta, on selvää, että tutkimuskysymyksiä riittää vielä seuraavillekin tutkijasukupolville.

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