JYU DISSERTATIONS 691

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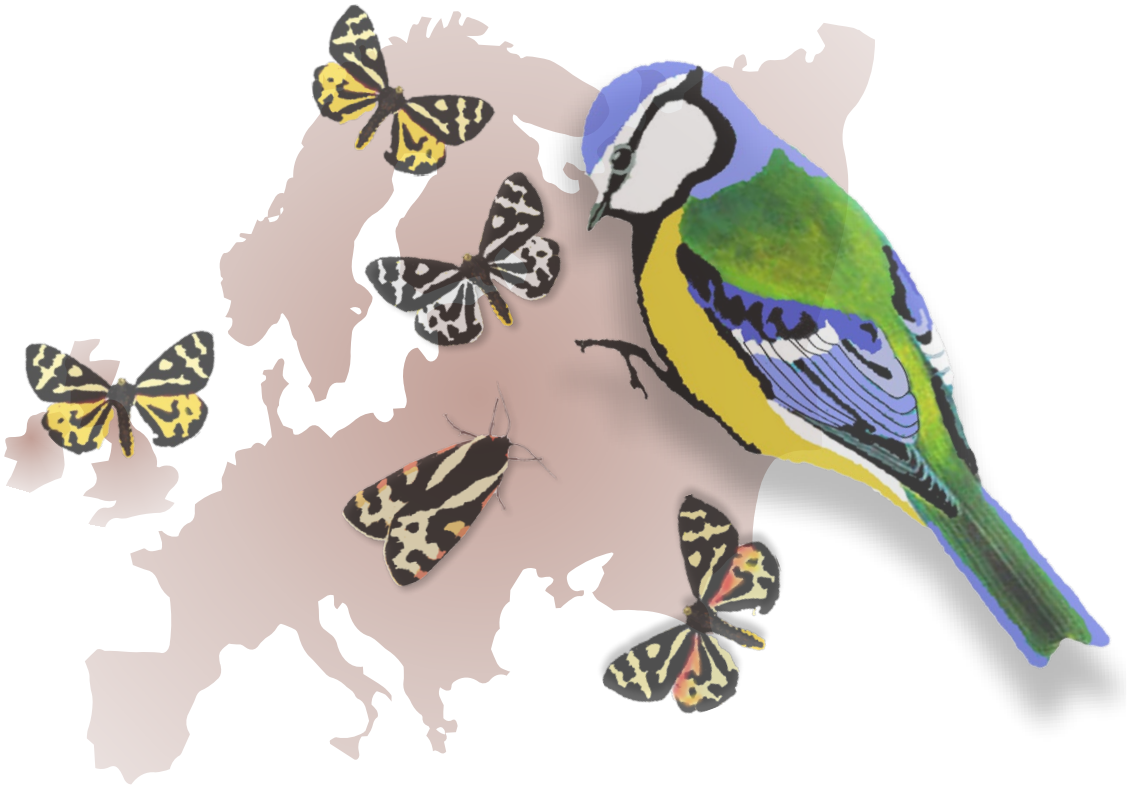
The Evolution and Ecological Drivers of Variation in Chemical Defences in the Wood Tiger Moth (*Arctia plantaginis*)



UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF MATHEMATICS
AND SCIENCE

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The Evolution and Ecological Drivers of Variation in Chemical Defences in the Wood Tiger Moth (*Arctia plantaginis*)

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ABSTRACT

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Aposematic warning signals and repellent chemical compounds are costly defences aimed at deterring predators' attacks. However, despite the selective pressure from predators, the strength of chemical defences exhibits substantial and unexpected variation within and across species. This thesis aims to better understand the evolutionary drivers of this variation in chemical defence. The wood tiger moth, *Arctia plantaginis,* is a chemically defended species with conspicuous hindwing colouration that differs both locally and geographically. A major component of the moth's chemical defences is produced *de novo* and secreted in response to attacks by avian predators. These secretions contain two methoxypyrazines: SBMP (2-sec-butyl-3-methoxypyrazine) and IBMP (2-iso-butyl-3-methoxypyrazine). In this thesis, I measured the variation in methoxypyrazine production across different wood tiger moth populations and tested how this variation influences predators' behaviour. Furthermore, I asked whether early life resources, such as proteins, play a key role in the production of this moth's chemical defences and warning signal. Thus, using diet manipulations, I investigated how dietary resources are distributed between growth, chemical defence, and colour pigmentations in male and female wood-tiger moths, and whether trade-offs between those traits occur. I found that the chemical defence of wild moths partly reflects local predation pressure, and both genetic and environmental components influence the strength of chemical defence. Male and female moths reared on a high-resource diet had more deterrent defensive fluids than individuals raised on low-resource or food-deprived treatments and, while the pigment components of the warning signals were only marginally influenced by food availability, there was a positive correlation between the strength of the visual component and the chemical toxins (suggesting so-called "signal honesty"). In conclusion, the resources available in early life have an important role in the efficacy of chemical defences, but warning signals are more genetically robust under variable environmental conditions.

Keywords: Chemical defences; honest signalling; melanin; pyrazine; resource allocation; warning signals; wood tiger moth.

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TIIVISTELMÄ

Ottocento, Cristina
Täpläsiilikkään (*Arctia plantaginis*) kemiallisen puolustautumisen evoluutio ja siihen vaikuttavat ympäristötekijät
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Saalistajien hyökkäyksiä torjuvat aposemaattiset varoitussignaalit ja myrkylliset kemialliset yhdisteet ovat kalliita puolustusmekanismeja. Huolimatta saalistajien tuottamasta valintapaineesta, puolustuskemikaalien vahvuudessa on merkittävää ja odottamatonta vaihtelua niin lajien sisällä kuin niiden välillä. Väitöskirjassani teen selkoa evolutiivisista voimista kemiallisen puolustautumisen vaihtelun taustalla. Täpläsiilikäs, *Arctia plantaginis*, on kemiallisesti puolustautuva perhoslaji, jonka takasiipien huomiota herättävä väritys vaihtelee sekä paikallisella tasolla että globaalisti. Laji tuottaa kemiallisen puolustautumisen keskeistä komponenttia *de novo*, ja erittää sitä vasteena lentävien saalistajien hyökkäyksiin. Puolustuserite sisältää kahta eri metoksipyratsiinia: SBMP:tä ja IBMP:tä. Väitöskirjassani tutkin puolustautumisen vaihtelua täpläsiilikäspopulaatioiden välillä ja vaihtelun vaikutuksia lintujen käyttäytymiseen. Lisäksi selvitin, vaikuttavatko varhaisen kasvuympäristön resurssit, kuten proteiinit, merkittävästi kyseisen lajin kemiallisen puolustautumisen ja varoitusvärityksen muodostumiseen. Tutkin miten ravintoresurssien käyttö jakautui kasvun, kemiallisen puolustautumisen ja pigmentaation välillä sekä mahdollisia allokaatiokustannuksia naaras- ja koirastäpläsiilikkäissä säätelemällä saatavilla olevaa ravintoa. Havaitsin, että villien yksilöiden kemiallinen puolustautuminen on osittain mukautunut paikalliseen saalistuspaineeseen, ja sekä geneettiset tekijät että ympäristötekijät vaikuttavat kemiallisen puolustautumisen vahvuuteen. Osoitin myös, että koiras- ja naarastäpläsiilikkäät, jotka kasvoivat runsasravinteisella ruokavaliolla, erittivät tehokkaampaa puolustusnestettä kuin matalaravinteisella tai rajoitetulla ruokavaliolla kasvaneet. Ravinnon saatavuus vaikutti varoitusvärityksen pigmentaatioon vain osittain, mutta varoitusvärin ja myrkyllisten kemikaalien voimakkuuksien välillä oli positiivinen korrelaatio (viitaten niin kutsuttuun "rehelliseen signalointiin"). Yhteenvetona varhaisessa kasvuympäristössä saatavilla olevat resurssit ovat tärkeitä kemiallisen puolustautumisen tehokkuuden kannalta, mutta varoitussignaalit ovat vakaita ympäristötekijöiden vaihtelusta huolimatta.

Avainsanat: Kemiallinen puolustautuminen; melaniini; pyratsiini; rehellinen signalointi; resurssien jako; täpläsiilikäs; varoitussignaalit.

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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 Ottocento, C., Winters, A. E., Rojas, B., Mappes, J., & Burdfield-Steel, E. 2022. Not just the sum of its parts: Geographic variation and nonadditive effects of pyrazines in the chemical defence of an aposematic moth. *Journal of Evolutionary Biology* 00, 1–12. https://doi.org/10.1111/jeb.14142

II Ottocento, C., Rojas, B., Burdfield-Steel, E., Furlanetto, M., Nokelainen, O., Winters, S., & Mappes, J. 2023. Diet influences resource allocation in chemical defence but not melanin synthesis in an aposematic moth. Pre-print *bioRxiv* (2023): 2023-02 https://doi.org/10.1101/2023.02.24.529866

III Burdfield-Steel, E., Ottocento, C., Furlanetto M., Rojas, B., Nokelainen, O., & Mappes J. 2023. Honest signalling in predator-prey interactions: testing the resource allocation hypothesis. Manuscript.

Table of author contribution to the original publications.

Study	**I**	**II**	**III**
Original idea	EB**,** JM, BR	JM, BR, EB	JM, BR, EB
Study design	EB, BR, JM, CO	BR, EB, JM	BR, EB, JM
Data collection	CO, EB, AW	CO, BR, MF	CO, MF, BR, EB
Data analysis	EB, CO, AW	BR, CO, ON, SW, MF	EB, CO, ON
Writing	CO, AW, JM, EB, BR	CO, JM, BR	EB, CO, JM, BR

JM = Johanna Mappes, BR = Bibiana Rojas, EB = Emily Burdfield-Steel, CO = Cristina Ottocento, AW = Anne Winters, MF = Miriam Furlanetto, ON = Ossi Nokelainen, SW = Sandra Winters

1 INTRODUCTION

1.1 Prelude - The complexity of variation in defence within and among populations of aposematic organisms

The concept of aposematism was first introduced independently by the English naturalists Alfred Russel Wallace (1867, 1889) and Edward Bagnall Poulton (1890) to describe a phenomenon whereby conspicuously coloured animals are repellent or unpalatable to predators. Aposematism comes from the Greek origin terms *apo* (away) and *sema* (sign), and it refers to an anti-predator strategy that combines a primary defence (e.g., a colour, an odour, a sound) which operates before the attack of the predator, with secondary defences (e.g., a chemical, morphological, behavioural attributes) which deter the predators only after the attack (Poulton 1890, Rojas *et al.* 2015). The distinctive warning signals in aposematic organisms indicate their unprofitability to the predators and direct them to more profitable prey (Ruxton *et al*. 2019). The association between the signal and unprofitability facilitates predators' avoidance learning, especially when prey are easy to detect and remember (Mappes *et al.* 2005). Avoidance learning has been demonstrated to occur more rapidly when the warning signal is highly conspicuous (Lindström *et al.* 1999) and when the prey species employing warning signals are present in high densities (Lindström *et al.* 2001). This phenomenon can create a positive frequency dependent selection (+FDS) and is expected to purge any variation in signal salience. However, it is important to note that still numerous aposematic species exhibit significant variation in their warning signals (Briolat *et al*. 2018).

Polymorphism (the occurrence of two or more morphs present in the same populations) in aposematic species, has been well documented in several taxa such as frogs (e.g., blue poison dart frog *Dendrobates tinctorius*, Rojas and Endler 2013), beetles (e.g., leaf beetle *Chrysomela lapponica*, Zvereva *et al.* 2002), butterflies (e.g., plain tiger butterfly *Danaus chrysippus*, (L.), Smith *et al.* 1993), and moths (e.g., large yellow underwing moth *Noctua pronuba* (L.), Cook and Sarsam 1981). Polymorphic aposematic individuals are known to exhibit variation in their primary defences in the form of different patterns, (e.g., the fire salamander *Salamandra Salamandra*, Najbar *et al.* 2018), colours (e.g., the hibiscus harlequin

bug *Tectocoris diophthalmus*, Fabricant *et al.* 2018), or in the expression of a particular trait, such as the intensity of colouration (e.g., cichlid fish *Cichlasoma citrinellum*, Webber *et al.* 1972).

Due to the expectation that predators learn to avoid warningly coloured prey more quickly when the consequences of attacking them are severe, it is expected that predator selection would diminish the variation in chemical defence as well. Increasing evidence, however, points that variation in secondary defences is common among aposematic organisms (Speed *et al.* 2012). Such variation can be present, for example, in the chemical profile, in the amount of specific chemicals, or both (Speed *et al.* 2012). There are numerous aposematic organisms in nature that exhibit variation in their chemical defences such as poison frogs, *Dendrobates tinctorius* (Lawrence *et al.* 2019); ladybirds *Harmonia axyridis* (Bezzerides *et al.* 2007); nudibranchs *Goniobranchus splendidus* (Winters *et al.* 2019); monarch butterflies *Danaus plexippus* (Alonso-Mejía and Brower 1994). Variation in chemical traits can manifest in different ways, including differences in concentration for a specific chemical compound (as observed in species like monarchs) or variations in the composition of chemical bouquets (as seen in examples such as poison frogs). In some cases, both the amount and consistency of chemical traits may exhibit variation (Ottocento *et al.* 2022). However, the causes and consequences of variation in the chemical defences are not yet well understood: the study of chemical defences is often complicated by non-additive interactions between chemical components, meaning their effects on predators may not simply mirror measures of the pure compounds. Therefore, to better understand the significance of chemical variation it is fundamental to investigate the extent of such variation and test the efficacy of defences against relevant predators.

1.2 Primary defences: warning colours

Structural colours and pigments in animals serve as dynamic visual communication to convey messages to other organisms, such as conspecific or predators (Cuthill *et al.* 2017). Structural colouration, such as iridescence, is caused by the variation in hue with the angle of view (Stavenga *et al.* 2011). For example, in the chemically defended jewel beetle, *Sternocera aequisignata*, the iridescence guarantees protection against avian predators' attack (Kjernsmo *et al*. 2021). Structural colours offer the capability to directly control the way that light reflects from the organism. On the opposite, pigments (e.g., flavonoids, pterins, and melanin) reflect (or absorb) the wavelengths of light, independently of the angle (Vukusic and Stavenga 2009). Pigments are constrained by chemistry (Stoddard and Prum 2011, Cuthill *et al.* 2017), thus they are expected to be more stable and genetically robust than structural colours. However, pigment can be costly to synthesise and often macronutrients, such as proteins, are needed from the dietary resources. For example, proteins are the basis for the biosynthesis pathway of melanin expression (Lee *et al.* 2008), which has been shown to be

genetically based in both invertebrates (Ellers and Boggs 2002, Lindstedt *et al.* 2009, van't Hof *et al.* 2019) and vertebrates (McNamara *et al.* 2021). Melanin pigment also plays an important physiological function in many aposematic organisms (Fabricant *et al.* 2013, Lindstedt *et al.* 2020): it has a protective function against UV radiation in vertebrates (Jablonski and Chaplin 2017, Nicolaï *et al.* 2020), while in invertebrates is important in the thermoregulation process (True, 2003, Trullas *et al.* 2007, Lindstedt *et al.* 2009). It also plays a fundamental function in wound healing (Bilandžija *et al.* 2017), predation (Majerus 1998, Hegna *et al.* 2013), and immune defence (McNamara *et al.* 2021), in insects. Since pigments are often resource limited, warning signals can be costly to produce and thus, variation in signal expression may be maintained via physiological trade-offs.

1.3 Secondary defences: chemical defences

While many predators can benefit from eating aposematic prey when the advantages of obtaining nutrients overcome the costs of ingesting toxins (Sherratt *et al.* 2004, Rowland *et al.* 2010), some organisms can be unpalatable enough to trigger a disgust behaviour of the predators and escape. The burnet moths, *Zygaena filipendulae*, the European swallowtail larvae, *Papilio machaon*, the large white *Pieris brassicae*, the firebug *Pyrrhocoris apterus*, and the ladybird, *Coccinella septempunctata* are such examples (see Wiklund and Järvi 1982).

The nutrients gathered from the diet are a fundamental part of the acquisition of substances needed for the production or sequestration chemical defences. Some animals, such as longwing butterflies (genus Heliconius) (Brown and Benson 1974), monarch butterflies *Danaus plexippus* (L.) (Agrawal *et al.* 2012), sequester the defensive compounds from their host plants. Nudibranch molluscs of the genus *Chromodoris* store the repellent compound latrunculin A, sequestered from sponges, in their mantle (Cheney *et al.* 2016). These animals have evolved the ability to selectively sequester and accumulate toxic compounds without breaking their own physiological equilibrium. However, several organisms can synthesize the chemical compounds *de novo* (Pasteels 1984, Pasteels 1989, Burdfield-Steel *et al.* 2018), without a direct supply of defensive compounds derived from food plants or other organisms, even though some plant compounds (e.g., sterols, sugars) or specific macromolecules (such as proteins) may still be an initial source for the *de novo* synthesis. For example, the leaf beetle *Oreina gloriosa* synthesises a mixture of cardenolides *de novo* as defence (Rowell-Rahier *et al.* 1994) and the American Acraeinae butterflies (Lepidoptera: Nymphalidae) produces the cyanogenic glucoside linamarin *de novo* (Brown and Francini 1990). Interestingly, there are also animals able to simultaneously sequester and produce their defences *de novo*: both in the burnet moth *Zygaena filipendulae* and in the red postman *Heliconius erato* (Lepidoptera) the sequestration of certain compounds is combined with the *de novo* synthesis of cyanogenic glucosides (Fürstenberg-Hägg *et al.* 2014, Mattila *et al.* 2021). In *Z. filipendulae* the evolution of the sequestration strategy from cyanogenic plants as

a food source was preceded by the *de novo* CNglcs biosynthesis. Larvae that sequester the toxic compound from plants can preserve energy and nitrogen for metabolic activity, and the biosynthesis of chitin (Fürstenberg-Hägg *et al.* 2014). Indeed, the sequestration of plant allelochemicals is predicted to be more beneficial and less physiologically and energetically costly than the production of *de novo* chemical defences. Likewise, organisms that sequester their chemical compounds benefit from the other nutrients acquired from the plants (Zvereva and Kozlov 2016). Studies on the costs of *de novo* defences are still relatively scarce (Zvereva and Kozlov 2016), but it has been suggested that the expense of producing these types of defences may cause physiological trade-offs with other traits such as development, survival, and production of new offspring (Camara 1997, Ruxton *et al.* 2019, Zvereva and Kozlov 2016).

The variation in chemical defences within and among populations of aposematic species is crucial to determine the degree of protection provided against pathogens and predators. This variation can be present not only in the amount of toxins or distasteful compounds, but also in the specific chemical profile of these defensive compounds (Speed *et al.* 2012) and can reflect (1) environmental effects, such as the age of the organism or competition for resources available in the environment (Speed *et al.* 2012, Burdfield-Steel *et al.* 2019); (2) genetic effects, or (3) the genotype-by-environment interactions, which can arise when specific genotypes react in distinct ways to the different environmental conditions encountered. There are however, only few studies that have shown how genetic effects and genotype-by-environment interactions have a strong impact on heritable variation in chemical defences (Sculfort *et al.* 2020)
The costs of producing and maintaining chemical defences may vary among individuals of the same species, or even populations, leading to a great variation in the type and efficacy of defences used. A classic example is automimicry, which emerges when some individuals of the same populations have chemical defences while others do not (Brower *et al.* 1967, Speed *et al.* 2012). This intriguing case can possibly be explained as a form of cheating, where those individuals that do not have chemical defences benefit from not investing in them, while still gaining protection from their resemblance to their defended conspecifics. Such a strategy could risk becoming too common, at which point predators would cease to avoid any individuals in the population, although this may be avoided if predators have the capability to detect cheating individuals. The rhizosphere bacterium *Pseudomonas fluorescens* is an example of automimicry regulated by frequency-dependent selection: when the population density is high the secondary metabolites to repel predators are secreted, on the other hand, when the metabolite production is not necessary, the non-toxic strains grow faster (Jousset *et al.* 2009, Jousset, 2011, Speed *et al.* 2012).

Variation in the biochemical profile of defended species can be a consequence of competition for food resources or the use of the same chemical defences for other purposes such as pheromones. In some arctiid moths the pyrrolizidine alkaloids (PAs) not only function as a defence against other invertebrates, but also as pheromones (Rothschild *et al.* 1979, Weller *et al.* 1999) or even as nuptial gifts in *Utetheisa ornatrix* (Dussourd *et al.* 1991). The variability

of defensive toxins demonstrates the complexity of the interactions between organisms, their life history and the different selective pressures acting in different environments and highlights the importance of investigating how the different selective pressures shape the evolution of the diversity of life.

1.4 Resource allocation, trade-offs, and honest signalling

1.4.1 How do animals use and allocate their (finite) resources?

Life history theory explains how organisms allocate resources in various traits such as reproduction, survival, and growth during their lifetime (Stearns 1992). According to the theory, organisms that can invest more resources in growth and reproduction, at the expense of their survival, will have a higher number of offspring. On the contrary, organisms that invest fewer resources in reproduction, therefore having fewer offspring, will be more likely to survive longer and have more opportunities to reproduce again in the future. Consequently, there should be a trade-off between reproduction and survival when the total resources available for individuals are the same. This is, however, not always the case, as several studies have demonstrated that individuals who manage to gain a greater amount of resources can have higher reproductive allotment without having a reduced survival, compared to individuals with a low amount of resources (Reznick *et al.* 2000). For instance, positive correlations have been found between female survival and egg production in the carabid beetles *Calathus melanocephalus* and *Pterostichus versicolor* (van Dijk 1994), and between female reproductive success and survival in the song sparrow *Melospiza melodia* (Smith 1981). A key prediction formulated by van Noordwijk and De Jong (1985) states that a positive correlation between life history traits could be observed if the genetic variance for resource acquisition is larger than the genetic variance for resource allocation. This scenario may however be restricted to specific environmental conditions (Reznick *et al.* 2000). Trade-offs are the result of multiple strategies that organisms adopt, and resource allocation affects the ability of organisms to adapt to changing environments, in the form of positive or negative correlations between different traits. In aposematic organisms, maintaining the strength of their secondary defences and the conspicuousness of their colouration is usually energetically costly. Moreover, not only may these traits draw from the same pool of resources (such as amino acids and proteins), but these primary resources may be also required for growth and reproduction.

1.4.2 Resource allocation in aposematic organisms

The production of bright and conspicuous warning colouration, secondary defences (e.g., chemical defences), and life-history traits (e.g., growth, reproduction) in aposematic organisms is a complex and dynamic process that requires energy. But do all these traits compete for the same pool of resources

and evolve simultaneously? The resource-allocation hypothesis provides a possible answer to question, assuming that when resources are limited, they need to be distributed to various traits, leading to trade-offs (Stearns 1989, Glazier 2002). Thus, it is necessary to first verify whether these traits are costly and attained from the same pool of resources. Measuring the costs may be difficult, but it is possible to measure trade-offs in resource allocation when acquiring and maintaining defences may compete for the same elements or biomolecules (e.g., nitrogen and proteins) allocated for other vital functions such as development and reproduction (Camara 1997, Ruxton *et al.* 2019). Several experimental studies have tested the resource allocation hypothesis between warning signal, life-history traits, and chemical defences. For example, in *Heliconius erato* there is a positive correlation between toxicity and life history traits such as developmental time and mass (Mattila *et al.* 2021); in *Harmonia axyridis* the secretion of the chemical defences (reflex bleeding response) and the warning colouration do not correlate, but the costly reflex bleeding negatively affects life history traits such as developmental time and size (Grill and Moore 1998). Recent work on *Danaus plexippus* showed that the sequestration of cardenolides increases oxidative damage and decreases conspicuousness (Blount *et al*. 2023), while Lindstedt *et al.* (2010) found that *Arctia plantaginis* have a paler and weaker warning signal when reared on high concentrations of iridoid glycosides (IG). Altogether, this suggests that not only do trade-offs in resource allocation affect primary and secondary defences, but also that the excretion costs of those defences may influence and conflict with the cost of conspicuousness (Lindstedt *et al.* 2010).

1.4.3 Honest signalling in aposematic organisms

The concept of honest signalling refers to a particular characteristic or behaviour of an organism that conveys a message that truly represents its quality or ability. For example, the bright and colourful plumage of male peacocks is a handicap trait that makes it more difficult to escape from predators. However, when choosing a mate, females can use that trait as a reliable indicator of the male's quality: only the healthy individuals that can afford such a disadvantageous trait have better fitness (Gadagkar 2003). The handicap hypothesis was first introduced by Amotz Zahavi (1975, 1977) to explain how organisms evolve costly behaviours or structures that provide honest information about their fitness, and states that only high-quality organisms can survive to the costs of those traits. This hypothesis has been rejected (e.g., Kirkpatrick 1986) and then supported (Grafen 1990) over the years. However, more recent studies (Penn and Szamado 2019, Fromhage and Henshaw 2021) have criticised the Zahavian principle (which considered handicap traits as wasteful and inefficient because they would only reduce survival) and highlight the importance of consider the reproduction advantages of those traits.

Aposematic signalling has not received so much attention in the context of honest signalling theory in the past, compared to other types of communications (Summers *et al.* 2015), however, in recent years, the number of studies investigating honest signalling in aposematic species is increasing.

There are two different ways to describe honesty in aposematic signals (Summer *et al.* 2015). *Qualitative honesty*, is the concept on which the foundation of aposematism lies, and occurs when signal conspicuousness correlates positively with the presence of a secondary defences. *Quantitative honesty* refers instead to the strength of warning signals that accurately reflects the efficacy of the secondary defences (Summers *et al.* 2015).

A major critique of the concept of quantitative honesty has been the lack of a clear biological link that demonstrates a positive correlation between the warning signal and the chemical defences. Indeed, Guilford and Dawkins, (1993) dismissed the idea of a possible mechanistic link that would identify aposematic honest signalling as a strict Zahavian mechanism. However, it has been suggested that the energy used to sequester or synthesise the defences, or to synthesise the pigments of the warning signal, can play an essential part in maintaining honesty in aposematic signals (Holloway *et al.* 1991, Srygley 2004, Blount *et al.* 2009). In a broader context, the role of shared resources allocated to primary and secondary defences can be tested in the context of trade-offs of resources allocation (Holen and Svennungsen 2012). This is important because it highlights the necessity to experimentally test predators' response the primary and secondary defences (White and Umbers 2021) and observing the predators' behaviour in response to variation chemical defences rather than just measuring the amount or concentration of defensive compounds (Lawrence *et al*. 2019).

1.5 The aposematic wood tiger moth *Arctia plantaginis* as a study species

The aposematic wood tiger moth *Arctia plantaginis* (Erebidae: Arctiinae), formerly *Parasemia plantaginis* (Rönkä *et al.* 2016), displays a conspicuous colouration that differs both geographically and locally (Watson and Goodger 1986, Chinery 1993, Nokelainen 2013) throughout the Holarctic region (Hegna *et al.* 2015). This species is a well-suited system for studies on resource allocation and honest signalling as both its primary (warning signal) and secondary (chemical) defences have been identified and studied (Lindstedt *et al.* 2011, Nokelainen *et al.* 2014, Rönkä *et al.* 2018, Rojas *et al.* 2017, Burdfield-Steel *et al.* 2018). The wood tiger moth is a polyphagous species, thus the nutrients available to it are strongly dependent on the host plants, and a capital breeder, so additional resources cannot be acquired in the adult life-stage, when reproduction takes place (Tammaru and Haukioja 1996).

Adult wood tiger moths have conspicuously coloured hindwings to repel the attacks of avian predators (Lindstedt *et al.* 2011, Nokelainen *et al.* 2012). In males, the warning signal colouration varies across the range of the species distribution from red to yellow, or white (Dubatolov and Zahiri 2005, Hegna *et al.* 2015, Fig. 1). In females, the wings display continuous variation in colouration from red to yellow (Lindstedt *et al.* 2011, Hegna *et al.* 2015). The black melanin

patterning is present in both sexes, and it is a dopamine-derived eumelanin, while the red, yellow, and white pigments are partly produced by pheomelanin (Brien *et al*. 2022). Melanin plays a crucial role not only in the visual warning signals but also in other processes such as thermoregulation (Lindstedt *et al*. 2009), immunity (Nokelainen *et al.* 2013), and warning signal (Hegna *et al.* 2013). *A. plantaginis* also shows geographic variation in the amount of melanin, which increases in the alpine region (Hegna *et al.* 2013) presumably providing thermoregulation benefits, as well as varying between and within populations.

FIGURE 1 Range of variation in hindwing colouration in Georgian male wood tiger moths. Photo: Cristina Ottocento

The secondary defences of the wood tiger moth are enemy specific. The abdominal fluid is targeted towards invertebrate predators such as ants, while the thoracic fluid is targeted against avian predators (Rojas *et al.* 2017). The thoracic fluid contains two types of methoxypyrazines, SBMP (2-sec-butyl-3-methoxypyrazine) and IBMP (2-iso-butyl-3-methoxypyrazine, which are produced *de novo*. This means that these compounds are not sequestered from the diet but are produced by the moth itself (Burdfield-Steel *et al.* 2018). The chemical defences of female wood tiger moths are known to be costly and affected by resource limitation. Experimental manipulation of the nutrients in the diet at the larval stage have shown that food deprivation has negative effect on the efficacy of the chemical defences (Burdfield-Steel *et al.* 2019). This has provided new opportunities to investigates the investment of dietary resources, such as proteins, in primary and secondary defences.

1.6 Aim of the thesis

Are primary and secondary defences linked? Are their expensive costs paid in the currency of energy, leading to trade-offs, such as one trait being prioritised over the other? To tackle these questions, it has been proposed that part of the variation seen in aposematic populations could be explained by both traits obtained resources from the same pool, possibly leading to a trade-off between warning signal and chemical defence. However, few studies have tested both chemical defence and signal efficacy in the same system and observed the natural predator response to this variation in the defences.

The main aims of this thesis were to investigate the variability of chemical defences within and between populations in *A. plantaginis*, and whether the production of the chemical defences has the same or greater sensitivity to the variation in early-life resources than the warning signal.

In study I, I examined the strength and variation of the chemical defences of the Estonian, Finnish, Georgian and Scottish populations of wood tiger moth (which have different predation pressures, as has been shown in previous works by Nokelainen *et al.* 2014 and Rönkä *et al.* 2020) and explore whether the differences in these defences between populations could have a genetic basis. In addition, this work tested the role of the main pyrazine compounds on deterring ecologically relevant predators.

In study II, I manipulated the protein availability in the larval diet of *A. plantaginis*, to test how resources are distributed between growth, defence, and melanin pigmentation. According to the resource allocation hypothesis, I predicted a trade-off between the costly melanin and the chemical defence (i.e., individuals with low-melanin amounts have more resources to allocate to chemical defence).

