Identification of Glutamic Acid as a Host Marking Pheromone of the African Fruit Fly Species Ceratitis rosa (Diptera: Tephritidae)

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ABSTRACT: Host marking pheromones (HMPs) deposited by female fruit flies deter other females from overexploiting the same fruit for egg laying. Using a bioassay-guided approach, we identified the HMP of the Natal fruit fly species Ceratitis rosa as glutamic acid, I, from the aqueous fecal matter extract of ovipositing females by liquid chromatography–quadrupole time-of-flight–mass spectrometry (LC-QTOF-MS). Dual choice oviposition assays showed that both the fecal matter extract and I significantly reduced oviposition responses in conspecific females of C. rosa. Glutamic acid levels were 10–20 times higher in fecal matter than in the ovipositor or hemolymph extracts of females. Identification of I as a host marking pheromone in females of C. rosa improves our understanding of fruit fly chemical ecology and provides evidence that it could be used as a potential component in the integrated management of this fruit fly species.

KEYWORDS: Ceratitis rosa, host marking pheromone, ovipositional deterrent, fruit fly

INTRODUCTION

Fruit flies in the family Tephritidae (Diptera) are major pests of fruits in Africa, causing losses worth $2 billion annually. Several management techniques including early harvesting, fruit bagging, odor-baited trapping, biological control agents (parasitoids, predators, and pathogens), chemical sprays, and orchard sanitation are used to control fruit flies. Additionally, because semiochemicals mediate communication in many species of fruit flies, they can be exploited for fruit fly control. During recent chemical ecology studies of African indigenous fruit flies in the genus Ceratitis, we documented host marking behavior in C. cosyra, C. capitata, C. rosa, and C. fasciventris, but this behavior was not observed in C. ananae.

Host marking behavior involves dragging a protracted ovipositor on the surface of an oviposition substrate by females immediately after oviposition, and in the process, the female deposits the host marking pheromone (HMP). HMPs inform conspecifics, and at times heterospecifics, of an already used oviposition substrate, thereby minimizing over-exploitation of the substrate as well as time spent by females in oviposition attempts. Some tephritid fruit flies are known to perceive HMPs using sensilla found on the ventral side of the second, third, and fourth tarsomeres of the prothoracic tarsi and the short hairs on the labelum and meso- and metathoracic legs. These sensilla contain contact-chemoreceptor cells which are sensitive to HMPs and are influenced by several factors such as concentration of the pheromone, physiological state of the fly, nature of the fly (e.g., whether wild or laboratory reared), and the type of host.

Previous research has identified and used HMPs for the control of a few fruit fly species. For instance, the fatty acid glycoside, N-[15(β-glucopyranosyl)oxy-8-hydroxypalmitoyl]-taurine, identified from the cherry fruit fly Rhagoletis cerasi was evaluated in cherry orchards to control this fruit fly species in Switzerland. Additionally, the fatty acid amide, N-(2,14-dimethyl-1-oxopentadecyl)-glutamic acid, identified from the Mexican fruit fly Anastrepha ludens was evaluated in mango and plum orchards to control this fruit fly species in Mexico. These HMPs were identified from fecal matter extracts of females of the respective fruit fly species. Recently, we identified the HMP of C. cosyra from aqueous fecal matter extract of ovipositing females as glutathione (GSH). In laboratory bioassays, GSH reduced oviposition responses in conspecifics of C. cosyra and the heterospecific species C. rosa, C. fasciventris, C. capitata, and Zeugodacus cucurbitae.

In the current study, we report the identification of the HMP from the fecal matter extract of females of the Natal fruit fly C. rosa. We used the same protocol previously described for the identification of the HMP of C. cosyra.

