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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;

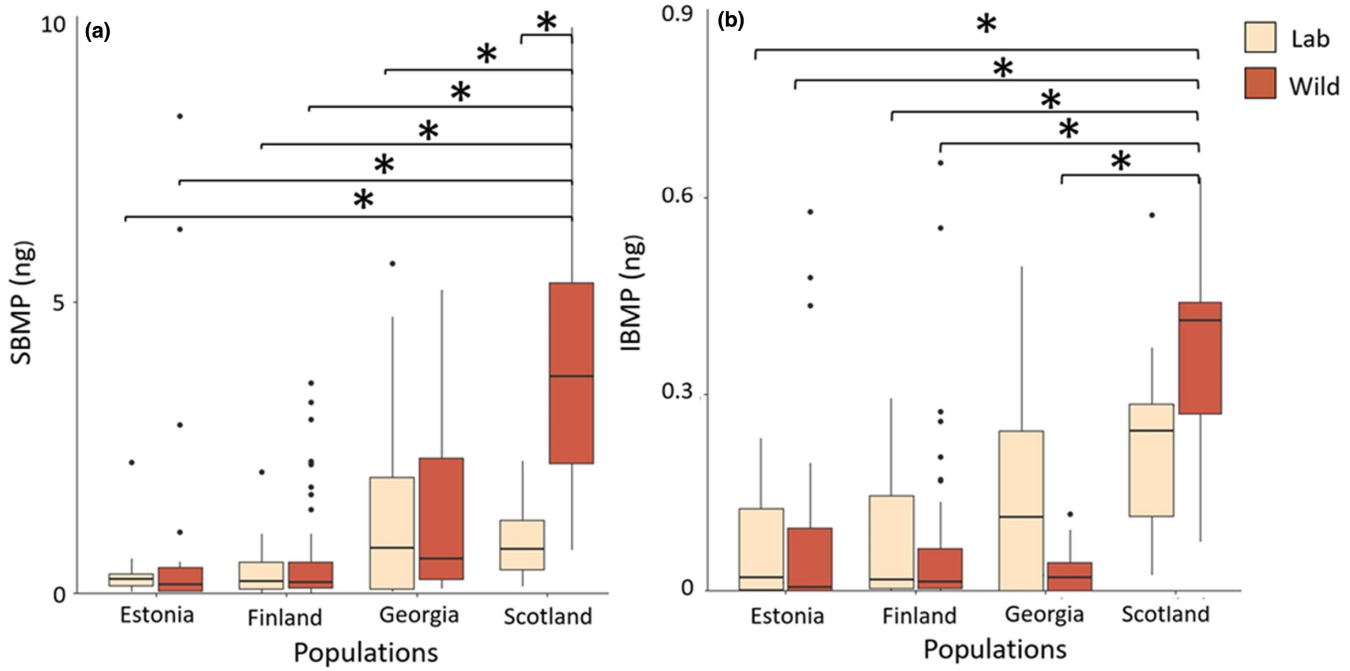

FIGURE 1 (a) SBMP amount in nanograms for each population (note this is the total amount of SBMP and IBMP in the thoracic fluids in nanograms, not the concentration). Wild individuals from Scotland had a higher amount of SBMP than the wild and laboratory Estonian, Finnish and laboratory-raised Scottish 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.

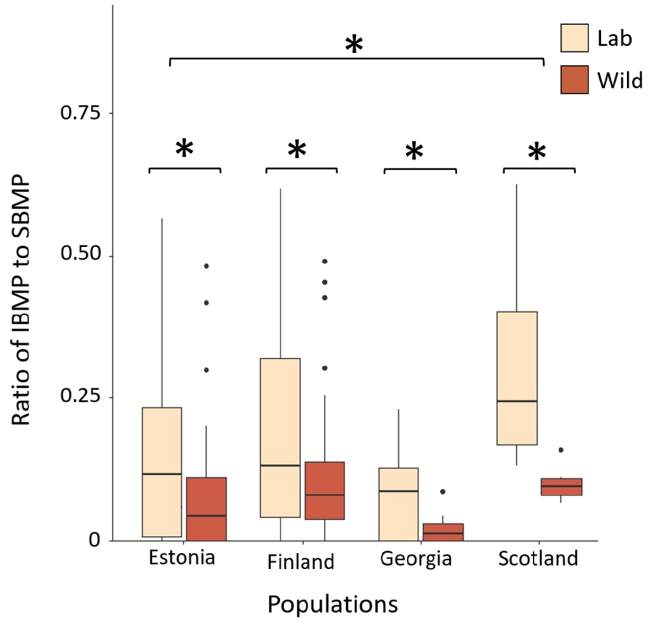

FIGURE 2 Ratio of IBMP to SBMP for each population. The laboratory populations had higher ratio of IBMP to SBMP compared to the wild populations. Boxes show the median and the 25th and 75th percentiles of data distribution. Vertical lines show the data range.