Finally, in study III, I investigated if resource-allocation trade-offs can create honest correlations between warning signals and defence in female wood tiger moths. As previous experiments have shown, both the quality of the warning signal (the conspicuous red colouration the hindwings) and the pyrazine content in the defensive fluids are under selection by avian predators, thus this provides a unique opportunity to test trade-offs in resource allocation.

2 METHODS

2.1 Insects rearing and larval diet

The wild male moths used for the experiments in Chapter I, were collected by either pheromone baited traps or nets from Finland (Tornio; Jyväskylä; Tvärminne), Estonia (Pärnu), Georgia (Zekari Pass; Fig. 2B), and Scotland (Findlater Castle; Findochty and Portknockie path) between 2015 and 2021. The laboratory-reared moths were from individuals raised in laboratory conditions (greenhouse) and originated from laboratory stock founded from the same populations. After capture in the wild, moths were either sampled for chemical fluids immediately or transported to the University of Jyväskylä in Central Finland (62°N, 26°E) for their fluids to be collected there.

The Finnish and Estonian laboratory populations were overwintered every third generation during the third instar. Larvae were grown in family groups of no more than 30 and fed with *Taraxacum spp.* (dandelion) and misted with water daily. The Georgian populations did not survive with a *Taraxacum spp.* diet only, so it was supplemented with *Plantago sp.* and *Rumex sp.* The individuals that reached the pupation stage were kept separately in vials at 25°C until the adults emerged. The chemical defence fluid was extracted by squeezing just below the prothoracic section of the male moths with tweezers, collected with a glass capillary, and stored in Eppendorf tubes at -18°C (Furlanetto 2017).

FIGURE 2 A) Georgian male wood tiger moth in the wild. B) Zekari pass (41°49′39″N 42°51′43″E) located in Lesser Caucasus mountains in Georgia, where we collected *Arctia plantaginis* Georgian population. Photos: Cristina Ottocento

The wood tiger moths used in the experiments of chapters II and III were obtained from a laboratory stock founded in 2013 with wild-caught individuals from Estonia and kept at the greenhouse of the University of Jyväskylä. The stock was supplemented annually with Estonian wild-caught individuals, to preserve genetic diversity. For chapter II, I selected families among which the variation in male melanin amount, existed and, for chapter III, I picked families the cover the full range of variation in female colouration see in the stock. After hatching, larvae were kept together for 14 days and fed with lettuce and dandelion. Then, using a split family design (for chapter II), they were divided into two different treatments of an artificial diet differing in their protein content (high, low). For chapter III they were split into four different treatments: 1) fed *ad libitum* with *Taraxacum spp*.; 2) fed with *Taraxacum spp*., but food deprived for one day once per week; 3) fed on an artificial low protein diet; 4) and fed on an artificial high protein diet.

For each treatment, larvae were kept in boxes of 10 individuals until reaching the pupa stage. Each box was checked and watered daily and cleaned when needed. Larvae were fed daily, *ad libitum*, with their diet treatment (except for the food deprived treatment). Life-history traits (time to pupation, pupal weight) and the degree of melanin (high, low) were recorded for each individual. When the male and female adults emerged from pupae, they were given water and stored at 4°C to slow their metabolic rate down to maintain their condition for further use. The thoracic defence fluid was extracted as described previously in chapter I.

2.2 Chemical analysis

Prior to GC-MS analysis, the chemical defence samples collected in chapters I, III were thawed and mixed with a 200-µl NaCl solution (3%). Measurement of the pyrazines was done as described in Burdfield-Steel *et al.* (2018). Pyrazine volatiles were extracted using a solid phase microextraction fiber and the analyses were carried out on an Agilent 6890 series GC system. The oven temperature was programmed as follows: 3 min at 60°C then ramped to 170°C at a rate of 7°C/min and from 170 to 260°C at a rate of 20°C/min and kept at that temperature for an additional 5 min. The amount of the two methoxypyrazines in the sample was calculated by comparison with known amounts of the SBMP and IBMP standards, run in the same manner as the fluid samples.

2.3 Predator assay

For the predator response assay in chapters I, II, and III, I used blue tits (*Cyanistes caeruleus*). This species is common in Finland, possible to keep in captivity for brief time periods during the experiments, and it has already been used in different studies on the chemical defences of wood tiger moths (Rojas *et al.* 2017; Burdfield-Steel *et al.* 2018).

The birds used for the experiment were caught at Konnevesi Research Station (Central Finland). The experimental box (see Fig. 3 A) was fitted with a light bulb showing the entire visible daylight spectrum (Nokelainen *et al.* 2012), a water bowl, a perch and a mesh fabric barrier that allowed observers to clearly see when the birds first noticed the food item presented. This barrier was in front of a movable hatch on which the food was placed. During the training session, I offered each bird four baits (oat flakes) and three sunflower seeds on a small white plate. Only when the birds ate all the baits in the training phase did the experiment begin.

Each bird experienced four trials, one after the other, at 5-minute intervals. The first and last trials were done with baits soaked in tap water to ensure that the bird was motivated to eat (first) and that the bird was still hungry (last). In the second and third trials, the bird was presented with either the defensive fluids of the wood tiger moth (chapters I, II, III) or pure pyrazines SBMP and IBMP (chapter I) soaked in the baits. The trial ended two minutes after the bird had eaten the baits, or after a maximum duration of 5 minutes if the bird did not eat the whole baits. In each trial, I recorded the reaction of the birds to the chemical defences of the moths: the proportion of bait eaten (Fig. 3 B), the beak cleaning frequency, the latency to approach (how long it took for the bird to get close to the plate on which the bait was offered), the latency to eat after approaching (the hesitation time between approach and attacking) and the latency to eat.

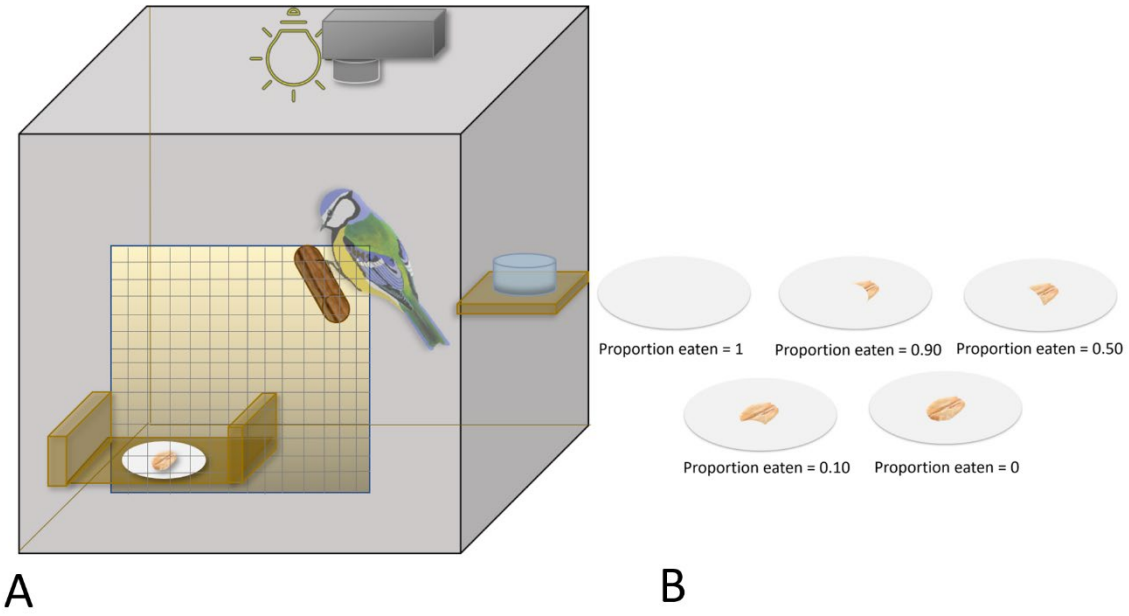

FIGURE 3 A) Experimental setup of the predator assay illustrating the perch, water, camera, light source, mesh opening for observation and hatch for inserting the plate with the bait (oat flake) into the enclosure. 3 B) Proportion of bait (oat soaked with defensive fluid or water) eaten by the predators. The palatability was classified into five classes according to the proportion of the bait consumed by the birds: 100% (1), 90% (0.90), 50% (0.50), 10% (0.10), and 0%. (Study II).

2.4 Image analysis

For chapters II and III, after emerging as adults and being sampled for their chemical defences, all moths were stored in Eppendorf tubes and kept at -18°C for further colour analysis. Before starting to spread the moths' wings for photography, frozen individuals were put in one large snap-top Tupperware container. The bottom of the container was covered with paper towels soaked in water, to ensure that the wings and the specimen were moisturised to prevent them from breaking during the spreading process. After 24 hours I spread the moth wings using needles on a wooden board. Spread moths were photographed in a dark room, under controlled visible light. A standard light source 75W Exo-terra Sunray (mimicking sunlight across the spectrum) was used. Photographs were taken with a Samsung NX1000 digital camera, customised to record across the full-spectrum range and equipped with a Nikon EL-Nikkor 80 mm enlarging lens, in a dark room under controlled conditions using an established protocol (Troscianko and Stevens 2015, Nokelainen *et al.* 2017). The camera was positioned directly above the moth (i.e., at a 90-degree angle to the surface of the wings). A ruler was also photographed alongside the moths to correctly scale the image during the analysis. The images were scaled to the same resolution (pixels per mm), based on the ruler measurements (20 mm). Photographs were taken in

RAW format (Troscianko and Stevens 2015; Nokelainen *et al.* 2017). I used ImageJ (v. 1.50f) for image analysis. In chapter (II) from every image, a set of regions of interest (ROIs) were selected from the dorsal side of the male's wings: (a) whole forewing area, (b) forewing melanised region, (c) whole hindwing area, and (d) hindwing melanised region. These were reported in a dataset using the batch image analysis tool (ImageJ). The relative proportion of the wings that were melanised was calculated by dividing the area of the melanised ROIs by the whole wing ROIs, for the forewings: [(forewing melanised region area) / (whole forewing area)]; for the hindwings: [(hindwings melanised region area)/ (whole hindwings area)]. For chapter (III) image analysis was performed with ImageJ (v. 1.50f), creating multispectral images that combined the photographs of the female moths taken in the UV and visible spectra. From every image, a set of regions of interest was selected: (a) whole forewing area (b) forewing melanised region (c) whole hindwing area (d) hindwing melanised region and (e) hindwing coloured region. These were reported in a dataset, selecting the Visible R luminance channel and running it through no visual system, in order to observe the raw camera responses as objective reflectance data. The RGB values were converted into hsv (hue-saturation-value) colour space using the rgb2hsv in R. As hue is a circular value, I added an offset to all hue values to make the red degrees comparable. The relative proportion of the melanised area was calculated as in chapter II for the male wings.

3 RESULTS AND DISCUSSION

3.1 Geographic variation in chemical defences (I)

The variation of the chemical defences in wood tiger moth partially reflects predator pressure. Populations of the same species living in different habitats can experience different predation rates and predator communities (Gibson 1984; Losey *et al.* 1997, Endler and Mappes 2004, Speed *et al.* 2012, Rönkä *et al.* 2020), and thus may evolve different types of defences or variation in their composition to survive the attack of their enemies. In wood tiger moths, the Estonian and Finnish populations have a lower predation pressure, while the Scottish population has the highest (Rönkä *et al.* 2020). The attacks of avian predators are a major driver of changes in the warning colouration of this species (Rönkä *et al.* 2020), so it is reasonable to think that they may also influence the chemical defences of the moths. Wild individuals from the Scottish population had the highest amount of SBMP compared to the wild and laboratory Estonian and Finnish populations, and the laboratory Scottish and Georgian moths. The variation in SBMP was higher in the wild Scottish population than in the wild and lab Finnish and Estonian populations. Regarding IBMP the Scottish wild population had higher amounts than the wild Georgian population and the wild and laboratory Finnish and Estonian moths. Overall, I found no difference in the IBMP variation between wild- and laboratory-raised moths. This confirms that the Scottish population may have indeed a higher amount of chemical defences because the local predation pressure is higher.

Measuring the amounts of defensive chemical compounds is not enough to determine their actual efficacy (Lawrence *et al.* 2019), as toxicity and unpalatability need not be the same (Winters *et al.* 2018). Thus, I assessed the response of an ecologically relevant predator, the blue tit, to the moths' chemical defences. In this experiment I used only Finnish and Georgian moths, as they showed significantly different pyrazine amounts, and I successfully managed to

raise both populations in the laboratory. I found that fluids from the laboratory moths triggered a stronger deterrent response than those from the wild populations. The laboratory moths were raised with a constant amount of food while the wild moths may have experienced food deprivation, indeed, a period with a shortage of resources has a strong influence on the efficacy of the chemical defences (Burdfield-Steel *et al.* 2019). The laboratory Georgian moths elicited a longer hesitation time compared to the wild Georgian and Finnish moths, and lower proportions of baits soaked with the fluids from Georgian laboratory moths were eaten in comparison to baits soaked in the fluids from Finnish and Georgian wild moths. I speculated that the chemical defences of Georgian laboratory moths were more deterrent to predators because there may be other chemical compounds, such as pyrrolizidine alkaloids (PAs) (Winters *et al.* 2021), that affect the wood tiger moth's chemical defences. PAs were not present in the laboratory diets used in this work, however, these compounds, may have been sequestered from the Georgian and Finnish wild moths tested in the study, and this may have led to trade-offs with the production of pyrazine (Winters *et al.* 2021). Moreover, the Georgian laboratory moths were exceptionally fed with *Plantago sp.* and *Rumex sp.* (because the *Taraxacum spp.*- diet was not enough for their development and survival). Plantago plants present iridoid glycosides, which can be sequestered by the moths in small amounts and can trigger a repellent behaviour in predators after eating the bait (Lindstedt *et al.* 2010, Reudler *et al.* 2015).

3.2 The role of methoxypyrazines in the wood tiger moth defences (I)

SBMP and IBMP are the methoxypyrazines mainly responsible for the deterrent response of avian predators when they approach wood tiger moths. I expected that those two pyrazines in combination would cause the strongest repellent response from the predators. Surprisingly, the results indicate that exposure to only one of the two compounds, SBMP, elicited the repellent bird response with an increase in concentration (in terms of hesitation time to approach the bait and proportion of bait eaten). In contrast, as the concentration rose, IBMP became palatable (see Fig. 4). However, IBMP caused the birds to drink more water, which is another sign of the baits' distastefulness (Rojas *et al.* 2017), which may indicate that IBMP provides moths with protection at the last line of defence, i.e., after attack.

More surprisingly, the increase in concentration of both pyrazines SBMP and IBMP showed a synergistic effect: the number of the birds' disgust behaviours (reducing the proportion eaten, increasing drinking behaviour, and potentially also beak wiping) that were negatively affected by the combination of SBMP and IBMP together, was higher than the effect of the two methoxypyrazines alone. This indicates that more is not always better in term of

eliciting a predator response aversion to the chemical defence (I), and that different chemical components may work together to produce the reaction seen in predators.

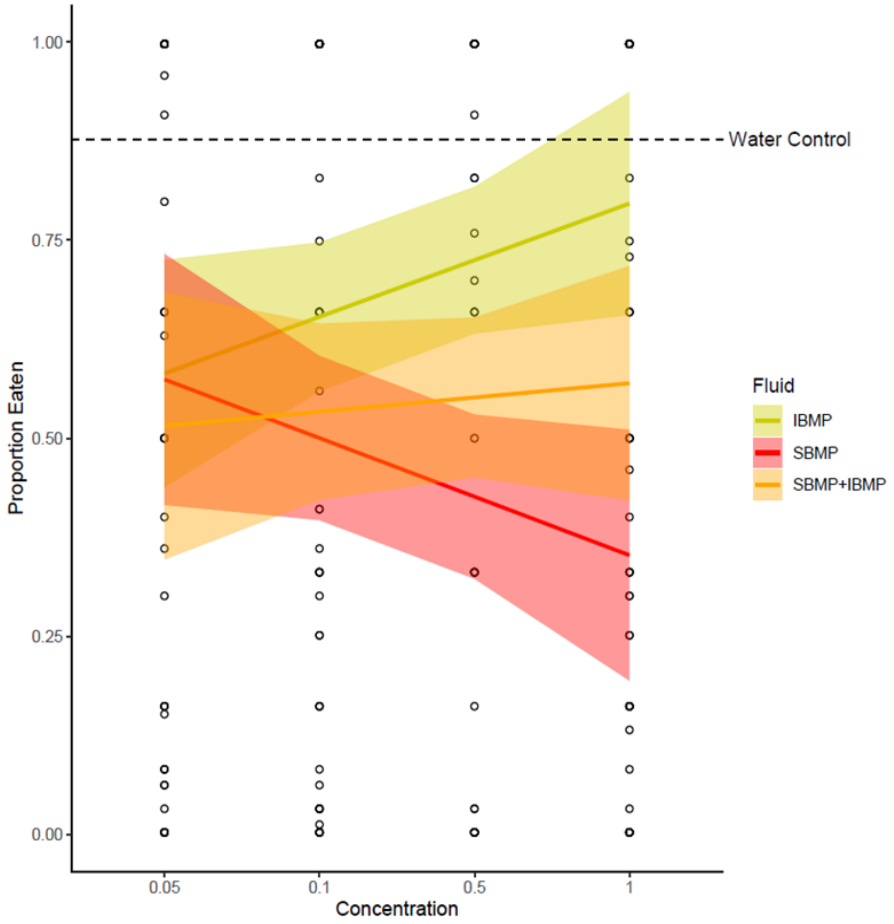

FIGURE 4 Proportion of bait eaten by predators to increasing concentrations (ng/µl) of each pyrazine type (SBMP = red, IBMP = yellow, SBMP + IBMP = orange). Shaded area represents standard error. Average bird response to the water control is indicated by the dotted line (Study I).

3.3 Effect of diet manipulation on the chemical defences (II, III)

I hypothesised that the chemical defences of male and female wood tiger moths raised on a high-protein or *ad libitum* natural diets would be a stronger deterrent to predators than the defences of moths raised on a low-protein or food-deprived diets. I found that the defences of the moths were affected by the constant amount of natural resource (*ad libitum* diet) and by the protein content of the food: the higher amount of protein, the better. The protein (i.e., nitrogen) level in the diet is essential for the synthesis of pyrazine compounds (Hodge *et al.*1972; Wong and Bernhard 1988), which have a repulsive smell (Rothschild *et al.* 1984, Guilford *et*

al. 1987, Kaye *et al.* 1989, Moore *et al.* 1990) that serves as a shield to repel avian predators' attacks (Marples and Roper 1996, Lindström *et al.* 2001, Siddall and Marples 2011) as shown in study I. Indeed, predators found the chemical defences of moths raised on the high-protein and *ad libitum* treatments unpalatable: the proportion of bait eaten by the predators was lower, hence the defences more deterrent, compared to the control. However, in study II I did not find any differences in the latency to approach the baits soaked with fluids from male moths raised on high-protein diet, compared to the control, which indicates that the predators may not perceive any difference in the odour of the volatile compounds from male moths raised on different diets. I found a difference in the predators' latency to approach to the chemical defences of females raised in high- and low- protein diet (III). Wood tiger moths are sexually dimorphic and, while males are quite active, females often rest on the vegetation, thus they may need to increase their protection against predators. Females are also the larger sex, which may also affect their detectability and acceptability as prey. Thus, to increase protection, females may invest more in the production of the chemical defences (Blount *et al.* 2012, Briolat *et al.* 2018), possibly increasing the repellent odour effect.

3.4 Effect of diet manipulation on the melanin and warning signal (II, III)

Animals, such as *A. plantaginis*, which synthesise their chemical defences *de novo* rely on essential nutrients present in their food. Resources (such as proteins and nitrogen), and the energy necessary to acquire them, are important not only for the defences but also to grow and develop during the organism's life, and to build the nitrogen-based pigments which shape the colouration of the wings (Talloen *et al.* 2004). When I assessed how the diet, and specifically its protein content, influence the moths' primary and secondary defences and life-history traits, I expected melanin, a nitrogen-rich pigment, to increase or decrease in amount according the protein content of the diet. In insect species such as the Egyptian cotton leafworm, *Spodoptera littoralis*, individuals raised on a low-quality nitrogen diet have less melanised cuticles than individuals reared on a high-protein diet (Lee *et al.* 2008), whereas in the forest moth, *Malacosoma disstria*, low-nitrogen availability leads to a decrement in melanin pigments (Ethier *et al.* 2015). Interestingly, I found that the variation in the protein content (high/low) in the diet does not affect the melanin level in male and female wood tiger moths (II, III), which indicates that this trait in *A. plantaginis* is not directly influenced by the resources available during the development. On the contrary, this indicates that the amount of melanin in the hindwings, which has a crucial signalling role against potential predators (Sargent 1978, Kang *et al.* 2017, Rönkä *et al.* 2018), has a strong genetic component.

Female wood tiger moths have a continuous variation in the aposematic hindwing colouration from yellow to red. Red is considered the most deterrent to predators, as it elicits lower attack rates and longer hesitation time than orange (Lindstedt *et al.* 2011). Therefore, it is expected that directional selection will favour darker, redder, hindwings. However, in natural populations, this is not always the case, as other selective forces can take place in maintain different colour morphs (i.e., thermoregulation: Lindstedt *et al.* 2009, protection from pathogens: Friman *et al.* 2009, sexual selection: De Pasqual *et al.* 2022). Moreover, the excretion/detoxification process is costly and can influence the aposematic colouration: a diet rich in iridoid glycosides, for example, can constrain the warning signal leading to a light orange wing colouration (Lindstedt *et al.* 2010). I hypothesised that the high-protein and *ad libitum* treatments will facilitate the production of the most conspicuous colouration of the hindwings (redder hue, higher saturation, and lower brightness) compared to the in low-protein or food-deprived diets. Female moths raised on the food-deprived diet did not have any difference in hindwing colouration compared to individual raised in *ad libitum* diet, possibly because moths can get enough resources from their natural diet, even if scarce, to build their genetically determined red pigmentation. However, I found that individuals reared in low-protein diet displayed a decrease in saturation and an increase in the brightness of the hindwings when compared to those raised on the high-protein diet. Given that signal intensity (saturation) has a key role in the effectiveness of warning colouration, the reduction in saturation similarly points to a weaker visual signal. This is important as the heritability in the production of red pigments (pheomelanin; M. Brien, personal communication) is high. In *A. plantaginis*, therefore, genetic constrains can hinder the effect of environmental variation.

3.5 Testing the resource allocation hypothesis (II, III)

The resource allocation hypothesis imposes a significant evolutionary consideration: should aposematic individuals invest more in enhancing their conspicuous visibility, increasing their secondary defenses, or should they invest equally in both traits?

I hypothesised that the production of the costly melanin pigments in *A. plantaginis* would compete with the synthesis of the pyrazine compounds, resulting in a trade-off between melanin and the effectiveness of secondary defences (II). However, I found the opposite: the most melanised male moths from both the high- and low-protein diet treatments had more deterrent defences (in terms of proportion of bait eaten and latency to eat; Fig. 5) than those with lower amounts of melanin. Thus, these results indicate a positive correlation between melanin and pyrazine defences while highlighting the sensitivity of the chemical defences to environmental conditions: despite being produced *de novo* (Burdfield-Steel *et al.* 2018) rather than sequestered from plants, their production

is still affected by the food intake. Melanin pigments, in contrast, are not influenced by resource variation.

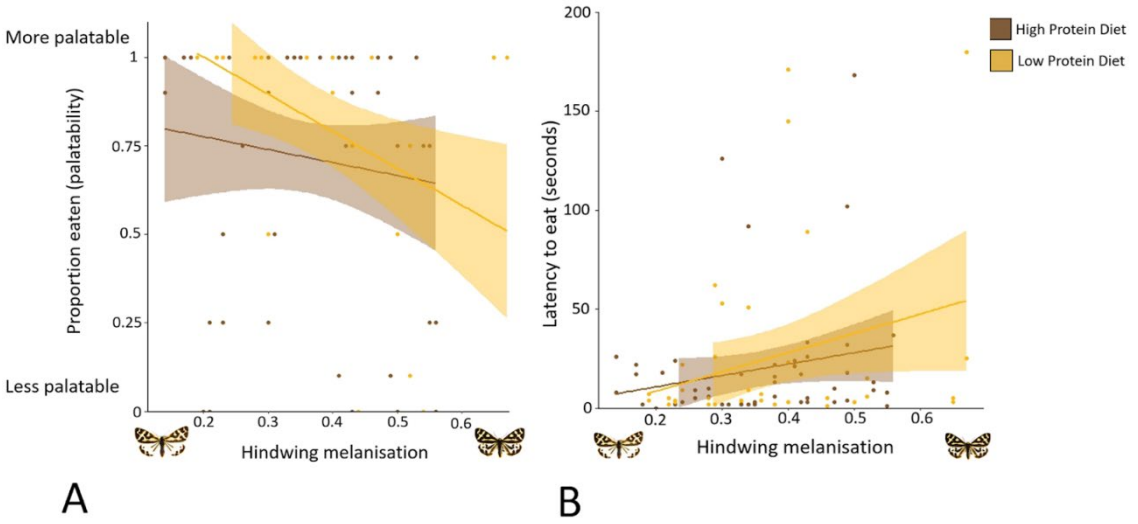

FIGURE 5 A) Palatability (proportion of defence fluid-soaked bait eaten by birds) and B) latency to eat the defence fluid-soaked bait compared to hindwing melanisation for male moths raised on high- and low-protein diets (Study II).

I expected that the aposematic warning signal and the toxic chemical defence in female moths would show "*quantitative honesty*" when resources are limited, such that individuals with access to fewer resources during development, but not those raised in a high-resource environment, show a positive correlation between the visual signal and the chemical defences (III). According to the model formulated by Blount *et al.* 2009, when resources are abundant, a negative correlation is predicted between the aposematic signal and the chemical defences: animals should potentially invest more in toxins rather than conspicuous colouration because the latter could potentially cause high detectability by predators (Blount *et al.* 2009).

Contrary to the expectations, in the natural diet treatments (*ad libitum* and food-deprived) I did not observe a correlation between aposematic traits. Instead, I found that the high-protein diet clearly represents the optimal amount of supply for the wood tiger moth, as females raised on it not only had the most conspicuous warning signals (in terms of hue and saturation), but also the most deterrent defences (Fig. 6). There are different explanations that support the positive correlation between primary and secondary defences, such as sexual selection or antioxidant molecules. For example, when males display a conspicuous morph to attract females (Andersson and Iwasa 1996) they may incur in high detection costs which could then lead to an increase in the chemical defences in response to the need for greater protection against predators. Another example are the antioxidant compounds used as a protection against the sequestration of defensive toxins, which may function as pigments in the visual

signal (Blount *et al.* 2009). Positive correlation in primary and secondary defences has previously been demonstrated in different aposematic organisms such as ladybirds *Coccinella septempunctata* (see Blount *et al.* 2012) and the strawberry poison frog, *Dendrobates pumilio* (Maan and Cummings 2012). However, and most importantly, whether or not predators perceive the variation in chemical defences has often been more difficult to test and thus less explored. The precise metabolic pathways that may link the production of pigments and chemical compounds in *A. plantaginis* has not been described yet. However, our results support the resource allocation hypothesis, as manipulation of a single resource, in this case, protein content in the diet, caused changes in both the strength of warning colouration and chemical defence, and influenced predators' behaviour.

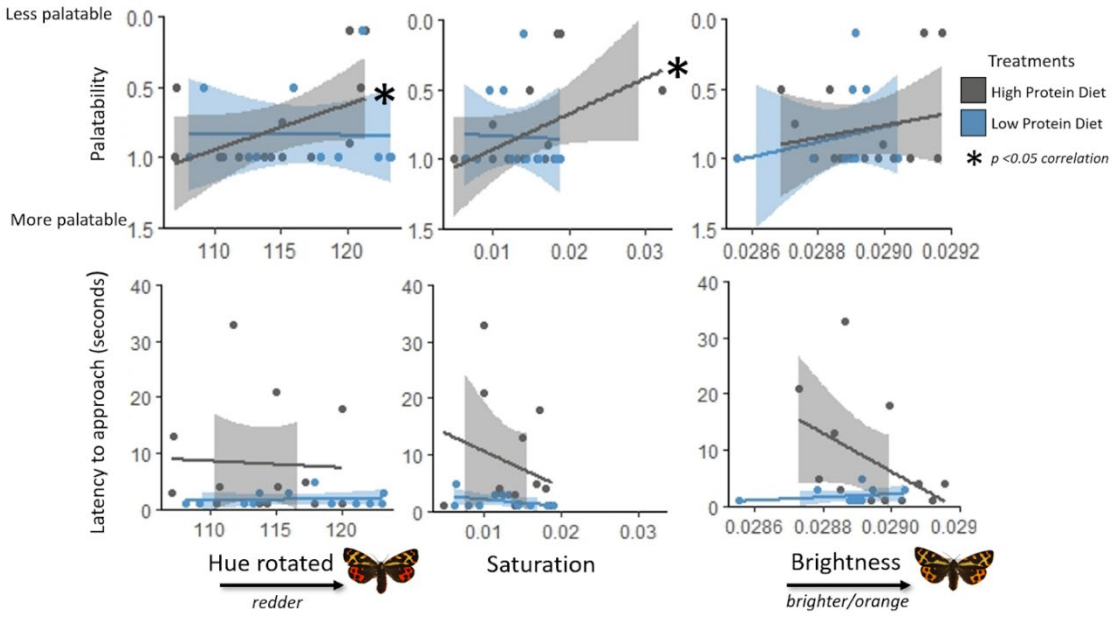

FIGURE 6 Top: correlation between the proportion of bait soaked in female moths' fluid (raised in high- and low-protein diets) eaten by predators (palatability) with hue, saturation, and brightness. Bottom: correlation between the latency to approach (in seconds) the bait soaked in female moths' fluid (raised in high- and low-protein diets) with hue, saturation, and brightness (Study III).

4 CONCLUSION AND FUTURE DIRECTIONS

The nonadditive interactions between the chemical components of the defence can introduce complexity to the study of chemical defences, calling for caution when extrapolating predators' responses from chemical measurements. Bioassays, and the use of model organisms such as *Daphnia* or mice (White and Umbers 2021), often do not represent the full picture of the natural conditions and can fail to represent the perception and behavioural responses of relevant predators.

Furthermore, the costs of chemical compounds are less obvious than their benefits, and the expenses paid to build and maintain the defences have a key role when considering the evolution and ecological drive of variation in aposematic traits. The resources available in early life affect and limit the production of chemical defences, especially in lepidopteran species which accumulate their defences or sequester the nutrients to produce their precious toxins during the development to guarantee the protection during the adult life (Summers *et al.* 2015). In wood tiger moths, the synthesis of melanin and colour pigments in males and females is less regulated by the fluctuation of environmental conditions than the production of chemical defences. This may indicate that primary and secondary defences are not constrained by the same pool of resources in this species. To test whether these trade-offs are present, it has been essential to manipulate the diet content, however, considering only one level of dietary manipulation in the laboratory can overlook the variation present in natural conditions: wild individuals may experience different levels of resource limitation, and studies on honesty signalling measured in laboratory populations may have misleading interpretations.