MATERIALS AND METHODS

Insects. Ceratitis rosa previously collected from the wild plant Lettowianthus stellatus at the coastal region of Kenya, Mrima Hill (4°29'32" S; 39°15'27" E; 290 m ASL), was maintained at the International Centre of Insect Physiology and Ecology (icipe), Kenya (1.2219° S; 36.8967° E; 1600 m ASL). The colonies have been maintained since 1997 (over 300 generations) with yearly infusions with wild-caught C. rosa to reduce inbreeding and loss of genetic variation.
variability. The fruit flies were reared in a clear Perspex cage (30 × 30 × 30 cm), with fine netting ventilation on one side. They were provided with water, mangoes (oviposition substrate) and a 4:1 mixture of sugar (Mumias Sugar Co., Nairobi, Kenya) and enzymatic yeast hydrolysate (USB Corporation, Cleveland, OH) as described previously. The rearing room was maintained at 23–25 °C and 40–60% RH with a photoperiod of 12:12 h (L:D) cycle.

Collection and extraction of fecal matter were performed from both sexes as previously described. One hundred and fifty fruit flies of a given sex of either males or females of a known age (starting from day 1 after hatching up to day 30) were placed in a clean glass bottle (200 mL) and covered with a net lid tied to the rim. Fecal matter was collected daily and pooled after 5 days, washed with 5 mL of distilled water, and then freeze-dried based on sex. Each collection yielded 10–15 mg of fecal matter which was stored at –80 °C until sufficient quantities were obtained for both bioassays and chemical analysis.

**Cage Bioassays.** The bioassay procedures were identical to previously described methods, using sexually mature female fruit flies (10–21 days old), and a ripe mango (apple variety) as an oviposition substrate. Apple mango slices were prepared by cutting them lengthwise into two equal halves. The endocarp and mesocarp were carefully scooped out and the remaining exocarp thoroughly washed with distilled water, dried with paper towel, and fitted in covers of 50 mm-diameter Petri dishes with the rims on the top surface. The experimental samples (tests and controls) were applied to the entire mango slices using new cotton swabs (one per mango slice). Oviposition reduction response in the fruit flies was studied in dual-choice tests, and female fruit flies in 10 batches of 10 (n = 100) were observed individually to choose between ovipositing on:

(a) mango slice treated with 1 mL of aqueous fecal matter solution (10 mg/mL),

(b) mango slice treated with 1 mL of known concentrations of glutamic acid (1 mg/mL, 5 or 10 mg/mL) and a control (treated with 1 mL distilled water) tested against .

In all the experiments, total oviposition time was measured as previously described, beginning 30 s after introduction of a female into the observation cage and stopped when she displayed host marking behavior after egg laying. Released females were only allowed to oviposit once and the maximum total oviposition time allowed per observation was 5 min. After an observation session was terminated, the female fruit fly was returned to the rearing cage. Nonresponding females which failed to make a choice between the treated substrate and control after 30 min were replaced with fresh ones.

It was observed that immediately following oviposition Ceratitis female fruit flies marked a surface area of ca. 2 cm². Therefore, we sampled an equivalent surface area in treated and control mango slices and analyzed for glutamic acid to determine the concentration of the compound that female fruit flies encounter during oviposition. The mango slices were prepared as described for the dual choice oviposition assay and 10 mg/mL of glutamic acid in 1% formic acid/dd H₂O, 1 mL) evenly applied to the entire outer surface using cotton swabs and allowed to dry. Each mango slice was further cut into small pieces, each measuring 2 cm² (one-half of the mango slice produced 18–22, 2 cm² pieces). The 2 cm² pieces of mango were each held with a pair of forceps and thoroughly rinsed 30 times on the outer surface with 1 mL (1% formic acid/dd H₂O) into a clean 30 mL beaker using a 1 mL 200–1000 µL Eppendorf pipette. This was repeated 10 times using different 2 cm² pieces randomly selected giving rise to 10 samples.

The samples were transferred into a 1.5 mL Eppendorf tubes, vortexed for 30 s, sonicated for 10 min, and centrifuged at 14 000 rpm for 5 min to remove any insoluble material after which 1 µL of the supernatant was analyzed by LC-MS.