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

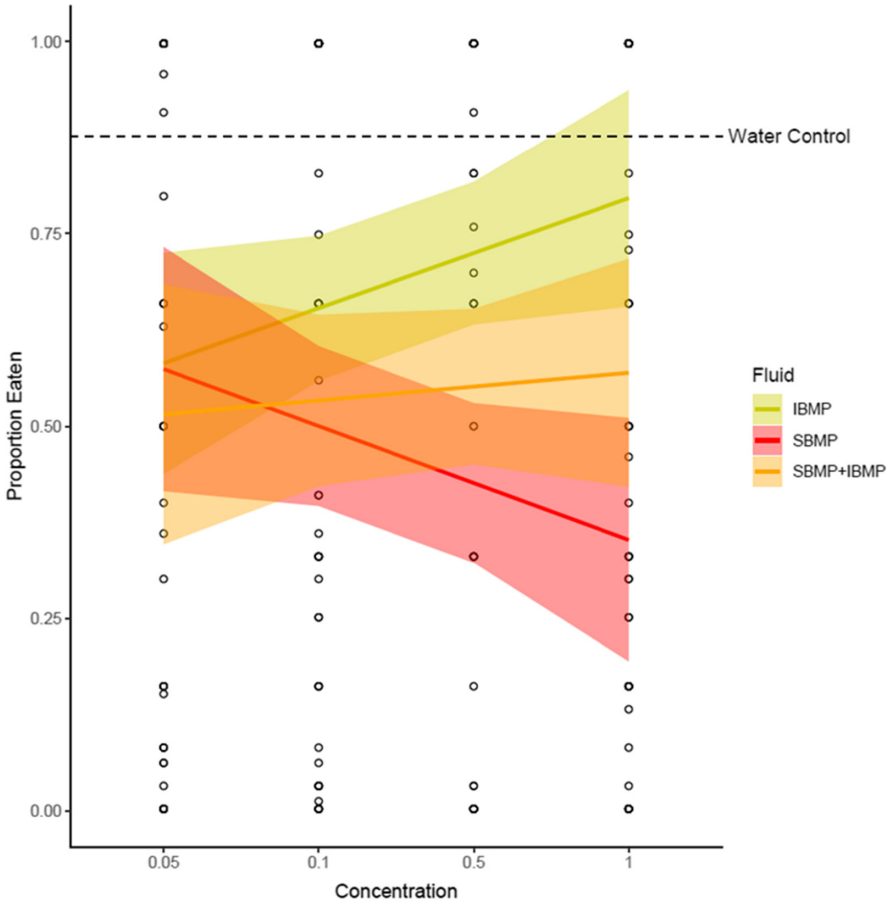

FIGURE 3 Proportion of fluid-soaked oats eaten in response to increasing concentrations (ng/μl) of each pyrazine type (SBMP = red, IBMP = yellow, SBMP + IBMP = orange). Shaded area represents standard error. Average bird response to the water control is indicated by a dotted line.