Finally, although not investigated in this work, prey density in natural populations can influence their aposematic traits and the correlation between primary and secondary defences. When the density of the aposematic prey is high, predators would be generally more exposed to and aware of the chemical defences of prey, thus their attacks will be less frequent than prey living in low density populations, leading to a decrease in investment in aposematic displays

(Speed and Ruxton 2007). Hence, a positive correlation between aposematic traits would be expected if the population size varies.

There is still a lot to explore to understand the variation in chemical defences in aposematic organisms and further studies will hopefully provide more accurate and robust information on the ecological and physiological linkage between aposematic traits.

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REFERENCES

Agrawal, A. A., Petschenka, G., Bingham, R. A., Weber, M. G., & Rasmann, S. 2012. Toxic cardenolides: chemical ecology and coevolution of specialized plant–herbivore interactions. *New Phytol*. 194: 28-45.

Alonso-Mejia, A., & Brower, L. P. 1994. From model to mimic: age-dependent unpalatability in monarch butterflies. *Experientia* 50: 176-181.

Andersson, M., & Iwasa, Y. 1996. Sexual selection. *Trends ecol. evol.* 11: 53-58.

Arenas, L. M., Troscianko, J., & Stevens, M. 2014. Color contrast and stability as key elements for effective warning signals. *Frontiers Ecol. Evol.* 2: 25.

Bates, H. W. 1862 Contributions to an insect fauna of the Amazon valley (Lepidoptera : Heliconidae). *Trans. Linn. Soc. Lond*. 23: 495-56

Bezzerides, A. L., McGraw, K. J., Parker, R. S., & Husseini, J. 2007. Elytra color as a signal of chemical defense in the Asian ladybird beetle *Harmonia axyridis*. *Behav. Ecol. Sociobiol.* 61: 1401-1408.

Bilandžija H., Laslo M., Porter M. L. & Fong D. W. 2017. Melanization in response to wounding is ancestral in arthropods and conserved in albino cave species. *Sci. Rep-UK* 7: 17148 https://doi.org/10.1038/s41598-017-17471-2

Blount, J. D., Speed, M. P., Ruxton, G. D., & Stephens, P. A. 2009. Warning displays may function as honest signals of toxicity. *Proc. Royal Soc. B*. 276: 871-877.

Blount, J.D., Rowland, H.M., Drijfhout, F.P., Endler, J.A., Inger, R., Sloggett, J.J., Hurst, G.D., Hodgson, D.J. and Speed, M.P., 2012. How the ladybird got its spots: effects of resource limitation on the honesty of aposematic signals. *Funct. Ecol.* 26: 334-342.

Blount, J. D., Rowland, H. M., Mitchell, C., Speed, M. P., Ruxton, G. D., Endler, J. A., & Brower, L. P. 2023. The price of defence: toxins, visual signals and oxidative state in an aposematic butterfly. *Proc. Royal Soc. B.* 290: 20222068.

Brien M. N., Orteu A., Yen E. C., Galarza J. A., Kirvesoja J., Pakkanen H., Wakamatsu K., Jiggins C. D., Mappes J. 2022 Colour polymorphism associated with a gene duplication in male wood tiger moths. *bioRxiv* 2022.04.29.490025 doi.org/10.1101/2022.04.29.490025

Brower, L. P., Van Brower, J., & Corvino, J. M. 1967. Plant poisons in a terrestrial food chain. *PNAS* 57: 893-898.

Brown Jr, K. S., & Benson, W. W. 1974. Adaptive polymorphism associated with multiple Müllerian mimicry in *Heliconius numata* (Lepid. Nymph.). *Biotropica* 1: 205-228.

Brown, K. S., & Francini, R. B. 1990. Evolutionary strategies of chemical defense in aposematic butterflies: cyanogenesis in Asteraceae-feeding American Acraeinae. *Chemoecology* 1: 52-56.

Burdfield-Steel, E., Pakkanen, H., Rojas, B., Galarza, J.A. and Mappes, J., 2018. De novo synthesis of chemical defenses in an aposematic moth. *J. Insect Sci.* 18: 28.

Burdfield-Steel, E., Brain, M., Rojas, B. and Mappes, J., 2019. The price of safety: food deprivation in early life influences the efficacy of chemical defence in an aposematic moth. *Oikos* 128: 245-253.

Brien, M. N., Orteu, A., Yen, E. C., Galarza, J. A., Kirvesoja, J., Pakkanen, H., ... & Mappes, J. 2022. Colour polymorphism associated with a gene duplication in male wood tiger moths. *bioRxiv*, 2022-04.

Briolat, E. S., Burdfield-Steel, E. R., Paul, S. C., Rönkä, K. H., Seymoure, B. M., Stankowich, T., & Stuckert, A. M. 2019. Diversity in warning coloration: selective paradox or the norm?. *Biol. Rev.* 94: 388-414.

Cheney, K. L., White, A., Mudianta, I. W., Winters, A. E., Quezada, M., Capon, R. J., Mollo E., Garson, M. J. 2016. Choose your weaponry: Selective storage of a single toxic compound, latrunculin A, by closely related nudibranch molluscs. *PLoS One*, 11: e0145134.

Chinery, 1993 Insects of Britain and Northern Europe, 3rd edn. 353 p., Collins, London

Cook, L. M., & Sarsam, V. 1981. Polymorphism in the moth Noctua pronuba (L.). *Heredity*, 46: 443-447.

Cuthill, I. C., Allen, W. L., Arbuckle, K., Caspers, B., Chaplin, G., Hauber, M. E., ... & Caro, T. 2017. The biology of color. *Science*, 357:eaan0221.

Darst, C. R., Cummings, M. E., & Cannatella, D. C. 2006. A mechanism for diversity in warning signals: conspicuousness versus toxicity in poison frogs. *PNAS* 103: 5852-5857.

De Pasqual, C. 2022. On the maintenance of intrapopulation genetic variation: the interplay between sexual selection, chemical communication, and fitness in the wood tiger moth (*Arctia plantaginis*). JYU dissertations.

Dubatolov, V. V., & Zahiri, R. E. Z. A. 2005. Tiger-moths of Iran. Atalanta, 36: 481-525.

Dussourd, D. E., Harvis, C. A., Meinwald, J., & Eisner, T. 1991. Pheromonal advertisement of a nuptial gift by a male moth (*Utetheisa ornatrix*). *PNAS* 88: 9224-9227.

Ellers, J., & Boggs, C. L. 2002. The evolution of wing color in Colias butterflies: heritability, sex linkage, and population divergence. *Evolution*, 56: 836-840.

Endler, J. A., & Mappes, J. 2004. Predator mixes and the conspicuousness of aposematic signals. *Am. Nat.*, 163: 532-547.

Ethier, J., Gasse, M., Lake, K., Jones, B. C., Evenden, M. L., & Despland, E. 2015. The costs of colour: plasticity of melanin pigmentation in an outbreaking polymorphic forest moth. *Entomol. Exp. Appl*, 154: 242-250.

Fabricant, S. A., Kemp, D. J., Krajíček, J., Bosakova, Z., & Herberstein, M. E. 2013. Mechanisms of color production in a highly variable shield-back stinkbug, *Tectocoris diopthalmus* (Heteroptera: Scutelleridae), and why it matters. *PLoS One* 8: e64082.

Fabricant, S. A., Burdfield-Steel, E. R., Umbers, K., Lowe, E. C., & Herberstein, M. E. 2018. Warning signal plasticity in hibiscus harlequin bugs. *Evol. Ecol.* 32: 489-507.

Friman, V. P., Lindstedt, C., Hiltunen, T., Laakso, J., & Mappes, J. 2009. Predation on multiple trophic levels shapes the evolution of pathogen virulence. *PloS one* 4: e6761.

Fromhage, L., & Henshaw, J. M. 2022. The balance model of honest sexual signaling. *Evolution* 76: 445-454.

Fürstenberg-Hägg, J., Zagrobelny, M., Jørgensen, K., Vogel, H., Møller, B. L., & Bak, S. 2014. Chemical defense balanced by sequestration and de novo biosynthesis in a lepidopteran specialist. *PLoS One* 9: e108745.

Furlanetto M. 2017. Investigating the cost of visual signals and chemical defences in *Arctia plantaginis*. Unpublished MSc Thesis, University of Padua – University of Jyväskylä

Gadagkar, R. 2003. Is the peacock merely beautiful or also honest? *Curr. Sci.* 10: 1012-1020.

Glazier, D. S. 2002. Resource-allocation rules and the heritability of traits. *Evolution* 56: 1696-1700.

Grafen, A. 1990. Biological signals as handicaps. *J. Theor Biol* 144: 517-546.

Grill, C. P., & Moore, A. J. 1998. Effects of a larval antipredator response and larval diet on adult phenotype in an aposematic ladybird beetle. *Oecologia* 114: 274-282.

Guilford, T., & Dawkins, M. S. 1993. Are warning colors handicaps?. *Evolution* 47: 400-416.

Guilford, T. I. M., Nicol, C., Rothschild, M., & Moore, B. P. 1987. The biological roles of pyrazines: evidence for a warning odour function. *Biol. J. Linn. Soc. Lond.* 31: 113-128.

Hodge J. E., Mills F. D., Fisher B. E. 1972 Compounds of browned flavor derived from sugar-amine reactions. *J. Cereal Sci*. 17: 34-40

Holen, O. H. and Svennungsen, T. O. 2012 'Aposematism and the handicap principle.', *Am. Nat*. United States 180: 629–641. doi: 10.1086/667890.

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. *Proc. Royal Soc. B*. 280: 2012-2812

Hegna, R. H., Galarza, J. A., & Mappes, J. 2015. Global phylogeography and geographical variation in warning coloration of the wood tiger moth (*Parasemia plantaginis*). *J. Biogeog.* 42: 1469-1481.

Jablonski, N. G., & Chaplin, G. 2017. The colours of humanity: the evolution of pigmentation in the human lineage. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* 372: 20160349.

Jousset, A., Rochat, L., Péchy-Tarr, M., Keel, C., Scheu, S., & Bonkowski, M. 2009. Predators promote defence of rhizosphere bacterial populations by selective feeding on non-toxic cheaters. *ISME J.* 3: 666-674.

Jousset, A. 2012. Ecological and evolutive implications of bacterial defences against predators. *Environ. microbio.* 14: 1830-1843.

Kang, C., Moon, H., Sherratt, T. N., Lee, S. I., & Jablonski, P. G. 2017. Multiple lines of anti-predator defence in the spotted lanternfly, *Lycorma delicatula* (Hemiptera: Fulgoridae). *Biol. J. Linn. Soc. Lond* 120: 115-124.

Kaye, H., Mackintosh, N. J., Rothschild, M., & Moore, B. P. 1989. Odour of pyrazine potentiates an association between environmental cues and unpalatable taste. *Anim. Behav.* 37: 563-568.

Kirkpatrick, M. 1986. The handicap mechanism of sexual selection does not work. *Am. Nat*. 127: 222-240.

Kjernsmo, Karin, Anna M. Lim, Rox Middleton, Joanna R. Hall, Leah M. Costello, Heather M. Whitney, Nicholas E. Scott-Samuel, and Innes C. Cuthill. 2022. Beetle iridescence induces an avoidance response in naïve avian predators. *Anim. Behav.* 188: 45-50.

Lawrence J.P., Rojas B., Fouquet A., Mappes J., Blanchette A., Saporito R.A., Bosque R. J., Courtois E.A., Noonan B.P. 2019 Weak warning signals can persist in the absence of gene flow. *PNAS* 116: 19037-19045.

Lee K. P., Simpson S. J. & Wilson K. 2008 Dietary protein-quality influences melanization and immune function in an insect. *Funct. Ecol*. 22: 1052–1061

Lindstedt C. Lindström L, Mappes J, McMillan W. O. 2009 Thermoregulation constrains effective warning signal expression. *Evolution* 63: 469–478

Lindstedt, C., Talsma, J. H. R., et al. 2010 'Diet quality affects warning coloration indirectly: Excretion costs in a generalist herbivore', *Evolution*. 64: 68–78. doi: 10.1111/j.1558-5646.2009.00796.x.

Lindstedt, C. et al. 2011 'Direction and strength of selection by predators for the color of the aposematic wood tiger moth', *Behav. Ecol.* 22: 580–587. doi: 10.1093/beheco/arr017.

Lindstedt, C. et al. 2016 'Evolutionary constraints of warning signals: A genetic trade-off between the efficacy of larval and adult warning coloration can maintain variation in signal expression', *Evolution*. [Society for the Study of Evolution, Wiley], 70: 2562–2572. doi: 10.1111/evo.13066.

Lindstedt, C., Suisto, K. and Mappes, J. 2020 'Appearance before performance? Nutritional constraints on life-history traits, but not warning signal expression in aposematic moths', *J. Anim.l Ecol*. 89: 494–505. doi: 10.1111/1365-2656.13103.

Lindström, L., Alatalo, R. V., Mappes, J., Riipi, M., & Vertainen, L. 1999. Can aposematic signals evolve by gradual change? *Nature* 397: 249-251.

Lindström, L., Alatalo, R. V., Lyytinen, A., & Mappes, J. 2001. Strong antiapostatic selection against novel rare aposematic prey. *PNAS* 98: 9181-9184.

Lindström, L., Rowe, C., & Guilford, T. 2001. Pyrazine odour makes visually conspicuous prey aversive. *Proc. Royal Soc. B*. 268: 159-162.

Losey, J. E., Harmon, J., Ballantyne, F., & Brown, C. 1997. A polymorphism maintained by opposite patterns of parasitism and predation. *Nature* 388: 269-272.

Maan, M. E., & Cummings, M. E. 2012. Poison frog colors are honest signals of toxicity, particularly for bird predators. *Am. Nat*, 179: E1–E14. https://doi.org/10.1086/663197

Marples, N. M., & Roper, T. J. 1996. Effects of novel colour and smell on the response of naive chicks towards food and water. *Anim. Behav.* 51: 1417-1424.

Majerus MEN 1998 Melanism: evolution in action. Oxford University Press, Oxford

McNamara, M. E., Rossi, V., Slater, T. S., Rogers, C. S., Ducrest, A. L., Dubey, S., & Roulin, A. 2021. Decoding the evolution of melanin in vertebrates. *Trends ecol. evol.* 36: 430-443.

Mappes, J., Marples, N., & Endler, J. A. 2005. The complex business of survival by aposematism. *Trends ecol. evol.* 20: 598-603.

Mattila, A. L., Jiggins, C. D., Opedal, Ø. H., Montejo-Kovacevich, G., McMillan, W. O., Bacquet, C., & Saastamoinen, M. 2021. Evolutionary and ecological processes influencing chemical defense variation in an aposematic and mimetic Heliconius butterfly. *PeerJ*, 9: e11523.

Moore, B. P., Brown, W. V., & Rothschild, M. 1990. Methylalkylpyrazines in aposematic insects, their host plants and mimics. *Chemoecology* 1: 43-51.

Najbar, A., Konowalik, A., Najbar, B., & Ogielska, M. 2018. Yellow patterns polymorphism of the fire salamander *Salamandra salamandra* in Poland. *Acta Herpetol.* 13: 101-108.

Nokelainen, O., Hegna, R. H., Reudler, J. H., Lindstedt, C., & Mappes, J. 2012. Trade-off between warning signal efficacy and mating success in the wood tiger moth. *Proc. Royal Soc. B*. 279: 257-265.

Nokelainen O. 2013 Many forms of the wood tiger moth (*Parasemia plantaginis*): selective heterogeneity favours polymorphic warning signals, University of Jyväskylä.

Nokelainen, O., Valkonen, J., Lindstedt, C., & Mappes, J. 2014. Changes in predator community structure shifts the efficacy of two warning signals in Arctiid moths. *J Anim. Ecol.* 83: 598-605.

Nokelainen O., Hubbard N., Lown A.E., Wood L.E., Stevens M. 2017 Through predators' eyes: phenotype–environment associations in shore crab coloration at different spatial scales. *Biol. J. Linn. Soc. Lond.* 122: 1–14.

Ottocento, C., Winters, A. E., Rojas, B., Mappes, J., & Burdfield-Steel, E. 2022. Not just the sum of its parts: Geographic variation and nonadditive effects of pyrazines in the chemical defence of an aposematic moth. *J. Evol. Biol.*

Pasteels, J. M., Rowell-Rahier, M., Braekman, J. C., Daloze, D., & Duffey, S. 1989. Evolution of exocrine chemical defense in leaf beetles (Coleoptera: Chrysomelidae). *Experientia* 45: 295-300.

Penn, D. J., & Számadó, S. 2020. The Handicap Principle: how an erroneous hypothesis became a scientific principle. *Biol. Rev.* 95: 267-290.

Poulton E. B. 1890 The colours of animals, their meaning and use, especially considered in the case of insects. New York, D. Appleton and Company

Reudler, J. H., Lindstedt, C., Pakkanen, H., Lehtinen, I., & Mappes, J. 2015. Costs and benefits of plant allelochemicals in herbivore diet in a multi enemy world. *Oecologia* 179: 1147-1158.

Reznick, D., Nunney, L., & Tessier, A. 2000. Big houses, big cars, superfleas and the costs of reproduction. *Trends ecol. evol*. 15: 421-425.

Rojas, B., & Endler, J. A. 2013. Sexual dimorphism and intra-populational colour pattern variation in the aposematic frog *Dendrobates tinctorius*. *Evol. Eco.* 27: 739-753.

Rojas, B., Valkonen, J. and Nokelainen, O. 2015 'Aposematism', *Curr. Biol*. 25: 350–351

Rojas, B., Burdfield-Steel, E., Pakkanen, H., Suisto, K., Maczka, M., Schulz, S., & Mappes, J. 2017. How to fight multiple enemies: target-specific chemical defences in an aposematic moth. *Proc. Royal Soc. B*. 284: 20171424.

Rojas, B., Mappes, J. and Burdfield-Steel, E. 2019 'Multiple modalities in insect warning displays have additive effects against wild avian predators'. *Behav. Ecol. Sociobiol.* 73: 1-12. doi: 10.1007/s00265-019-2643-6.

Rönkä, K., Mappes, J., Kaila, L., & Wahlberg, N. 2016. Putting Parasemia in its phylogenetic place: A molecular analysis of the subtribe Arctiina (lepidoptera). *Syst. Entomol*. 41: 844–853. https://doi.org/10.1111/syen.12194

Rönkä, K et al. 2018 'Can multiple-model mimicry explain warning signal polymorphism in the wood tiger moth, *Arctia plantaginis* (Lepidoptera: Erebidae)?', *Biol. J. Linn. Soc. Lond* 124: 237–260. doi: 10.1093/biolinnean/bly042.

Rönkä, K. et al. 2020 'Geographic mosaic of selection by avian predators on hindwing warning colour in a polymorphic aposematic moth', *Ecol. Lett*. 11: 1654-1663 doi: 10.1111/ele.13597.

Rothschild, G. H. L. 1979. A comparison of methods of dispensing synthetic sex pheromone for the control of oriental fruit moth, *Cydia molesta* (Busck)(Lepidoptera: Tortricidae), in Australia. *Bull. Entomol. Res.* 69: 115-127.

Rothschild M., Moore B. P., Brown W. V. 1984 Pyrazines as warning odour components in the monarch butterfly, *Danaus piexippus*, and in moths of the genera Zygaena and Amata (Lepidoptera). *Biol. J. Linn. Soc. Lond.* 23: 375-380

Rowell-Rahier, M., Pasteels, J. M., Alonso-Media, A., & Brower, L. P. 1995. Relative unpalatability of leaf beetles with either biosynthesized or sequestered chemical defence. *Anim. Behav.* 49: 709-714.

Rowland, H. M., Hoogesteger, T., Ruxton, G. D., Speed, M. P., & Mappes, J. 2010. A tale of 2 signals: signal mimicry between aposematic species enhances predator avoidance learning. *Behav. Ecol.* 21: 851-860.

Ruxton, G. D., Allen, W. L., Sherratt, T. N., & Speed, M. P. 2019. Avoiding attack: the evolutionary ecology of crypsis, aposematism, and mimicry. Oxford university press.

Sargent, T. D. 1978. On the maintenance of stability in hindwing diversity among moths of the genus Catocala (Lepidoptera: Noctuidae). *Evolution* 1: 424-434.

Sculfort, O., de Castro, E. C., Kozak, K. M., Bak, S., Elias, M., Nay, B., & Llaurens, V. 2020. Variation of chemical compounds in wild Heliconiini reveals ecological factors involved in the evolution of chemical defenses in mimetic butterflies. *Ecol. Evo.* 10: 2677-2694.

Sherratt, T. N., Speed, M. P., & Ruxton, G. D. 2004. Natural selection on unpalatable species imposed by state-dependent foraging behaviour. *J Theor. Biol.* 228: 217-226.

Siddall, E. C., & Marples, N. M. 2011. The effect of pyrazine odor on avoidance learning and memory in wild robins Erithacus rubecula. *Curr. Zool.* 57: 208-214.

Smith, J. N. 1981. Cowbird parasitism, host fitness, and age of the host female in an island song sparrow population. *The Condor* 83: 152-161.

Smith, D. A., Owen, D. F., Gordon, I. J., & Owiny, A. M. 1993. Polymorphism and evolution in the butterfly *Danaus chrysippus* (L.)(Lepidoptera: Danainae). *Heredity* 71: 242-251.

Speed, M. P., & Ruxton, G. D. 2007. How bright and how nasty: explaining diversity in warning signal strength. *Evolution* 61: 623-635.

Speed, M. P., Ruxton, G. D., Mappes, J., & Sherratt, T. N. 2012. Why are defensive toxins so variable? An evolutionary perspective. *Biol. Rev.* 87: 874-884.

Stavenga, D. G., Wilts, B. D., Leertouwer, H. L., & Hariyama, T. 2011. Polarized iridescence of the multilayered elytra of the Japanese jewel beetle, *Chrysochroa fulgidissima*. *Philos. Trans. R. Soc. Lond. B, Biol. Sci.* 366: 709-723. https://doi.org/10.1098/rstb.2010.0197

Stearns, S. C. 1989. Trade-offs in life-history evolution. *Funct. Ecol.* 3: 259-268.

Stearns, S. C. 1992 The Evolution of Life Histories. Oxford University Press, London.

Stoddard, M. C., & Prum, R. O. 2011. How colorful are birds? Evolution of the avian plumage color gamut. *Behav. Ecol.* 22: 1042-1052.

Sugumaran M. 2002 Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. *Pigment Cell Res.* 15 :2-9

Summers, K., Speed, M. P., Blount, J. D., & Stuckert, A. M. M. 2015. Are aposematic signals honest? A review. *J. Evol. Biol.* 28: 1583-1599.

Srygley, R. B. 2004. The aerodynamic costs of warning signals in palatable mimetic butterflies and their distasteful models. *Proc. Royal Soc. B*, 271: 589-594.

Talloen, W., Dyck, H. V., & Lens, L. 2004. The cost of melanization: Butterfly wing coloration underenvironmental stress. *Evolution*, 58: 360-366.

Tammaru, T., & Haukioja, E. 1996. Capital breeders and income breeders among Lepidoptera: consequences to population dynamics. *Oikos* 1: 561-564.

Troscianko, J., and M. Stevens. 2015. Image Calibration and Analysis Toolbox - a free software suite for objectively measuring reflectance, colour and pattern. *Methods in Ecol. and Evo* 6: 1320–1331.

True, J. R. 2003. Insect melanism: the molecules matter. *Trends ecol. evol*. 18: 640-647.

Trullas S. C., Wyk J. H.V., Spotila J. R. 2007 Thermal melanism in ectotherms. *J. therm. Biol.* 32: 235–245

Van Dijk, T. S. 1994. On the relationship between food, reproduction and survival of two carabid beetles: *Calathus melanocephalus* and *Pterostichus versicolor*. *Ecol. Entomol*, 19: 263-270.

Van Noordwijk, A. J., & De Jong, G. 1986. Acquisition and allocation of resources: their influence on variation in life history tactics. *Am. Nat*. 128: 137-142.

van't Hof Arjen E., Reynolds Louise A., Yung Carl J., Cook Laurence M. and Saccheri Ilik J. 2019. Genetic convergence of industrial melanism in three geometrid moths. *Biol. Lett*. 15: 20190582

Vukusic, P. 2006. Structural colour in Lepidoptera. *Curr. Biol.* 16: 621-623.

Vukusic, P., & Stavenga, D. G. 2009. Physical methods for investigating structural colours in biological systems. *J. R. Soc. Interface.* 6: 133-148.

Wallace, A.R. 1867. Mimicry, and other protective resemblances among animals. Westminster Quart. *Foreign Quart. Rev*. July 1: 1–48.

Wallace, A.R. 1889. Darwinism: An Exposition of the Theory of Natural Selection with Some of its Applications. London: Macmillan & Company.

Watson, A., & Goodger, D. T. 1986. Catalogue of the Neotropical tiger-moths. British Museum (Natural History).

Webber, R., Barlow, G. W., & Brush, A. H. 1976. Pigments of a color polymorphism in a cichlid fish. *Investigations of the Ichthyofauna of Nicaraguan Lakes*. 1: 29.

Weller, S. J., Jacobson, N. L., & Conner, W. E. 1999. The evolution of chemical defences and mating systems in tiger moths (Lepidoptera: Arctiidae). *Biol. J. Linn. Soc. Lond*. 68: 557-578.

White, T. E. and Umbers, K. D. L. 2021 'Meta-analytic evidence for quantitative honesty in aposematic signals', *Proc. Royal Soc. B*. 288: 20210679. doi: 10.1098/rspb.2021.0679.

Wiklund, C., & Järvi, T. 1982. Survival of distasteful insects after being attacked by naive birds: a reappraisal of the theory of aposematic coloration evolving through individual selection. *Evolution* 1: 998-1002.

Winters, A. E., White, A. M., Cheney, K. L., & Garson, M. J. 2019. Geographic variation in diterpene-based secondary metabolites and level of defence in an aposematic nudibranch, *Goniobranchus splendidus*. *J. Molluscan Stud.* 85: 133-142.

Winters, A.E., Wilson, N.G., van den Berg, C.P., How, M.J., Endler, J.A., Marshall, N.J., White, A.M., Garson, M.J. and Cheney, K.L., 2018. Toxicity and taste: unequal chemical defences in a mimicry ring. *Proc. Royal Soc. B*. 285: 20180457.

Winters, A. E., Lommi, J., Kirvesoja, J., Nokelainen, O., & Mappes, J. 2021. Multimodal aposematic defenses through the predation sequence. *Front. Ecol. Evol*. 9: 512.

Wong J. M., Bernhard R. A. 1988 Effect of Nitrogen Source on Pyrazine Formation. *J. Agric. Food Chem*. 36: 129-133

Zahavi, A. 1975. Mate selection—a selection for a handicap. *J. Theor. Biol.* 53: 205-214.

Zahavi, A. 1977. Reliability in communication systems and the evolution of altruism. *Evol. ecol.* 253-259.

Zvereva, E. L., Kozlov, M. V., & Kruglova, O. Y. 2002. Colour polymorphism in relation to population dynamics of the leaf beetle, *Chrysomela lapponica*. *Evol. eco.* 16: 523-539.

Zvereva, E. L., & Kozlov, M. V. 2016. The costs and effectiveness of chemical defenses in herbivorous insects: A meta-analysis. *Ecol. Monogr*. 86: 107-124.

ORIGINAL PAPERS

I

NOT JUST THE SUM OF ITS PARTS: GEOGRAPHIC VARIATION AND NONADDITIVE EFFECTS OF PYRAZINES IN THE CHEMICAL DEFENCE OF AN APOSEMATIC MOTH

by

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RESEARCH ARTICLE

Not just the sum of its parts: Geographic variation and nonadditive effects of pyrazines in the chemical defence of an aposematic moth

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Abstract

Chemical defences often vary within and between populations both in quantity and quality, which is puzzling if prey survival is dependent on the strength of the defence. We investigated the within- and between-population variability in chemical defence of the wood tiger moth (*Arctia plantaginis*). The major components of its defences, SBMP (2-sec-butyl-3-methoxypyrazine) and IBMP (2-isobutyl-3-methoxypyrazine), are volatiles that deter bird attacks. We hypothesized that (1) variation in the chemical defences of male wood tiger moths reflects the local predation pressure; (2) observed differences in quantity and quality of defence among populations have a genetic basis; and (3) increasing concentrations of SBMP and IBMP will elicit greater aversive reactions in predators, with the two pyrazines having an additive effect on predators' avoidance. We found that (1) the chemical defence of wild moths partly reflects local predator selection: high predation pressure populations (Scotland and Georgia) had stronger chemical defences, but not lower variance, than the low-predation populations (Estonia and Finland). (2) Based on the common garden results, both genetic and environmental components seem to influence the strength of chemical defence in moth populations; and (3) IBMP alone did not provide protection against bird predators but worked against bird attacks only when combined with SBMP, and while SBMP was more effective at higher concentrations, IBMP was not. Altogether this suggests that, when it comes to pyrazine concentration, more is not always better, highlighting the importance of testing the efficacy of chemical defence and its components with relevant predators, as extrapolating from chemical data may be less than straightforward.

KEYWORDS
antipredatory strategy, lepidoptera, multicomponent defence, predator–prey interactions, pyrazine, wood tiger moth

Cristina Ottocento and Anne E. Winters shared first authorship.

1 | INTRODUCTION

Chemical defences are one of the most common types of secondary defences used by aposematic species (Eisner et al., 2005; Speed et al., 2012). Variation in chemical defences both between- and within-populations exists (e.g., in ladybirds *Harmonia axyridis*, see Bezzerides et al., 2007 and Arenas et al., 2015; in poison frogs, *Dendrobates tinctorius*, see Lawrence et al., 2019; in nudibranchs *Goniobranchus splendidus*, see Winters et al., 2019; in Heliconiini butterflies, see Sculfort et al., 2020). However, given the crucial role of chemical defences in predator avoidance, such variation may have important consequences for prey survival and, thus, both its causes and consequences are of great interest to those studying the evolution of antipredator defences.

Between- and within-population variation in chemical defences may reflect: (1) solely environmental conditions without any genetic effects, for example, age, local differences in nutrient availability and/or competition for resources that are necessary for the production of the chemical defence (Burdfield-Steel et al., 2019; Speed et al., 2012); (2) genetic differences in individuals' capacity in sequestering, gathering or synthesizing compounds for chemical defence. In this case, there may be differences between sexes or we may find size-dependent variation in chemical defence (Alonso-Mejia & Brower, 1994; Hudson et al., 2021); or (3) that different genotypes may react differently to variable environmental conditions creating genotype-by-environment interactions. Evidence for the first mechanism has now been found both in species that sequester their defences (Brower et al., 1982), and in some that produce their defences themselves (i.e., de novo; Burdfield-Steel et al., 2018). Evidence for genetic effects and genotype-by-environment interactions are less common, but some studies have found results consistent with heritable variation in defence (e.g., Sculfort et al., 2020).