**Chemical Analyses.** Fecal matter, ovipositor, and hemolymph obtained from sexually mature C. rosa females and/or males (10–15 days old) were prepared and analyzed using a Waters Synapt G2-Si LC-QTOF-MS in full scan MS⁺ positive mode following the procedure and instrument parameters previously described for the identification of the HMP of C. cosyra. Briefly, 0.2 µL of the sample was automatically injected into a Waters UPLC and separated on a 250 mm × 4.6 mm i.d., 5 µm, ACE C-18 column (Advance Chromatography Technologies, Aberdeen, Scotland) using a gradient program with mobile phases of water (A) and acetoniitrite (B), each with 0.01% formic acid.

Data acquisition was achieved with a Waters MassLynx version 4.1 SCN 712. The mass spectrum was generated for every peak and potential assignments done using monoisotopic masses with tolerance of 10 ppm. The generated empirical formula was used to predict compound identities from online databases (METLIN, ChemSpider, ChemCalc, and fragment ion calculator), literature, and where available confirmed with authentic sample through coinjections. The application manager ChromaLynx, a module of MassLynx software, was used to investigate the presence of nontarget compounds in samples. Library searches were performed using the commercial NIST MS/MS library.

A similar procedure was used to analyze the extracting solvents and the rearing diet. The rearing diet was analyzed using two methods: (a) first dissolved and prepared in the same solvent as the one used to extract fecal matter and analyzed by LC-QTOF-MS to establish the presence of any free amino acids and (b) acid hydrolysis before analysis by LC-QTOF-MS. The amino acids were identified by comparison of mass spectrometric data, retention time, and coinjection of the natural extract with an authentic standard mixture of amino acids. The amino acid standard solution (AAS 18) was obtained from Sigma-Aldrich (Chemie GmbH, Munich, Germany). All samples were analyzed in triplicate, with each replicate collected from different batches of both sexes.

**Relationship between Glutamic Acid Amount in Female Fecal Matter and Age of C. rosa.** The fecal matter (10 mg) of C. rosa was obtained from females as earlier described, with collections from 1 day old females after eclosion and then 3 day intervals until day 30, when most of the fruit flies no longer survived. Fecal matter was also collected from males at similar ages. Both samples were separately extracted and analyzed using an Agilent 1260 Infinity HPLC system (Agilent Technologies, Palo Alto, CA) coupled to an Agilent 6120 mass detector MS with a single quadrupole analyzer (Agilent Technologies, Palo Alto, CA) following previously developed methods for the identification of the HMP of C. cosyra. Serial dilutions of glutamic standard (10-glutamic acid, >99% purity) (Sigma-Aldrich, St. Louis, MO) (1–100 ng/mL) were also analyzed by LC-MS to generate a linear calibration curve (peak area vs concentration) with the following equation: [y = 3137x − 1353.1 (R² = 0.9993)] which was used for external quantitation.

**Large Arena Bioassay.** This experiment was carried out in an enclosed room measuring 3 m × 1.5 m and fitted with a large clear glass sealed window. The room conditions were like the one used in fruit fly rearing. A photoperiod of 12:12 h (L:D) cycle was maintained in the room, illuminated using four cool daylight fluorescent tubes (Osram L 58W/765) hung from the ceiling (2.4 m from the floor). Mangoes purchased from the local market in Nairobi were thoroughly cleaned using Teepol liquid detergent soap and tap water to remove surface dirt, rinsed with distilled water, and then dried with a paper towel before being stored at −20 °C for 48 h to kill any residual fruit fly egg/larvae carried from the field. The mangoes were then thawed overnight in a laminar flow hood, dried using an internal fan in the hood followed by UV-light sterilization for 6 h. Subsequently, the mangoes were grouped into seven categories which were treated with either different concentrations of glutamic acid (0.1, 1, 5, 10, and 20 mg/mL; n = 125 mangoes; 25 mangoes for each concentration tested) or 1 mL of distilled water (experimental control, n = 25), or they were untreated (this served to check for any residual field infested fruit flies, n = 10) and placed in an enclosed rearing cage. All the treated mangoes were air-dried before being placed individually in a 50-mm-
diameter Petri dish and arranged on a table (different mango treatments covered the entire width of the table). Mangoes were randomized in subsequent experiments. The mangoes were placed on three clean tables, each measuring 51 × 107 × 107 cm (Figure 1).

