ANTENNAL RESPONSES OF WEST INDIAN AND CARIBBEAN FRUIT FLIES (DIPTERA: TEPHRITIDAE) TO AMMONIUM BICARBONATE AND PUTRESCINE LURES

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Abstract

Efforts to monitor and detect tephritid fruit flies in the genus *Anastrepha* currently involve MultiLure traps baited with two food-based synthetic attractants, ammonia (typically formulated as ammonium acetate or ammonium bicarbonate) and putrescine (1,4-diaminobutane). These baits are used in Central America, Florida, Texas, and the Caribbean to target/capture economically important *Anastrepha* spp. within each region. The efficacy of these baits varies by region and by species. Antennal responses to these compounds have been quantified for *A. suspensa* populations in Florida, but not elsewhere. This is the first report of antennal responses of Puerto Rican populations of *A. obliqua* and *A. suspensa* to the bait odors emitted from ammonium bicarbonate and putrescine lures. Responses to lure volatiles (tested separately and in combination), as measured by electroantennography (EAG), were dose dependant for both species and both sexes. Although the average response to ammonium bicarbonate in combination with putrescine was always numerically higher than responses to ammonium bicarbonate alone within a species and a sex, this result was never statistically significant. Males of *A. obliqua* were less sensitive than females, while males of *A. suspensa* were more sensitive than females to all volatiles and volatile mixtures, but these differences were not statistically significant at any dose. Female *A. obliqua* were more sensitive than *A. suspensa* females at all doses for all volatiles and all volatile mixtures, and these differences were statistically significant at the two highest doses of ammonium bicarbonate and ammonium bicarbonate plus putrescine. Our results are broadly similar with the electrophysiological studies conducted on Florida populations of *A. suspensa*, but there are important differences, most notably that the Florida study detected significantly lower responses by males than females to putrescine and ammonium bicarbonate plus putrescine. The implications of our results are discussed with respect to monitoring practices in different regions.

Key Words: *Anastrepha obliqua*, *Anastrepha suspensa*, electroantennography, putrescine, ammonium bicarbonate

Resumen

Los esfuerzos para monitorear y detectar las moscas de la fruta tefrítidas del género *Anastrepha* implica la utilización de trampas Multilure que contienen dos atrayentes sintéticos, el amoniaco (formulado como acetato de amonio o bicarbonato de amonio) y putrescina (1,4-diamino butano). Estos cebos se utilizan en América Central, Florida, Texas y el Caribe para identificar y capturar especies de *Anastrepha* de importancia económica en estas regiones. La eficacia de estos cebos varía según la región y especie. Respuestas de las antenas de moscas a estos compuestos se han cuantificado para poblaciones de *A. suspensa* en Florida, pero no para otras regiones. Este es el primer reporte de respuestas de antenas de moscas fruteras en poblaciones de *A. obliqua* y *A. suspensa* a olores emitidos por cebos conteniendo bicarbonato de amonio y putrescina en trampas en Puerto Rico. Las respuestas a los volátiles de estos compuestos (por separado y en combinación), medido por electroantenografía (EAG), dependieron de la dosis en ambas especies y ambos sexos. Aunque la respuesta a bicarbonato de amonio en combinación con putrescina fue siempre mayor dentro de una especie y sexo que la respuesta utilizando bicarbonato de amonio solo, este resultado no fue estadísticamente significativo. Los machos de *A. obliqua* fueron menos sensitivos que las hembras, mientras que los machos de *A. suspensa* fueron más sensitivo a todos los volátiles y mezclas de estos, pero estas diferencias tampoco fueron estadísticamente significativas en ningúna de las dosis. Hembras de *A. obliqua* fueron más sensitivas que las de *A. suspensa* a todas las dosis de los volátiles y mezclas de éstos, y estas diferencias fueron estadísticamente significativas en las dos dosis más altas de bicarbonato de amonio y bicarbonato de amo-

nio más putrescina. Nuestros resultados son muy similares con estudios electrofisiológicos realizados en poblaciones de *A. suspensa* en Florida pero existen diferencias importantes; el estudio de la Florida detectó respuestas significativamente menores en machos a putrescina y a bicarbonato de amonio más putrescina que en las hembras. Las implicaciones de nuestros resultados se discuten en relación a las prácticas de monitoreo de moscas fruteras utilizadas en diferentes regiones.