In addition to the causes of such variation, there may also be consequences of variation, which are perhaps harder to study. Not surprisingly, direct tests of chemical defences with relevant predators are still rare. For example, in several poison frog studies (e.g., Cummings & Crothers, 2013; Darst et al., 2006; Darst & Cummings, 2006; Maan & Cummings, 2012) the toxicity of chemical defences was tested via injecting frog alkaloids in mice. Another common approach is to test the strength of chemical defence by mixing chemical compounds into the water and survey the mortality of water fleas (*Daphnia sp.*) (e.g., Arenas et al., 2015). Although those measurements may give a good "proxy" about prey's toxicity, such assays do not show the connection between toxicity and palatability or true toxicity to relevant predators (Lawrence et al., 2019). For example, predators may ignore or be unable to detect the variation present in the chemical defence (Lawrence et al., 2019) or different compounds may be effective only against one predator type (Rojas et al., 2017). Therefore, measuring the response(s) of relevant predators is crucial for understanding how intraspecific variation in chemical defences is maintained. The sensitivity of predators to any existing variation will determine if such variation is visible to selection. Moreover, variation in the chemical defences of prey can be itself a form of defence. A study by Barnett et al. (2014) shows that predators are more willing to eat prey with a constant level of defence, as opposed to prey with variability in their defences. Thus, while we hypothesized that greater predation pressure would select for reduced variation in the strength of the chemical defence, this may not always be the case. Nevertheless, if we wish to increase our understanding of how chemical defences do respond to predation pressure, we need to continue to examine wild populations as accurately as possible. Chemical defences in living organisms may be associated both with a distasteful flavour and an unpleasant odour for the predators (Clucas, 2010). One of the prevalent types of deterrent odorants known are pyrazines, heterocyclic aromatic organic compounds and organoleptic agents involved in the release of a warning smell in many aposematic insects (Guilford et al., 1987; Moore & Brown, 1981; Rothschild & Moore, 1987). Pyrazines have a distinctive smell that has been shown to help predators learn the association between a warning signal and a secondary defence (Rowe & Guilford, 1996). Furthermore, studies suggest they can also produce reactions in predators consistent with an unpleasant taste (Rojas et al., 2017, 2019).

One species that uses pyrazines in its defence is the wood tiger moth, *Arctia plantaginis* (Erebidae: Arctiinae), which is a chemically defended, warningly coloured species. The major components of this moth's defences, the methoxypyrazines SBMP (2-sec-butyl-3-methoxypyrazine) and IBMP (2-isobutyl-3-methoxypyrazine), are synthesized de novo (Burdfield-Steel et al., 2018) and secreted as reflex blood in response to attacks by avian predators (Rojas et al., 2017; Winters et al., 2021). Here, we aim to investigate the within- and between-population variation in the amount and composition of methoxypyrazines from male wood tiger moths collected in Estonia, Finland, Scotland and Georgia, and test the hypotheses that (1) both variation in chemical defences and predator response towards defensive fluids reflect differences in predation pressure among populations, being weaker and more variable in populations with low predation pressure (Estonia and Finland; Rönkä et al., 2020), and stronger with less variability in populations with high predation pressure (Scotland and Georgia; Rönkä et al., 2020); (2) the observed differences in defence have a genetic basis; (3) there is an adverse reaction of wild predators to increased concentrations of the two pure (synthetic) methoxypyrazines SBMP and IBMP, both separately and combined. We tested these hypotheses by comparing the amount of pyrazines in defensive fluids of moths originating from different populations, rearing moths in a common garden environment to detect whether differences in defence have a genetic basis, and by testing the responses of wild-caught birds towards defensive fluids and pure pyrazines in different doses.

2 | MATERIALS AND METHODS

2.1 | Study species

The wood tiger moth *Arctia plantaginis* (formerly *Parasemia plantaginis*; Rönkä et al., 2016) is a diurnal aposematic species, that presents two different types of chemical fluids: one is produced from the

abdomen, and it is a deterrent to ants; the other is released from the thoracic area and is an effective deterrent to birds (Rojas et al., 2017) thanks to two methoxypyrazines: SBMP and IBMP. These methoxypyrazines are not sequestered directly from plants but are produced de novo (Burdfield-Steel et al., 2018). In Finland, wood tiger moths produce only one generation per year in the wild (Lindstedt et al., 2010; Ojala et al., 2005). Under greenhouse conditions, however, it is possible to obtain up to three generations per year. The larvae are polyphagous, feeding on a variety of different plants such as *Taraxacum sp*. (dandelion), *Plantago sp*., *Rumex sp*. and *Vaccinium uliginosum* (Ojala et al., 2005).

2.2 | Measurement of pyrazine levels across populations

2.2.1 | Collection of thoracic fluids

Male moths from wild populations were collected between 2015 and 2021 in four countries, Finland (Tornio; Jyväskylä; Tvärminne), Estonia (Pärnu), Georgia (Zekari pass) and Scotland (Findlater Castle; Findochty and Portknockie path). Moths were caught either with nets or using pheromone traps baited with laboratory-reared females. Upon capture, the moths were kept in individual containers and either had their thoracic fluids sampled the day after capture or were transported back to the University of Jyväskylä for sampling. Laboratory-reared moths were taken from populations founded from individuals from the same countries and maintained at the University of Jyväskylä. All laboratory-reared individuals originated from eggs laid by wild-caught females were reared in the greenhouse, although the length of time that the stock they originated from was kept in the laboratory varied. Greenhouse conditions followed roughly the outdoor temperatures, approximately 25°C during the day, dropping to 15–20°C at night. Daylight lasted for approximately 20 h.

For the Finnish and Estonian populations, larvae were overwintered every third generation at 5°C during the third instar. Larvae were housed in clear plastic tubs in family groups of no more than 30, fed *ad libitum* with *Taraxacum spp*. (dandelion) and misted with water daily. The only exception to this was the Georgian population, whose diet in the laboratory was supplemented with *Plantago sp*. and *Rumex sp*. because a *Taraxacum sp*.-only diet is not sufficient for their development and survival (own observation). Tubs were cleaned daily as needed and uneaten food was replaced. Upon pupation, individuals were kept individually in vials at 25°C until eclosion. When the adults emerged, they were given water and stored at 4°C to slow their metabolic rate.

In all cases, the protocol for sampling the thoracic fluids was the same and followed the method previously described in Rojas et al. (2017) and Burdfield-Steel et al. (2019). Moths were stored in chilled conditions (4°C) until 1 h prior to sampling, at which point they were provided with water (either in droplets or on a damp paper towel) to rehydrate and placed at room temperature to become active. Thoracic fluids were collected by pinching just below the prothoracic section of the moths with a pair of tweezers. This stimulated the release of the defence fluid, which was then collected with 10-µl glass capillaries and the volume was measured with a calliper. Samples were then transferred to glass vials and stored at −20°C until analysis.

2.2.2 | Measurement of methoxypyrazines

Prior to GC–MS analysis, samples were thawed and mixed with a 200-µl NaCl solution (3%). Measurement of the pyrazines was done following the methods of Cai et al. (2007) as described in Burdfield-Steel et al. (2018). Pyrazines were extracted from the headspace of fluid samples using SPME fibres (StableFlex 1-cm fibres with Divinylbenzene/Carboxen/Polydimethylsiloxane coating, Sigma- Aldrich, Darmstadt, Germany) for 30 min at 37°C. GC/MSD and analyses were carried out on an Agilent 6890 series GC system equipped with a Zebron ZB-5HT Inferno (Phenomenex Inc., Torrance, CA) column (length 30 m, 0.25 mm I.D. with a film thickness of 0.25 µm) connected to an Agilent 5973 N MSD. Fibres were manually loaded into the injector using a splitless injection mode, and the inlet temperature was set to 260°C. Helium was used as a carrier gas at a constant flow rate of 0.8 mL/min. The oven temperature was programmed as follows: 3 min at 60°C then ramped to 170°C at a rate of 7°C/min and from 170 to 260°C at a rate of 20°C/min and kept at that temperature for an additional 5 min. SBMP and IBMP were detected using selected ion monitoring of ions 124, 138 and 151. The chromatograms and mass spectra were evaluated using Agilent Chemstation (v. G1701CA) software and the Wiley 8th edition mass spectral database and the methoxypyrazines were identified using the ratio of these detected ions from the NIST webbook page (Stein), as well as by comparison with standards of SBMP and IBMP. The amount of the two methoxypyrazines in the sample was calculated by comparison with known amounts of the standards, run in the same manner as the fluid samples.

2.2.3 | Measure of methoxypyrazine statistical analysis

All statistical analyses were carried out with the software R v. 4.1.2 (R Core Team, 2022) using the RStudio v. 1.2.1335 interface (RStudio Team, 2019). We tested the effect of population (Finnish, Estonian, Georgian, Scottish) and rearing environment (hereafter referred to as origin: wild vs. laboratory) on the amount (ng) of SBMP, IBMP using linear mixed-effects models with a normal distribution in the package lme4 (Bates et al., 2015). In each model, population, "origin" and the interaction between the two were set as the explanatory variables and "year" was included as a random factor, to account for the nonindependence of data gathered within the same year. A Watson–Williams F-test (pairwise comparison) was applied to compare variance in the amount SBMP and IBMP from wood tiger moths raised in the laboratory versus wild, and Bartlett test (Bartlett, 1937) was applied to compare variance in the amount of each pyrazine across populations. To look for any confounding effects of body

size, we tested the effect of the pupal weight (a proxy of adult body size) of laboratory-reared individuals and populations on the amount (ng) of SBMP and IBMP pyrazines using linear mixed-effects models with a normal distribution in the package lme4 (Bates et al., 2015). In each model, pupal weight and population and the interaction between the two were set as the explanatory variables and "year" was included as a random factor, to account for the nonindependence of data gathered within the same year. Next, because the pupal weight did not explain the laboratory-raised population differences in SBMP and IBMP amount, it was removed from further analyses. The differences in the amount of SBMP and IBMP between population (wild and laboratory Finnish, Estonians, Georgians and Scottish) were then compared using Tukey–Kramer post hoc test for multiple comparisons. p values <0.05 were considered significant.

2.3 | Pure pyrazine assay

A previous study found the concentration of SBMP and IBMP in the fluid of the moths to be between 0.1 and 1 ng/μl (Burdfield-Steel et al., 2018). Therefore, synthetic SBMP and IBMP (Supelco, Sigma-Aldrich) were diluted in water at the University of Jyväskylä to the following concentrations: 0.05, 0.1, 0.5 and 1 ng/μl. A 50/50 blend of SBMP and IBMP was also made such that each dilution (0.05, 0.1, 0.5 and 1 ng/μl) was the total additive concentration of the two pyrazines combined. These dilutions were then refrigerated at ~4°C for no more than one month before use in the experiment.

We used blue tits (*Cyanistes caeruleus*) as model predators to test their response to the pure methoxypyrazines. This species is common in Finland, easy to capture and possible to keep in captivity for short periods of time necessary for the experiments (Rönkä et al., 2018). Furthermore, blue tits are thought to be natural predators of wood tiger moths, they have an overlapping distribution range and have already been used in other studies on the chemical defences of wood tiger moths (Burdfield-Steel et al., 2019; Rojas et al., 2017; Rojas et al., 2019; Rönkä et al., 2018).

The birds used for the experiment were caught at Konnevesi Research Station, in Central Finland (62.6164° N, 26.3459° E), from January to March in the years 2017–2019, maintained individually in plywood cages with a perch, water bowl and *ad libitum* food, and kept on a 12:12 h light:dark cycle. We based the predator assay training on those of Burdfield-Steel et al. (2019).

A total of 79 blue tits were used to measure bird responses to pure pyrazines. Each bird was used in the experiment only once and was assigned a single treatment. Birds were first trained to eat sunflower seeds to improve habituation by offering them familiar food into the experimental cage, and later seeds were mixed with the oats to motivate birds to learn eating the water-soaked oats before the assay. Each assay consisted of five trials. In the first trial, birds were offered water-soaked oats to ensure they were motivated to feed and, in the last trial, birds were again offered water-soaked oats to rule out satiation. During trials 2, 3 and 4 each bird was presented with 3 oats per trial on a small white dish. Each oat was covered with 8 μl of either water (as a control treatment) or one of the pure pyrazine treatments. Therefore, only trials 2, 3 and 4 are used in the analysis. In each trial we recorded hesitation time (measured as time in seconds from seeing the oat to pecking/eating the first oat), the proportion of the oats eaten (to the nearest 10%), beak cleaning (a disgust behaviour measured as the number of bouts where the bird wiped its beak against a surface such as the perch), drinking (the number of times the bird drank water, which is a behaviour that can increase in response to distasteful food) and trial duration (from the time the oats were seen by the bird until they were consumed—or max 300 s if some of the oats remained). All trials were video recorded using a hole at the top of the experimental enclosure.

2.3.1 | Pure pyrazine assay statistical analysis

All analyses were conducted using R version 4.1.2 (R Core Team, 2022). To test whether bird hesitation time differed among treatments, we used a cox proportional hazards model using the package coxme (Therneau, 2020). To test whether the proportion of oats eaten (the less birds eat, the more unpalatable the bait is) differed among treatments, we used a beta regression model using package glmmTMB (Brooks et al., 2017) and included trial duration as an offset term in the model. To test whether counts of bird beak cleaning and water drinking behaviours differed among treatments, we first excluded observations from birds that did not eat any of the oats and included trial duration as an offset term in the models. We then used Generalized Linear Mixed Models (GLMM) with Poisson distribution using package lme4 (Bates et al., 2015).

For each bird response variable, we first tested whether pyrazine treatments differed from the control treatment. In each model, the predictor variables included the chemical treatment (a categorical variable with different levels for each pyrazine and concentration) and trial number (2,3,4) to test whether birds altered their behaviour as the trials progressed. Each model also included bird age, sex and weight as co-variates and bird ID as a random factor. Automated model selection was performed using the dredge function of the MuMIn package (Barton, 2022) with chemical treatment and trial number set as fixed in all models. In all cases, the simplest model within delta 2 of the top model contained only chemical treatment and trial number as fixed factors and bird ID as a random factor, and this was chosen as the final model. If bird response significantly differed from the water control for any pyrazine treatments, we then created a new model excluding the water control to test the effect of concentration as an ordered factor, pyrazine type and their interaction. In this model, concentration is an ordinal variable with orthogonal polynomial contrasts. Statistical significance was set at $p<0.05$.

2.4 | Bird response to moths' defensive fluid

Blue tits (*C. caeruleus*; $n = 116$) were used for the predator assay as described in the previous section (pure pyrazine analysis). We

used fluids from 34 wild Finnish males, 21 laboratory Finnish males, 44 wild Georgian males and 8 laboratory Georgian males. The collection of the thoracic fluid was done as described above (see "Measurement of pyrazine levels across populations"). In addition, we offered water to 9 birds which were used as controls. Because the moths had different volumes of thoracic fluid, the fluid of each individual fluid was diluted proportionally with water to reach a total volume of 15 μl of fluid. Then, the 15 μl were divided into two samples of 7 μl each, which were offered to the same bird. The same amount of water was offered to control birds. During bird training sessions, we put 4 oat flakes and 3 sunflower seeds on a small white plate. Only when the birds ate all the oats in the training phase, did the experiment begin.

Each bird experienced 4 trials, consecutive, with 5-min intervals. In each trial, the bird was presented with a plate containing one oat flake. Following the methodology of Burdfield-Steel et al., 2019, we ran only four trials with one oat flake each (compared to the five trials and three oat flakes per trial in the pure pyrazine assay) because the volume of chemical defence fluid released by each individual moth did not allow for more. The first and last trials were done with oats soaked in water to ensure that the bird was motivated to eat (first) and that the bird was still hungry (last). In the second and third trials, the bird was presented with an oat soaked in 7 μl of the defensive fluids of the same moth. The trial ended two minutes after the bird had eaten the whole oat, or after a maximum duration of 5 min if the bird did not eat the whole oat. In each trial, we recorded the hesitation time (the time in seconds that occurs from the moment when the bird sees the oat to when they pecking/eating it); the proportion of oat eaten (to the nearest 10%); beak cleaning (number of times the bird wiped its beak against a surface, e.g., the perch); the drinking (number of times the bird drinks water, as a response to the distaste of the food); the trial duration (from the time the oats were seen by the bird until they were consumed—or max 300 s if the oat was not eaten). All trials were video recorded.

2.4.1 | Predator assay statistical analysis

The statistical analyses were conducted using the software R v. 4.1.2 (R Core Team, 2022) using the RStudio v. 1.2.1335 interface (RStudio Team, 2019). The behaviours of the birds were first compared to a water-only control to determine if the thoracic fluid of the moths elicited an adverse reaction in the predators. To test the difference in hesitation time in response to thoracic fluids from wild and laboratory Finnish and Georgian wood tiger moths, we used a cox proportional hazards model using the package coxme (Therneau, 2020). The proportion of oat eaten was tested using package glmmTMB with family = beta_family(link = "logit"). The birds' beak cleaning and water drinking behaviours, were tested using general linear mixed-effects model with Poisson distribution using package lme4 (Bates et al., 2015). Each model included bird ID as a random factor. The interaction (country: origin) between the population and the origin (wild/laboratory) and the trial were set as fixed factors. Also, the trial with duration as an offset was included as an explanatory variable, while the proportion of oat eaten, and the beak wiping and drinking were set as response variables. These models only looked at observations where the proportion eaten was greater than zero. The treatments that showed a different reaction to the water control (hesitation time, proportion of oat eaten and water drinking) were then compared using Tukey–Kramer post hoc test for multiple comparisons and excluding the water control group. Statistical significance was set at $p < 0.05$.

3 | RESULTS

3.1 | Geographic variation in pyrazines

3.1.1 | Differences in pyrazines across populations

Wild individuals from Scotland had a higher amount of SBMP than the wild and laboratory Estonian, Finnish and laboratory-raised Scottish moths ($p < 0.05$; Table S2, Figure 1a), but not from wild moths from Georgia ($p > 0.05$; Table S2, Figure 1a). Thus, the quantity of SBMP in the thoracic fluids of wood tiger moths was significantly different between populations (Chisq = 24.52; df = 3; Pr(>Chisq) = 1.949 e-05). Wild individuals from Scotland had the higher amount of IBMP than the wild Georgian moths and laboratory and wild individuals from Estonia and Finland ($p < 0.05$; Table S4, Figure 1b), but not from the laboratory-raised Georgian and Scottish ($p > 0.05$; Table S4, Figure 1b). Thus, the quantity of IBMP in the thoracic fluids of wood tiger moths was significantly influenced by the interaction between population and origin (Chisq = 10.68; df = 3; Pr(>Chisq) = 0.014, Table S3, Figure 1b). The pupal weight of the laboratory-raised moths did not explain population differences in the amount of SBMP, and it was therefore removed from further analyses (Chisq = 7.59, df = 3, Pr(>Chisq) = 0.055) and IBMP (Chisq = 5.17, df = 3, Pr(>Chisq) = 0.15).

Wild wood tiger moths had a lower variance in the amount of SBMP than those reared in the laboratory (F = 0.53, num df = 63, denom df = 91, $p = 0.0077$; see Table S5), but we found no difference in the variance of the amount of IBMP between laboratory and wild wood tiger moths (F = 0.99, num df = 63, denom df = 91, $p = 0.99$; see Table S5). The measure of variability (variance) of SBMP (Bartlett's K-squared = 15.34, df = 3, $p = 0.00155$) and IBMP (Bartlett's K-squared = 10.31, df = 3, $p = 0.016$) was different between populations. The Scottish wood tiger moth population presented the highest variance in the amount of SBMP, and the Finnish population the lowest, while the Finnish population presented the highest IBMP variance, followed by the Scottish population (see Table S6).

There were significant differences in the ratio of IBMP to SBMP between populations (Chisq =27.25; df = 3; Pr(>Chisq; Figure 2) = 5.224 e-06) and origin (Chisq =11.08; df = 1; Pr(>Chisq) = 0.0008; Figure 2), but not in the interaction between population and origin (Chisq =5.73; df = 3; Pr(>Chisq) = 0.125;

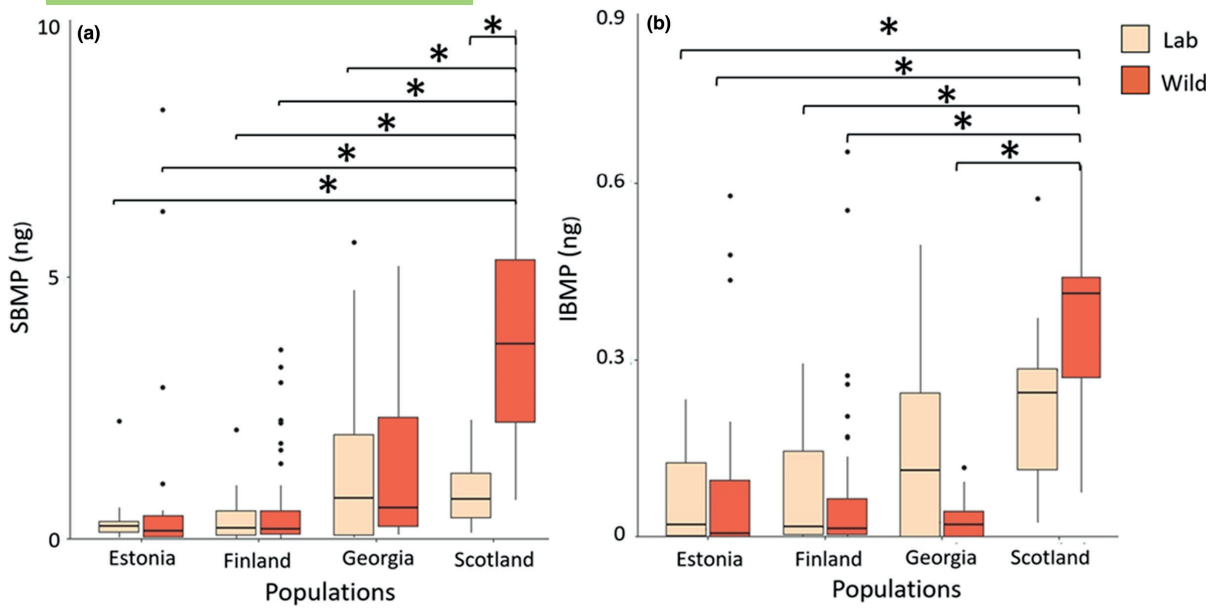

FIGURE 1 (a) SBMP amount in nanograms for each population (note this is the total amount of SBMP and IBMP in the thoracic fluids in nanograms, not the concentration). Wild individuals from Scotland had a higher amount of SBMP than the wild and laboratory Estonian, Finnish and laboratory-raised Scottish moths but not from wild moths from Georgia. (b) IBMP amount in nanograms for each population. Wild individuals from Scotland had higher amount of IBMP than the wild Georgian moths and laboratory and wild individuals from Estonia and Finland, but not from the laboratory-raised Georgian and Scottish moths. Boxes show the median and the 25th and 75th percentiles of data distribution. Vertical lines show the data range.

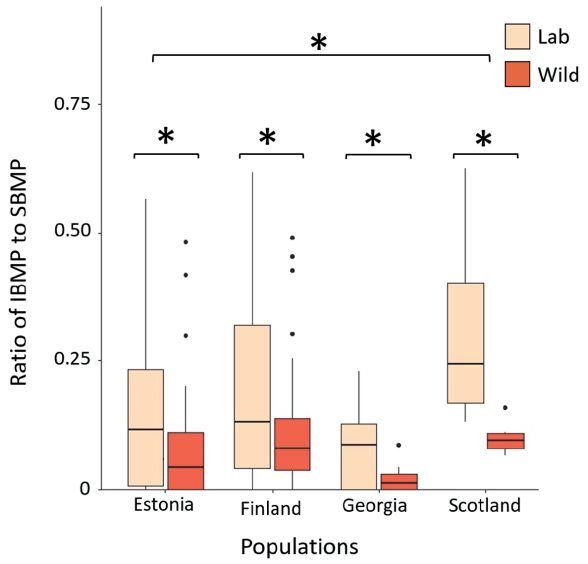

FIGURE 2 Ratio of IBMP to SBMP for each population. The laboratory populations had higher ratio of IBMP to SBMP compared to the wild populations. Boxes show the median and the 25th and 75th percentiles of data distribution. Vertical lines show the data range.

Figure 2). The laboratory populations had higher ratio of IBMP to SBMP compared to the wild populations (diff = −0.079; lwr = −0.12; upr = −0.04; *p* adj = 0.0001; Figure 2).

3.2 | Pure pyrazine assay

Birds hesitated longer to eat oats in later trials (coef ± *SE* = −0.21 ± 0.09, *z* = −2.28, *p* = 0.023), but none of the pyrazine treatments differed significantly from the control (Table S8, Figure S1). However, there was a trend for birds to hesitate longer before eating oats of SBMP 0.1 ng/μl (coef ± *SE* = −1.33 ± 0.68, *z* = −1.95, *p* = 0.051) and 1.0 ng/μl (coef ± *SE* = −1.34 ± 0.69, *z* = −1.95, *p* = 0.052) concentrations compared to the control (Figure S1).

Birds ate a smaller proportion of the oats in later trials (estimate ± *SE* = −0.07 ± 0.01, *z* = −5.60, *p* < 0.001, Table S9). In addition, the proportion of oats birds ate was significantly less than the control for SBMP at the three highest concentrations: 0.1 ng/μl (estimate ± *SE* = −2.70 ± 1.28, *z* = −2.11, *p* = 0.04), 0.5 ng/μl (estimate ± *SE* = −4.76 ± 1.31, *z* = −3.64, *p* < 0.001), and 1.0 ng/μl (estimate ± *SE* = −3.01 ± 1.28, *z* = −2.34, *p* = 0.019) and for the 50/50 blend of SBMP + IBMP at the 0.05 ng/μl (estimate ± *SE* = −2.57 ± 1.28, *z* = −2.00, *p* = 0.05, Figure 3) and 1.0 ng/μl concentrations (estimate ± *SE* = −2.35 ± 1.20, *z* = −1.96, *p* = 0.050, Figure 3), but no concentrations of IBMP differed from the control (Table S9, Figure 3). Next, testing the independent variables of concentration and fluid, there was a significant interaction. As fluid concentration increased, birds decreased consumption of SBMP, but increased consumption of IBMP (estimate ± *SE* = −2.87 ± 1.45, *z* = −1.98, *p* = 0.048, Table S10, Figure 3).

Bird beak wipes did not change across trials (estimate ± *SE* = −0.02 ± 0.05 *z* = −0.43, *p* = 0.668), and none of the pyrazine

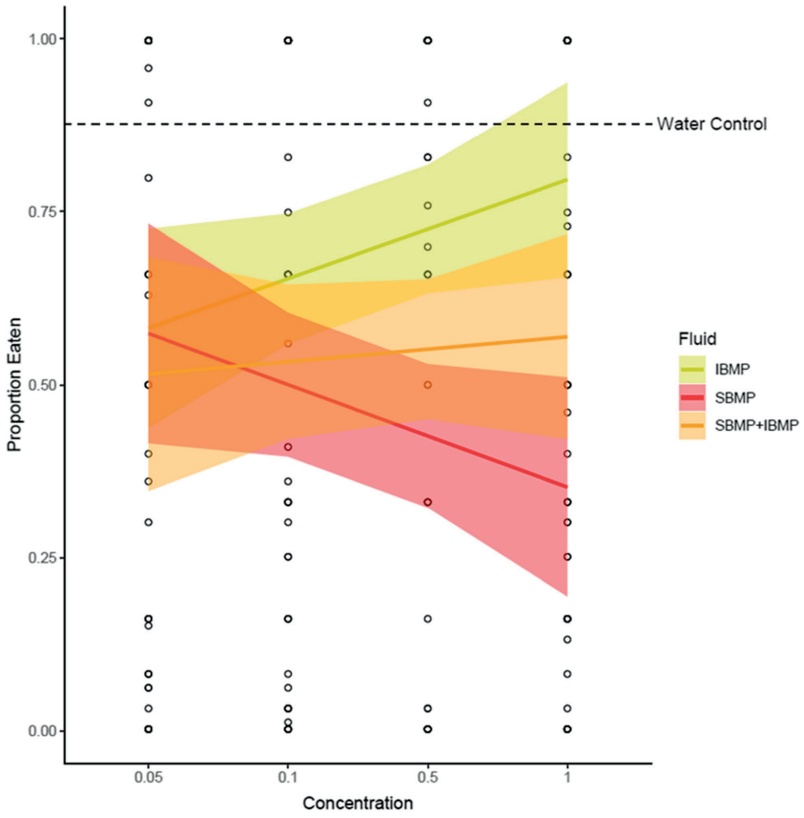

FIGURE 3 Proportion of fluid-soaked oats eaten in response to increasing concentrations (ng/μl) of each pyrazine type (SBMP = red, IBMP = yellow, SBMP + IBMP = orange). Shaded area represents standard error. Average bird response to the water control is indicated by a dotted line.

treatments differed significantly from the control (Table S11, Figure S3). However, there was a trend for birds to wipe their beaks more after eating oats of the SBMP + IBMP 0.5 ng/μl concentration compared to the control (estimate ± *SE* = 1.58 ± 0.82, $z = 1.93$, $p = 0.054$, Figure S3).

Birds drank more water in later trials (estimate ± *SE* = 0.34 ± 0.07, $z = 4.60$, $p < 0.001$). In addition, birds drank more water in response to the SBMP + IBMP 0.5 ng/μl concentration compared to the control (estimate ± *SE* = 3.06 ± 1.41, $z = 2.17$, $p = 0.030$). There was also a trend for birds to drink more water in response to the IBMP 0.05 ng/μl concentration compared to the control (estimate ± *SE* = 2.46 ± 1.42, $z = 1.74$, $p = 0.083$), but no concentrations of SBMP differed from the control (Table S12, Figure S4). Next, testing the independent variables of concentration and fluid, there was no effect of fluid, concentration or their interaction (Table S13).

