The Evolution of Tyramides in Male Fungus-Growing Ants (Formicidae: Myrmicinae: Attini: Attina)

Amy R. Luo1 · Madeline F. Hassler2 · Tappey H. Jones2 · Robert K. Vander Meer3 · Rachelle M. M. Adams4

Received: 24 May 2022 / Revised: 22 August 2022 / Accepted: 27 August 2022 / Published online: 20 September 2022
© The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2022

Abstract
Ants use a variety of semiochemicals for essential activities and have been a source for many novel natural products. While ant taxa produce a wide variety of chemicals, the chemistry and ecology of male ants have remained understudied. Tyramides are a class of compounds that have been found only in males of the Myrmicinae ant subfamily. Tyramides found in the fire ant Solenopsis invicta are transferred to gynes during mating where they are converted to tyramine, leading to rapid reproductive development. To further understand the evolution of tyramide production in male ants, we determined the tyramide composition in males of 15 fungus-growing ant species (Formicidae: Myrmicinae: Attini: Attina) and a Megalomyrmex species (Formicidae: Myrmicinae: Solenopsidini). Thirteen tyramides were identified, four for the first time in natural sources, and their percent composition was mapped to the fungus-growing ant phylogeny.

Keywords Myrmicinae · Fungus-farming · Reproduction · Mating · Tyramide · Male alates

Introduction
Ants (Formicidae Latreille, 1809) occupy virtually every ecological niche in the world. Thus, the over 16,500 described species have evolved a variety of mechanisms to solve similar problems (AntWeb 2022). Ant colonies depend on an array of semiochemicals for crucial functions, including food procurement, defense, and pheromonal communication (Vander Meer et al. 1998). Taxonomic and chemical divergence is evident within the two largest ant sub-families, Myrmicinae Lepeletier de Saint-Fargeau, 1835 (147 genera, 7,071 species) (Bolton 2022) and Formicinae Latreille, 1809 (52 genera, 3,220 species) (Bolton 2022). For example, Myrmicinae species have a well-developed sting apparatus (Kugler 1979) and produce a wide variety of bioactive alkaloid structural types that are injected or wiped onto adversaries (Fox and Adams 2022). In contrast, Formicidae species have no sting and spray formic acid for defense (Touchard et al. 2016).

The biology and chemistry of male ants are poorly understood compared to that of female alates (i.e., winged reproductive females or queens) and workers (Vargo and Laurel 1994; Bernadou and Heinze 2013). But males, like female alates, contribute to their natal colony’s reproductive output and play an essential role in fitness. Males usually die soon after mating, often through predation in the air or desiccation on the ground (Hölldobler and Wilson 1990; Boomsma et al. 2005; Helms 2018). Male aggregation behavior can occur hundreds of meters above the ground to attract females during brief mating flights (Helms et al. 2016; Helms 2018), while species with “female calling” often remain on or close to the ground (Helms 2018). In species with female calling, males can survive outside the colony for months, waiting for females (Shik et al. 2012). Despite variation in mating strategies, males’ relatively short lifespan and the difficulty of studying mating flights (but see Helms 2018 review) has limited research on male ants. But there has recently been a positive trend in male-focused research, including taxonomy (Boudinot 2013; Boudinot and Fisher 2013; Macgown et al. 2014; Probst et al. 2015) and chemical ecology (Vander Meer et al. 2021).
Males in the sub-family Myrmicinae produce a class of compounds called tyramides (Jones et al. 2010; Adams et al. 2010; Chen and Grodowitz 2017). A breakthrough in our understanding of how male produced tyramides are used by newly-mated fire ant queens was recently published (Vander Meer et al. 2021). Males transfer tyramides to female alates during mating, and female alates produce a tyramide-specific hydrolase enzyme in their reproductive system that quickly converts tyramides to tyramine, a biogenic amine. Tyramine floods the hemolymph of newly mated queens and triggers rapid reproductive development. In addition, artificial injection of tyramine into unmated female alates resulted in rapid dealation, ovariole development, and queen pheromone production. Vander Meer et al. (2021) conclude that male-produced tyramides enable young queens to rapidly shift to a reproductive stage after leaving their natal nest where reproduction is suppressed by their mother queen’s primer pheromone. They further suggest that variations of this mechanism are likely to occur in other ant species.

Fungus-growing ants (Formicidae: Myrmicinae: Atta: Attina) are obligate symbionts that cultivate a fungal garden as their only food source. Attines are a diverse group composed of over 250 species, that vary in natural history traits, including mating frequencies and colony size (Branstetter et al. 2017; Murakami et al. 2000; Solomon et al. 2019). Before this paper, only two fungus-growing ant species Cyphomyrmex faunulus and Trachymyrmex septentrionalis, were shown to produce tyramides (N-[2-(4-hydroxyphenyl)ethyl]-propanamide and N-[2-(4-hydroxyphenyl)ethyl]-2-oxobutanamide, respectively) (Adams et al. 2010).

