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Short Communication

De novo Synthesis of Chemical Defenses in an Aposematic Moth

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Abstract

Many animals protect themselves from predation with chemicals, both self-made or sequestered from their diet. The potential drivers of the diversity of these chemicals have been long studied, but our knowledge of these chemicals and their acquisition mode is heavily based on specialist herbivores that sequester their defenses. The wood tiger moth (*Arctia plantaginis*, Linnaeus, 1758) is a well-studied aposematic species, but the nature of its chemical defenses has not been fully described . Here, we report the presence of two methoxypyrazines, 2-sec-butyl-3-methoxypyrazine and 2-isobutyl-3-methoxypyrazine, in the moths' defensive secretions. By raising larvae on an artificial diet, we confirm, for the first time, that their defensive compounds are produced de novo rather than sequestered from their diet. Pyrazines are known for their defensive function in invertebrates due to their distinctive odor, inducing aversion and facilitating predator learning. While their synthesis has been suspected, it has never previously been experimentally confirmed. Our results highlight the importance of considering de novo synthesis, in addition to sequestration, when studying the defensive capabilities of insects and other invertebrates.

Key words: pyrazine, insect, chemical defense

Many animals protect themselves from predation using a large variety of chemicals, both produced by themselves or sequestered from their diet. The potential drivers of this diversity have been subjected to considerable research by evolutionary biologists. However, knowledge of these chemicals and their acquisition mode is heavily based on species that rely on their sequestration (e.g., Optiz and Müller 2009). Wood tiger moths (*Arctia plantaginis*, formerly *Parasemia plantaginis:* Rönkä et al. 2016) have been extensively studied due to their wing color polymorphism and are experimental model species for the study of predator–prey interactions . Despite this, the nature of their chemical defenses has yet to be fully described. Their larvae are polyphagous; in Finland alone, they are known to feed on plants from four different genera spanning four families: *Taraxacum*, *Plantago*, *Rumex*, and *Vaccinium* (pers. obs.). Unlike other tiger moths (subfamily Arctiinae: Lepidoptera: Erebidae), they do not sequester pyrrolizidine alkaloids, nor do they appear to utilize any dietary sources of other well-known defense compounds in the group, such as cardenolides or lichen phenolics. Furthermore, previous studies have shown that *A. plantaginis* does not seem to sequester iridoid glycosides (IGs), although it is capable of feeding on plants with high IG content (Lindstedt et al. 2010).

Adult moths release two defensive fluids when threatened: one from the cervical gland at the base of the head (henceforth referred to as 'neck fluid') and one from the anus. Recent work by Rojas et al. (2017) suggests that these two fluids are targeted toward different predator types; neck fluids are particularly effective against birds. Neck fluids are secreted in response to pressure, and, because birds typically attack the head of the moth (pers. obs.), they are likely to come into direct contact with the fluid. Previous research with wild-caught birds has demonstrated the deterrent effect of this fluid even without wing color cues (Rojas et al. 2017). Furthermore, chemical assays found that the neck fluids contain 2-sec-butyl-3-methoxypyrazine (SBMP), which is suggested to underlie the aversive reaction seen in bird predators. *A. plantaginis* is a capital breeder, so adults do not feed. Thus, toxin sequestration from the diet could only take place during the larval stage. Given that the moth population used in this study was fed predominantly on dandelion, which is not known to contain pyrazines (Schütz et al. 2006), this compound is unlikely to have been sequestered from the moths' diet. However, populations are sometimes supplemented with lettuce, some cultivars of which do contain 3-alkyl-2-methoxypyrazines (Murray and Whitfield 1975); therefore, in order to confirm the de novo production of methoxypyrazines in this species, the exact diet contents must be known. Here, we raised wood tiger moths from the same population on an artificial diet to test the hypothesis that the methoxypyrazines found in their chemical defenses are not sequestered from their

diet. We used a solid-phase micro extraction (SPME) technique for sampling, and gas chromatography with mass selective detection (GC/MSD) to detect the presence/absence of methoxypyrazines in the neck fluids.

Materials and Methods

Larvae were taken from six matings of unrelated adults from a laboratory stock founded from moths collected in Southern and Central Finland in 2011. Upon hatching, families were split into two treatments: one fed on dandelion (*Taraxacum* sp.) and one on an artificial diet. Dandelion was collected from the area surrounding the University of Jyväskylä, central Finland. Artificial diet was made on site, under sterile conditions, and a 250-g batch contained the following: 150-ml distilled water, 4.58-g agar, 32.1-g semolina, 8.58-g yeast, 8.3-g wheat germ, and 1.75-g vanderzant vitamin mix. All individuals were reared under laboratory conditions with an average temperature of 25°C during the day and 15–20°C at night. Upon emergence, all adult moths were stored in a climate cabinet at 4°C.