3.3 | Bird response to moths' defensive fluid

Following the measurement of the pyrazine levels across populations and the bioassay testing the response of wild-caught predators to the pure pyrazine, we tested bird response to the thoracic fluid of Finnish and Georgian laboratory and wild populations. We used the thoracic fluid of moths from Finland and Georgia as they showed significantly different chemical compositions, and both populations could be successfully reared in the laboratory. The chemical defence fluid from Georgian wood tiger moths reared in the laboratory provoked longer hesitation times compared to the control (coef = −1.91, *SE* = 0.56, $z = -3.38$, $p = 0.00071$; Figure 4a), whereas that of both Finnish laboratory-raised (coef = −0.599, *SE* = 0.46, $z = -1.30$, $p = 0.19$) and wild moths (coef = −0.50, *SE* = 0.43, $z = -1.16$, $p = 0.25$), and wild Georgian moths (coef = −0.64, *SE* = 0.42, $z = -1.51$, $p = 0.13$) did not differ significantly from the control. When we analysed the proportion of oats eaten, which can be used as a proxy for distastefulness, fluids from all four groups were eaten less than the water control (Finnish laboratory: coef = −3.74, *SE* = 0.88, $z = -4.24$, $p = 2.22\text{e-}05$; Georgian laboratory: coef = −4.315, *SE* = 1.085, $z = -3.98$, $p = 6.99\text{e-}05$; Finnish wild: coef = −2805, *SE* =0.83, $z = -3.39$, $p = 0.0007$, and Georgian wild: coef = −2.84, *SE* = 0.81, $z = -3.51$, $p = 0.0004$, see Figure 4b). We found no significant difference in the beak wiping behaviour between birds exposed to oats soaked in either fluid from laboratory and wild Finnish and Georgian wood tiger moths, and those exposed to water-soaked oats ($p > 0.05$, see Figure S5, Table S16, supplementary material). Birds drank more water after tasting the thoracic fluid from laboratory (coef = −3.40, *SE* = 0.94, $z = -3.63$, $p = 0.000281$) and wild (coef = −3.99, *SE* = 0.94, $z = -4.24$, $p = 2.21\text{e-}05$) Finnish wood tiger moths and wild Georgian wood tiger moths (coef = −2.22, *SE* = 0.86, $z = -2.53$, $p = 0.011420$, Figure 5) compared to oats soaked in water.

Next, we tested the difference between laboratory and wild Finnish and Georgian moths without the water control. The thoracic defence from laboratory-raised Georgian wood tiger moths elicited longer hesitation times in the predators' than the defence fluid from Georgian wild moths, and both Finnish laboratory and

wild moths ($p < 0.05$, Figure 4a, see Table S18; supplementary material). When we tested the proportion of oat eaten, the thoracic fluid from the Georgian laboratory moths was eaten less than Finnish and Georgian wild moths ($p < 0.05$, Figure 4b, see Table S19; supplementary material). Predators did not differ in water drinking behaviour after experiencing the chemical defences of Finnish and Georgian moths ($p > 0.05$, Figure 5, see Table S20; supplementary material).

4 | DISCUSSION

Predation is acknowledged as one of the strongest selective pressures influencing the ecology and evolution of prey populations. Thus, we can assume that variation in predator community structure should reflect the antipredator adaptations of prey. When looking at chemical variation across different wood tiger moth populations,

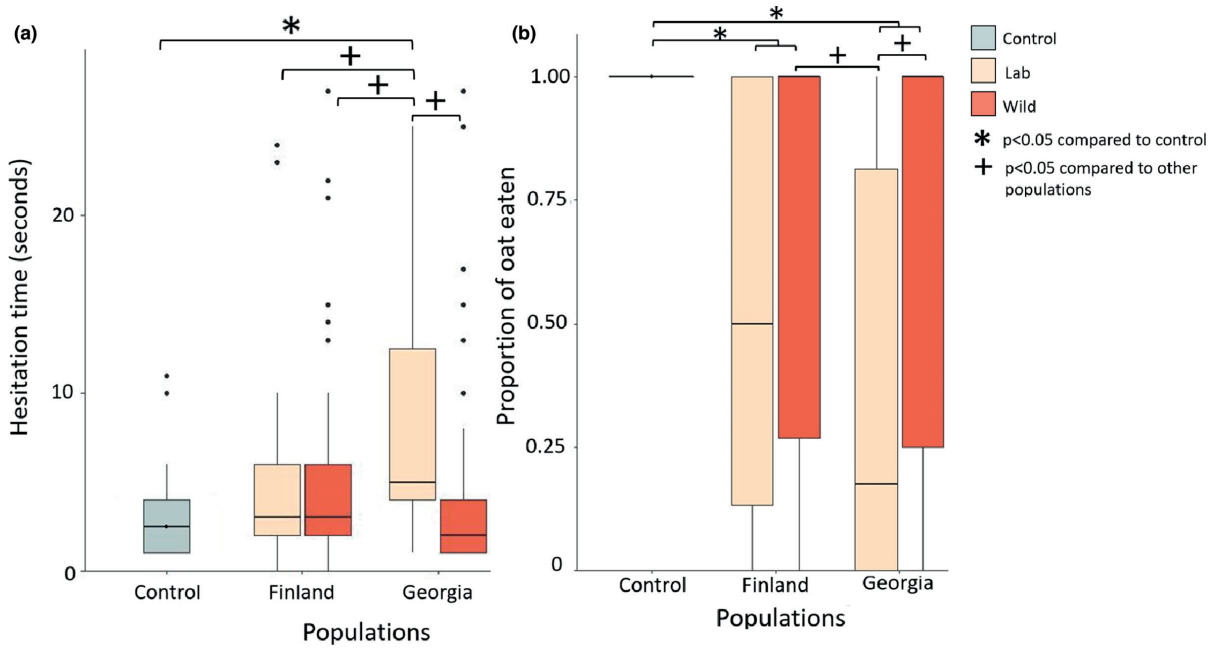

FIGURE 4 (a) Predators hesitate longer when exposed to fluids of Georgia moths raised in laboratory conditions compared to water. Boxes show the median and the 25th and 75th percentiles of data distribution. Vertical lines show the data range. (b) Proportion of oat eaten is lower when predators are exposed to fluids of moths raised in a laboratory and wild conditions from both populations compared to water. Boxes show the median and the 25th and 75th percentiles of data distribution. Vertical lines show the data range.

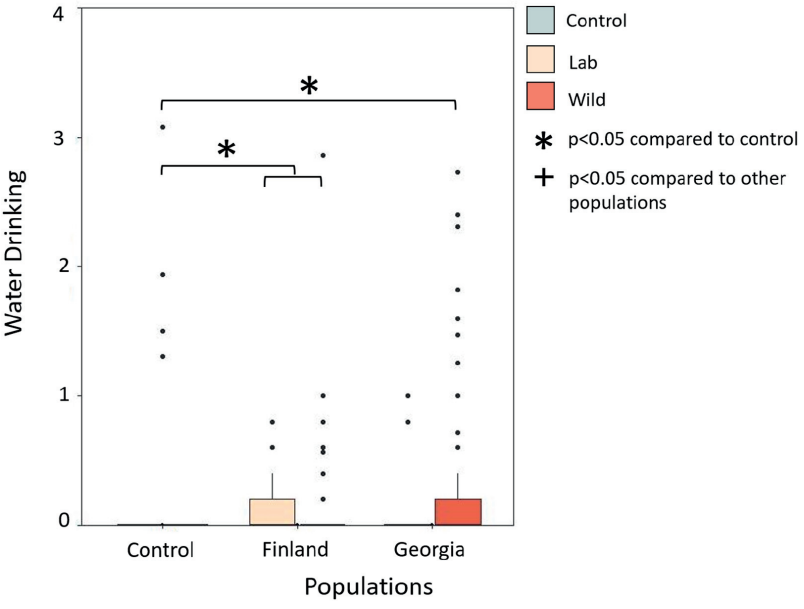

FIGURE 5 Water drinking increased when predators were exposed to fluids of wild and laboratory Finnish populations and wild Georgian moths compared to water. Water drinking did not differ between the two populations and wild and laboratory-raised moths. Boxes show the median and the 25th and 75th percentiles of data distribution. Vertical lines show the data range.

we predicted that the thoracic fluid of populations with higher predation pressure would have stronger defences with less variability, and populations with lower predation pressure would have weaker defences with a greater variability.

Previous studies have shown that wood tiger moths experience higher predation pressure in Scotland and Georgia than in other populations (Nokelainen et al., 2014; Rönkä et al., 2020), and that wild Scottish wood tiger moths have a higher likelihood of being attacked because their surrounding environment is more open and visible (Nokelainen et al., 2014). Our analyses show that moths from the Scottish and Georgian populations indeed had higher amounts of SBMP in their defensive fluids compared to the other populations. Our pure pyrazine assays showed that higher concentrations of SBMP, but not IBMP, elicit stronger disgust responses from birds, suggesting that the Scottish and Georgian populations are indeed better protected. The amounts of SBMP found in the laboratory Georgian population did differ from the Finnish populations and birds hesitated longer to "attack" oats soaked with the defensive fluids of Georgian laboratory-raised moths than those soaked with fluids from the wild Georgian and wild and laboratory-raised Finnish population. The proportion of oat eaten (used as a proxy for distastefulness) also differed between the laboratory-raised Georgian population and the wild Georgian and wild Finnish populations. The Georgian population showed clear differences from the other populations in the amounts of SBMP, IBMP and ratio of the two pyrazines. The variation in chemical defences also partially reflects differences in populations' predation pressure, with greater variability of IBMP in the population with the lowest predation pressure (Finland; Rönkä et al., 2020). However, the variability of SBMP and IBMP was also higher in the Scottish population. Thus, our results support the hypothesis that stronger predation pressure may have selected for stronger chemical defences, but not necessarily reduced variation in the strength of defence within populations.

Second, we predicted population-level differences would be genetic in origin. As we found the same pattern both in wild-caught and laboratory-raised moths, we infer that this difference is likely to have a genetic basis. However, in comparing moths reared in different diets, we were also interested in testing if laboratory-reared moths that were kept on an *ad libitum* diet would have higher level of chemical defence and lower variance than their wild counterparts, which may experience much more variation in food availability and quality during development. We found that laboratory-raised moths indeed had higher amounts of SBMP and IBMP compared to wild-caught moths but also higher variation in SBMP abundance compared to the wild moths. This sounds counterintuitive, but the variation seen in the laboratory-raised moths may just reflect the absence of predation pressure and the relaxed selection in the lab. Finally, it should be noted that wood tiger moths are capital breeders, meaning that adults do not eat and all resources must be acquired at the larval stage. For that reason, it is unknown how effectively moths can recover their chemical defences after they have released them. Moths can certainly produce defensive fluids multiple times over their lifespan, but the amount of pyrazine may decrease with each release. Evidence from enclosure experiments with wild-caught birds and live moths suggests that ca. 30% of moths that are attacked and taste-rejected by birds can survive the attack (Rönka et al. unpublished; Winters et al., 2021). Thus, if wild-caught moths have previously been attacked and released their defensive fluids, this may contribute to the lower level of defence seen. While we cannot rule this out, if prior attacks were indeed driving the pattern of reduced defence in the wild populations, we would expect this to be most noticeable in the Scottish population, where prior studies suggest bird predation is highest, and much reduced in the Estonian population where attack rates are low (Rönkä et al., 2020). However, this is not the pattern we see (Figure 3), as the Scottish population is in fact the only population that present the higher amount of SBMP and IBMP in wild moths than laboratory-reared.

When we tested the moths' chemical defence against wild blue tits, we found that laboratory-raised moths triggered a stronger response than wild-caught moths. This is in line with a previous study showing that food deprivation results in lowered defence against birds in this species (Burdfield-Steel et al., 2018). Thus, variation in the efficacy of chemical defences from individuals of the same population (but raised in different environments, e.g.: in wild vs. laboratory conditions) may be due to food deprivation or competition for resources (Speed et al., 2012) in wild moths during the early life stages. It should be noted that because the Georgian laboratory-raised population was additionally fed with *Plantago sp.* and *Rumex sp.*, this may also have influenced their stronger chemical defence. The main difference found between the Georgian laboratory populations and the Finnish was a longer hesitation time for birds to attack the fluids. It has been previously shown (Burdfield-Steel et al., 2019) that resource limitation in early life indeed impacts the efficacy of the wood tiger moth's chemical defences in terms of bird hesitation time, which was lower when the birds experienced the defences of moths raised with reduced access to food (Burdfield-Steel et al., 2019). Thus, we cannot rule out that this may be an effect of the more varied diet eaten by the laboratory Georgian population. Another possibility is that fluids of Georgian moths contained small amounts of iridoid glycosides that *A. plantaginis* are able to sequester in low amounts from *Plantago* plants (Lindstedt et al., 2010; Reudler et al., 2015). Although previous studies found only trace amounts of iridoids from moths, those doses were sufficient to trigger disgust behaviour (after tasting) of birds. It is unknown, however, if birds can smell iridoids and avoid attacking such prey.

We hypothesized that the two pyrazines combined together would have an additive effect on predator avoidance. However, our analysis of wild blue tit responses to pure pyrazines suggests that SBMP alone was a more effective defence than IBMP: birds ate a smaller proportion of oats soaked with SBMP and there was a trend for birds to hesitate longer to approach SBMP oats compared to the control. In contrast, IBMP was a weak defence on its own, although there was a trend for IBMP to cause birds to drink more water, which suggests that birds may find IBMP more aversive after tasting it. Despite having no effect on bird hesitation to approach, the 50/50

blend of IBMP + SBMP influenced the greatest number of bird behaviours compared to the control: reducing the proportion eaten, increasing drinking behaviour and there was a trend to increase beak wipes. Rather than the combination having an intermediate effect between that of the two pure pyrazines, as we would expect if the effects of the two were purely additive, this suggests the combination of the two instead has a nonadditive, synergistic, effect. The efficacy of this combination during the subjugation stage of attack could explain why moths use IBMP in combination with SBMP even when IBMP alone is mostly ineffective. Similarly, a recent study by Yan et al. (2021) found that subthreshold pyrazines, which are not detected at the given concentration on their own, can nonetheless contribute synergistically to the organoleptic properties of a chemical mixture as suggested by Maga et al. (1973). Interestingly, Yan et al. (2021) also found that subthreshold pyrazines reduced the odour thresholds of suprathreshold pyrazines, which could explain why the combination of SBMP + IBMP did not affect bird hesitation to approach the defensive odour. Altogether these results suggest that the aversion of a chemical mixture is not the same as the sum of its parts, and chemical defences should, therefore, be presented in natural combinations to account for potential synergistic and antagonistic relationships that influence the sensory responses of predators.

We also hypothesized that an increased concentration of the methoxypyrazines SBMP and IBMP would elicited stronger aversive reactions in bird predators. In support of our hypothesis, we found that birds ate a smaller proportion of oats as the concentration of SBMP increased. However, surprisingly the efficacy of SBMP + IBMP did not increase with concentration, and the efficacy of IBMP decreased with concentration. Wood tiger moths produce between 0.5 and 2 μl of fluid, so the average concentration of the fluids is in the lower range of the concentrations tested (based on the abundances shown in Figure 1). Overall, we found an unexpected relationship with pyrazine concentration, where more is not always better—especially for IBMP. This finding is in line with research on pyrazines in food science, where concentration has been found to change the quality rather than just the intensity of sensory perception. For example, Evers et al. (1972) described 5,7-dihydrothieno (3,4,6)—pyrazine as resembling roasted nuts, baked goods or fresh milk, depending on the concentration and evaluation medium (Maga et al., 1973). This means that aversion towards a chemical mixture cannot always be extrapolated from the concentration of its contents, and predator responses to defence fluids at natural concentrations should also be measured.

When examining the match between the response to the pure pyrazines and the thoracic fluids of the moths we also have to consider the possibility that the moths' defensive fluids may contain additional compounds. A recent study found that sequestered pyrrolizidine alkaloids (PAs) of wood tiger moths can provoke disgust reactions in wild birds (Winters et al., 2021). The presence of PAs alone did not deter the predators, but the combination of both pyrazines and PAs confers better defences to the moths (Winters et al., 2021). Predators, especially birds, can detect the smell of pyrazine from a distance (Guilford et al., 1987), which plays a role in the antipredator defences of aposematic prey, so this may also explain why laboratory moths seem to allocate more resources to the production of pyrazine when they are raised with a constant amount of resources (i.e., in the laboratory) and on a diet from which they cannot sufficiently sequester defensive toxins such as PAs. Predators can indeed use more than one cue to assess the toxicity of prey, so multiple defensive compounds can be used as a multimodal signal (Marples et al., 1994; Rojas et al., 2019). However, our finding that Finnish moths reared in the laboratory, which did not have access to PAs in their diet, were not less defended than those from the wild suggests pyrazines are indeed the main contributor to the aversive power of the thoracic fluids.

Overall, chemical variation in wood tiger moths appears to correlate with previously measured predation pressure, suggesting that natural selection may also drive investment in chemical defences in this species. Clearly, the study of chemical defences may be complicated by nonadditive interactions between the chemical components of the defence, and caution must be used when extrapolating from chemical measurements to predator responses.

AUTHOR CONTRIBUTIONS

JM and EB-S conceived and designed the study. EB-S, CO, AW and BR performed field work and data collection. CO, EB-S and AW carried out all the chemical and statistical analyses. CO and EB-S wrote a first draft of the manuscript with input from AW, JM and BR. All authors critically reviewed the manuscript, approved the submitted version, and agreed to be held accountable for the content therein.

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CONFLICT OF INTEREST

None.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/jeb.14142.

DATA AVAILABILITY STATEMENT

The supporting data will be archived in an appropriate public repository (jyx.jyu.fi) and the data DOI will be included upon acceptance.

PERMITS

Wild birds were used with permission from the Central Finland Centre for Economic Development, Transport and Environment and licence from the National Animal Experiment Board (ESAVI/9114/04.10.07/2014) and the Central Finland Regional Environment Centre (VARELY/294/2015).

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REFERENCES

Alonso-Mejia, A., & Brower, L. (1994). From model to mimic: Age-dependent unpalatability in monarch butterflies. *Cellular and Molecular Life Sciences*, *50*, 176–181.

Arenas, M. L., Walter, D., & Stevens, M. (2015). Signal honesty and predation risk among a closely related group of aposematic species. *Scientific Reports*, *5*(1), 1–12. https://doi.org/10.1038/srep11021

Barnett, C. A., Bateson, M., & Rowe, C. (2014). Better the devil you know: Avian predators find variation in prey toxicity aversive. *Biology Letters*, *10*(11), 20140533.

Bartlett, M. S. (1937). Properties of sufficiency and statistical tests. *Proceedings of the Royal Society of London Series A-Mathematical and Physical Sciences*, *160*(901), 268–282. https://doi.org/10.1098/rspa.1937.0109

Barton, K. (2022). *MuMIn: Multi-model inference*. R package version 1.47.1. https://CRAN.R-project.org/package=MuMIn

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*(1), 1–48. https://doi.org/10.18637/jss.v067.i01

Bezzerides, A. L., McGraw, K. J., Parker, R. S., & Husseini, J. (2007). Elytra color as a signal of chemical defense in the Asian ladybird beetle *Harmonia axyridis*. *Behavioral Ecology and Sociobiology*, *61*(9), 1401–1408. https://doi.org/10.1007/s00265-007-0371-9

Brooks, M. E., Kristensen, K., van Benthem, K. J., Magnusson, A., Berg, C. W., Nielsen, A., Skaug, H. J., Mächler, M., & Bolker, B. M. (2017). Modeling zero-inflated count data with glmm TMB. *bioRxiv*, 132753. https://doi.org/10.1101/132753

Brower, L. P., Seiber, J. N., Nelson, C. J., Lynch, S. P., & Tuskes, P. M. (1982). Plant-determined variation in the cardenolide content, thin-layer chromatography profiles, and emetic potency of monarch butterflies, *Danaus plexippus* reared on the milkweed, *Asclepias eriocarpa* in California. *Journal of Chemical Ecology*, *8*, 579–633. https://doi.org/10.1007/BF00989631

Burdfield-Steel, E., Brain, M., Rojas, B., & Mappes, J. (2019). The price of safety: Food deprivation in early life influences the efficacy of chemical defence in an aposematic moth. *Oikos*, *128*(2), 245–253. https://doi.org/10.1111/oik.05420

Burdfield-Steel, E., Pakkanen, H., Rojas, B., Galarza, J. A., & Mappes, J. (2018). De novo synthesis of chemical defenses in an aposematic moth. *Journal of Insect Science*, *18*(2), 28. https://doi.org/10.1093/jisesa/iey020

Cai, L., Koziel, J. A., & O'Neal, M. E. (2007). Determination of characteristic odorants from Harmonia axyridis beetles using in vivo solid-phase microextraction and multidimensional gas chromatography-mass spectrometry-olfactometry. *Journal of Chromatography A*, *1147*, 66–78. https://doi.org/10.1016/j.chroma.2007.02.044

Clucas, B. (2010). Defensive chemicals. In M. D. Breed, & J. Moore (Eds.), *Encyclopedia of Animal Behavior* (pp. 481–486). Academic Press. https://doi.org/10.1016/B978-0-08-045337-8.00293-X

Cummings, M. E., & Crothers, L. R. (2013). Interacting selection diversifies warning signals in a polytypic frog: An examination with the strawberry poison frog. *Evolutionary Ecology*, *27*(4), 693–710. https://doi.org/10.1007/s10682-013-9648-9

Darst, C. R., & Cummings, M. E. (2006). Predator learning favours mimicry of a less-toxic model in poison frogs. *Nature*, *440*(7081), 208–211. https://doi.org/10.1038/nature04297

Darst, C. R., Cummings, M. E., & Cannatella, D. C. (2006). A mechanism for diversity in warning signals: Conspicuousness versus toxicity in poison frogs. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(15), 5852–5857. https://doi.org/10.1073/pnas.0600625103

Eisner, T., Eisner, M., & Siegler, M. (2005). *Secret weapons: Defenses of insects, spiders, scorpions, and other many-legged creatures*. Belknap Press. https://doi.org/10.1007/s10841-006-9007-z

Evers, W. J., Wilson, R. A., Theimer, E. T., & Katz, I. (1972). (U.S. Patent No. 3,647,792). U.S. Patent and Trademark Office.

Guilford, T. I. M., Nicol, C., Rothschild, M., & Moore, B. P. (1987). The biological roles of pyrazines: Evidence for a warning odour function. *Biological Journal of the Linnean Society*, *31*(2), 113–128. https://doi.org/10.1111/j.1095-8312.1987.tb01984.x

Hudson, C. M., Brown, G. P., Blennerhassett, R. A., & Shine, R. (2021). Variation in size and shape of toxin glands among cane toads from native-range and invasive populations. *Scientific Reports*, *11*(1), 1–11. https://doi.org/10.1038/s41598-020-80191-7

Lawrence, J. P., Rojas, B., Fouquet, A., Mappes, J., Blanchette, A., Saporito, R., Bosque, R. J., Courtois, E., & Noonan, B. P. (2019). Weak warning signals can persist in the absence of gene flow. *PNAS*, *116*, 19037–19045. https://doi.org/10.1073/pnas.1901872116

Lindstedt, C., Talsma, J. H. R., Ihalainen, E., Lindström, L., & Mappes, J. (2010). Diet quality affects warning coloration indirectly: Excretion costs in a generalist herbivore. *Evolution*, *64*(1), 68–78. https://doi.org/10.1111/j.1558-5646.2009.00796.x

Maan, M. E., & Cummings, M. E. (2012). Poison frog colors are honest signals of toxicity, particularly for bird predators. *The American Naturalist*, *179*(1), E1–E14. https://doi.org/10.1086/663197

Maga, J. A., Sizer, C. E., & Myhre, D. V. (1973). Pyrazines in foods. *Critical Reviews in Food Science & Nutrition*, *4*(1), 39–115. https://doi.org/10.1080/10408397309527153

Marples, N. M., van Veelen, W., & Brakefield, P. M. (1994). The relative importance of colour, taste and smell in the protection of an aposematic insect *Coccinella septempunctata*. *Animal Behaviour*, *48*(4), 967–974. https://doi.org/10.1006/anbe.1994.1322

Moore, B. P., & Brown, W. V. (1981). Identification of warning odour components, bitter principles and antifeedants in an aposematic beetle: *Metriorrhynchus rhipidius* (coleoptera: Lycidae). *Insect Biochemistry*, *11*(5), 493–499. https://doi.org/10.1016/0020-1790(81)90016-0

Nokelainen, O., Valkonen, J., Lindstedt, C., & Mappes, J. (2014). Changes in predator community structure shifts the efficacy of two warning signals in arctiid moths. *Journal of Animal Ecology*, *83*, 598–605. https://doi.org/10.1111/1365-2656.12169

Ojala, K., Julkunen-Tiitto, R., Lindström, L., & Mappes, J. (2005). Diet affects the immune defence and life-history traits of an arctiid moth Parasemia plantaginis. *Evolutionary Ecology Research*, *7*(8), 1153–1170.

R Core Team. (2022). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. https://www.R-project.org/

Reudler, J. H., Lindstedt, C., Pakkanen, H., Lehtinen, I., & Mappes, J. (2015). Costs and benefits of plant allelochemicals in herbivore diet in a multi enemy world. *Oecologia*, *179*(4), 1147–1158.

Rojas, B., Burdfield-Steel, E., Pakkanen, H., Suisto, K., Maczka, M., Schulz, S., & Mappes, J. (2017). How to fight multiple enemies: Target-specific

chemical defences in an aposematic moth. *Proceedings of the Royal Society B*, *284*, 20171424. https://doi.org/10.1098/rspb.2017.1424

Rojas, B., Mappes, J., & Burdfield-Steel, E. (2019). Multiple modalities in insect warning displays have additive effects against wild avian predators. *Behavioral Ecology and Sociobiology*, *73*(3), 37. https://doi.org/10.1007/s00265-019-2643-6

Rönkä, K., De Pasqual, C., Mappes, J., Gordon, S., & Rojas, B. (2018). Colour alone matters: No predator generalization among morphs of an aposematic moth. *Animal Behaviour*, *135*, 153–163. https://doi.org/10.1016/j.anbehav.2017.11.015

Rönkä, K., Mappes, J., Kaila, L., & Wahlberg, N. (2016). Putting *Parasemia* in its phylogenetic place: A molecular analysis of the subtribe Arctiina (lepidoptera). *Systematic Entomology*, *41*, 844–853. https://doi.org/10.1111/syen.12194

Rönkä, K., Valkonen, J. K., Nokelainen, O., Rojas, B., Gordon, S., Burdfield-Steel, E., & Mappes, J. (2020). Geographic mosaic of selection by avian predators on hindwing warning colour in a polymorphic aposematic moth. *Ecology Letters*, *23*(11), 1654–1663.

Rothschild, M., & Moore, B. (1987). Pyrazines as alerting signals in toxic plants and insects. In V. Labeyrie, G. Fabres, & D. Lachaise (Eds.), *Insects-Plants* (pp. 97–107). W Junk. https://doi.org/10.1111/ele.13597

Rowe, C., & Guilford, T. (1996). Hidden colour aversions in domestic chicks triggered by pyrazine odours of insect warning displays. *Nature*, *383*(6600), 520–522.

Sculfort, O., de Castro, E. C., Kozak, K. M., Bak, S., Elias, M., Nay, B., & Llaurens, V. (2020). Variation of chemical compounds in wild Heliconiini reveals ecological factors involved in the evolution of chemical defenses in mimetic butterflies. *Ecology and Evolution*, *10*(5), 2677–2694. https://doi.org/10.1002/ece3.6044

Speed, M. P., Ruxton, G. D., & Mappes, J. (2012). Why are defensive toxins so variable? An evolutionary perspective. *Biological Reviews*, *87*(4), 874–884. https://doi.org/10.1111/j.1469-185X.2012.00228.x

Therneau, T. 2020. *Coxme: Mixed effects cox models*. R package version.2. http://CRAN.R-project.org/package=coxme

Winters, A. E., Lommi, J., Kirvesoja, J., Nokelainen, O., & Mappes, J. (2021). Multimodal aposematic defenses through the predation sequence. *Frontiers in Ecology and Evolution*, *9*, 657740. https://doi.org/10.3389/fevo.2021.657740

Winters, A. E., White, A. M., Cheney, K. L., & Garson, M. J. (2019). Geographic variation in diterpene-based secondary metabolites and level of defence in an aposematic nudibranch, *Goniobranchus splendidus*. *Journal of Molluscan Studies*, *85*(1), 133–142. https://doi.org/10.1111/1365-2656.13643

Yan, Y., Chen, S., Nie, Y., & Xu, Y. (2021). Quantitative analysis of pyrazines and their perceptual interactions in soy sauce aroma type baijiu. *Food*, *10*(2), 441. https://doi.org/10.3390/foods10020441

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Supplementary material

Contents: **Pages**

Differences in pyrazine levels across populations

TableS1. The amount of SBMP (ng) in laboratory and wild populations of wood tiger moths was analysed using linear mixed-effects model with a normal distribution. In the model, SBMP variation was set as dependent variable and the different populations, the origin and the interactions between populations and origin as explanatory variable. Each model also included year as a random factor.

	Chisq	Df	Pr(>Chisq)
population	24.52	3	1.949e-05
origin (laboratory/wild)	0.90	1	0.34
population:origin	4.73	3	0.19

Table S2. Differences in the amount of SBMP (ng) in laboratory and wild populations of wood tiger moth using Tukey–Kramer post hoc test for multiple comparisons. Statistical significance was set at $p < 0.05$.

	diff	lwr	upr	p adj
fin.lab-est.lab	0.03	-0.41	0.48	0.99
geo.lab-est.lab	0.415	-0.097	36.46	0.20
sco.lab-est.lab	0.34	-0.22	34.47	0.22
est.wild-est.lab	0.16	-0.29	0.62	0.96
fin.wild-est.lab	0.09	-0.29	0.47	0.99
geo.wild-est.lab	0.46	-0.075	1.00	0 .15
sco.wild-est.lab	1.24	0.58	1.91	0.0000015
geo.lab-fin.lab	0.38	-0.11	0.88	0.27
sco.lab-fin.lab	0.31	-0.23	0.85	0.66
est.wild-fin.lab	0.13	-0.31	0.57	0.98
fin.wild-fin.lab	0.06	-0.3	0.42	0.99
geo.wild-fin.lab	0.43	-0.09	0.96	0.19
sco.wild-fin.lab	1.21	0.56	1.87	0.0000019
sco.lab-geo.lab	-0.07	-0.67	0.52	0.99

est.wild-geo.lab	-0.25	-0.76	0.25	0.79
fin.wild-geo.lab	-0.32	-0.76	0.115	0.32
geo.wild-geo.lab	0.05	-0.53	0.63	0.99
sco.wild-geo.lab	0.83	0.13	1.53	0.009
est.wild-sco.lab	-0.18	-0.73	0.375	0.98
fin.wild-sco.lab	-0.25	-0.74	0.24	0.77
geo.wild-sco.lab	0.12	-0.50	0.75	0.99
sco.wild-sco.lab	0.90	0.16	1.64	0.005
fin.wild-est.wild	-0.07	-0.44	0.30	0.99
geo.wild-est.wild	0.30	-0.23	0.83	0.67
sco.wild-est.wild	1.08	0.42	1.74	0.00004
geo.wild-fin.wild	0.37	-0.1	0.84	0.23
sco.wild-fin.wild	1.15	0.54	1.76	0.0000011
sco.wild-geo.wild	0.78	-18.11	15.98	0.998

TableS3. The amount of IBMP (ng) in laboratory and wild populations of wood tiger moths was analysed using linear mixed-effects model with a normal distribution. In the model, IBMP variation was set as dependent variable and the different populations, the origin and the interactions between populations and origin as explanatory variable. Each model also included year as a random factor.