The genus *Anastrepha* (Diptera: Tephritidae) is a group of approximately 200 species of flies restricted in their natural range to the tropics and subtropics of the western hemisphere (Norrbom 2004). While fewer than 10% of these species are considered economically important, several, including *A. obliqua* (Macquart), *A. suspensa* (Loew), *A. ludens* (Loew), *A. fraterculus* (Wiedemann), *A. grandis* (Macquart), *A. serpentina* (Wiedemann) and *A. striata* Schiner have a profound impact on the production of tropical and subtropical fruits (White & Elson-Harris 1992; Aluja 1994). Growers in areas where any of these flies are known to occur may be restricted from exporting to certain markets, or may be forced to subject their fruit to expensive and potentially damaging post-harvest sterilization measures (Simpson 1993). Regulatory agencies have long been interested in tools to detect the arrival of economically important species and to monitor population fluctuations of species that already occur in a given area.

For all species of frugivorous Tephritidae studied to date, adult females are anautogenous (require protein consumption for ovary development) at eclosion (Drew & Yuval 2000). Consequently, females are highly attracted to volatile chemical cues indicative of protein-rich meals. In the past, surveillance programs used glass McPhail traps baited with liquid protein, usually consisting of hydrolyzed torula yeast with borax as a preservative/buffering agent (see review in Heath et al. 1993). Research has shown that ammonia vapor is the key attractant escaping from proteinaceous baits (Bateman & Morton 1981; Mazor et al. 1987) and this has led to the development of ammonia-based synthetic lures, including ammonium bicarbonate (Robacker & Warfield 1993) and ammonium acetate (Heath et al. 1995; Epsky et al. 1995), but female tephritids are also attracted to natural sources, such as avian feces and urine (Piñero et al. 2003). Amines, including putrescine (1,4-diaminobutane), were shown to enhance the efficacy of ammonia-baited traps for capture of *A. ludens* (Heath et al. 1995, 2004) and *A. suspensa* (Kendra et al. 2008). However, putrescine, a metabolic product of the amino acids ornithine and arginine (Robinson 1991), is not attractive alone (Heath et al. 1995, 2004). Current monitoring and detection programs use the plastic Multilure trap baited with ammonium acetate and putrescine lures (Anonymous 2010), but the effectiveness of these baits and this trap recently has been called into question (Díaz-Fleischer

et al. 2009). In particular, Díaz-Fleischer et al. (2009) point out that the response of flies to the baits is influenced by a number of variables, including species, sex, and nutritional status. This variability in response to baits is highlighted by similar variability in the responses of laboratory-strain flies versus wild flies (Díaz-Fleisher et al. 2009; Kendra et al. 2010). Similarly, Kendra et al. (2005a, 2009) demonstrated strong effects of physiological status (stage of ovary development) on both electroantennographic (EAG) and behavioral responses of female *A. suspensa* to lure volatiles. Understanding how these variables influence trap catch may help improve trap efficiency.

Our aim was to quantify the antennal olfactory response of feral Puerto Rican *A. obliqua* and *A. suspensa* to commercially available lures of ammonium bicarbonate and putrescine, both separately and in combination. Ammonium bicarbonate lures release equimolar amounts of two potential attractants, ammonia vapor and carbon dioxide (Kendra et al. 2005b); putrescine lures release a combination of putrescine and 1-pyrroline (an oxidative byproduct of putrescine, Robacker 2001). These EAG responses were compared to those obtained previously with *A. suspensa* populations in Florida (Kendra et al. 2005a, 2005b, 2009). This approach will allow us to assess geographical variations in EAG responses of one species (*A. suspensa* populations in Florida and Puerto Rico) as well as variations between 2 closely-related species (*A. obliqua* and *A. suspensa*) that occur sympatrically in Puerto Rico.

