

# Addition of Ammonium Acetate to Protein-Borax Baited Traps Does Not Improve Attraction of *Anastrepha obliqua* or *Anastrepha serpentina* (Diptera: Tephritidae)

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## Abstract

Ammonia is considered a key component in the attraction of tephritid flies to protein-based lures. The addition of ammonium acetate to improve hydrolyzed protein-borax mixtures used to monitor tephritids has not been evaluated, although it has improved attraction to toxic baits. The presence of ammonium acetate crystals in a ventilated tube inside a trap containing hydrolyzed protein + borax, did not improve the capture of *Anastrepha obliqua* or *Anastrepha serpentina* flies in field experiments when compared with hydrolyzed protein + borax alone. In contrast, the addition of 1% ammonium acetate into the drowning solution of a hydrolyzed protein + borax mixture resulted in significantly reduced captures of both pests. Laboratory tests indicated that the emission of ammonia gas was increased 1.6–4.5-fold from traps that included ammonium acetate. These results confirm the hypothesis that a higher release of ammonia does not improve the attraction of tephritids when protein-derived odors are also present.

**Key words:** *Anastrepha*, hydrolyzed protein, ammonia, attractant

The West Indian fruit fly, *Anastrepha obliqua* Macquart, is an important pest of mango, *Mangifera indica* L. and tropical plum, *Spondias* spp., whereas the sapote fruit fly, *Anastrepha serpentina* (Wiedemann), (Diptera: Tephritidae) is a pest of sapodilla, *Manilkara zapota* (L.) P. Royen and other tropical fruits (Hernández-Ortiz and Aluja 1993). Liquid hydrolyzed proteins and ammonium salts are currently recommended as lures to monitor *Anastrepha* spp. (IAEA 2018). Ammonia, a by-product of protein degradation, is known to modulate the response of tephritid flies to food-based attractants (Mazor 1987, Flath et al. 1989, Bateman and Morton 1981). Fruit fly species, the fly's physiological state, and the protein source may interact with fly's response to compounds that release ammonia (Díaz Fleischer et al. 2014; Piñero et al. 2011; Piñero et al. 2017). Ammonium acetate is the most widely used ammonium salt for attraction of *Anastrepha* (Heath et al. 1995, Robacker et al. 1996), but fly responses to this compound can be highly variable among different *Anastrepha* species (Thomas et al. 2008). Combinations of ammonium acetate and yeast or protein hydrolysate were more attractive than protein hydrolysates alone for *Rhagoletis pomonella* (Walsh) (Diptera: Tephritidae), *Rhagoletis fausta* (Osten Sacken) (Diptera: Tephritidae), *Rhagoletis cingulata* (Loew) (Diptera: Tephritidae) (Moore 1969; Reissig 1974, 1976), and *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) (Gothilf and Levin 1989, Piñero et al. 2015). The addition of ammonium acetate to GF120<sup>®</sup>, a bait formulation of spinosad, also increased fly attraction and feeding on droplets offered to *R. pomonella*

(Yee 2007), *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), and *Zeugodacus cucurbitae* (Coquillett) (Diptera: Tephritidae) (Piñero et al. 2011).

Recently, we reported that in the absence of other stimuli, ammonia was highly attractive for *A. obliqua*, but the number of flies captured in traps baited with mixtures of hydrolyzed protein + borax was not correlated with the amount of ammonia released from the trap (Lasa and Williams 2021). Protein-borax mixtures have been extensively used for monitoring tephritids, not only because borax preserves trapped flies for later identification (Lopez and Becerril 1967), but also because it raises the pH of the mixture, resulting in an increased release of ammonia (Epsky et al. 2014).

Despite its apparent value as a component of tephritid attractants, the effect of adding ammonium acetate to protein-borax mixtures has not been evaluated in any species of tephritid, with the aim of improving fly attraction to monitoring traps. Consequently, the present study evaluated the attraction of *A. obliqua* and *A. serpentina* to hydrolyzed protein + borax enriched with ammonium acetate in solution, or as separate crystals placed within the same trap.