	Chisq	Df	Pr(>Chisq)
population	5.425	3	0.14
origin (laboratory/wild)	0.11	1	0.74
population:origin	10.68	3	0.01

Table S4. Differences in the amount of IBMP (ng) in laboratory and wild populations of wood tiger moth using Tukey–Kramer post hoc test for multiple comparisons. Statistical significance was set at $p < 0.05$.

	diff	lwr	upr	p adj
fin.lab-est.lab	0.06	-0.086	0.20	0.92
geo.lab-est.lab	0.07	-0.095	0.24	0.89
sco.lab-est.lab	0.15	-0.03	0.33	0.21
est.wild-est.lab	0.025	-0.12	0.17	0.99
fin.wild-est.lab	0.02	-0.10	0.14	0.99
geo.wild-est.lab	-0.03	-0.20	0.15	0.99
sco.wild-est.lab	0.25	0.03	0.46	0.014
geo.lab-fin.lab	0.01	-0.15	0.17	0.99
sco.lab-fin.lab	0.09	-0.09	0.26	0.79
est.wild-fin.lab	0.02	-0.1	0.14	0.99
fin.wild-fin.lab	-0.04	-0.15	0.08	0.98
geo.wild-fin.lab	-0.09	-0.26	0.08	0.78
sco.wild-fin.lab	0.19	-0.025	0.4	0.013
sco.lab-geo.lab	0.07	-0.12	0.28	0.93
est.wild-geo.lab	0.04	-0.12	0.21	0.99
fin.wild-geo.lab	0.05	-0.09	0.19	0.96
geo.wild-geo.lab	-0.1	-0.28	0.09	0.75
sco.wild-geo.lab	0.17	-0.05	0.40	0.26
est.wild-sco.lab	0.12	-0.06	0.3	0.43
fin.wild-sco.lab	-0.12	-0.29	0.03	0.24
geo.wild-sco.lab	-0.17	-0.37	0.03	0.15
sco.wild-sco.lab	0.1	-0.14	0.34	0.9
fin.wild-est.wild	-0.003	-0.12	0.12	1.00

geo.wild-est.wild	-0.05	-0.23	0.12	0.98
sco.wild-est.wild	0.22	0.006	0.43	0.04
geo.wild-fin.wild	-0.04	-0.2	0.1	0.97
sco.wild-fin.wild	0.22	0.026	0.42	0.01
sco.wild-geo.wild	0.27	0.039	0.51	0.01

TableS5. Variance in SBMP and IBMP in wild and laboratory wood tiger moth populations

	variance WILD	variance LAB
SBMP	0.18	0.335
IBMP	0.0245	0.0247

TableS6. Variance in SBMP and IBMP in Estonia, Finland, Georgia and Scotland wood tiger moth populations

	variance Estonians	variance Finnish	variance Georgians	variance Scottish
SBMP	0.255	0.14	0.385	0.41
IBMP	0.013	0.03	0.015	0.019

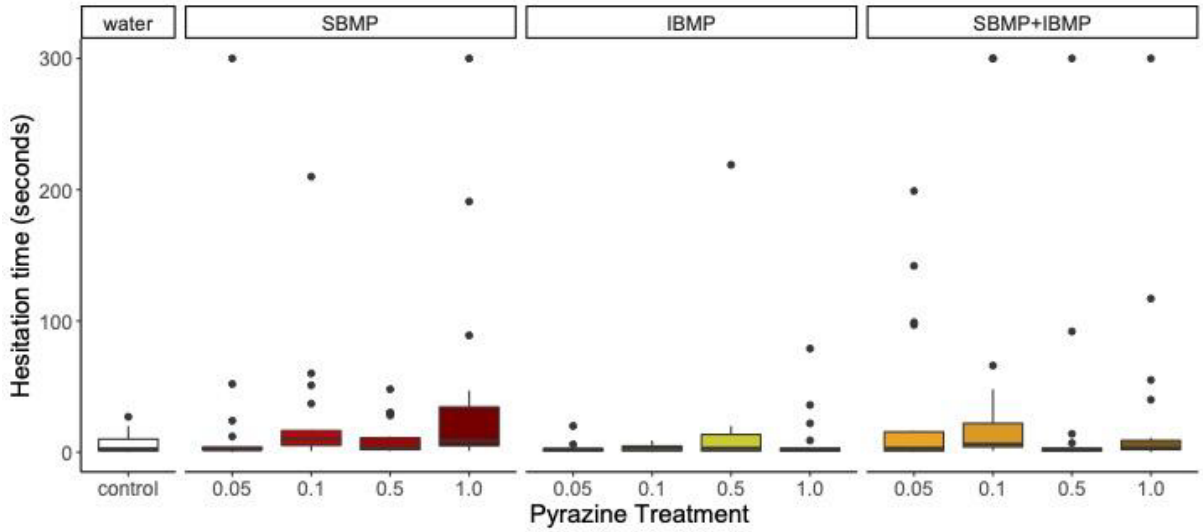
TableS7. The ratio of IBMP to SBMP in laboratory and wild populations of wood tiger moths was analysed using linear mixed-effects model with a normal distribution. The ratio was set as dependent variable and the different populations, the origin and the interactions between populations and origin as explanatory variable, year was included as a random factor.

	Chisq	Df	Pr(>Chisq)
population	27.25	3	5.224e-06
origin (laboratory/wild)	11.08	1	0.0008
population:origin	5.73	3	0.125

Bird response to pure pyrazine treatments

Hesitation time compared to control (water)

FigS1. Bird hesitation to approach fluid-soaked oats (per second) for each pyrazine type (SBMP = red, IBMP = yellow, SBMP+IBMP = orange) and ng/ul concentration (higher concentrations shown in darkening shades) compared to the water control. Boxes show the median and the 25th and 75th percentiles of data distribution. Vertical lines show the data range.



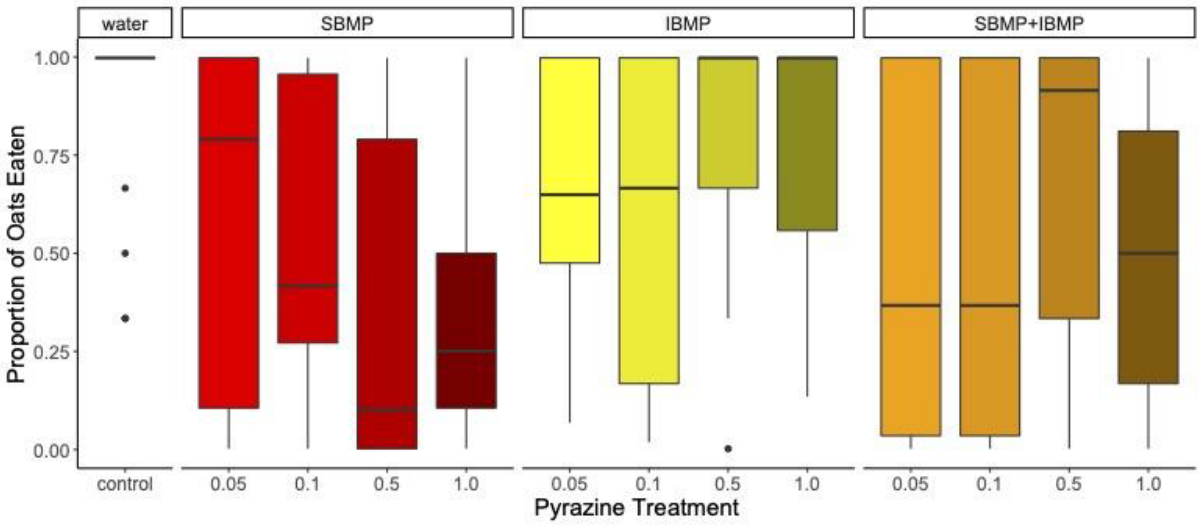
TableS8. Hesitation time was analyzed using a cox proportional hazards model. Hesitation time was set as the dependent variable with chemical treatment and trial number as explanatory variables. Trial duration was included as an offset term and bird ID was included as a random factor.

	coef	exp(coef)	se(coef)	z	p
0.05.SBMP	0.02	1.02	0.696	0.03	0.98
0.1.SBMP	-1.33	0.26	0.69	-1.95	0.05
0.5.SBMP	-0.51	0.597	0.68	-0.75	0.45
1.SBMP	-1.34	0.26	0.69	-1.95	0.05
0.05.IBMP	0.58	1.78	0.68	0.85	0.40
0.1.IBMP	0.24	1.275	0.68	0.36	0.72
0.5.IBMP	-0.33	0.72	0.72	-0.45	0.65
1.IBMP	-0.10	0.90	0.68	-0.15	0.88
0.05.SBMP+IBMP	-0.44	0.64	0.69	-0.64	0.52
0.1.SBMP+IBMP	-1.075	0.34	0.69	-1.55	0.12

0.5.SBMP+IBMP	-0.30	0.74	0.68	-0.44	0.66
1.SBMP+IBMP	-0.59	0.55	0.64	-0.93	0.35
trial	-0.21	0.81	0.09	-2.28	0.02

Percentage of oats eaten per minute compared to control (water)

FigS2. Percentage of fluid-soaked oats eaten for each pyrazine type (SBMP = red, IBMP = yellow, SBMP+IBMP = orange) and ng/ul concentration (higher concentrations shown in darkening shades) compared to the water control. Boxes show the median and the 25th and 75th percentiles of data distribution. Vertical lines show the data range.



TableS9. The proportion of oats was analyzed using a Generalized linear mixed model. The proportion of oats eaten was set as the dependent variable with chemical treatment and trial number as explanatory variables. Trial duration was included as an offset term and bird ID was included as a random factor.

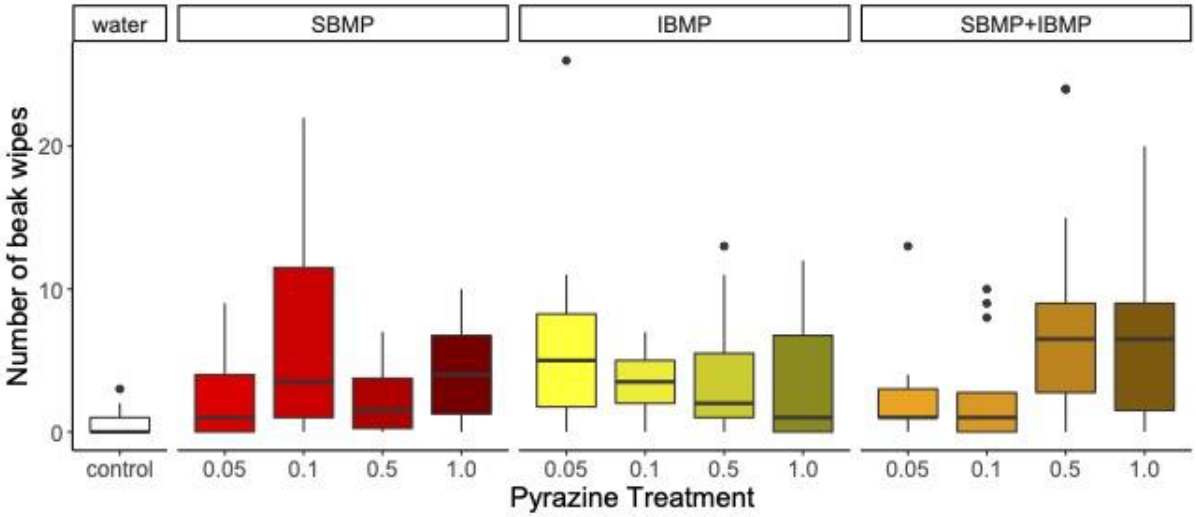
	Estimate	Std. Error	z-value	Pr(>|z|)
Intercept	1.92	0.91	2.115	0.03 *
0.05.SBMP	-1.73	1.28	-1.35	0.18
0.1.SBMP	-2.7	1.28	-2.105	0.035 *
0.5.SBMP	-4.76	1.31	-3.64	0.0003 ***
1.SBMP	-3.006	1.28	-2.34	0.019 *
0.05.IBMP	-1.53	1.28	-1.19	0.23
0.1.IBMP	-2.10	1.28	-1.64	0.10
0.5.IBMP	-0.95	1.35	-0.70	0.48
1.IBMP	0.003	1.28	0.003	0.998
0.05.SBMP+IBMP	-2.57	1.28	-1.999	0.046 *
0.1.SBMP+IBMP	-1.93	1.29	-1.50	0.13
0.5.SBMP+IBMP	-1.64	1.28	-1.28	0.20
1.SBMP+IBMP	-2.35	1.20	-1.96	0.05 .
trial	-0.07	0.01	-5.60	2.12e-08 ***

TableS10. The proportion of oats eaten was analyzed using a Generalized linear mixed model using template model builder. The proportion of oats eaten was set as the dependent variable with concentration, fluid, concentration:fluid, and trial number as explanatory variables. Concentration is an ordinal variable with orthogonal polynomial contrasts. Trial duration was included as an offset term and bird ID was included as a random factor.

	Estimate	Std. Error	z-value	Pr(>|z|)
Intercept	-1.66	0.67	-2.465	0.01 *
conc.L	1.63	1.025	1.59	0.11
conc.Q	0.44	1.07	0.41	0.68
conc.C	-0.68	1.11	-0.62	0.54
fluidSBMP	-1.89	0.74	-2.55	0.01 *
fluidSBMP+IBMP	-0.98	0.735	-1.33	0.18
trial	-0.24	0.13	-1.89	0.06
conc.L:fluidSBMP	-2.87	1.45	-1.98	0.05 *
conc.Q:fluidSBMP	0.29	1.48	0.20	0.845
conc.C:fluidSBMP	0.895	1.51	0.59	0.55
conc.L:fluidSBMP+IBMP	-1.81	1.42	-1.28	0.20
conc.Q:fluidSBMP+IBMP	-1.36	1.47	-0.93	0.35
conc.C:fluidSBMP+IBMP	0.07	1.52	0.045	0.96

Beak wipes per minute compared to control (water)

FigS3. Number of beak wipes per minute after eating fluid-soaked oats for each pyrazine type (SBMP = red, IBMP = yellow, SBMP+IBMP = orange) and ng/ul concentration (higher concentrations shown in darkening shades) compared to the water control. Boxes show the median and the 25th and 75th percentiles of data distribution. Vertical lines show the data range.



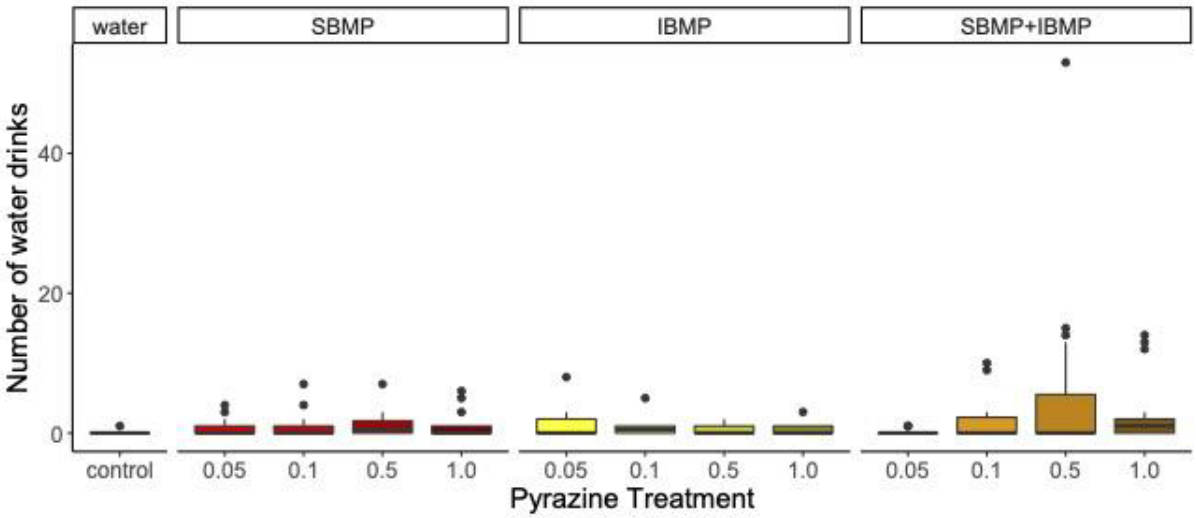
TableS11. The number of beak wipes per minute was analyzed using a Generalized linear mixed model. The number of beak wipes was set as the dependent variable with chemical treatment and trial number as explanatory variables. Trial duration was included as an offset term and bird ID was included as a random factor.

	Estimate	Std. Error	z-value	Pr(>|z|)
Intercept	-3.87	0.65	-5.95	2.69E-09 ***
0.05.SBMP	0.35	0.86	0.41	0.68
0.1.SBMP	0.22	0.83	0.26	0.795
0.5.SBMP	-0.36	0.93	-0.39	0.695
1.SBMP	0.46	0.825	0.555	0.58
0.05.IBMP	1.33	0.83	1.60	0.11
0.1.IBMP	0.97	0.82	1.18	0.24
0.5.IBMP	1.39	0.86	1.625	0.10
1.IBMP	1.09	0.875	1.24	0.21

0.05.SBMP+IBMP	0.79	0.83	0.95	0.34
0.1.SBMP+IBMP	-0.22	0.91	-0.24	0.81
0.5.SBMP+IBMP	1.57	0.82	1.93	0.05 .
1.SBMP+IBMP	0.61	0.795	0.77	0.44
trial	-0.019	0.046	-0.43	0.67

Water drinks per minute compared to control (water)

FigS4. Number of water drinks per minute after eating fluid-soaked oats for each pyrazine type (SBMP = red, IBMP = yellow, SBMP+IBMP = orange) and ng/ul concentration (higher concentrations shown in darkening shades) compared to the water control. Boxes show the median and the 25th and 75th percentiles of data distribution. Vertical lines show the data range.



TableS12. The number of water drinks per minute was analyzed using a Generalized linear mixed model. The number of water drinks was set as the dependent variable with chemical treatment and trial number as explanatory variables. Trial duration was included as an offset term and bird ID was included as a random factor.

	Estimate	Std. Error	z-value	Pr(>|z|)
Intercept	-7.91	1.29	-6.13	8.76E-10 ***
0.05.SBMP	1.89	1.45	1.30	0.19
0.1.SBMP	1.44	1.42	1.015	0.31
0.5.SBMP	2.09	1.47	1.42	0.15

1.SBMP	1.79	1.42	1.26	0.21
0.05.IBMP	2.46	1.42	1.73	0.08 .
0.1.IBMP	1.78	1.43	1.245	0.21
0.5.IBMP	1.71	1.49	1.15	0.25
1.IBMP	1.55	1.52	1.02	0.31
0.05.SBMP+IBMP	0.35	1.60	0.22	0.82
0.1.SBMP+IBMP	2.35	1.46	1.605	0.11
0.5.SBMP+IBMP	3.06	1.41	2.17	0.03 *
1.SBMP+IBMP	1.98	1.39	1.425	0.15
trial	0.34	0.07	4.60	4.15E-06 ***

TableS13. The number of water drinks per minute was analyzed using a Generalized linear mixed model. The number of water drinks was set as the dependent variable with concentration, fluid, concentration:fluid, and trial number as explanatory variables. Concentration is an ordinal variable with orthogonal polynomial contrasts. Trial duration was included as an offset term and bird ID was included as a random factor.

	Estimate	Std. Error	z-value	Pr(>|z|)
Intercept	-6.05	0.43987	-13.752	< 2e-16 ***
conc.L	-0.07	0.50	-0.15	0.88
conc.Q	0.06	0.52	0.112	0.91
conc.C	-0.62	0.73	-0.86	0.39
fluidSBMP	0.26	0.72	0.36	0.72
fluidSBMP+IBMP	-0.16	0.71	-0.22	0.83
trial	0.34	0.07	4.64	3.48e-06 ***
conc.L:fluidSBMP	0.71	0.99	0.71	0.48
conc.Q:fluidSBMP	1.87	1.07	1.75	0.08 .

conc.C:fluidSBMP	-0.18	0.99	-0.19	0.85
conc.L:fluidSBMP+IBMP	-1.79	1.03	-1.735	0.08 .
conc.Q:fluidSBMP+IBMP	-0.30	0.99	-0.31	0.76
conc.C:fluidSBMP+IBMP	0.04	0.99	0.04	0.97

Bird response to moths' defensive fluid

Table S14. Hesitation time (in seconds) with control, water. To test the difference in hesitation time in response to thoracic fluids from wild and lab Finnish and Georgian wood tiger moths, we used a cox proportional hazards model using the package coxme. The model included bird ID as a random factor. The interaction (country:origin) between population and the origin (wild/laboratory) and the trial were set as fixed factors and included as explanatory variables, while the hesitation time was set as a response variable.

	coef	exp(coef)	se(coef)	z	p
countryoriginFinnish laboratory	-0.60	0.55	0.46	-1.30	0.19
countryoriginGeorgian laboratory	-1.91	0.15	0.56	-3.38	0.0007***
countryoriginFinnish wild	-0.5	0.61	0.43	-1.16	0.25
countryoriginGeorgian wild	-0.64	0.53	0.42	-1.51	0.13
Trial 3	-0.02	0.98	0.15	-0.13	0.9

TableS15. Proportion of oat eaten with control (water). The proportion of oats eaten was tested using package glmmTMB with family =beta_family(link= “logit”). The model included bird ID as a random factor. The interaction (country:origin) between the population and the origin (wild/laboratory) and the trial were set as fixed factors. Also, the trial with duration as an offset was included as an explanatory variable, while the proportion of oat eaten was set as a response variable.

	Estimate	Std. Error	z-value	Pr(>|z|)
Intercept	-5.02	0.74	-6.80	1.02e-11 ***
countryorigFinnish laboratory	-3.74	0.88	-4.24	2.22e-05 ***
countryorigGeorgian laboratory	-4.315	1.085	-3.98	6.99e-05 ***
countryorigFinnish wild	-2.805	0.83	-3.39	0.0007 ***
countryorigGeorgian wild	-2.84	0.81	-3.51	0.0004 ***
Trial 3	0.17	0.10	1.68	0.093 .

Fig S5. Beak cleaning with control, water. There are no differences in the beak cleaning behaviour when predators are exposed to fluids of moths raised in a laboratory and wild conditions from both populations compared to water. Boxes show the median and the 25th and 75th percentiles of data distribution. Vertical lines show the data range

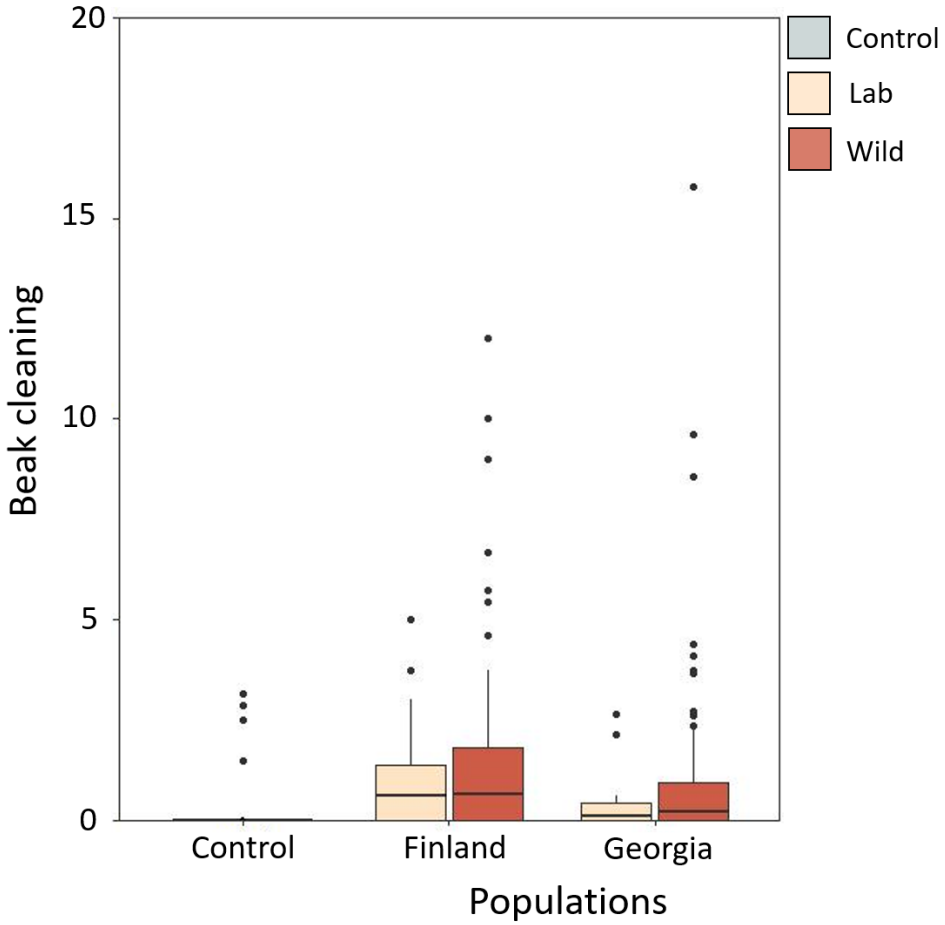


Table S16. Beak cleaning with control (water). The beak cleaning was tested using general linear mixed-effects model with Poisson distribution. The model included bird ID as a random factor. The interaction (country:origin) between the population and the origin (wild/laboratory) and the trial were set as fixed factors. Also, the trial with duration as an offset was included as an explanatory variable, while the beak cleaning per minute was set as a response variable.

	Estimate	Std. Error	z-value	Pr(>|z|)
Intercept	-2.84	0.795	-3.58	0.00035 ***
countryorigFinnish laboratory	-0.65	0.88	-0.74	0.47

countryorigGeorgian laboratory	-0.88	1.07	-0.821	0.43
countryorigFinnish wild	0.02	0.85	0.02	0.98
countryorigGeorgian wild	-0.05	0.84	-0.055	0.96
Trial3	-0.23	0.11	-2.14	0.0325

Table S17. Water drinking with control (water). The water drinking was tested using general linear mixed-effects model with Poisson distribution. The model included bird ID as a random factor. The interaction (country:origin) between the population and origin (wild/laboratory) and the trial were set as fixed factors. Also, the trial with duration as an offset was included as an explanatory variable, while the water drinking per minute was set as a response variable.

	Estimate	Std. Error	z-value	Pr(>|z|)
Intercept	-2.15	0.81	-2.67	0.008 **
countryoriginFinnish laboratory	-3.40	0.94	-3.63	0.0003 ***
countryoriginGeorgian laboratory	-22.73	512	-0.04	0.965
countryoriginFinnish wild	-3.98	0.94	-4.24	2.21e-05 ***
countryoriginGeorgian wild	-2.18	0.86	-2.53	0.011 *
Trial 3	0.03	0.21	0.16	0.88

TableS18. Hesitation time without control. We compared differences between populations using Tukey–Kramer post hoc test for multiple comparisons and excluding the water control group. Statistical significance was set at $p < 0.05$.

	diff	lwr	upr	p adj
geo.lab-fin.lab	49.28	4.99	93.57	0.02*
fin.wild-fin.lab	-13.04	-42.62	16.55	0.66
geo.wild-fin.lab	4.291126	-23.98	32.565	0.98
fin.wild-geo.lab	-62.32	-104.21	-20.43	0.0009***

geo.wild-geo.lab	-44.99	-85.96	-4.015	0.025*
geo.wild-fin.wild	17.33	-7.01	41.67	0.26

Table S19. Proportion of oat eaten without control. We compared differences between populations using Tukey–Kramer post hoc test for multiple comparisons and excluding the water control group. Statistical significance was set at $p < 0.05$.

	diff	lwr	upr	p adj
geo.lab-fin.lab	-15.515	-46.53	15.50	0.57
fin.wild-fin.lab	15.74	-4.98	36.46	0.20
geo.wild-fin.lab	14.68	-5.13	34.47	0.22
fin.wild-geo.lab	31.25	1.92	60.59	0.03*
geo.wild-geo.lab	30.19	1.495	58.88	0.035*
geo.wild-fin.wild	-1.07	-18.11	15.98	0.998

Table S20. Water drinking without control. We compared differences between populations using Tukey–Kramer post hoc test for multiple comparisons and excluding the water control group. Statistical significance was set at $p < 0.05$.

	diff	lwr	upr	p adj
geo.lab-fin.lab	-0.43	-1.54	0.685	0.75
fin.wild-fin.lab	-0.34	-1.08	0.40	0.63
geo.wild-fin.lab	0.14	-0.57	0.85	0.96
fin.wild-geo.lab	0.08	-0.97	1.135	0.996
geo.wild-geo.lab	0.56	-0.465	1.59	0.49
geo.wild-fin.wild	0.48	-0.13	1.09	0.18

II

DIET INFLUENCES RESOURCE ALLOCATION IN CHEMICAL DEFENCE BUT NOT MELANIN SYNTHESIS IN AN APOSEMATIC MOTH

by

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Diet influences resource allocation in chemical defence but not melanin synthesis in an aposematic moth

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Abstract

For animals that synthesise their chemical compounds *de novo*, resources, particularly proteins, can influence investment in chemical defences and nitrogen-based wing colouration such as melanin. Competing for the same resources often leads to trade-offs in resource allocation. We manipulated protein availability in the larval diet of the wood tiger moth, *Arctia plantaginis*, to test how early life resource availability influences relevant life history traits, melanin production, and chemical defences. We expected higher dietary protein to result in more effective chemical defences and a higher amount of melanin in the wings. According to the resource allocation hypothesis, we also expected individuals with less melanin to have more resources to allocate to chemical defences. We found that protein-deprived moths had a slower larval development, and their chemical defences were less unpalatable for bird predators, but the expression of melanin in their wings did not differ from that of moths raised on a high-protein diet. The amount of melanin in the wings, however, unexpectedly correlated positively with chemical defences, irrespective of the diet they were raised on. Our findings demonstrate that the resources available in early life have an important role in the efficacy of chemical defences, but melanin-based warning colours are less sensitive to resource variability than other fitness-related traits.

1. Introduction

Organisms need to invest simultaneously in various life-history traits such as growth, energy maintenance, reproduction, predator avoidance and protection from pathogens (Stearns, 1992; Roff & Fairbairn, 2007). According to life-history theory, limited resources need to be distributed among various competing traits, inevitably leading to trade-offs in resource allocation (i.e., resource-allocation hypothesis; Bazzaz et al., 1987; Stearns, 1989; Glazier, 2002).

Examples include trade-offs between larval and adult traits (Stevens et al., 1999), or between predator-avoidance and immune function (Rigby & Jokela, 2000). In general, allocation costs lead to trade-offs between traits that shape the demography of populations (McCauley et al.,1990; Boggs, 1992) and can significantly impact eco-evolutionary dynamics in nature (Schroderus et al. 2010; Farahpour et al., 2018). Testing the effect of only a few (one or two) traits on the resource allocation strategy may overlook critical information about the costs, especially if those traits are prioritised by the organism for fitness investment in a specific life stage (Lindstedt et al., 2020; de Jong, 1993). In organisms with complex life cycles, such as insects, considering how resources are allocated across and within life stages is essential to understanding how trade-offs shape fitness and different traits (Burdfield-Steel et al., 2019; Lindstedt et al., 2016).