Materials and Methods

Insects

Anastrepha suspensa and *A. obliqua* adults were reared from fruit collected beneath or on host trees at the USDA-ARS Tropical Agricultural Research Station in Mayagüez, Puerto Rico; *A. obliqua* emerged from *Mangifera indica* and *Spondias mombin* (Anacardiaceae) and *A. suspensa* emerged from *Psidium guajava* and *Eugenia uniflora* (Myrtaceae). Fruit was placed on a plastic mesh suspended above a layer of vermiculite approximately three cm deep. All pupae were removed from the vermiculite after two weeks and placed into plastic Petri dishes with moistened vermiculite (no more than 50 pupae per dish). The pupae were then monitored daily

for the emergence of adults. Emerged adults were placed into a 30 × 30 × 30 cm square cage (Bugdorm, Bioquip, Rancho Dominguez, California) with approx. 50:50 sex ratio. Flies were fed *ad libitum* a mixture of refined cane sugar and yeast hydrolysate (4:1); water was provided using agar blocks. The cages were stored at 26 °C (± 1) and 75% RH at 12:12 h L:D. All flies subjected to electrophysiological tests were 18 - 24 d post eclosion. Ovary dissections confirmed that flies of this age were sexually mature (ovarian stages 5 and 6, Kendra et al. 2006).

Electroantennography

Olfactory responses were recorded using a Syntech EAG system (Hilversum, Netherlands) and EAG 2000 software. The EAG system consisted of probe/micromanipulator (MP-15), data acquisition interface box (serial IDAC-232), and a stimulus air controller (CS-5). Antennal preparations were made as described previously (Kendra et al. 2009). Briefly, freshly severed fly heads were mounted onto a spot of salt-free electrode gel (Spectra 360; Parker Laboratories, Fairfield, New Jersey) at the tip of a glass capillary electrode. The ends of the antennae were stuck onto a separate spot of salt-free electrode gel at the tip of a separate glass capillary reference electrode. The electrodes were drawn from thin-walled borosilicate glass capillaries (1.5 mm o.d., World Precision Instruments, Sarasota, Florida) and filled with 0.1 N KCl solution and silver wire (0.5 mm, World Precision Instruments) interfaced with the EAG probes. Changes in potential were recorded and analyzed using the Syntech EAG 2000 program.

Electroantennogram studies are typically coupled with behavioral assays. Because there are published accounts of the behavioral responses of these species to ammonium acetate and putrescine (Jenkins et al. 2011; Kendra et al. 2005b), we did not include such an assay in this study.