Gravid females of *C. rosa* 10 days old (*n* = 100/table, total number of females for the three tables *n* = 300) contained in 200 mL bottles were introduced at the center of each table and allowed to oviposit for 24 h. Each mango from the various treatments was incubated in 20 × 12.5 × 8 cm³ plastic containers (Kenpoly, Nairobi, Kenya) which were covered with perforated plastic lids for 10 days. The inner bottom of these containers was lined with a thin layer of paper towel to absorb any sap produced by decaying fruits and collect emerged larvae. The paper towels were replaced every 2 days with clean, dry towels.

On day 10, the time it takes for *C. rosa* to reach the fourth instar stage, the paper towels were removed, and the larvae-infested mango individually washed under running tap water into a second plastic container. Fourth-instar larvae were recovered by sieving the water collected in the plastic container. The recovered larvae were introduced into another clean plastic container filled with water to immobilize them and to facilitate easy counting. This experiment was repeated three times with different batches of insects and mangoes.

**Statistical Analyses.** The number of insects responding to the treatments and controls in the dual choice assays was analyzed by Chi-square goodness of fit to assess: (a) *C. rosa* female discrimination to aqueous fecal matter extracts of conspecifics compared to control and (b) *C. rosa* female discrimination to different concentrations of glutamic acid, 1, against control. Nonrespondents were not included in the analysis. We used the two-sample Wilcoxon test to test for differences in the median oviposition time between the control and various treatments. Analyses of LC-QTOF-MS profiles involved quantitation and comparison of peak areas and mass spectra. Concentration of 1 was determined from fecal matter at different female ages calculated from the corresponding peak areas, and the data were fit with third-order polynomials. In the large arena bioassay, the number of larvae counted in all mangoes with similar treatment was expressed as mean larvae/mango ± standard error, and the data were fit with second-order polynomials. Analysis of variance was carried out for all the concentrations for the various ages, number of larvae and means were separated using Tukey’s studentized HSD. R-statistical program version 2.11.0 software¹⁹ was used to perform the statistical analyses, and all tests were performed at 5% significance level.

### RESULTS AND DISCUSSION

**Bioactivity of *C. rosa* Fecal Matter Extract.** Females of *C. rosa* significantly preferred to oviposit into mango slices treated with water than into mango slices treated with 10 mg/mL fecal matter extract of conspecifics (χ² = 17.64, df = 1, *P* < 0.001) (Figure 2A) in agreement with previous results.⁵,¹⁶,²⁰ As expected, ovipositing females required twice as long to...
assess the suitability of the treated oviposition substrate than controls (two sample Wilcoxon test, \( W = 259, P < 0.001 \)), (Table 1).

Thirty eight percent of ovipositing fruit flies chose to lay eggs in mango slices treated with fecal matter extract, but they took relatively double the time to assess the oviposition substrate compared to controls. This indicates that females recognized the treated hosts but factors such as innate sensitivity to HMPs\(^\text{21}\) and physiological state of the fruit fly could have contributed to the observed oviposition pattern. It is known that ovipositing females carrying more mature eggs or those that have experienced a longer elapsed time since the last oviposition are often less responsive to HMPs.\(^\text{22}\) It is also known that the nature of the fruit fly, for instance, laboratory reared flies of \( C.\) capitata females kept for over 200 generations were found to be 3-fold less sensitive to the same concentration of HMP compared to the wild-caught fruit flies.\(^\text{23}\) Additionally, the type of host substrate used could play a role in oviposition site selection.\(^\text{24}\) The preferred host for \( C.\) rosa is \textit{Lettuwianthus stellatus}.\(^\text{25}\) Future studies should investigate the influence of the preferred host on HMP responses in both wild- and laboratory-reared fruit flies.