Defensive neck fluids of adult moths were sampled between 0 and 10 d after eclosion. Before sampling, all moths were removed from the climate cabinet and sprayed with water. They were then given 1 h to drink and become active. We stimulated fluid release by pinching the anterior end of the thorax, behind the head and the neck, using tweezers. Individual secretions of three males from each treatment were collected with 10-µl glass capillaries, and immediately transferred to glass vials containing 200-µl NaCl solution (3%). Secretions from a further 46 individuals (both male and female) were pooled in groups of two in 15 µl of autoclaved double-distilled H_2O. Samples from stock individuals were included in the dandelion treatment, as their rearing conditions were identical.

Measurement of the pyrazines was done following the methods of Cai et al. (2007). Pyrazines were extracted from the headspace of fluid samples using SPME fibers (StableFlex 1-cm fibers with Divinylbenzene/Carboxen/Polydimethylsiloxane coating, Sigma-Aldrich, Darmstadt, Germany) for 30 min at 37°C. GC/MSD 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 5973N MSD. Fibers 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. Methoxypyrazines were detected using selected ion monitoring of ions 124, 138, and 151. Following the findings of Rojas et al. (2017), standards of SBMP were measured in a scan mode and we then chose the targeted ions based on the ion responses. The chromatograms and mass spectra were evaluated using Agilent Chemstation (v. G1701CA) software and the Wiley 8th edition mass spectral database. Methoxypyrazines were identified using the ratio of these detected ions from the NIST webbook page (see Supp Material [online only] for electron impact mass spectrum of both pyrazines detected) (Stein), as well as by comparison with standards of SBMP and 2-isobutyl-3-methoxypyrazine (IBMP) (see Supp Material [online only]). To confirm that SBMP and IBMP were not being produced in the artificial diet, approximately 400 mg of food was transferred into glass vials and allowed to sit at room temperature for 1 and 2 h. The headspace was then tested with the same methods described earlier. Additional tests using agar made with known concentrations of IBMP confirmed our ability to detect pyrazines from the medium, if present.

Results

Individually analyzed neck fluids from *A. plantaginis* (both male and female) from both treatments (N = 3 for each) were found to contain IBMP and SBMP (Fig. 1, see Supp Material [online only] for ion chromatograms). Both ranged in concentration from 0.1 to 1 ng/µl. SBMP was found in all pooled samples (11 from the artificial diet and 12 from the dandelion diet). IBMP was found in all but three pooled samples: one from the artificial and two from the dandelion diet, although this absence may be due to poor sample quality in these cases. Neither pyrazine was detected from the headspace of the artificial diet.

Discussion

Our results confirm that, unlike other well-studied tiger moths, wood tiger moths can produce at least part of their defensive compounds, methoxypyrazines, de novo instead of sequestering them from their diet. This novel finding has important implications for our understanding of chemically defended insects. While pyrazines are known to play an important role in insect defense (Guilford et al. 1987) and have been previously described in tiger moths (Rothschild et al. 1984), experimental tests of their acquisition mode have been lacking. Early work on pyrazines often failed to find clear dietary sources of the compounds. Indeed, Rothschild et al. (1984) reported that they sometimes detected pyrazines in butterflies whose host plants lacked them, insinuating a role of de novo synthesis, as did Moore et al. (1990) who further noted that methoxypyrazines were found in the adults of