Antennae under test were positioned at the outlet of the air delivery tube, in an orientation transverse to the airstream. A stream of humidified air, purified by activated charcoal, was delivered at a flow rate of 400 mL/min. Volatiles were manually injected into the airstream, using gastight syringes (VICI Precision Sampling, Baton Rouge, Louisiana) through a port in the delivery tube 13 cm from the antennal preparations. All volatiles tested were prepared by placing the chemical/commercial bait in an airtight 500 mL glass jar with a rubber septum to allow sampling of the volatiles in the jar. Jars were left for at least 24 hours at room temperature (25 °C ±1) prior to sampling to ensure that the headspace became saturated at the vapor pressure of the respective bait(s). 2-Butanone (Aldrich) was used as the standard in all tests because it elicited a strong EAG response in other Tephritidae, including *Bactrocera tryoni* (Hull & Cribb 2001), *A. suspensa* (Kendra et al. 2005a), and *Ceratitis capitata* (Niogret et al. 2011). Although ethyl butyrate and ethyl hexanoate have elicited strong EAG responses in *A. obliqua* specifically (Cruz-Lopez et al. 2006), a principle goal of this study was to compare our results with studies conducted on *A. suspensa* and these studies had used 2-butanone as their standard; using a different standard would make comparison of relative response values impossible. Furthermore, previous trials have demonstrated that 2-butanone elicited strong EAG responses in *A. obliqua* (DAJ, unpublished data). Ammonium bicarbonate and putrescine were obtained from commercial lures (Suterra, LLC; Bend, Oregon). Although ammonium acetate is more commonly used in the field, we used ammonium bicarbonate for two reasons: 1) EAG response to ammonium bicarbonate has been published previously for *A. suspensa* (Kendra et al. 2005a, 2005b, 2009), facilitating comparative analysis; and 2) the acetic acid liberated by ammonium acetate lures tends to interfere with electrophysiological recordings (P. E. K., unpublished data). Each antennal preparation was first standardized by addition of 20 µL 2-butanone, then subjected to a series of test injections (0.125 mL, 0.25 mL, 0.5 mL, 1.00 mL, 2.00 mL, and 4.00 mL) of the volatiles to be tested (ammonium bicarbonate, putrescine, and mixtures of ammonium bicarbonate and putrescine). To control for diminishing activity of antennae during the experiments, each series of volatile injections was followed by a second injection of the standard. The lures tested were selected in random order, and dose levels were administered in decreasing order for some antennal preparations and in increasing order for others. To control for the effects of injecting gas from the syringe, test injections were also made of ambient air at the same dose volumes as the tested volatiles. Depolarization of antennae, recorded as changes in potential in mV and adjusted for responses to injections of ambient air, were normalized as percentages of the response to the standard, using a software adjustment in SYNTECH EAG 2000 to compensate for declines in response to the standard. Data from antennal preparations responding less than 1.2 mV to the standard were discarded, since such antennal preparations tended to lose responsiveness entirely before tests were completed.

Statistical Analyses

The responses of the flies to the different volatiles were compared separately at each dose using multivariate analyses of variance (MANOVA) (SAS Institute 1985), with the variables being fly species, sex, and the 3 volatiles or volatile mixtures. Commercial graphing software (SigmaPlot

10.0, Systat Software Inc., Chicago, Illinois) was used to evaluate several regression models, including logistic, sigmoidal, and hyperbolic, to fit the dose responses. Ultimately a hyperbolic regression model was used as it resulted in the most physiologically reasonable fits to the data. Hyperbolic equations are commonly used for ligand-binding studies and have been shown previously to serve well for characterization of EAG dose-response relationships in tephritid species (Kendra et al. 2009; Niogret et al. 2011). In addition, this regression model allowed for more consistent comparisons to previously reported results with *A. suspensa* (Kendra et al. 2009). The hyperbolic model was of the form

$$y = \frac{a\,x}{b + x} \qquad (1)$$

where *y* is the normalized antennal response, *x* is the dose of the attractant in mL, and *a* and *b* are empirical coefficients representing maximal EAG response and receptor binding affinity, respectively (Table 1).

Results

Flies of both species and sexes responded in a dose dependent manner to ammonium bicarbonate, putrescine, and the combined presentation of ammonium bicarbonate with putrescine (Figs. 1 and 2). The responses to ammonium bicarbonate plus putrescine were consistently numerically higher than responses to ammonium bicarbonate alone within species and sexes, though it was only statistically significant at the 1 mL and 2 mL doses (at the 1 mL dose: $F = 10.7$, $P = 0.002$, df = 108,4; at the 2 mL dose: $F = 4.9$, $P = 0.029$, df = 108,4). *Anastrepha obliqua* were consistently more sensitive than *A. suspensa* to ammonium bicarbonate and ammonium bicarbonate with putrescine, although this was only significant for females at the doses of 2 and 4 mL ($F = 7.36$; $P = 0.0078$; df = 108,4: $F = 11.97$; $P = 0.0008$; df = 108, 4: and AB: $P = 0.0092$; $F = 7.97$; df = 108,4; AB+Pt: $P = 0.0336$; $F = 4.98$; df = 108, 4 [for 2 mL and 4mL, respectively]). Male *A. suspensa* responded at numerically higher levels than females and the converse was true for *A. obliqua*, though we did not detect statistical differences in the responses of the sexes.