Here, we sampled males from 15 fungus-growing species in seven genera (Acromyrmex [two spp.], Apterostigma [one sp.], Atta [two spp.], Cyphomyrmex [three spp.], Mycetommollerius (formally Trachymyrmex) [three spp.], Paratrachymyrmex (formally Trachymyrmex) [two spp.], and Sericomyrmex [two spp.]), representing a wide range within the subtribe Attina (Branstetter et al. 2017; Solomon et al. 2019). Megalomyrmex milenae (Formicidae: Myrmicinae: Solenopsidini) was included as an outgroup. The aim of this study is to characterize the diversity of tyramide structural types produced by males across the fungus-growing ant subtribe in a phylogenetic context that will become the foundation for tyramide functional investigations within the Attina in the future.

Methods and Materials

Colony Collections Live queenright colonies and queenless sub-colonies were collected from the Panama Canal region from 2015–2019 during May, June, and July. Atta sexdens males were collected immediately preceding a mating flight in May of 2019, then placed directly into vials of methanol. Collections were from: Barro Colorado Island (9.1522, -79.8465), Gamboa (9.1197, -79.6948), El Llano (9.2825, -78.9614), Plantation Road (9.07634, -79.6598), Creek Site on 16E (9.1631, -79.7449), Rio La Seda (9.1562, -79.7345), Fort Sherman (9.3653, -79.9593), Cardenas Creek (9.1685, -79.7519), and Parque Metropolitano (8.9915, -79.5442) (Table 1). Queenright colonies and queenless sub-colonies were maintained in a USDA containment facility (Permit P526P-19–02,323) at Ohio State University in Columbus, Ohio, for up to four years before the collection of males.

Chemical Extraction Live male ants (whole) were placed in vials with 40–150 µL of HPLC grade methanol (Table 1). Other males were trisected in a glass dish (head, thorax, and abdomen). The trisected parts were each placed in their own vial with methanol and passively extracted for at least two weeks. The dish and tools used for trisection were rinsed in ethanol, methanol, and then pentane between trisections. See Table 1 for the extraction procedure used for each male collection.

Gas Chromatography-Mass Spectrometry GC–MS was carried out in the electron impact (EI) mode using a Shimadzu QP-2010 GC–MS or a Shimadzu QP-2020 GC–MS equipped with an RTX-5, 30 m x 0.25 mm i.d. column. The instrument was programmed from 60°C to 250°C at 10°C/min. High-resolution mass spectrometry (HRMS) measurements were obtained by the Mass Spectrometry Laboratory of the School of Chemical Sciences at the University of Illinois Urbana-Champaign using a Waters Q-TOF Ultima ESI mass spectrometer or a Waters Micromass VG 70-VSE mass spectrometer. Previously identified tyramide compounds (1, 2, 3, 4, 6, 10, 11, 12, and 13) were determined through comparison with authentic samples (see Jones 2010; Adams 2010). Unidentified tyramides 5, 7, 8, and 9 were synthesized by the methods described below and had identical gas chromatographic retention times and mass spectra as the natural compounds. Retention indices were calculated by direct comparison with a Restek System Performance Test Standard Mixture of n-Alkanes (16 components) (Restek Pure Chromatography). Comparison of standard 0.6 µg/L and 60 µg/L solutions of N-[2-(4-Hydroxyphenyl)ethyl]-propanamide, 2, in methanol to the methanol extracts of Mycetomollerius zeteki, Cyphomyrmex costatus, and C. muelleri showed that they had approximately 1.1 µg/ant, 0.3 µg/ant, and 0.5 µg/ant, respectively, of their most abundant tyramides.

NMR and Chemical Synthesis N-[2-(4-Hydroxyphenyl)ethyl]-2-oxobutanamide (5). Compound 5 was prepared according to Jones et al. 2010 using 0.21 g (2 mmol) of 2-ketobutyric acid and 0.137 g (1 mmol) of tyramine in the presence of an equivalent of 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDCI) to provide 0.235 g of 5 after...
the usual work up. NMR spectra were obtained using a JEOL Ltd. 400 YH NMR spectrometer. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.05 (1H, br-s) 7.02 (2H, d, $J = 4.2$ Hz), 6.78 (2H, d, $J = 4.2$ Hz), 5.9 (1H br-s), 3.51 (2H, q, $J = 6.8$ Hz), 2.93 (2H, q, $J = 6.8$ Hz), 2.76 (2H, t, $J = 6.8$ Hz), 1.07 (2H, t, $J = 7.2$ Hz). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 199.6, 160.3, 154.9, 129.9, 115.7, 40.9, 34.6, 30.4, 7.1; EIMS $m/z$ 221 [M$^+$] (1), 220 (2), 164 (2), 121 (36), 120 (100), 107 (34), 103 (4), 93 (5), 91 (9), 77 (11), 57 (16), 51 (2), 41 (2); HRMS $m/z$ 222.1134([M+1]$^+$), was calculated for C$_{12}$H$_{16}$NO$_3$, 222.1130.