Melanin plays an important role in the warning signals of many aposematic organisms (Fabricant et al., 2013; Lindstedt et al. 2020), those in which a primary defence (e.g., a warning signal such as colour, odour, sound) is coupled with secondary defences (chemical, morphological, behavioural) (Poulton 1890). Melanin is a phenolic biopolymer widely present across the animal kingdom, whose function in organisms as distant as mammals and insects is similar despite differences in the enzymes and substrates that regulate its production across species (Sugumaran, 2002). In vertebrates, including humans, melanin has a pivotal role in protection against UV radiation (Jablonski & Chaplin 2017; Nicolaï et al. 2020), as well as in sexual and protective colouration (Cuthill et al. 2017). In insects, specifically, melanin is also crucial in physiological and ecological processes such as thermoregulation (True, 2003; Trullas et al., 2007; Lindstedt et al. 2009), immune defence (Wilson et al., 2001), wound healing (Bilandžija et al. 2017) and

protection from predators (Hegna et al. 2013; Majerus, 1998). The expression of melanin is often genetically based (Ellers and Boggs, 2002; Lindstedt et al. 2009; van't Hof et al. 2019) and tightly related to protein resources (Lee, Simpson & Wilson, 2008). Thus, the proteins necessary for the synthesis of melanin pigments can constrain the expression of life-history traits and chemical defences (Lindstedt et al., 2020; Galarza 2021), making aposematic signals that require melanin good candidates for testing the resource-allocation hypothesis.

To date, several studies have focused on testing how the production of secondary defences may imply trade-offs in the allocation of resources needed for the primary defences, but experimental studies testing both the melanin component of warning signals and the chemical defences in the same system are lacking. A study on the Asian lady beetle, *Harmonia axyridis*, found no phenotypic correlation between the reflex bleeding response (frequency of secretion of the defensive chemicals) and the beetle colouration, even though reflex bleeding is costly and affects life-history traits (Grill and Moore, 1998). Recent work on monarch butterflies, *Danaus plexippus*., showed a link between toxin sequestration and warning signals, where male conspicuousness was inversely correlated with oxidative damage (due to an increase in concentrations of sequestered cardenolides) (Blount et al., 2023). Previous work by Lindstedt et al. (2010) showed that the aposematic, generalist herbivore *Arctia plantaginis* (hereafter referred to as the wood tiger moth) develops a paler, orange warning signal when reared on *Plantago lanceolata* with high concentrations of iridoid glycosides (IG) in comparison to the more conspicuous, dark red warning signal of individuals fed on a strain of the same plant with low IG concentrations. This suggests that the cost of conspicuousness arose via higher excretion costs rather than via resource-allocation costs (Lindstedt et al. 2010).

Here, we use the aposematic wood tiger moth, which has a black melanin pattern that covers approximately 20 - 70 % of its hindwings and 50 - 80% of its forewings (Hegna et al., 2013), to investigate how early-life resource availability influences melanin expression in the wings, the efficacy of chemical defences, and life history traits. In this species, melanin synthesis competes against the production of chemical defences for energy and resources from diet precursors (nitrogen from proteins). While the metabolic pathway in the wood tiger moth chemical defence has not been identified yet, the thoracic defence fluid present two methoxypyrazines which are, similarly to the eumelanin components, heterocyclic compounds based on nitrogen (Higasio & Shoji, 2001). We manipulated the protein content of the diet of male wood tiger moths, to investigate how wild-caught, natural predators (blue tits, *Cyanistes caeruleus*) respond to their chemical defences, and whether the production of such defences leads to trade-offs with the production of other traits, such as wing melanisation. We predicted that, compared to males raised on a high-protein diet, males raised on a low-protein diet would have (1) higher melanisation, resulting from a trade-off between melanin production and the effectiveness of secondary defences (i.e. more melanised individuals have less deterrent chemical defences, and vice versa); (2) less effective chemical defences, rendering them more palatable to bird predators; and (3) higher life-history costs in terms of size or developmental time.

2. Materials & Methods

Study species

The aposematic wood tiger moth *Arctia plantaginis*, formerly *Parasemia plantaginis* (Rönkä et al., 2016), displays a conspicuous colour polymorphism (Watson and Goodger, 1986; Chinery,

1993; Nokelainen, 2013) throughout the Holarctic region (Hegna, Galarza, Mappes, 2015). Male hindwings across the species distribution range can have a red, yellow or white background with black patterning that covers variable proportions (ca 20-70%) of the wing. White and yellow colourations are partly produced by pheomelanin, whereas black is a dopamine-derived eumelanin (Brien et al. 2022). Previous research has scored melanisation (the amount of eumelanin) of the hindwings as "plus" (with stripes, high melanin) or "minus" (without stripes, low melanin) (see Fig. 1), but this arbitrary categorisation does not necessarily represent the true variation, in which subtle differences are not detectable by the human eye. Long-term natural frequencies of male wood tiger moth melanin morphs in Estonia (classified by human eye) are about 47% high-melanin and 53% low-melanin (O. Nokelainen, unpublished data; see Fig. 1).

As capital breeders, wood tiger moth adults do not feed; thus, resource acquisition only occurs during the larval stage. To avoid predation by avian and terrestrial predators, these moths produce two different types of defensive fluids: abdominal secretions deter predators such as ants, and thorax secretions deter birds with no adverse effects on ants (Rojas et al., 2017). The thoracic fluid contains two methoxypyrazines: 2-sec-butyl-3-methoxypyrazine (SBMP) and 2-isobutyl-3-methoxypyrazine (IBMP), which are produced *de novo* (Burdfield-Steel et al., 2018).

Insects rearing, larval diet and thoracic fluid collection

Male wood tiger moths were obtained from a laboratory stock founded in 2013 with wild-caught individuals from Estonia and kept at the greenhouse of the University of Jyväskylä. The stock was supplemented annually with Estonian wild-caught individuals to preserve genetic diversity. The greenhouse conditions approximately followed the outdoor temperatures and natural

daylight from May to August in Central Finland: between 20°C and 30°C during the day and decreased to 15°C-20°C during the night. We picked 10 families that contained both low- and high-melanin male morphs in the parental and grandparental generations, as classified by eye (see Fig. 1). Laboratory crosses show that wing melanisation (both pattern and amount) is strongly heritable (personal observation). After hatching, larvae of each of the 10 families were kept together for 14 days and fed with lettuce and dandelion (*Taraxacum spp.)*. Then, using a split family design, they were divided into two artificial diet treatments that differed in protein content (high protein, low protein; see recipe in supplementary material). For each diet treatment, we used 240 individuals which were kept in boxes of 10 individuals until reaching the pupal stage. Each box was checked and watered daily and cleaned when needed. Larvae were fed daily, *ad libitum*, with their corresponding diet treatment. Life-history traits (time to pupation, pupal weight) and the degree of melanin (high +, low -) were recorded for each individual. In total, 480 larvae were followed from eggs to adulthood or death, 177 of which emerged as females and 184 as males. All larvae were grown by Furlanetto (2017) from May to August 2017. When the male adults emerged from pupation, they were given water and stored at 4°C to slow their metabolic rate and to maintain their condition; thoracic chemical fluid was then collected in June and July 2017. Prior to fluid collection, moths were kept at 20-25°C for thirty minutes. Then fluid was extracted by squeezing just below the prothoracic section with tweezers and collecting expelled fluid in a 10 µl glass capillary. Fluid samples were stored in glass vials at -18°C (Furlanetto, 2017).

Effect of diet manipulation on melanin

To measure variation in wing melanisation, we photographed the 53 male moths whose defensive fluids were used in the predator response assay. The degree of melanisation varies continuously and thus we measured the proportion of the hindwing that was melanised on a continuous scale using image analysis.

Figure 1. Hindwing and forewing melanin variation in male wood tiger moths from Estonia. Left, (+) high-melanin morph; right, (-) low-melanin morph. See text for details. Image from Nokelainen et al. 2013 (reprinted with permission).

Spread male moths were photographed using an established protocol (Nokelainen et al. 2017) and scaled to the same resolution (pixels per mm). We used the MICA toolbox (Troscianko & Stevens 2015) in ImageJ (v. 1.50f) for image analysis. From every image, a set of regions of interest (ROIs) were selected from the dorsal side of the wings: (a) whole forewing area, (b) forewing melanised region, (c) whole hindwing area, and (d) hindwing melanised region. The relative proportion of the wings that were melanised was calculated by dividing the area of the melanised ROIs by the whole wing ROIs, for the forewings:[(forewing melanised region area) / (whole forewing area)]; and for the hindwings: [(hindwings melanised region area)/(whole hindwings area)].

Effect of diet manipulation on the predator response to male's defensive fluid

Blue tits (*Cyanistes caeruleus*) are generalist feeders that have a similar distribution as *Arctia plantaginis* in Europe and are known to attack this species. Blue tits are common in Finland and

can be kept briefly in captivity for experiments (e.g., Rojas et al., 2017). For the predator response assay (*C. caeruleus; n* = 62) we used fluids from 53 male moths fed on high- and low-protein diets; baits soaked with water were offered to 9 birds as a positive control. The volume of wood tiger moth thoracic fluid has no effect on blue tit responses (Burdfield-Steel et al. 2019), so we diluted each fluid sample with water to reach a total volume of 15 µl. The 15 µl was then divided into two samples of 7 µl each, which were used as bait for the same bird. The same amount of water was offered to control birds. The birds used for the experiment were caught at Konnevesi Research Station (Central Finland) from February to April 2018 using feeders with peanuts as bait (Ham et al. 2006), and then housed individually in plywood cages for the duration of the assays (see Ottocento et al. 2022).

Birds were first familiarised with the experimental boxes and trained to eat a bait (oat flakes) (see Ottocento et al. 2022). Each bird then experienced four sequential trials, at 5-minute intervals, in which they were presented with a plate containing one bait (see Fig. 2 A). The first and last trials were done with baits soaked in tap water to ensure that the bird was motivated to eat (first) and that the bird was still hungry (last). In the second and third trials, the bird was presented with a bait soaked in 7µl of the defensive fluids of the same moth (30 raised under a high protein diet, 23 raised under a low protein diet). The trial ended two minutes after the bird had eaten the whole bait, or after a maximum duration of 5 minutes if the bird did not eat the whole bait. In each trial, we recorded the proportion of the bait eaten (see Fig. 2 B), the beak cleaning frequency (number of times the bird wiped its beak against a surface, e.g., the perch), the latency to approach (how long it took for the bird to get close to the plate on which the bait

was offered), the latency to eat after approaching (the hesitation time between approach and attacking) and the latency to eat.

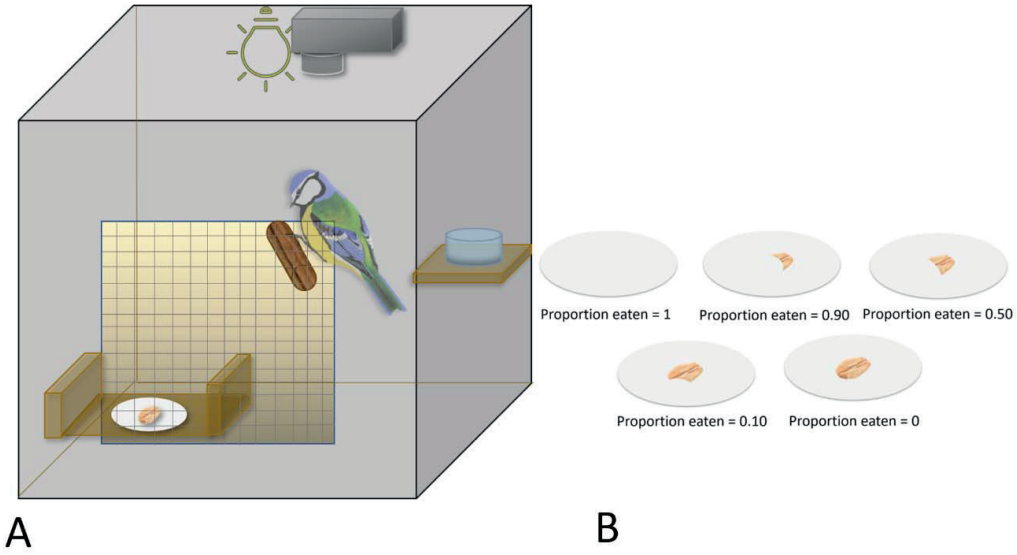

Figure 2 A). Experimental setup of the predator assay illustrating the perch, water, camera, light source, mesh opening for observation and hatch for inserting the plate with the bait (oat flake) into the enclosure. **2 B)** Proportion of bait (oat soaked with defensive fluid or water) eaten by the predators. The palatability was classified into five classes according to the proportion of the bait consumed by the birds: 100% (1), 90% (0.90), 50% (0.50), 10% (0.10), and 0%.

Statistical analyses

All statistical analyses were done with the software R v. 4.1.2 (R Core Team, 2022) using the RStudio v. 1.2.1335 interface (RStudio Team, 2019). The level of significance in all analyses was set at $p < 0.05$.

Effect of diet manipulation on melanin

Our results show that the amount of melanin in the hindwings of wood tiger moths is best measured as a continuous variable (see supplementary material), so we analysed how protein content in the diet influences the proportion of the wing that is melanised and the size of the wings using a linear mixed models with treatment as a fixed factor and family as a random factor, using the lme4 package (Bates et al. 2015). The total area of the forewings, the total area of the hindwings, the amount of melanin in the forewings (melanised area forewing/total area forewing), and the amount of melanin in the hindwings (melanised area hindwing/total area hindwing) were set as response variables (in separate models); the different types of diet (high/low protein content) were set as predictors.

Effect of diet manipulation on the predator response to male's defensive fluid

To test the proportion of bait eaten, we used beta regression models using package glmmTMB (Brooks et al., 2017) with family = beta_family(link= "logit); diet (low protein, high protein), the amount of melanin, and trial set as fixed effects; bird ID as a random effect; and an offset included to account for differences in observation time. To test for differences in beak wiping events per minute, we used generalised linear mixed-effects models (GLMM; Knudson et al., 2022) with a log link and Poisson distribution, fit by maximum likelihood (Laplace approximation). The interaction between diet (low protein, high protein), the amount of melanin, and trial were set as fixed effects, beak wiping frequency was set as the response variable, and bird ID was set as a random effect. To test the latency to approach, the latency to eat after approaching and the latency to eat, we used a Cox mixed-effects model using package *coxme* (Therneau, 2020), fit by maximum likelihood. These three behaviours were set as response variables; the interaction between diet (low protein, high protein), the amount of melanin and

trial were set as fixed effects; and bird ID was set as a random effect. The behaviours of the predators were first compared to a water-only control to determine whether moth chemical defences elicited adverse predator reactions. We used Spearman's rank correlations (Ojala et al., 2005) to test the correlation between the amount of melanin and predator responses to chemical defences (proportion of bait eaten, beak wiping, latency to approach, latency to eat after approaching, latency to eat).

Effect of diet manipulation and hindwing melanin on life-history traits

To test whether male moths' development time is affected by dietary protein and the amount of melanin, we used a mixed-effects Cox model (package coxme, Therneau et al., 2020), with development time included as the response variable, diet treatment and melanisation included as the predictor variables, and family as a random factor. We assessed the effect of diet treatment on pupae weight with a generalised mixed-effects model (package lme4, Bates et al. 2015) with a Gamma (link="log") distribution, with diet treatment and melanisation as the predictor variables and family as a random factor. We used a linear mixed-effects model (package lme4, Bates et al. 2015) with diet and melanisation as predictor variables and family as a random factor to test the effect of diet on the volume of the thoracic fluid. We tested the correlation between the pupal weight and the volume of the thoracic fluid using a Pearson correlation. Spearman's rank correlations were used to test the relationships between the amount of melanin and the developmental time, pupal weight, and volume of thoracic fluid.

Results

Effect of diet manipulation on melanin

In moths raised in low-protein diet, neither the wing melanin content of the hindwings (coef ± s.e =0.001± 0.32, t = 0.265, $p = 0.96$) and the forewings (coef ± s.e = -0.02± 0.03, t = -0.85, $p = 0.40$) nor the size of the hindwings (coef ± s.e = -95528± 81245, t = -1.18, $p = 0.246$) or forewings (coef ± s.e = -104081±89682, z = -1.16, $p = 0.25$) differed to moths raised on a high-protein diet.

Effect of diet manipulation and hindwing melanin on the predator response to male's defensive fluid

To test if the thoracic fluid of the moth evoked an adverse reaction in the birds, we first examined predator behaviour in response to baits soaked in water (control). Baits soaked in the chemical defences of moths raised on a high-protein diet were eaten by the predators in lower proportions than baits soaked in water (control) (coef ± s.e -2.54 ± 0.87, z = -2.91, $p = 0.004$), while baits soaked in fluid from moths fed with low protein diet did not differ to the predators' response to baits soaked in water (coef ± s.e -1.56 ± 0.87, z = -1.79, $p = 0.074$). We found no significant differences in the other predator behaviours recorded (i.e., latency to approach; latency to eat after approach; latency to eat; frequency of beak wiping) between birds exposed to baits soaked in either fluid from moths raised on high-protein or low-protein diets, and those exposed to water-soaked baits ($p > 0.05$ for all comparisons, see Table S1A, S2A, S3, S4A; supplementary material).

Irrespective of diet type, the chemical defences of more melanised individuals were also more deterrent against predators (Spearman's rank correlation, $r_s = 0.27$; $p = 0.005$, Fig. 3A), as baits soaked in these defences were eaten in lower proportions than those from less melanised males

(coef ± s.e -5.20 ± 1.96, z = -2.66, $p = 0.007$, Fig. 3A). Similarly, the predators' latency to eat the bait increased with higher amounts of melanin (Spearman's rank correlation, $r_s = 0.26$; $p = 0.012$, Fig. 3B), indicating an adverse reaction of the predators to the chemical defence of highly melanised individuals, regardless of the diet they were raised on (coef ± s.e =-4.92 ± 2.17, z = -2.26, $p = 0.024$, Fig. 3B). There was no correlation between the amount of melanin and other behavioural variables (latency to approach; latency to eat after approach; frequency of beak wiping; $p > 0.05$ for all comparisons; supplementary material).

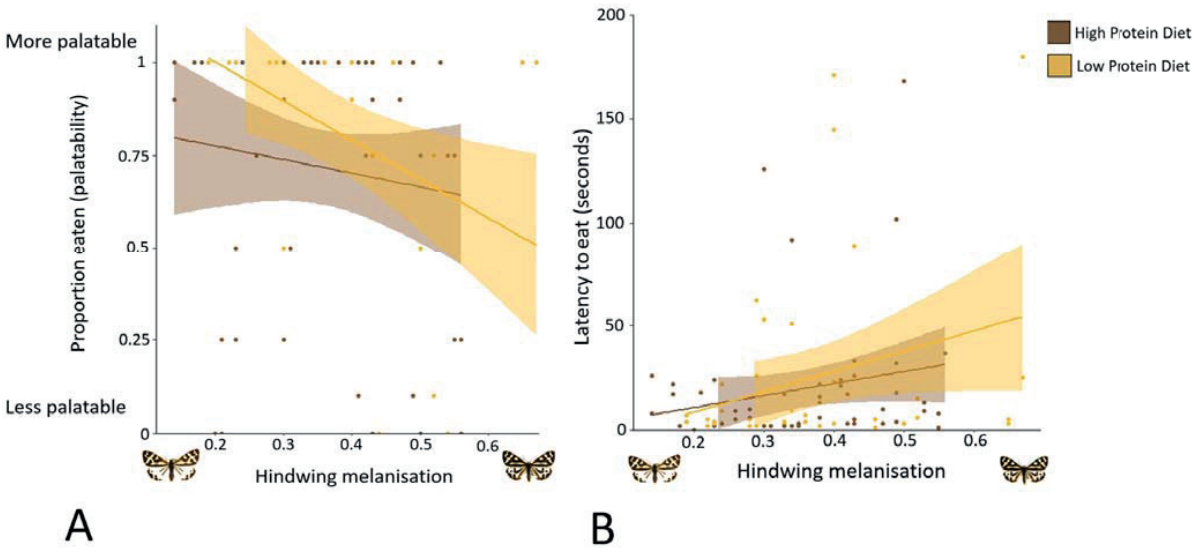

Figure 3 A) Palatability (proportion of defence fluid-soaked bait eaten by birds) and **B)** latency to eat the defence fluid-soaked bait compared to hindwing melanisation for male moths raised on high- and low-protein diets.

Effects of diet manipulation and hindwing melanin on the life-history traits

The developmental time from egg to pupa varied among diet treatments: individuals raised on a low-protein diet took significantly longer to pupate than those raised on a high-protein diet (coef ± s.e =-0.92± 0.32, z = -2.87, $p = 0.004$). There were no differences in body mass (coef ± s.e= 0.03 ± 0.04, t = 0.75, $p = 0.47$, see Table S6) or in the volume of the thoracic defensive fluid (coef ± s.e =1.05± 0.87, t = 1.2, $p = 0.23$) between individuals raised on high- and low-protein diets. The volume of the chemical defence did not correlate with the pupae weight (t = 0.96, df = 188, $p = 0.34$). There was no correlation between the melanin amount and the larval developmental time (r_s = 0.11; $p = 0.435$), the body mass (r_s = 0.003; $p = 0.98$), or the volume of defensive fluid (r_s = -0.03; $p = 0.79$).

Discussion

Investigating how resource allocation affects life-history traits and defences is vital for understanding which factors drive variation in the warning signals and chemical defences of aposematic organisms. Phenotypic variation in aposematic animals is puzzling because predators avoid defended prey that are common but attack rarer ones (Müller 1878). Trade-offs in resource allocation have been suggested to account for some of the variation found in natural aposematic populations (Wang, 2011; Burdfield-Steel et al., 2019; Blount et al., 2023). However, the phenotypic correlations among traits that characterise aposematic organisms such as melanin and chemical defences do not necessarily prove this connection, as interactions between such complex traits can be shaped by genetic correlations, pleiotropic effects or simply variable environmental conditions (e.g., food availability, environmental stochasticity, presence of predators). Therefore, to test the resource allocation hypothesis, we need to first test whether the production of defences is costly and then, whether their development is linked to other traits. To

investigate whether those traits are costly, and whether a specific trait (melanin or chemical defence) is prioritised over others, we manipulated specific constituents of the early-life resources of male wood tiger moths. The diet used in the study varied in the amount of protein, which is an essential compound in different aspects of the larval life history of this species, and which has been shown to influence the efficacy of the species' warning signal, immunity and life-history traits (Lindstedt et al., 2020).

Surprisingly, dietary protein levels had no effect on the amount of melanin in the fore- or hindwings, suggesting that this trait may not be directly influenced by early-life environment. However, Lindstedt et al. (2020) recently found that the protein content of the diet fed to larval wood tiger moths directly influenced the amount of forewing melanin of adult males in the closely related Finnish population. The apparent mismatch between Lindstedt et al.'s (2020) and our results could be due to the study design: Lindstedt et al. (2020) used larvae from selection lines for low and high larval melanisation, such that the phenotypic variation was larger than the natural variation (and, consequently, the average phenotypes were either rare or missing completely), likely making the possible costs of melanin easier to detect. In this study, by contrast, we used the natural variation of phenotypes. Interestingly, Lindstedt et al. did not find differences in the amount of hindwing melanin between individuals fed with high- and low-protein diets. Thus, hindwing melanisation and patterning seem to be under strong genetic control, which is unsurprising because moth hindwings commonly have an important signalling function (Sargent 1978, Kang et al. 2017; Rönkä et al. 2018). It is also possible that the Finnish and Estonian populations differ in their degree of plasticity of melanin production, but this hypothesis requires further investigation. Other studies in invertebrates have shown that

melanisation is influenced by the amount of protein content in the diet (Lee et al., 2008; Ethier et al., 2015), while in vertebrates melanin is less affected by dietary changes (Hill and Brawner, 1998; Lee et al., 2008). For example, in the African cotton leafworm, *Spodoptera littoralis*, a diet with high protein content led to more melanised cuticles, faster growth and better antibacterial activity and survival (Lee et al., 2008). In the forest moth *Malacosoma disstria,* individuals raised under low-nitrogen availability had decreased melanic pigmentation and smaller size, highlighting the high costs of melanisation (Ethier et al., 2015). In contrast, in vertebrates such as the tawny owl, *Strix aluco*, the genetic control of melanin deposition appears to be strictly regulated (Roulin & Dijkstra, 2003; Mundy & Kelly, 2003; Bize et al., 2006; Hoekstra, 2006; Emaresi et al., 2011). However, even in vertebrates, this is not always the case and environmental conditions may alter melanised traits in some organisms (Fargallo et al., 2006).

The amount of melanin in the wood tiger moth wings increases with latitude and altitude (Hegna et al. 2013), as predicted by the thermal melanism hypothesis, which states that darker individuals have an advantage under low temperatures because they can warm up faster than light individuals (Trullas et al. 2007). Convincing support for the fitness advantage of dark colouration in cold environments has been found in many invertebrates (e.g., Kingsolver 1995; Ellers & Boggs 2004; but see Rosa and Saastamoinen 2020), but also in cold-blooded vertebrates (Vences et al. 2002; Stuart-Fox et al. 2017; Azócar et al. 2020). Interestingly, more melanin in the wings results in a better capacity to absorb radiation and warm-up for flight at the expense of increased vulnerability to predator attacks due to a reduction in the size of the non-melanic component of the warning signal (yellow or white pigment in the hindwings) (Hegna et al. 2013). Similar results were found also in wood tiger moth larvae, where higher amounts of

melanin in the body resulted in improved thermoregulation at the cost of increased predation (Lindstedt, Lindström, & Mappes, 2008; 2009; Nielsen & Mappes, 2020). It is possible that the Estonian population, situated at lower latitudes than the Finnish population, is free from the (potentially genetic) constraints imposed by a colder climate. Moreover, while the melanin pattern in male wood tiger moths seems genetically regulated and the environmental component is low, our study shows that any dietary resources beyond what is required for the melanin patterns are allocated elsewhere, such as to more effective chemical defences.

We hypothesised that the thoracic fluid of males raised on a high-protein treatment would elicit a stronger predator response than the fluids from moths raised on a low-protein diet, as shown in a previous study (Furlanetto 2017) where the abundance of pyrazines was higher in fluids from moths raised on the high-protein artificial diet. An increase in protein content means an increase in the concentration of nitrogen, an essential element in the synthesis of the pyrazine molecule (Hodge, Mills & Fisher, 1972; Wong & Bernhard, 1988). Pyrazines have a characteristic repulsive odour (Rothschild, Moore & Brown, 1984; Guilford et al. 1987; Kaye et al. 1989; Moore, Brown & Rothschild, 1990) and a deterrent effect on birds (Marples & Roper, 1996; Lindström, Rowe & Guilford, 2001; Siddall & Marples, 2011). In this study, predators found the chemical secretions from male moths raised on the high protein diet more unpalatable (i.e., tasted worse) than those from males raised on the low protein diet, which confirms that the chemical defence is costly. However, we did not find any differences between the predators' hesitation time (i.e., latency) to "attack" the bait soaked with fluids from male moths raised on high-protein and the latency to attack baits with water (control), which suggests that the predators may not perceive a difference in the odour (volatile compounds) of both. These results

agree with previous studies suggesting that the defensive fluids of wood tiger moths may contain several repulsive compounds, not only pyrazines, that influence defence efficacy in concert (see also Winters et al. 2021; Ottocento et al. 2022) and affect predator response in different ways (Rojas et al. 2019; Winters et al. 2021). Moreover, as Ottocento et al. (2022) recently showed, the relative amount of the two methoxypyrazines (SBMP, IBMP) present in the wood tiger moth defensive fluids is more relevant to predator deterrence than the total amount of pyrazines. Unfortunately, however, it is not possible to quantify the amount of pyrazines and conduct bird assays with the defensive fluids of the same individual. Interestingly, the same study (Ottocento et al., 2022) showed that predator responses are stronger towards the chemical defences of individuals originating from populations with high predation pressure (e.g., Scotland; Rönkä et al 2020) than towards individuals from populations with lower predation pressure (e.g., Estonia; Rönkä et al 2020), even if the total amount of pyrazines in the defensive fluids does not differ among populations.

Our results show that highly melanised male moths from both diet treatments have more effective chemical defences than those with less melanin, hinting at a positive correlation between costly melanin pigments and chemical defences. While this is not what we predicted, particularly in a scenario of low resource availability in early life, environmental conditions may have a greater influence on chemical defences than the pigmentary composition of the melanin hindwings, which is less sensitive to the variability of resources than other fitness-related traits. Although in this species the chemical defence compounds are not sequestered directly from plants but produced *de novo* (Burdfield-Steel et al., 2018), the main elements that the moths require to build their defences are still collected from their food intake.

When looking at how early-life resource availability influences wood tiger moths' size, we hypothesised that males raised on a low-protein diet would have both smaller pupae and smaller hind- and forewings than those raised on a high-protein diet, as environmental fluctuations and environmental stress (in this case due to the low amount of protein in the diet) may strongly affect both insect wing (Bitner-Mathé & Klaczko, 1999) and pupal size (Nguyen et al., 2019). Our findings, however, reveal no differences in size between males raised on high- and low-protein diets. This could be because individuals raised on a low-protein diet had a longer development time than those raised on a high-protein diet, which might facilitate the acquisition of more resources at the larval stage, even if of poorer quality, allowing these individuals to ultimately reach the same size as those on a richer diet, as reported by Lindstedt et al. (2017). A longer developmental time, however, may also lead to increased predation risk (Clancy & Price,1987; Häggström, & Larsson 1995), higher vulnerability to environmental perturbations (Tammaru et al., 2001), reduced fecundity (Saastamoinen, Hirai, & van Nouhuys, 2013), and difficulties to escape a risky environment (Cowan, Houde & Rose, 1996).

In sum, experimental evidence suggests that the production and maintenance of chemical defences are affected and limited by the resources available in early life, but melanin synthesis is not. This implies that, at least in wood tiger moths, melanin synthesis in adult wings seems to be less environmentally regulated than pyrazine production, implying that these traits are not limited by the same resource pool. Our findings also confirm that resource-dependent variation in chemical defences is perceived by natural predators, which has seldom been shown when variation in chemical defences is investigated (White and Umbers, 2021). Studying the response

of natural predators, which are the selective agents for defended prey, is key when aiming to understand how variation in defences is maintained in aposematic species.

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Author contributions

JM, BR and EB-S conceived and designed the study. MF, EB-S and BR raised the larvae under the different treatments. MF, ON, CO and SW did the colour analyses. CO, BR and EB-S did the unpalatability assays with birds. CO and BR wrote a first draft of the manuscript with input from JM and EB-S. All authors critically reviewed the manuscript, approved the submitted version, and agreed to be held accountable for the content therein.