**LC-QTOF-MS Identification of \( C.\) rosa HMP.** Chemical analysis of the aqueous fecal matter extracts of both females and males, by LC-QTOF-MS, identified glutamic acid, 1, as specific to the fecal matter extract of females. \( I \) eluted from the column at 1.7 min (Figure 3) with a molecular ion peak [M +H] \(^+\) at \( m/z \) 148.0607, corresponding to a molecular formula of \( C_5H_{10}NO_4 \). The amino acid identity was supported by the presence of the expected fragments with monoisotopic mass ions at \( m/z \) 102.0549 [M +H − HCOOH] \(^+\) and 130.0503 [M +H − H_2O] \(^+\), (Figure 4A). The identity of \( I \) was confirmed by comparison of mass spectrometric data, retention time, and

<table>
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<th>test</th>
<th>treatment median (range) (minutes)</th>
<th>control median (range) (minutes)</th>
<th>( P )-value</th>
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<td>4.4 (3.3–21.4)</td>
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<td>concentration (mg/mL)</td>
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<td></td>
<td>5</td>
<td>7.1 (4.2–23.2)</td>
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<tr>
<td></td>
<td>10</td>
<td>9.5 (7.3–28.6)</td>
<td>&lt;0.001</td>
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**Table 1. Oviposition Time of \( C.\) rosa Females on Mango Slices Treated with Aqueous Solution of Crude Fecal Matter, and Solution of Glutamic acid \( n = 100 \) Females/Treatment**

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Figure 3. Representative overlaid total ion chromatogram showing \( C.\) rosa profile of (A) female fecal extract, (B) male fecal extract, (C) ovipositor extract, (D) male hemolymph extract, (E) female hemolymph extract, (F) undigested rearing diet, (G) digested rearing diet, (H). glutamic acid standard, and (I) extracting solvent. * glutamic acid peak.
coinjection of the natural extract with an authentic standard (Figure 4B). Additionally, 11 other compounds common to fecal matter extracts of males, females, and the undigested rearing diet were identified (Table 2).

Comparison of the LC-QTOF-MS amino acid profiles of the nonhydrolyzed rearing diet and acid digested rearing diet, revealed the presence of several amino acids including glutamic acid, cysteine, glycine, alanine, arginine, lysine, histidine, proline, valine, methionine, tyrosine, isoleucine, leucine, and phenylalanine in the acid digested diet. These amino acids were not detected in the nonhydrolyzed rearing diet (Figure 3). This indicates that 1 is likely a product from digestion of a protein derived from the rearing diet, which may occur in the gut of females.

Bioactivity of Glutamic Acid. 1 reduced oviposition responses of females of C. rosa following a concentration-dependent response (Figure 2B). There was a significant preference for the mango slices treated with water compared to the mango slices treated with glutamic acid, especially at the highest concentration of 10 mg/mL ($\chi^2 = 40.96$, df = 1, $P < 0.001$ (Figure 2B)). Ovipositing females on average required 5 times longer to assess the suitability of the treated mango slices than controls (two sample Wilcoxon test, $W$ = 369, $P < 0.001$ (Table 1)). These results confirmed the bioactivity previously observed for the fecal matter extract and presence of a HMP in the extract.