N-[2-(4-Hydroxyphenyl)ethyl]-2-hydroxybutanamide (8).
A solution containing 20 mg of ketotyramide 5 in 3 mL of methanol was treated with three drops of 1 M NaOH and 60 mg of NaBH$_4$ and stirred overnight at room temperature. The solvent was removed under reduced pressure, and the residue was carefully acidified with 10% HCl and extracted.

Table 1 Collection information: Collection codes contain the collector’s initials, date of collection (year/month/day), and colony number; males were stored in MeOH as whole (W) or abdomen (A) samples

| Genus          | Species        | Collection Code | Collection Location | Males in vial | Sample Type |
|----------------|----------------|-----------------|---------------------|---------------|-------------|
| Acromyrmex     | echinatior     | KAK190508-01    | Gamboa              | 5             | W           |
|                | octospinosus   | KAK190509-01    | Gamboa              | 3             | W           |
| Apterostigma   | dentigerum     | JM1M70506-03    | Plantation Rd       | 5             | W           |
|                |                | JM1M70511-01    | 16E                 | 5             | W           |
|                |                | ARL190522-04    | La Seda             | 4             | W           |
|                |                | ARMA190506-08   | La Seda             | 6             | W           |
| Atta           | cf. cephalotes | RMMA100615-01   | Gamboa              | 1             | W           |
|                | sexdens        | RMF190501-02    | Gamboa              | 2             | W           |
|                |                | RMF190501-03    | Gamboa              | 2             | W           |
| Cyphomyrmex    | costatus       | IMP170506-02    | Plantation Rd       | 10            | W           |
|                |                | CTH170506-04 (1)| Plantation Rd       | 9             | W           |
|                |                | CTH170506-03    | Plantation Rd       | 9             | W           |
|                | longiscapus    | RMMA180620-01   | La Seda             | 6             | W           |
|                |                | RMMA180623-05   | El Llano            | 5             | A           |
|                |                | RMMA180623-13   | El Llano            | 3             | A           |
|                |                | RMMA180622-03   | 16E                 | 3             | A           |
|                |                | RMMA180622-09   | 16E                 | 7             | A           |
|                | muelleri       | NMI170515-02    | 16E                 | 5             | A           |
| Sericomymex    | amabilis       | AC150523-01     | 16E                 | 5             | W           |
|                |                | TK150507-06     | La Seda             | 5             | W           |
|                |                | AMK150511-17    | La Seda             | 5             | W           |
|                |                | MRB180715-01    | Barro Colorado      | 8             | A           |
|                | opacus         | RMMA150508-01   | La Seda             | 9             | A           |
|                |                | MLD170522-01    | Plantation Rd       | 10            | A           |
|                |                | MRB180725-04    | Parque Metro        | 9             | A           |
| Mycetomollerius| opulentus      | MRF190523-03    | Fort Sherman        | 6             | W           |
|                | mikromelanos   | CRC170508-01    | La Seda             | 5             | A           |
|                |                | CRC170505-05    | 16E                 | 4             | A           |
|                |                | CRC170513-10    | La Seda             | 5             | A           |
|                |                | MRF190520-01    | La Seda             | 5             | W           |
|                | zeteki         | CRC170519-01    | Cardenass Creek     | 6             | W           |
| Paratrachymyrmex| bugnioni      | APH150510-15    | La Seda             | 5             | W           |
|                | corniti        | MRB180728-01    | Gamboa              | 10            | A           |
|                |                | MRF190522-05    | La Seda             | 9             | W           |
|                |                | MRF190522-04    | La Seda             | 4             | W           |
| Megalomyrmex   | milenae        | JMR190522-02    | La Seda             | 5             | W           |
twice with 5 mL portions of CH₂Cl₂. The combined CH₂Cl₂ extracts were dried over anhydrous MgSO₄, and removal of the solvent provided 2-hydroxybutanoyltyramide 8 as a single component by GC–MS. ¹H NMR (400 MHz, D₆-DMSO) δ 7.61 (1H, t, J = 8 Hz), 6.95 (2H, d, J = 8 Hz), 6.63 (2H, d, J = 8 Hz), 3.73 (1H, q, J = 4 Hz), 3.21 (2H, m, J = 8 Hz), 2.57 (2H, t, J = 8 Hz), 2.47 (1H, s), 2.26 (1H, s) 1.59 (1H, m), 1.43 (1H, m), 0.78(2H, t, J = 8 Hz). ¹³C NMR (100 MHz, CDCl₃) δ 174.0, 156.1, 130.0, 126.0, 115.6, 72.5, 35.0 27.9, 23.5, 20.6, 17.9, 14.0; EIMS m/z 235[M⁺](1), 233 (1), 120 (100), 114 (60), 107 (15), 97 (90), 77 (9), 69 (45), 41 (47); HRMS m/z 234.1484[(M+1)⁺], was calculated for C₁₄H₂₂NO₂, 234.1494. In the cases of those specimens containing 9, a slow stream of hydrogen was bubbled through a few drops of the methanolic extract from the ants, containing 1–2 mg of PtO₂, until the catalyst turned black. Subsequent GC/MS analyses showed the disappearance of the tyramide peak corresponding to 9 and an increase in the amount of the tyramide peak corresponding to 7.