Data Accessibility Helsinki repository

Conflicts of Interest - None

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References

Azócar, D. L. M., Nayan, A. A., Perotti, M. G., & Cruz, F. B. (2020). How and when melanic coloration is an advantage for lizards: the case of three closely-related species of Liolaemus. Zoology, 141, 125774

Barek, H., Sugumaran, M., Ito, S., & Wakamatsu, K. (2018). Insect cuticular melanins are distinctly different from those of mammalian epidermal melanins. Pigment Cell & Melanoma Research, 31(3), 384-392.

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software, 67(1), 1–48. https://doi.org/10.18637/jss.v067.i01

Bazzaz, F. A., Chiariello, N. R., Coley, P. D., & Pitelka, L. F. (1987). Allocating resources to reproduction and defense. BioScience, 37, 58-67.

Bilandžija H., Laslo M., Porter M. L. & Fong D. W. (2017) Melanization in response to wounding is ancestral in arthropods and conserved in albino cave species. Scientific Reports 7:17148 https://doi.org/10.1038/s41598-017-17471-2

Bitner-Mathé, B. C., & Klaczko, L. B. (1999). Heritability, phenotypic and genetic correlations of size and shape of Drosophila mediopunctata wings. Heredity, 83(6), 688-696.

Bize, P., Gasparini, J., Klopfenstein, A., Altwegg, R., & Roulin, A. (2006). Melanin□based coloration is a nondirectionally selected sex□specific signal of offspring development in the alpine swift. Evolution, 60(11), 2370-2380.

Blount, J. D., Rowland, H. M., Mitchell, C., Speed, M. P., Ruxton, G. D., Endler, J. A., & Brower, L. P. (2023). The price of defence: toxins, visual signals and oxidative state in an aposematic butterfly. Proceedings of the Royal Society B, 290(1991), 20222068.

Boggs, C. L. (1992). Resource allocation: exploring connections between foraging and life history. Functional Ecology, 6(5), 508-518.

Brooks, M. E., Kristensen, K., van Benthem, K. J., Magnusson, A., Berg, C. W., Nielsen, A., Skaug, H. J., Mächler, M., & Bolker, B. M. (2017). Modeling zero-inflated count data with glmm TMB. bioRxiv, 132753. https://doi.org/10.1101/132753

Burdfield-Steel E., Pakkanen H., Rojas B., Galarza J. A., Mappes J. (2018). De novo synthesis of chemical defenses in an aposematic moth. Journal of Insect Science 18.

Burdfield-Steel E., Brain M., Rojas B., Mappes J., (2019). The price of safety: food deprivation in early life influences the efficacy of chemical defence in an aposematic moth. Oikos 128, 245-253. doi.org/10.1111/oik.05420

Brien M. N., Orteu A., Yen E. C., Galarza J. A. , Kirvesoja J., Pakkanen H., Wakamatsu K., Jiggins C. D., Mappes J. (2022) Colour polymorphism associated with a gene duplication in male wood tiger moths. bioRxiv 2022.04.29.490025 doi.org/10.1101/2022.04.29.490025

Chinery, M. (1993) Collins guide to insects of Britain and Western Europe. 3rd ed. Harper-Collins. London

Clancy, KM. & Price, P.W. (1987) Rapid herbivore growth enhances enemy attack: Sublethal plant defenses remain a paradox. Ecology 68: 733-737

Cowan Jr, J. H., Houde, E. D., & Rose, K. A. (1996). Size-dependent vulnerability of marine fish larvae to predation: an individual-based numerical experiment. ICES Journal of Marine Science, 53(1), 23-37.

Cuthill, I. C., Allen, W. L., Arbuckle, K., Caspers, B., Chaplin, G., Hauber, M. E., & Caro, T. (2017). The biology of color. Science, 357(6350), eaan0221.

De Jong, G. (1993). Covariances between traits deriving from successive allocations of a resource. Functional Ecology, 75-83.

Ellers, J., & Boggs, C. L. (2004). Functional ecological implications of intraspecific differences in wing melanization in Colias butterflies. Biological Journal of the Linnean Society, 82(1), 79-87.

Ellers J. & Boggs C. (2002) The evolution of wing color in Colias butterflies: heritability, sex linkage, and population divergence. Evolution, Vol. 56, No. 4 (Apr., 2002), pp. 836-840

Emaresi, G., Bize, P., Gasparini, J., Piault, R., & Roulin, A. (2011). Plumage polymorphism in melanin-based coloration: a case study in the tawny owl Strix aluco. Ecology and conservation of European forest-dwelling raptors, 242-252.

Ethier, J., Gasse, M., Lake, K., Jones, B. C., Evenden, M. L., & Despland, E. (2015). The costs of colour: plasticity of melanin pigmentation in an outbreaking polymorphic forest moth. Entomologia Experimentalis et Applicata, 154(3), 242-250.

Fabricant, S. A., Kemp, D. J., Krajíček, J., Bosakova, Z., & Herberstein, M. E. (2013). Mechanisms of color production in a highly variable shield-back stinkbug, Tectocoris diopthalmus (Heteroptera: Scutelleridae), and why it matters. PLoS One, 8(5), e64082.

Farahpour, F., Saeedghalati, M., Brauer, V. S., & Hoffmann, D. (2018). Trade-off shapes diversity in eco-evolutionary dynamics. elife, 7, e36273.

Fargallo A. J., Laaksonen T., Korpimaki E., Wakamatsu K. (2006) A melanin based trait reflects environmental growth conditions of nestling male Eurasian kestrels. Evolutionary Ecology. 21:157–171

Furlanetto M. (2017) Investigating the cost of visual signals and chemical defences in *Arctia plantaginis*. Unpublished MSc Thesis, University of Padua – University of Jyväskylä

Galarza, JA. Comparative transcriptomics of albino and warningly-coloured caterpillars (2021) Ecol Evol. 11, 7507– 7517. https://doi.org/10.1002/ece3.7581

Glazier, D. S. (2002). Resource□allocation rules and the heritability of traits. Evolution, 56, 1696-1700.

Grill C. P., Moore A. J. (1998) Effects of a larval antipredator response and larval diet on adult phenotype in an aposematic ladybird beetle. Oecologia 114, 274–282.

Guilford T., Nicol C., Rothschild M., Moore B. P. (1987) The biological roles of pyrazines: evidence for a warning odour function. Biological Journal of the Linnean Society. 31, 2, 113-128

Ham, A., Ihalainen, E., Lindström, L., Mappes, J. (2006) Does colour matter? The importance of colour in avoidance learning, memorability and generalisation. Behavioural Ecology and Sociobiology 60: 482–491

Häggström, H. & Larsson, S. (1995) Slow larval growth on a suboptimal willow results in high predation mortality in the leaf beetle Galerucella lineola. Oecologia, 104, 308–315. http://www.jstor.org/stable/4221112

Hegna R. H., Galarza J. A., Mappes J. (2015) Global phylogeography and geographical variation in warning coloration of the wood tiger moth (*Parasemia plantaginis*) Journal of Biogeography 42, 1469–1481

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 280: 2012-2812

Higasio, Y. S., & Shoji, T. (2001). Heterocyclic compounds such as pyrroles, pyridines, pyrollidins, piperdines, indoles, imidazol and pyrazins. Applied Catalysis A: General, 221(1-2), 197-207.

Hill, G. E., & Brawner III, W. R. (1998). Melanin–based plumage coloration in the house finch is unaffected by coccidial infection. Proceedings of the Royal Society of London. Series B: Biological Sciences, 265(1401), 1105-1109.

Hodge J. E., Mills F. D., Fisher B. E. (1972) Compounds of browned flavor derived from sugar-amine reactions. Cereal Science Today.17, 2, 34-40

Hoekstra, H. E. (2006). Genetics, development and evolution of adaptive pigmentation in vertebrates. Heredity, 97(3), 222-234.

Jablonski, N. G., & Chaplin, G. (2017). The colours of humanity: the evolution of pigmentation in the human lineage. Philosophical Transactions of the Royal Society B: Biological Sciences, 372(1724), 20160349.

Kang, C., Zahiri, R., & Sherratt, T. N. (2017). Body size affects the evolution of hidden colour signals in moths. Proceedings of the Royal Society B: Biological Sciences, 284, 20171287.

Kaye H., Mackintosh N. J., Rothschild M., Moore, B. P. (1989) Odour of pyrazine potentiates an association between environmental cues and unpalatable taste. Animal Behaviour 37, 563-568

Kingsolver, J. G. (1995). Viability selection on seasonally polyphenic traits: wing melanin pattern in western white butterflies. Evolution, 49(5), 932-941.

Knudson, C., Geyer, C. J., & Benson, S. (2022). Package 'glmm'.

Lee K. P., Simpson S. J. & Wilson K. (2008) Dietary protein-quality influences melanization and immune function in an insect. Functional Ecology. 22, 1052–1061

Lindstedt, C., Lindström, L., & Mappes, J. (2008). Hairiness and warning colours as components of antipredator defence: additive or interactive benefits?. Animal Behaviour 75, 1703-1713.

Lindstedt C. Lindström L, Mappes J, McMillan W. O. (2009) Thermoregulation constrains effective warning signal expression. Evolution 63-2: 469–478

Lindstedt C., Talsma J. H. R., Ihalainen E., Lindström L., Mappes J. (2010) Diet quality affects warning coloration indirectly: excretion costs in a generalist herbivore. Evolution 64-1: 68–78

Lindstedt C., Eager H., Ihalainen E., Kahilainen A., Stevens M., Mappes J. (2011) Direction and strength of selection by predators for the color of the aposematic wood tiger moth. Behavioral Ecology 580-587

Lindstedt C., Suisto K., Mappes J. (2020) Appearance before performance? Nutritional constraints on life-history traits, but not warning signal expression in aposematic moths. Journal of Animal Ecology 2020,494–505.

Lindström, L., Rowe, C., & Guilford, T. (2001). Pyrazine odour makes visually conspicuous prey aversive. Proceedings of the Royal Society of London. Series B: Biological Sciences, 268(1463), 159-162.

Majerus MEN (1998) Melanism: evolution in action. Oxford University Press, Oxford

Marples, N. M., & Roper, T. J. (1996). Effects of novel colour and smell on the response of naive chicks towards food and water. Animal Behaviour, 51(6), 1417-1424.

McCauley, E., Murdoch, W. W., Nisbet, R. M., & William S. C. Gurney. (1990). The Physiological Ecology of Daphnia: Development of a Model of Growth and Reproduction. Ecology, 71(2), 703–715. https://doi.org/10.2307/1940324

Moore B. P., Brown W. V., Rothschild M. (1990) Methylalkylpyrazines in aposematic insects, their host plants and mimics. Chemoecology 1, 43-51

Müller F., Über die vortheile der mimicry bei schmetterlingen. Zool Anz 1, 54–55. German (1878).

Mundy, N. I., Kelly, J., Theron, E., & Hawkins, K. (2003). Evolutionary genetics of the melanocortin 1 receptor in vertebrates. Annals of the New York Academy of Sciences, 994(1), 307-312.

Nguyen, B., Ponton, F., Than, A., Taylor, P. W., Chapman, T., & Morimoto, J. (2019). Interactions between ecological factors in the developmental environment modulate pupal and adult traits in a polyphagous fly. Ecology and Evolution 9, 6342-6352.

Nielsen, M. E., & Mappes, J. (2020). Out in the open: behavior's effect on predation risk and thermoregulation by aposematic caterpillars. Behavioral Ecology, 31(4), 1031-1039.

Nokelainen O. (2013) Many forms of the wood tiger moth (*Parasemia plantaginis*): selective heterogeneity favours polymorphic warning signals, University of Jyväskylä.

Nokelainen O., Hubbard N., Lown A.E., Wood L.E., Stevens M. (2017) Through predators' eyes: phenotype–environment associations in shore crab coloration at different spatial scales. Biological Journal of the Linnean Society XX:1–14.

Ojala, K., Julkunen-Tiitto R., Lindstrom L., Mappes J. (2005) Diet affects the immune defense and life-history traits of an Arctiid moth *Parasemia plantaginis*. Evolutionary Ecology Research 7:1153–1170.

Ottocento, C., Winters, A. E., Rojas, B., Mappes, J., & Burdfield Steel, E. (2022). Not just the sum of its parts: Geographic variation and nonadditive effects of pyrazines in the chemical defence of an aposematic moth. Journal of Evolutionary Biology.

Poulton E. B. (1890) The colours of animals, their meaning and use, especially considered in the case of insects. New York, D. Appleton and Company

R Core Team. (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing. https://www.R-project.org/

Rigby M. C. & Jokela J. (2000) Predator avoidance and immune defence: costs and trade-offs in snails. Proceedings of the Royal Society 267, 171-176

Rojas B., Burdfield-Steel E., Pakkanen H., Suisto K., Maczka M., Schulz S., Mappes J. (2017). How to fight multiple enemies: target-specific chemical defences in an aposematic moth. Proceedings of the Royal Society B 284:20171424

Rojas, B., Mappes, J., & Burdfield-Steel, E. (2019). Multiple modalities in insect warning displays have additive effects against wild avian predators. Behavioral Ecology and Sociobiology 73, 1-12.

Rönkä K., Mappes J., Kaila L., Wahlberg N. (2016) Putting Parasemia in its phylogenetic place: a molecular analysis of the subtribe Arctiina (Lepidoptera). Systematic Entomology 41:44–853.

Rönkä, K., De Pasqual, C., Mappes, J., Gordon, S., & Rojas, B. (2018). Colour alone matters: no predator generalization among morphs of an aposematic moth. Animal Behaviour, 135, 153-163.

Rönkä, K., Valkonen, J. K., Nokelainen, O., Rojas, B., Gordon, S., Burdfield□Steel, E., & Mappes, J. (2020). Geographic mosaic of selection by avian predators on hindwing warning colour in a polymorphic aposematic moth. Ecology Letters, 23(11), 1654-1663.

Roff, D. A., & Fairbairn, D. J. (2007). The evolution of trade□offs: where are we?. Journal of evolutionary biology, 20(2), 433-447. https://doi.org/10.1111/j.1420-9101.2006.01255.x

Rosa, E., & Saastamoinen, M. (2020). Beyond thermal melanism: association of wing melanization with fitness and flight behaviour in a butterfly. Animal Behaviour, 167, 275-288.

Rothschild M., Moore B. P., Brown W. V. (1984) Pyrazines as warning odour components in the monarch butterfly, *Danaus piexippus*, and in moths of the genera Zygaena and Amata (Lepidoptera). Biological Journal of the Linnean Society 23:375-380

Roulin, A., & Dijkstra, C. (2003). Genetic and environmental components of variation in eumelanin and phaeomelanin sex-traits in the barn owl. Heredity, 90(5), 359-364.

Saastamoinen, M., Hirai, N., & van Nouhuys, S. (2013). Direct and trans-generational responses to food deprivation during development in the Glanville fritillary butterfly. Oecologia, 171, 93-104.

Sargent, T. D. (1978). On the maintenance of stability in hindwing diversity among moths of the genus Catocala (Lepidoptera: Noctuidae). Evolution, 424-434

Siddall, E. C., & Marples, N. M. (2011). The effect of pyrazine odor on avoidance learning and memory in wild robins Erithacus rubecula. Current Zoology 57, 208-214.

Schroderus, E., Jokinen, I., Koivula, M., Koskela, E., Mappes, T., Mills, S.C., Oksanen, T.A. and Poikonen, T., (2010). Intra-and intersexual trade-offs between testosterone and immune

system: implications for sexual and sexually antagonistic selection. The American Naturalist, 176(4), pp.E90-E97.

Stearns, S. C. (1989). Trade-offs in life-history evolution. Functional ecology, 3(3), 259-268.

Stearns, S. C. (1992) The Evolution of Life Histories. Oxford University Press, London.

Stevens J. D., Hansell M. H., Freel A. J. & Monaghan A. P. (1999) Developmental trade-offs in caddis flies: increased investment in larval defence alters adult resource allocation. Proceedings of the Royal Society 266, 1049-1054

Stuart-Fox, D., Newton, E., & Clusella-Trullas, S. (2017). Thermal consequences of colour and near-infrared reflectance. Philosophical Transactions of the Royal Society B: Biological Sciences, 372(1724), 20160345

Sugumaran M. (2002) Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects Pigment Cell Res. 2002 Feb;15(1):2-9. doi: 10.1034/j.1600-0749.2002.00056.x. PMID: 11837452.

Summers K., Speed M. P., Blount J. D., Stuckert A. M. M., (2015) Are aposematic signals honest? A review. Journal of Evolutionary Biology 1-17

Tammaru, T., Tanhuanpää, M., Ruohomäki, K., & Vanatoa, A. (2001). Autumnal moth–why autumnal?. Ecological Entomology, 26(6), 646-654.

Therneau, T. 2020. Coxme: Mixed effects cox models. R package version.2. http://CRAN.R-project.org/package=coxme

True, J. R. (2003). Insect melanism: the molecules matter. Trends in ecology & evolution, 18(12), 640-647.

Trullas S. C., Wyk J. H.V., Spotila J. R. (2007) Thermal melanism in ectotherms. J. therm. Biol. 32, 235–245

Troscianko, J., and M. Stevens. 2015. Image Calibration and Analysis Toolbox - a free software suite for objectively measuring reflectance, colour and pattern. Methods in Ecology and Evolution 6:1320–1331.

van't Hof Arjen E., Reynolds Louise A., Yung Carl J., Cook Laurence M. and Saccheri Ilik J. (2019). Genetic convergence of industrial melanism in three geometrid mothsBiol. Lett.152019058220190582

Vences, M., Galán, P., Vieites, D. R., Puente, M., Oetter, K., & Wanke, S. (2002). Field body temperatures and heating rates in a montane frog population: the importance of black dorsal pattern for thermoregulation. Annales Zoologici Fennici 39: 209-220.

Wang, I.J. (2011). Inversely related aposematic traits: reduced conspicuousness evolves with increased toxicity in a polymorphic poison-dart frog. Evolution 65: 1637–1649.

Watson A., Goodger D. T. (1986) Catalogue of the Neotropical tiger moths. Trustes of British Museum (Natural History), London. 71

White, T. E. and Umbers, K. D. L. (2021) 'Meta-analytic evidence for quantitative honesty in aposematic signals', Proceedings of the Royal Society B: Biological Sciences. Royal Society, 288(1949), p. 20210679. doi: 10.1098/rspb.2021.0679.

Wilson, K., Cotter, S. C., Reeson, A. F., & Pell, J. K. (2001). Melanism and disease resistance in insects. Ecology letters, 4(6), 637-649.

Winters AE, Lommi J, Kirvesoja J, Nokelainen O and Mappes J (2021) Multimodal aposematic defenses through the predation sequence. Front. Ecol. Evol. 9:657740. doi: 10.3389/fevo.2021.657740

Wong J. M., Bernhard R. A. (1988) Effect of Nitrogen Source on Pyrazine Formation. Journal of Agriculture and Food Chemistry 36: 129-133

Supplementary material

Title: Diet influences resource allocation in chemical defence but not melanin synthesis in an aposematic moth

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1. High and Low protein diet

High protein artificial diet	Low protein artificial diet
Agar g 18	Agar g 18
Wheat Germ g 72	Wheat Germ g 72
Teklad VitFree Casein g 32.4	**Teklad VitFree Casein g 10.8**
Sucrose g 28.8	Sucrose g 28.8
Salt Mix g 10.8	Salt Mix g 10.8
Torula Yeast g 14.4	Torula Yeast g 14.4
Cellulose g 0	**Cellulose g 21.6**
Cholesterol g 3.15	Cholesterol g 3.15
Sorbic Acid g 1.8	Sorbic Acid g 1.8
Ascorbic Acid g 3.6	Ascorbic Acid g 3.6
VanderZandt Vit Mix g 12.6	VanderZandt Vit Mix g 12.6
Water (cool) ml 450	Water (cool) ml 450
Water (boiling) ml 450	Water (boiling) ml 450
Linseed oil ml 6	Linseed oil ml 6

Important note:

The casein used is Teklad Vitamin-Free. In this diet, casein is the predominant source of protein. If another type of casein is used, the amount of vitamins may also vary.

2. Is hindwing melanisation continuous or discrete?

To evaluate the extent to which human-eye categorization of moth melanin morphs (high melanin and low melanin) captures variation in the degree of melanisation, we compared human-eye categories to the results from a cluster analysis of the proportion of the wing that was melanised. We used k-means clustering with k = 2 to sort melanisation measurements into two clusters. The resulting clusters were labelled high and low melanin clusters based on their mean melanisation scores (e.g., the cluster with the higher mean melanisation score was labelled the high melanin cluster). We then calculated the proportion of moths that were "correctly" classified – those for which the human-eye and cluster analysis scores were the same – as: [(human-eye high melanin moths clustered as high melanin) + (human-eye low melanin moths clustered as low melanin)] / (total number of moths).

A low correspondence between human-eye categorizations and cluster analysis results does not necessarily indicate that moth melanisation is best treated as a continuous variable; it is still possible that the proportion of the wing that is melanised is well captured in a small number of categories different from the high and low categories used previously (e.g., high, medium, and low; or high and low but with a different threshold). To test this possibility, we determined the optimal number of clusters in the proportion of the wing that is melanised using the silhouette method (Rousseeuw, 1987). This approach calculates a silhouette score for each tested k value, with higher silhouette scores indicating better support for that k value. The k value with the highest silhouette score best captures the structuring of the data (the clusters are maximally distinguishable from one another), and is therefore considered the optimal

number of clusters. We calculated silhouette scores for k = 2 through k = 10 (i.e., sorting proportion melanised into two to ten clusters).

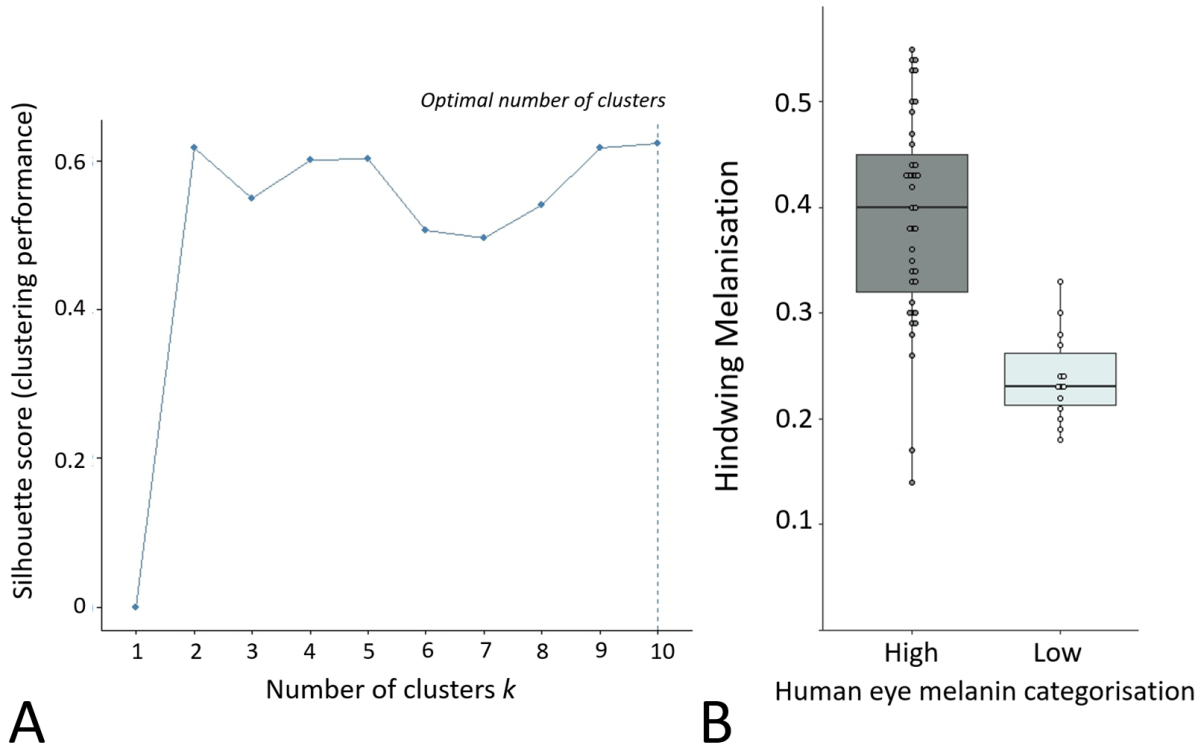

Figure 1 A) The k value with the highest silhouette score best captures the structuring of the data (optimal number of clusters). While an unimodal plot would have indicated a clearly optimal k value, this plot suggests more continuous data in the hindwing melanin amount. **1 B)** Objective hindwing melanisation measurements (the proportion of the wing that is melanised) for high- and low-melanin morphs as scored by the human eye. Note the overlap between high and low melanisation human-generated scores: subjective scoring may not be sensitive enough to detect patterns of variation.

When the proportion of the wing that was melanised was clustered into two categories, 70% of the resulting moth classifications corresponded with categorizations based on the human eye. To identify the optimal number of melanin clusters k we used the silhouette score, which represented the pairwise dissimilarity between and within cluster distances (clustering performance). The proportion of the wing that was melanised was best represented by 10 clusters, although other k values had very similar degrees of support (Fig. 1A); this high 'optimal' k value and the

similar degrees of support for multiple different k values indicates that wing melanisation is best treated as a continuous variable. Therefore, in the analyses we considered the melanin amount in the wings as continuous.

3. Effect of diet manipulation and hindwing melanin on the predator response to male's defensive fluid

3.1 Latency to approach

Table S1 A) Latency to approach the bait (oat) with control (water) was tested using cox mixed-effects model using package coxme, fit by maximum likelihood. The latency to approach was set as response variable; the different types of diet (low protein, high protein) were set as predictor variables. The diet and trial were set as fixed effects, while bird ID was set as a random effect. **S1 B)** Latency to approach the bait (oat) without control was tested using cox mixed-effects model using package coxme, fit by maximum likelihood. The latency to approach was set as response variable; the different types of diet were set as predictor variables. The interaction between diet (low protein, high protein), the amount of melanin and trial were set as fixed effects, while bird ID was set as a random effect.

S1A) With control	coeff	exp(coef)	se(coef)	z	p
High prot. treatment	-0.49	0.61	0.58	-0.85	0.39
Low prot. treatment	-0.22	0.80	0.57	-0.39	0.70
trial 3	0.29	1.34	0.25	1.14	0.25

S1B) Without control	coeff	exp(coef)	se(coef)	z	p
Low prot. treatment	0.34	1.41	1.25	0.27	0.78
Melanin hindwing	-3.52	0.03	2.41	-1.46	0.14
trial 3	0.17	1.19	0.22	0.78	0.44
Low protein diet:Hindwing melanin	1.02	2.78	3.16	0.32	0.75

Spearman's rank correlation (Correlation tested between the hindwing melanin and the predators' response (latency to approach) to the chemical defence) $r_s = 0.22$; $p = 0.265$

3.2 Latency to eat after approaching

Table S2 A) Latency to eat after approaching the bait (oat) with control (water) was tested using cox mixed-effects model using package coxme, fit by maximum likelihood.The latency to eat after approaching was set as response variable; the different types of diet (low protein, high protein) were set as predictor variables. The diet and trial were set as fixed effects, while bird ID was set as a random effect. **S2 B)** Latency to eat after approaching the bait (oat) without control was tested using cox mixed-effects model using package coxme, fit by maximum likelihood. The latency to eat after approaching was set as response variable; the different types of diet were set as predictor variables. The interaction between diet (low protein, high protein), the amount of melanin and trial were set as fixed effects, while bird ID was set as a random effect.

S2 A) With control	coeff	exp(coef)	se(coef)	z	p
High prot. treatment	0.27	1.32	0.34	0.80	0.42
Low prot. treatment	-0.15	0.86	0.34	-0.44	0.66
trial 3	-0.12	0.89	0.24	-0.48	0.63

S2 B) Without control	coeff	exp(coef)	se(coef)	z	p
Low prot. treatment	-0.06	0.94	0.25	-0.25	0.80
Melanin hindwing	-0.67	0.51	0.96	-0.70	0.48
trial 3	0.08	1.08	0.22	0.37	0.71

Spearman's rank correlation(Correlation tested between the hindwing melanin and the predators' response (latency to eat after approaching) to the chemical defence) r_s = 0.039; *p = 0.71*

3.3 Latency to eat

Table S3 Latency to eat the bait (oat) with control (water) was tested using cox mixed-effects model using package coxme, fit by maximum likelihood. The latency to eat was set as response variable; the different types of diet (low protein, high protein) were set as predictor variables. The diet and trial were set as fixed effects, while bird ID was set as a random effect.

S3 With control	coeff	exp(coef)	se(coef)	z	p
High prot. treatment	0.27	0.76	0.53	-0.52	0.60
Low prot. treatment	-0.42	0.65	0.52	-0.81	0.42
trial 3	-0.02	0.98	0.26	-0.07	0.94

3.4 Beak wiping

Table S4 A) Beak wiping reaction to the bait (oat) with control (water) was tested using a generalised linear mixed-effects models (GLMM) with a log link and Poisson distribution, fit by maximum likelihood (Laplace approximation) to test for differences in beak wiping events per minute. The diet (low protein, high protein) and trial were set as fixed effects, while the beak wiping frequency was set as response variable and bird ID as a random effect. **S4 B)** Beak wiping reaction to the bait (oat) without control was tested using a generalised linear mixed-effects models (GLMM) with a log link and Poisson distribution, fit by maximum likelihood (Laplace approximation) to test for differences in beak wiping events per minute. The interaction between diet (low protein, high protein), the amount of melanin, and trial were set as fixed effects, while the beak wiping frequency was set as response variable and bird ID as a random effect

S4 A) With control	Estimate	Std. Error	z value	Pr(>|z|)

Intercept	-4.41	0.70	-6.34	2.28e-10 ***
High prot. treatment	0.09	0.75	0.12	0.90
Low prot. treatment	0.03	0.76	0.04	0.97
trial 3	0.06	0.25	0.22	0.82

S4 B) Without control	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
Intercept	-3.53	0.87	-4.03	5.49e-05 ***
Treatment	-0.72	1.19	-0.60	0.55
Hindwing melanin	-3.40	2.27	-1.49	0.13
trial 3	0.008	0.20	0.04	0.97
Low protein diet:Hindwing melanin	3.13	2.95	1.06	0.29

Spearman's rank correlation (Correlation tested between the hindwing melanin and the predators' response (beak wiping) to the chemical defence) r_s = -0.025; *p = 0.8*

References

Rousseeuw, P. J. (1987). Silhouettes: a graphical aid to the interpretation and validation of cluster analysis. Journal of computational and applied mathematics, 20, 53-65

III

HONEST SIGNALLING IN PREDATOR-PREY INTERACTIONS: TESTING THE RESOURCE ALLOCATION HYPOTHESIS

by

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