In recovery studies, glutamic acid concentrations recovered ranged 3.5–4.4 $\mu$g/2 cm$^2$ of mango. This amount of 1 is 1.3

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**Table 2. Compounds Identified in Fecal Matter of Females, Males, and Undigested Rearing Diet**

<table>
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<tr>
<th>t$_R$ (min)</th>
<th>compound</th>
<th>molecular formula</th>
<th>[M+H]$^+$</th>
<th>key fragment ions</th>
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<th>M</th>
<th>U</th>
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<tr>
<td>1.68</td>
<td>glycylglycylglycyl-N-ethylglycinamide</td>
<td>C$<em>{20}$H$</em>{35}$N$<em>7$O$</em>{10}$</td>
<td>274.1516</td>
<td>84.9628,110.0131, 182.9704, 184.969, 198.9487, 214.9256, 238.1289</td>
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<td>+</td>
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<td>1.70</td>
<td>glutamic acid</td>
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<td>148.0607</td>
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<td>C$<em>{19}$H$</em>{17}$O$_4$</td>
<td>309.1790</td>
<td>175.1265, 180.1091, 225.133, 273.1562, 91.1675,295.1631, 310.1827,319.1748</td>
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<td>uridine</td>
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<td>102.0910, 136.0198, 158.1526</td>
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F = females, M = males, U = undigested rearing diet, + = present and − = not detected.

Figure 4. Representative mass spectrum of (A) natural glutamic acid from fecal matter extract and (B) synthetic glutamic acid.
and 4.2 times less than the amount found in 1 mg of ovipositor and fecal matter extract, respectively. Although, the observed effective oviposition deterrence concentration of 1 against C. rosa is 10 mg/mL for a mango slice, the actual concentration encountered by a fruit fly during oviposition is 4.20 ± 0.38 μg/2 cm². This result corroborates previous studies, which reported that up to 90 fruit flies make oviposition attempts in a mango fruit when deprived of an oviposition substrate for 24 h. Additionally, other research has reported effective HMP concentrations of 1−100 mg/mL, and that more than 75 ovipositor dragging circles are made by laboratory reared Ceratitis female fruit flies before detection of the presence of HMPs. This insensitivity of laboratory-reared fruit flies to HMP has been linked to population selection which favors high fecundity at the expense of HMP detection. These findings suggest that wild caught fruit flies may be more sensitive to HMPs. It would be interesting to compare responses of wild-caught and laboratory-reared fruit flies to field-realistic doses of HMPs, such as 1, to confirm previous work, and to also determine if contextual host plant volatiles are needed as background odors or for possible enhancement of the HMPs.

The fact that in our assays, ~30% of fruit flies tested did not respond (nonresponders) to fecal matter extract, is consistent with most laboratory assays involving semiochemicals using insects. For instance, previous research recorded nonresponders as 50% in oviposition assays with Rhagoletis mendax, and 5−40% in the bark beetle parasitoid Roptrocerus xylophagorum and sand fly Lutzomyia longipalpis.

**Figure 5.** Relationship between female age (days) and the amount (μg/mg) of glutamic acid levels detected in fecal matter of C. rosa.

As such, if 1 is a byproduct of the metabolism of food ingested by ovipositing females at this age, then its concentration would be expected to be high in both the ovipositor and fecal matter excreted by these females. If so, this may be advantageous to females since it would provide them with the opportunity to deposit the most potent levels of the HMP present in both the ovipositor and fecal matter on the oviposition substrate. Further research is needed to determine the possible physiological and/or behavioral mechanisms involved. As previously found for C. cosyra, the level of 1 was 10−20 times higher in fecal matter extract than in similar extracts of the ovipositor and hemolymph of females (Figure 3). These results suggest that 1 is transferred from the gut into the ovipositor via the hemolymph, with the excess amount excreted with the fecal matter. Experiments elucidating the transportation, stereochemistry and excretion of 1 in C. rosa are highly recommended.