N-[2-(4-Hydroxyphenyl)ethyl]-2-methylpentanamide (7). A solution containing 0.25 g (2 mmol) of 2-methyl-pentanoic acid in 20 mL of dichloromethane was treated with a slight excess of oxalyl chloride. After two hours, the solvent and excess oxalyl chloride were carefully removed by rotary evaporation. The residue was taken up in 10 mL of diethyl ether, and 0.27 g of tyramine (2 mmol) was added, followed by 15 mL of saturated NaHCO₃. The biphasic mixture was stirred overnight. The aqueous layer was separated, and the organic phase was dried over MgSO₄, filtered, and the solvent removed to provide 0.37 g of N-[2-(4-Hydroxyphenyl)ethyl]-2-methylpentanamide, prepared from 3-methyl- and 4-methylpentanoyltyramide, prepared from some Monomorium species (Jones et al. 2010). 2-Oxobutanoyltyramide, 5, was prepared from 2-oxobutanoic acid using carbodiimide coupling, and direct comparison of synthetic 5 with the ant extracts confirmed this structure. In those extracts containing 5, a later eluting tyramide, M⁺=221, whose mass spectrum had a diagnostic fragment at m/z = 121 (40%), along with the base peak at m/z = 120, similar to the mass spectrum of an α-ketotyramide found in males from some Monomorium species (Jones et al. 2010). 2-Oxobutanoyltyramide, 5, was prepared from 2-oxobutanoic acid using carbodiimide coupling, and direct comparison of synthetic 5 with the ant extracts confirmed this structure.

Results

Chemical Results Chemical analyses identified thirteen unique tyramide structures, nine of which were reported previously (Jones et al. 2010; Adams et al. 2010) (Table 2).

Four tyramides—5, 7, 8, and 9—are newly described compounds, identified by comparison to synthetic samples. Since the mass spectra of the tyramides found in various ant species are characterized by an intense base peak at m/z = 120 (Fig. 1) (Jones et al. 2010; Adams et al. 2010), they are easily visualized by a fragment ion search, even though they may co-elute with other compounds (e.g., fatty acids or their methyl esters) (Fig. 2). In addition, these compounds have discernable parent ions. Thus, the structures of novel tyramides 5, 7, 8, and 9 were identified from their mass spectra, microchemistry, and synthetic procedures. The four novel compounds include the first reported α-hydroxytyramide, 8, α,β-unsaturated tyramide, 9, and the second reported α-keto-tyramide, 5, from natural sources.

In several species, we observed a tyramide, M⁺ = 221, whose mass spectrum had a diagnostic fragment at m/z = 121 (40%), along with the base peak at m/z = 120, similar to the mass spectrum of an α-ketotyramide found in males from some Monomorium species (Jones et al. 2010). 2-Oxobutanoyltyramide, 5, was prepared from 2-oxobutanoic acid using carbodiimide coupling, and direct comparison of synthetic 5 with the ant extracts confirmed this structure.

In several species we observed a tyramide, M⁺ = 221, whose mass spectrum had diagnostic ions at m/z = 194 and m/z = 59, was present. These fragments would be expected from fragmentation on either side of the alcohol group of the α - hydroxytyramide corresponding to 5. An authentic sample of 2-hydroxybutanoyltyramide, 8, was prepared by NaBH₄ reduction of a few milligrams of 5. Direct comparison of synthetic 8 with the ant extracts confirmed this structure.