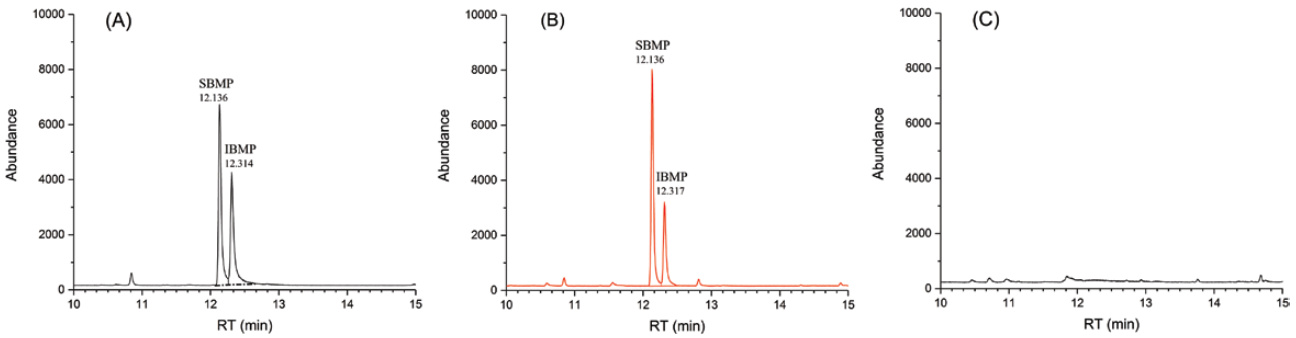

Fig. 1. Results of GC-MS analysis monitoring ions 124, 138, and 151. Methoxypyrazines detected from (A) male-fed artificial diet, (B) male-fed dandelion, and (C) artificial diet (1 h).

some species but not the larvae. This observation was, however, largely overlooked because lepidopteran larvae are known to frequently nibble chemically defended plants, other than their host plants, potentially using them as a 'drug source' for their own defense (Singer et al. 2009). Thus, the focus on plant-derived defenses may have led us to underestimate the prevalence of de novo synthesis, and its evolutionary implications. Our results support those early findings by Rothschild and Moore, by confirming de novo pyrazine production in an arctiid species.

Pyrazines have long been known for their defensive function in insects and other invertebrates due to their distinctive odor, not only inducing aversion (Rothschild et al. 1984, Guilford et al. 1987) but also facilitating predator learning (Rowe and Guilford 1996). Their presence in the moths' neck fluids may explain their antipredator function, as birds react similarly to SBMP within the concentration range reported from the fluids, even in the absence of color cues or previous experience (Rojas et al. 2017). The methoxypyrazine odor may make predators hesitate (allowing prey to escape) and induce predator avoidance learning when combined with a coloured warning signal. The biosynthesis of pyrazines is not well studied; however, in the laboratory, they can be produced from the reaction of amino acids and reducing sugars (Shu 1998) and glucose is thought to play a role in the formation of 2,5-dimethyl-3-alkylpyrazines in the defenses of leaf insects (Dossey et al. 2009). Potential biosynthesis pathways from amino acids to pyrazines in insect pyrazines have been suggested by David Morgan (2010).

The polyphagous nature of this species may have selected for the development of alternative sources of chemical defense. Given that much of the literature on chemical defense has focused on specialists (i.e., monarch butterflies, zygaenids, and arctiines, such as *A. caja*), it may have led to the systematic underestimation of the occurrence of de novo synthesis in defended species. For example, while describing the findings of D.W. Black's Ph.D thesis, Bowers (2008) reports low levels of cardenolides in *Syntomeida epilais* and *Composia fidelissima* fed on cardenolide-free diets, which she attributes to possible methodological problems. However, if valid, her finding would suggest that both species can produce at least small quantities of cardenolides de novo. Therefore, the assumption that sequestration is the main pathway by which species obtain defensive chemicals may have led us to overlook potential cases of de novo synthesis. Indeed, even in species which clearly can sequester a large proportion of their defenses from host plants, such as many *Heliconius* butterflies, de novo synthesis of cyanogens is widespread (Engler-Chaouat and Gilbert 2007), and this may occur in other Lepidoptera.

While sequestration of plant-produced compounds is likely to be the predominant mechanism for acquiring chemical defenses, especially in herbivorous insects, the ability to produce even low levels of defense de novo may greatly benefit nonspecialist species. It is also advantageous to use such species when testing 'cost of defense' hypotheses, as their chemical profiles are less influenced by the chemical profile of their food plants (Triponez et al. 2007). Moreover, as maintaining multiple detoxification mechanisms simultaneously is costly (Ali and Agrawal 2012), the underlying costs of defense may be easier to detect from generalist herbivores that have not co-adapted to use a specific host plant but instead maintain broad detoxification mechanisms (Lindstedt et al. 2010). Despite this, studies of defenses produced de novo are far rarer than those on sequestered defenses (Zvereva and Kozlov 2016). Thus, our finding not only sheds light on the defensive mechanisms of a well-studied aposematic species but also highlights the importance of considering de novo synthesis, and not merely sequestration, when studying the defensive capabilities of insects. Further studies will allow us to seek the biochemical and genetic mechanisms behind de novo synthesis and enable us to understand how the potential to supplement, or even replace, sequestered defenses with those produced de novo can influence the evolutionary trajectories of chemically defended species.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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