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,

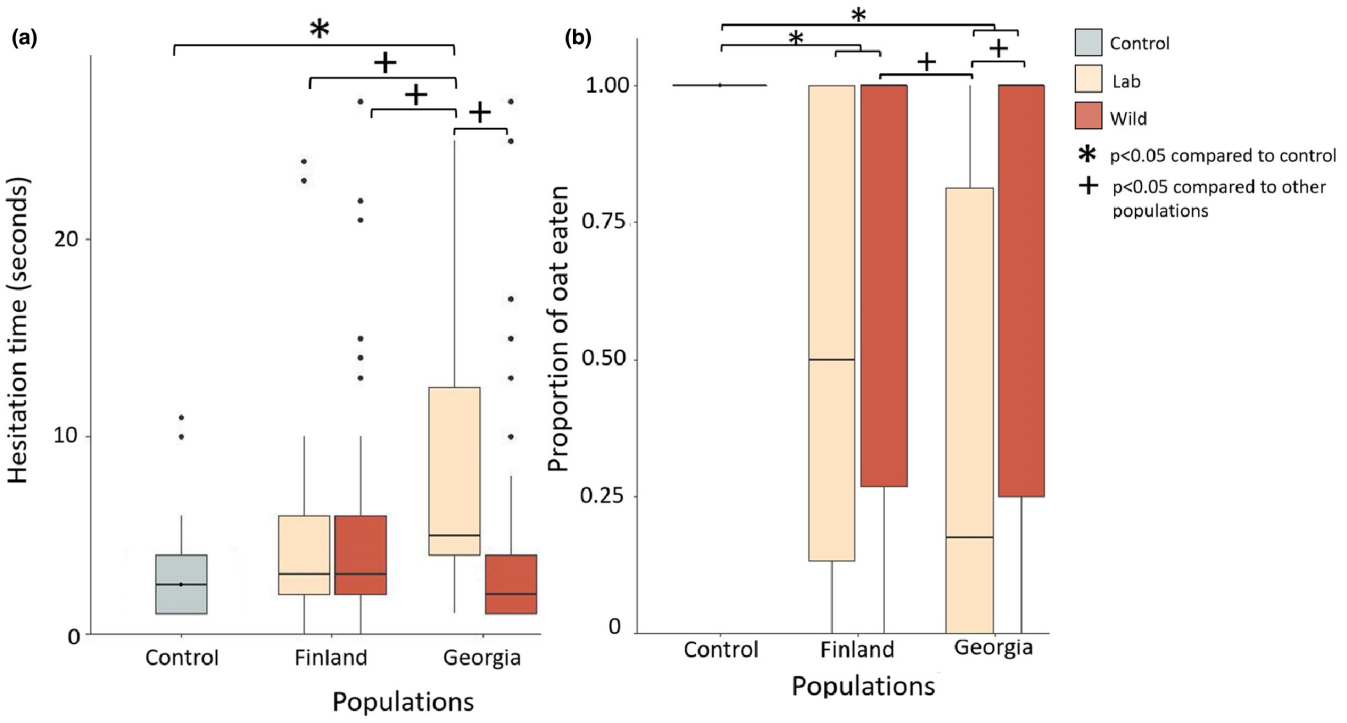

FIGURE 4 (a) Predators hesitate longer when exposed to fluids of Georgia moths raised in laboratory conditions compared to water. Boxes show the median and the 25th and 75th percentiles of data distribution. Vertical lines show the data range. (b) Proportion of oat eaten is lower when predators are exposed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

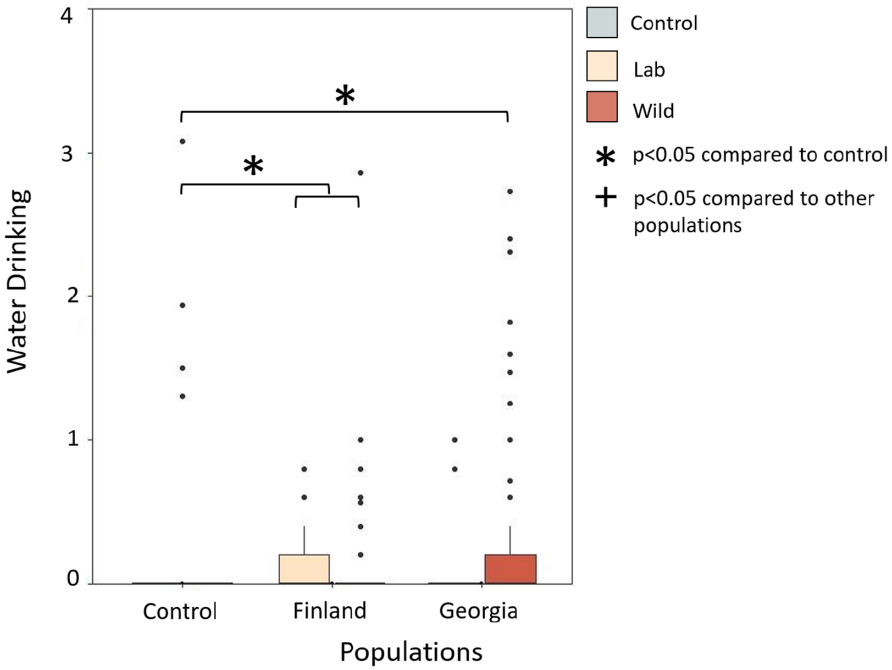

FIGURE 5 Water drinking increased when predators were exposed to fluids of wild and laboratory Finnish populations and wild Georgian moths compared to water. Water drinking did not differ between the two populations and wild and laboratory-raised moths. Boxes show the median and the 25th and 75th percentiles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

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

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