In several species we observed a tyramide, M⁺ = 235, corresponding to the molecular weight of the saturated n-hexanoyltyramide, but with a notably shorter retention time that
Table 2  Structures of tyramides identified in this study and in Adams et al. (2010): acetyltyramide, 1; propanoyltyramide, 2; methylpropanoyltyramide, 3; butanoyltyramide, 4; 2-oxobutanoyltyramide, 5; 2-methylbutanoyltyramide, 6; 2-methylpentanoyltyramide, 7; 2-hydroxybutanoyltyramide, 8; 2-methyl-(E2)-pentenoyltyramide, 9; pentanoyltyramide, 10; hexanoyltyramide, 11; heptanoyltyramide, 12; and octanoyltyramide, 13

| Tyramide | IUPAC Name                  | R       | M⁺  | Retention Index |
|----------|-----------------------------|---------|-----|-----------------|
| 1        | N-[2-(4-Hydroxyphenyl)ethyl]-acetamide | -CH₃    | 179 | 1859            |
| 2        | N-[2-(4-Hydroxyphenyl)ethyl]-propanamide | -C₂H₅  | 193 | 1939            |
| 3        | N-[2-(4-Hydroxyphenyl)ethyl]-2-methylpropanamide |      | 207 | 1966            |
| 4        | N-[2-(4-Hydroxyphenyl)ethyl]-butanamide | n-C₃H₇ | 207 | 2022            |
| 5        | N-[2-(4-Hydroxyphenyl)ethyl]-2-oxobutanamide |      | 221 | 1992            |
| 6        | N-[2-(4-Hydroxyphenyl)ethyl]-2-methylbutanamide |      | 221 | 2052            |
| 7        | N-[2-(4-Hydroxyphenyl)ethyl]-2-methylpentanamide |      | 235 | 2132            |
| 8        | N-[2-(4-Hydroxyphenyl)ethyl]-2-hydroxybutanamide |      | 223 | 2158            |
| 9        | N-[2-(4-Hydroxyphenyl)ethyl]-2-methyl-(E2)-pentenamide |      | 233 | 2220            |
| 10       | N-[2-(4-Hydroxyphenyl)ethyl]-pentanamide | n-C₄H₉ | 221 | 2120            |
| 11       | N-[2-(4-Hydroxyphenyl)ethyl]-hexanamide | n-C₅H₁₁ | 235 | 2228            |
| 12       | N-[2-(4-Hydroxyphenyl)ethyl]-heptanamide | n-C₆H₁₃ | 249 | 2324            |
| 13       | N-[2-(4-Hydroxyphenyl)ethyl]-octanamide | n-C₇H₁₅ | 263 | 2434            |
Fig. 1  Mass spectra of the novel compounds, a (5) 2-oxobutanoylt-yramide, M⁺ = 221, b (8) 2-hydroxybutanoyltymramide, M⁺ = 223, c (7) 2-methylpentanoyltymramide, M⁺ = 235, and d (9) 2-methyl-(E2)-pentenoyltymramide, M⁺ = 233, showing the characteristic base peaks at m/z = 120.

Fig. 2  a GC/MS of S. opacus male abdominal extract from colony MRB180725-04. The black line is the TIC. b Selected m/z = 120 ion chromatogram of S. opacus male abdominal extract from colony MRB180725-04. Only peaks corresponding to tyramides are labeled. For tyramide structures and retention indices, see Table 2. The asterisk indicates where the peak representing compound 4 would be, if present in this sample.
matched that of 2-methylpentanoyltyramide, 7. Samples of 3-methyl- and 4-methylpentanoyltyramide were also prepared from the corresponding 3 and 4-methylpentanoic acids, and their retention times did not match that of 7. In those species containing 7, we also observed a longer retention time compound, \( M^+ = 233 \), with intense ions at \( m/z = 97 \) and \( m/z = 114 \) that could be converted to tyramide 7 by microhydrogenation over PtO\(_2\). Direct comparison with a synthetic sample confirmed the structure of 2-methyl-(E2)-pentenoyltyramide, 9.

**Taxonomic Comparisons** Tyramides were observed in all ant samples examined in this study, except for *Apterostigma dentigerum*, where only one of four colonies sampled showed trace amounts of 1 and 2. *Apterostigma dentigerum* is unusual among the sampled Attina in that it appears to lack or have minimal amounts of tyramides.

Tyramide 2 was the most prevalent tyramide observed in this study and the largest profile component in the four sampled leaf-cutter species (two *Atta* spp. and two *Acromyrmex* spp.). It was present to some extent in all attine species sampled, but not in previously sampled *Cyphomyrmex faunulus* and *Trachymyrmex septentrionalis* (Adams et al. 2010). Tyramide 1 is found in smaller proportions within all species sampled in this study but absent in *C. faunulus* (Adams et al. 2010) (Fig. 3).