Discussion

Our EAG results with *A. suspensa* are broadly similar to those obtained by Kendra et al. (2009) in terms of the magnitude of response to identical doses of ammonium bicarbonate, putrescine, and ammonium bicarbonate in combination with putrescine. We noted higher sensitivity by all flies to ammonium bicarbonate in conjunction

Table 1. Empirical coefficients used in a hyperbolic equation to model EAG responses to ammonium bicarbonate, putrescine and ammonium bicarbonate in conjunction with putrescine.

| | *Anastrepha obliqua* | | | | | | *Anastrepha suspensa* | | | | | |
| | Ammon. bicarb. | | Putrescine | | Ammon. bicarb. + Putr. | | Ammon. bicarb. | | Putrescine | | Ammon. bicarb. + Putr. | |
	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
a	176.0	166.7	58.9	54.0	172.7	191.5	104.7	102.9	41.8	47.3	112.4	137.8
b	2.179	2.404	2.566	1.606	1.428	2.387	1.391	0.756	1.732	1.178	1.204	1.141
R^2	0.7616	0.7266	0.3815	0.3532	0.4967	0.7882	0.6634	0.7431	0.3623	0.4299	0.7228	0.6617

a and b are empirical coefficients representing maximal EAG response and receptor binding affinity, respectively.
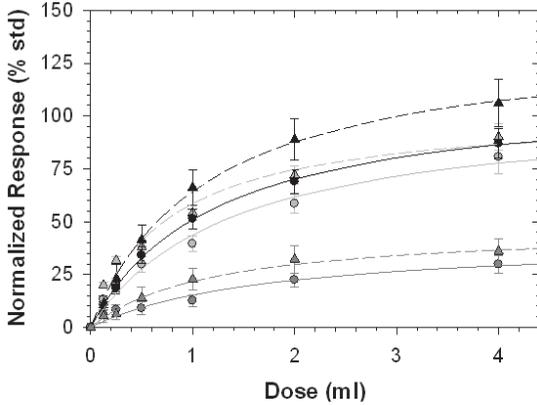
Fig. 1. Mean response (± SEM), normalized as a percent of the response to the standard, of female (circles) and male (triangles) *Anastrepha suspensa* to ammonium bicarbonate (light gray lines and shapes), putrescine (dark gray lines and shapes) and ammonium bicarbonate in combination with putrescine (black lines and shapes). Solid lines represent hyperbolic curves fitted to female responses and dashed lines represent hyperbolic curves fitted to male responses; see manuscript body for equation and coefficients.

with putrescine than to ammonium bicarbonate alone. However, we did not see a difference in the magnitude of response between males and females for any volatile at any dose. Kendra et al. (2009) recorded significant differences between male and female EAG responses to putrescine and to the combination of ammonium bicarbonate plus putrescine. As part of that same study,
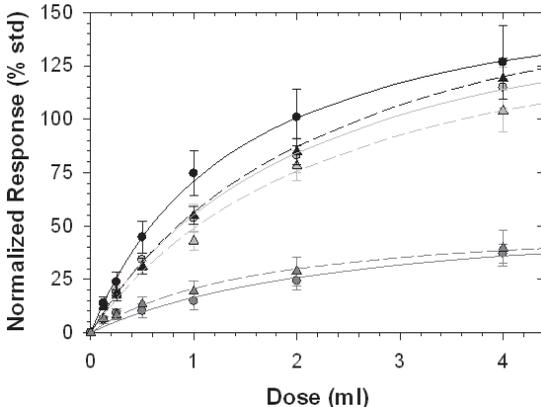

Fig. 2. Mean response (± SEM), normalized as a percent of the response to the standard, of female (circles) and male (triangles) *Anastrepha obliqua* to ammonium bicarbonate (light gray lines and shapes), putrescine (dark gray lines and shapes) and ammonium bicarbonate in combination with putrescine (black lines and shapes). Solid lines represent hyperbolic curves fitted to female responses and dashed lines represent hyperbolic curves fitted to male responses; see manuscript body for equation and coefficients.