**Large Arena Bioassay.** Treating mango slices with 1 directly reduced oviposition in females in a dose-dependent manner (R² = 0.9384), as well as the total number of larvae of C. rosa/mango that emerged (Figure 6). There was a significant difference in total number of larvae counted for all the different treatments compared to control (F(5, 12) = 49.45, P < 0.001). Mango slices treated with 20 mg/mL of 1 recorded the least total number of larvae, 366 (95% CI: 260.9−472.4), with an average number of larvae/fruit of 4.8 ± 1.63 larvae, followed by 10 mg/mL which had 391 (95% CI: 285.9−497.4), with an average number of larvae/fruit of 5.2 ± 1.94 larvae. The highest total number of larvae, 1168 (95% CI: 1062.6−1274.1), with an average number of larvae/fruit of 47.3 ± 1.64 larvae was recorded in the control treated with 1 mL of distilled water. No larvae were recorded in the untreated mangoes. Interestingly, these results mirror our laboratory results, suggesting that the HMP is a key source of chemical
information regarding host discrimination by ovipositing females. However, in the natural setting it is likely that other cues such as host odors, visual cues, or interaction of plant/fruit to HMP, may contribute to the host discrimination process, which would require additional research.

The present study identified glutamic acid, 1, as the HMP from the fecal matter extract of females of *C. rosa* and that the concentration of 1 influenced female oviposition response. Glutamic acid reduced oviposition in conspecific females in research arena assays which substantiated the laboratory results. This study also showed that 1 is present in the hemolymph and ovipositor of females and that age significantly affected female HMP production.

The presence of 1 in organisms has been of interest to researchers in the past. For example, 1 together with inosine and glutamine have been reported as components of the sex pheromone of the marine polychaete *Nereis succinea*. 1 is a constituent of volicitin, *N*-(17-hydroxylinolenoyl)-l-glutamine and other fatty acid amino acid conjugates found in the gut of *Manduca sexta*, and it is known to trigger elicitor activity in maize. In chemical synthesis, 1 has been employed as a useful chiral synthon in the synthesis of many pheromones. For example, synthesis of γ-caprolactone, a sex pheromone component of the dermestid beetle *N. fasciventris*, 1,6,10,43 as its component of volicitin, *N*-[(β-glucopyranosyl)oxy-8-hydroxypalmitoyl]-taurine, 14,44 Collectively, these results suggest that sibling species such as found in *Ceratitis* species may utilize the same pathway to make their HMPs, whereas nonrelated species may utilize different pathways, which may be associated with the evolution of fruit flies. Additional research would be needed to confirm this scenario.

Our studies on HMPs of *C. cosyra* and *C. rosa*, revealed that the HMP of *C. cosyra* reduced oviposition responses in conspecifics and the heterospecific species *C. rosa*, *C. fasciventris*, *C. capitata*, and *Zeugodacus cucurbitae*, while the HMP of *C. rosa* reduced oviposition responses in conspecific females of *C. rosa*, in agreement with previous findings using fecal matter. These findings suggest that over time *C. cosyra* females have evolved to produce the enzymes required for the biosynthesis of amino acids into the tripeptide glutathione, which has the advantage to reduce oviposition responses not only in conspecifics but also in heterospecifics. Evolutionary studies may provide an explanation for the utilization of similar molecular subunits in HMP production in sibling species. For instance, phylogenetic studies of the genus *Ceratitis* based on mitochondrial and nuclear gene found a more recent divergence of *C. rosa* compared with *C. cosyra*. Therefore, it appears that *C. cosyra* is at a more advanced stage than *C. rosa* in the evolutionary development of *Ceratitis* species. As such, it is not surprising that the advanced and complex evolution and preadaptive nature of *C. cosyra* compared to *C. rosa* could have contributed to the rapid spread of *C. cosyra* across Africa, attacking a wide range of fruits, which is second to the invasive fruit fly species *Bactrocera dorsalis*. Future research on competitive displacement and population genetics of other *Ceratitis* species is needed to validate this.

The identification of glutamic acid as a host marking pheromone in the Natal fruit fly *C. rosa* and the previous identification of glutathione as a HMP of *C. cosyra* improves our understanding of the chemical ecology of fruit flies, particularly in *Ceratitis* species. It remains to be established whether this pattern occurs in other *Ceratitis* species, and to assess their usefulness in the integrated management in fruit flies.

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