Generally, we found that closely related species had similar tyramide compositions. Based on our samples, the four leaf-cutter species are largely consistent; their tyramide profiles are comprised mainly of tyramide 2, relatively small proportions of tyramide 3 in *Acromyrmex* species, and trace or small amounts of tyramide 1 in *Atta* and *Acromyrmex*, respectively. *Mycetomollerius zeteki*, *My. opulentus*, and a single (out of five) *My. mikromelanos* colony (RMMA190506-03) have similar profiles to the leaf-cutter species, despite being more closely related to *Sericomyrmex*. The other four *My. mikromelanos* colonies were consistent with one another, but not with any other sampled taxa. *Paratrachyrmex cornetzi* and *P. bugnioni* are qualitatively similar, with only three minor non-overlapping tyramides. The three *Cyphomyrmex* species (*C. muelleri*, *C. longiscapus*, *C. faunulus*).

---

**Fig. 3** Tyramide composition—shown as a percentage of total peak area in the profile—across the phylogeny of the subtribe Attini (Branstetter et al. 2017). Data for *C. faunulus* and *T. septentrionalis* (indicated by *) are from Adams et al. (2010). *Megalomyrmex milenae* (Formicidae: Myrmicinae: Solenopsidini) is an outgroup. Each row represents the tyramide composition of the species, with darker shades representing larger proportions within the species; for species with multiple sampled colonies, the tyramide compositions are averaged across colonies. Absence of a compound is represented by dashes (——), trace amounts are indicated by (+)
and *C. costatus*), all members of the *C. wheeleri* species group, are qualitatively consistent (tyramides: 1, 2, 5, 6, 7, and 8), but vary in quantities (Fig. 3).

Using standard solutions of 2, we determined that *Mycetomolleria zeteki*, *Cynomyrmex costatus*, and *C. muelleri* had approximately 1.1 μg/ant, 0.3 μg/ant, and 0.5 μg/ant, respectively, of their most abundant tyramides (Fig. 3). By way of comparison, male *Solenopsis invicta* are reported to have 4.3 μg/ant (Vander Meer et al. 2021).

The two *Sericomyrmex* species, *S. amabilis* and *S. opacus*, showed the most variation between congeneric species, despite both being members of the *S. amabilis* species group. The tyramide profile of *S. amabilis* contains seven structures and is qualitatively similar to the *C. wheeleri* species group. In contrast, *S. opacus* contains 12 of the 13 tyramides found in this study, making its tyramide composition the most diverse (Fig. 3).

*Megalomyrmex milena*, a non-attine species and our outgroup, has a distinctly different profile from the other species in this study. While tyramide 7 is found in some of the attines as a minor compound, it is a major component in *Me. milena*. Additionally, only *Me. milena* produced greater-than-trace amounts of compound 4 (Fig. 3). *Megalomyrmex milena* also differs from the tyramide composition of *S. invicta* (composed almost entirely of tyramides 1 and 11), another *Solenopsidini* species (Vander Meer et al. 2021).

**Discussion**

In the current study, some genera (i.e., *Atta*, *Acromyrmex*, and *Cyphomyrmex*) show strong within-genus similarity in their tyramide profiles. Chemical profiles are, overall, most similar among closely related species; however, there was not a directional trend of structural diversity or profile complexity across our sampled taxa. Tyramides have been consistently observed in all Myrmicinae species examined thus far, though not without exception (i.e., *Apterostigma dentigerum*) (Jones et al. 2010; Adams et al. 2010; Chen and Grodowitz 2017; Vander Meer et al. 2021).

In the fire ant, *S. invicta*, tyramides stored in the male endophallic bladder are transferred to winged females during mating, where they are converted to the biogenic amine tyramine. Tyramine activates rapid reproductive development in the newly mated queen. This male/female sexual co-evolved system rapidly overcomes the effects of the queen’s primer pheromone that inhibits competitive reproductive development in her female sexual daughters (Vander Meer et al. 2021). While the tyramide compositions of *S. invicta* and fungus-growing ants differ, their hydrolysis product is the same biogenic amine, tyramine. It is probable that tyramine plays a similar role in fungus growing ants—initiating rapid reproductive development in newly mated queens. Interestingly, *S. invicta* queens mate only once (Ross and Fletcher 1985), but some leaf-cutter queens mate with multiple males (Murakami et al. 2000). These polyandrous queens have sperm from multiple males (Den Boer et al. 2009); however, each male is anticipated to transfer the same blend of tyramides to the new queen. In theory, the newly mated queens will hydrolyze the tyramide mix to tyramine regardless of which male or combination of males produced them, resulting in the rapid onset of reproductive development.

Multiple reproductive isolation mechanisms have evolved to prevent cross-species mating, but movement of species to new habitats may compromise some behavioral, phenological, physiological, or geographic mating isolation mechanisms (Vander Meer et al. 1985; Ross et al. 1987; Vander Meer and Lofgren 1989). We posit that species-specific tyramide hydrolysis could be another mechanism for reproductive isolation. In the event of cross-species mating, tyramide hydrolysis (tyramine production) would not occur, reducing the rate of founding success of the newly mated queen. Alternatively, variation in tyramide composition may be a result of genetic drift, which would be consistent with the general similarity of tyramide compositions in congeneric species. If there is selection for the presence of tyramides, but little to no selection on specific tyramide structures or compositions, species may vary in this trait.