two-choice bioassays indicated that more females were captured with ammonium bicarbonate plus putrescine than with ammonium bicarbonate alone. This difference was not observed in males, suggesting that the putrescine component is largely responsible for the female-biased attraction of the two-component lure. One explanation for the discrepancy in EAG results is that the *A. suspensa* assayed by Kendra et al. (2005a, 2005b, 2009) were all laboratory-reared flies, well synchronized in development (all with stage 5 ovaries, sexually mature but pre-ovipositional; Kendra et al. 2006). The current study used feral flies which were more variable in age (with ovarian development ranging from stage 5 to stage 6, ovipositional). Kendra et al. (2009) documented that the EAG response of *A. suspensa* to ammonium bicarbonate decreased in females from ovarian stage 5 to stage 6.

It is well known that protein lures attract higher proportions of females. Recent trials with Puerto Rican populations of *A. suspensa* and *A. obliqua* using corn protein hydrolysate and ammonium acetate and putrescine caught female-biased numbers of both species with percentages of females captured ranging from 53% to 95% (Jenkins et al. 2011). Antennal responses of *A. obliqua* to ammonium bicarbonate and putrescine and their combination have not been reported previously. The population of *A. obliqua* females we tested had significantly stronger EAG responses than females of *A. suspensa* at the two highest doses (2 mL and 4 mL) of ammonium bicarbonate alone and in combination with putrescine. This difference in antennal chemoreception of lure volatiles suggests that different doses of attractant chemicals may be needed for optimal captures of the two species. Thomas et al. (2008) demonstrated that higher doses of ammonia significantly reduced captures of *A. suspensa* and *A. ludens* in field tests, and Kendra et al. (2005a) observed decreased captures of immature (but not mature) female *A. suspensa* with increasing doses of ammonia in flight tunnel assays. This behavioral response was correlated with higher EAG responses (increased antennal sensitivity) recorded from immature females relative to mature females.

The need to detect and monitor tephritid species of economic importance has led to extensive research on attractants for these insects. The ideal fly trap would be one that is useful in multiple regions for detection of multiple species. The current trapping system for *Anastrepha* spp., namely plastic MultiLure traps baited with ammonium acetate and putrescine, has received criticism that it does not fulfill these criteria (Díaz-Fleischer et al. 2009). Criticisms of this trapping system include differential effectiveness of the baits in different regions (Epsky et al. 2003; Robacker and Thomas 2007; Thomas et al. 2008; Jenkins et al.

2011), differential attractiveness to different sexes (Díaz-Fleischer et al. 2009; Kendra et al. 2009; Jenkins et al. 2011), differential attractiveness to females of different physiological status (Kendra et al. 2005a; Díaz-Fleischer et al. 2009) and just plain ineffectiveness (Díaz-Fleischer et al. 2009). There has been some interest in the attractiveness of ovipositional host odors (Cruz-López et al. 2006). Trials using caged flies indicated that a nine-component lure of compounds from *Spondias mombin* (Anacardiaceae), a common host of *A. obliqua*, captured significantly more flies than a hydrolyzed protein bait (Cruz-López et al. 2006). However, the nine-component lure was equally attractive as hydrolyzed protein in one field trial (Toledo et al. 2009) and was less attractive than ammonium acetate and putrescine in another field trial (López-Guillen et al. 2010). Similarly, a four-component lure containing compounds from *Sargentia gregii* S. Wats. (Rutaceae) was not as attractive to *A. ludens* as ammonium acetate and putrescine lures (Robacker & Heath 1997). Even if ovipositional host odors were effective for a given species, it is likely that such cues would be far more species-specific than food-based odors indicating protein-rich resources. Despite its drawbacks, the MultiLure trap baited with ammonium acetate and putrescine remains the most useful tool available for monitoring and detection of *Anastrepha* spp. in North and Central America and the Caribbean.

Our results illustrate that there are measurable differences in the physiological responses of *A. obliqua* and *A. suspensa* to volatiles emitted from the two-component lure in Puerto Rico. This may explain some differences observed in the efficacy of the current trapping system in different regions where the 2 species co-occur or represent a large proportion of the trapped flies.

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