Enzymes often show a degree of substrate specificity (e.g., tyramide hydrolase found in the gynes of *S. invicta*) (Draganov et al. 2005; Horton et al. 2010; Kaur et al. 2014), but some attine males produce many tyramides with a diversity of structures, including a-ketoamides, simple alkyl amides, a-branched alkyl amides, and a, b-unsaturated amides. The surprising discovery of a diversity of tyramide structures will prompt future research to test the degree of tyramide hydrolysis specificity.

Within ants, evidence supports the specificity of tyramides to Myrmicinae males and its presence across the subfamily (Vander Meer unpublished). However, there may be exceptions. For example, it appears that *Ap. dentigerum* males produce little to no tyramides; however, we did not control for the age of males in this study. Sampling more Myrmicinae species will reveal broader phylogenetic tyramide patterns, and collecting males at light traps, outside the nest, will ensure sexual maturity. Such studies will provide further insight into the pervasiveness of male tyramides across ants and the functional link between tyramides and reproductive suppression of female alates inside natal nests.

**Acknowledgements** We thank the staff and researchers at the Smithsonian Tropical Research Institute (STRI) for logistical support, and the Autoridad Nacional del Ambiente y el Mar for permission to sample ants in Panama and export them to Ohio. For assistance in the field, we thank participants of the 2015 (Andrea-Pil Sussie Holm, Anne-Mette Kroner, Angelo Concilo, Thomas Kaare), 2017 (Mazie L. Davis, Natalie M. Hamilton, Conor T. Hogan, Jianmei Mah, Ian M. Pelfrey), and 2019 (Matthew R. Boot, Julian M. Roberts, Matthew R. Fisher, Kendall A. King) Tropical Behavioral Ecology and Evolution course, currently organized and financially supported by The Ohio State University in
coordination with STRI. RMMA was supported by The Ohio State University. Thank you to our two reviewers and journal editor for their invaluable feedback on this manuscript.

**Author Contributions** Conceptualization: RMMA and THJ. Methodology: RMMA and THJ. Formal analysis and investigation: ARL and MFH. Original draft preparation: ARL. Review and editing: all authors. Funding acquisition: RMMA and THJ. Resources: RMMA and THJ; Supervision: RMMA and THJ.

**Declarations**

**Competing Interests** The authors have no relevant financial or non-financial interests to disclose.

**References**

Adams RMM, Jones TH, Jeter AW (2010) Male specific tyramides from three additional myrmicine genera. Biochem Syst Ecol 38:454–456. https://doi.org/10.1016/j.bse.2010.03.008

Bernadou A, Heinze J (2013) Mating-associated changes in the behaviour of Leptothorax gredieli ant queens. Ethology 119:634–643. https://doi.org/10.1111/eth.12103

Bolton B (2022) An online catalog of the ants of the world. https://www.antcat.org. Accessed 4 Nov 2022

Boomsma JJ, Baer B, Heinze J (2005) The evolution of male traits in social insects. Annu Rev Entomol 50:395–420. https://doi.org/10.1146/annurev.ento.50.071803.130416

Boudinot B (2013) The male genitalia of ants: musculature, homology, and functional morphology (Hymenoptera, Aculeata, Formicidae). J Hymenopt Res 30:29–49. https://doi.org/10.3897/jhr.30.3535

Boudinot BE, Fisher BL (2013) A taxonomic revision of the Meranoplus F. Smith of Madagascar (Hymenoptera: Formicidae: Myrmicinae) with keys to species and diagnosis of the males. Zootaxa 3635:301–339. https://doi.org/10.11646/zootaxa.3635.4.1

Branstetter MG, Jessovnik A, Sosa-Calvo J, Lloyd MW (2017) Dry habitats were crucibles of domestication in the evolution of agrarian social insects. Westview Press, Boulder, pp 159–192

Chen J, Grodowitz MJ (2017) Tyramides in male alates of black imported fire ants Solenopsis richteri. Insect Sci 24:169–172. https://doi.org/10.1111/1744-7917.12304

Den Boer SPA, Baer B, Dreier S, Aron S (2009) Prudent sperm use by leaf-cutter ant queens. Proc R Soc B Biol Sci 276:3945–3953. https://doi.org/10.1098/rspb.2009.1184

Draganov DI, Teiber JF, Speelman A, Osawa Y (2005) Human paraoxonases (PON1, PON2, and PON3) are lactomases with overlapping and distinct substrate specificities. J Lipid Res 46:1239–1247. https://doi.org/10.1194/jlr.M400511-JLR200

Fox EGP, Adams RMM (2022) On the biological diversity of ant alkaloids. Annu Rev Entomol 67:367–385. https://doi.org/10.1146/annurev-ento-072821-063525

Helms J (2018) The flight ecology of ants (Hymenoptera: Formicidae). Myrmecological News 10:19–30

Helms JA, Godfrey AP, Ames T, Bridge ES (2016) Are invasive fire ants kept in check by native aerial insectivores? Biol Lett 12:20160059. https://doi.org/10.1098/rsbl.2016.0059

Hölldobler B, Wilson EO (1990) The ants. Belknap Press of Harvard University. Thank you to our two reviewers and journal editor for their invaluable feedback on this manuscript. Springer Nature or its licensor holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.

Horton JR, Upadhyay AK, Qi HH, Zhang X (2010) Enzymatic and structural insights for substrate specificity of a family of jumonji histone lysine demethylases. Nat Struct Mol Biol 17:38–44. https://doi.org/10.1038/nsmb.1753

Jones TH, Garraffo HM, Spande TF, Andriamaharavo NR (2010) Caste-specific tyramides from Myrmicine ants. J Nat Prod 73:313–316. https://doi.org/10.1021/np900697s

Kaur R, Kaur N, Gupta AK (2014) Structural features, substrate specificity, kinetic properties of insect α-amylase and specificity of plant α-amylase inhibitors. Pestic Biochem Physiol 116:83–93. https://doi.org/10.1016/j.pestbp.2014.09.005

Kugler C (1979) Evolution of the sting apparatus in the Myrmicine ants. Evolution 33:117–130

Macgown IA, Boudinot B, Deyrup M, Sorger DM (2014) A review of the Nearctic Odontomachus (Hymenoptera: Formicidae: Poneraeinae) with a treatment of the males. Zootaxa 3802:515–552. https://doi.org/10.11646/zootaxa.3802.4.6

Murakami T, Higashi S, Windsor D, et al (2000) Mating frequency, colony size, polyethism and sex ratio in fungus-growing ants (Attni) Behav Ecol Sociobiol 4:276–284

Probst RS, Guénard B, Boudinot BE (2015) Toward understanding the predatory ant genus Myopias (Formicidae: Poneraeinae), including a key to global species, male-based generic diagnosis, and new species description. Sociobiology 62:192–212. https://doi.org/10.13102/sociobiology.v62i2.192-212

Ross KG, Fletcher DJC (1985) Comparative study of genetic and social structure in two forms of the fire ant Solenopsis invicta (Hymenoptera: Formicidae). Behav Ecol Sociobiol 17:349–356. https://doi.org/10.1007/BF00293212

Ross KG, Meer Vander RK, Fletcher DJC, Vargo EL (1987) Biochemical phenotypic and genetic studies of two introduced fire ants and their hybrid (Hymenoptera: Formicidae). Evolution 41:280–293

Shik JZ, Flatt D, Kay A, Kaspari M (2012) A life history continuum in the males of a neotropical ant assemblage: Refuting the sperm vessel hypothesis. Naturwissenschaften 99:191–197. https://doi.org/10.1007/s00114-012-0884-6

Solomon SE, Rabeling C, Sosa-Calvo J, Lopes CT (2019) The molecular phylogenetics of Trachymyrmex Forel ants and their fungal cultivars provide insights into the origin and coevolutionary history of ‘higher-attine’ ant agriculture. Syst Entomol 44:939–956. https://doi.org/10.1111/syen.12370

Touchard A, Ailli S, Fox E et al (2016) The biochemical toxin arsenal from ant venoms. Toxins 8:30. https://doi.org/10.3390/toxins8010030

Vander Meer RK, Breed M, Winston M, Espelie KE (1998) Pheromone communication in social insects. Pheromone communication in social insects. Westview Press, Boulder, pp 159–192

Vander Meer RK, Chinta SP, Jones TH, O’Reilly EE (2021) Male fire ant neurotransmitter precursors trigger reproductive development in females after mating. Commun Biol 4:1400. https://doi.org/10.1038/s42003-021-02921-5

Vander Meer RK, Loefgren CS, Vander Meer RK, Lofgren CS, Alvarez FM (1985) Biochemical and behavioral evidence for hybridization between fire ants, Solenopsis invicta and Solenopsis richteri (Hymenoptera: Formicidae). J Chem Ecol 15:1757–1765. https://doi.org/10.1007/BF01012263

Vargo EL, Laurel M (1994) Studies on the mode of action of a queen pheromone of the fire ant Solenopsis invicta J Insect Physiol 40:601–610. https://doi.org/10.1006/jiphol.1994.1573

Springer Nature or its licensor holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.