## Material and Methods

### Traps, Attractants, and Experimental Sites

A bottle-shaped MS2 trap (Fitozoosanitaria SA de CV, Texcoco, Mexico), of 250 ml capacity, was used in all experiments. An identical

trap was modified to allow the introduction of a 15 ml polyethylene centrifuge tube attached to the lid inside the trap. The tube contained crystals of ammonium acetate and had a pair of 2 cm<sup>2</sup> rectangular openings located 2 cm below the screwcap. These openings were covered with 0.2 mm nylon mesh to allow ammonia to escape into the trap headspace and to prevent flies from entering the tube. Preliminary tests revealed that both types of traps capture similar numbers of flies when loaded with Captor + borax alone without ammonium acetate crystals. The chemically hydrolyzed protein Captor 300 (Química Lucava, Celaya, Mexico), borax (J.T. Baker, Mexico City, Mexico), and ammonium acetate (98% purity; Sigma Aldrich, Mexico City) were used in experiments. A preparation of 4% (vol/vol) Captor + 2% (wt/vol) borax was used for all experiments (Anonymous 1999). The first two experiments were performed during May–July 2016 in a mixed mango and tropical plum orchard mainly infested with *A. obliqua*, near Jalcomulco, Veracruz, Mexico (19°19'40" N, 96°45'26" W, 340 m altitude), whereas the third experiment was performed in June 2017 in a mixed sapodilla and mango orchard mainly infested with *A. serpentina* close to Apazapan, Veracruz (19°19'35" N, 96°43'23" W, 330 m altitude). The study site has a subtropical humid climate characterized by a mean temperature of 25°C (range 21–29 °C) and high mean relative humidity of 82% (range 65–100%) during the months of the experiments.

#### Attraction to Captor + Borax with Ammonium Acetate

In the first experiment, 250 ml Captor + borax was compared with 250 ml Captor + borax plus 2.5 g of ammonium acetate mixed in the same drowning solution. This concentration (1%) of ammonium acetate was selected based on previous findings (Piñero et al. 2011, 2015). In the second experiment, Captor + borax was compared with Captor + borax plus 2.5 g of ammonium acetate crystals placed in the additional tube attached to the lid. A ~6 ha area of orchard was divided into six blocks for experiment 1 and four blocks in experiment 2. Each block had several large mango trees (8–10 m diameter canopy). Two MS2 bottle traps baited with 250 ml of each attractant were hung from mango trees in each block a height of 3.5–4 m and with 10–12 m between adjacent traps within a block. Blocks were separated by 20–30 m. Following International Atomic Energy Agency guidelines (FAO/IAEA 2018), for experiment 1, traps were inspected and switched in position at 4-d intervals during a 16-d evaluation period i.e., each trap was sampled twice at each position within each block. Lures were renewed after 8 days. For experiment 2, traps were inspected and rotated in position every 7 d during a 4-wk evaluation period (twice at each position), in line with IAEA guidelines (FAO/IAEA 2018). In this case, lures were renewed weekly. At each inspection captured flies were preserved in 70% ethanol and were counted, sexed, and identified to species in the laboratory.

The third experiment in Apazapan evaluated the attraction of *A. serpentina* to each of three treatments: i) Captor + borax as the reference attractant, ii) Captor + borax plus 2.5 g ammonium acetate mixed with 250 ml of the drowning solution and, iii) Captor + borax plus 2.5 g of ammonium acetate crystals in the tube attached inside the trap. Baited traps were placed in four blocks in sapodilla trees at heights and distances similar to those of experiments 1 and 2. Traps were inspected and rotated in position every 7 d over a 3-wk period (sampled once at each position within the block). Attractants were replaced at each inspection. Captured flies were preserved, counted, sexed, and identified to species in the laboratory.

#### pH and Emission of Ammonia Under Laboratory Conditions

Attractants were prepared for pH determination and ammonia quantification after 24 h and 7 d under laboratory conditions. The pH was measured using a calibrated pH meter (Jenco Instruments, San Diego, CA) at 10 min after attractants had been prepared. The release of ammonia gas from traps containing 250 ml of attractant was quantified by placing each trap in a 5-l glass jar through which filtered air was pumped. Ammonia in the exhaust gas was trapped in a 10 ml tube of water at 24°C as described previously (Lasa and Williams 2021). The quantity of trapped ammonia was estimated through its reaction with Nessler reagent, quantified using an ammonia medium-range photometer (HI97715 model, Hanna Instruments Inc., Woonsocket, RI), and expressed in micrograms of ammonia per hour (Lasa and Williams 2021). Four replicate samples of each attractant were measured using this technique.

#### Statistical Analyses

Numbers of flies captured were transformed to flies per trap per day (FTD). The effects of attractants on mean FTD values and mean percentage of females were compared by paired *t*-test for *A. obliqua*. For *A. serpentina*, FTD values were subjected to one-way analysis of variance (ANOVA) and Tukey mean comparisons, whereas the percentage of females was subjected to Kruskal Wallis test and means compared by Dwass-Steel-Critchlow-Fligner (DSCF) pairwise comparisons. Quantities of ammonia released from traps were normalized by log transformation and compared by two-way ANOVA and Tukey test, with attractant and age (24 h and 7 d) as factors. All analyses were performed using the R-based software Jamovi v.1.0.7.0 (Jamovi 2021).

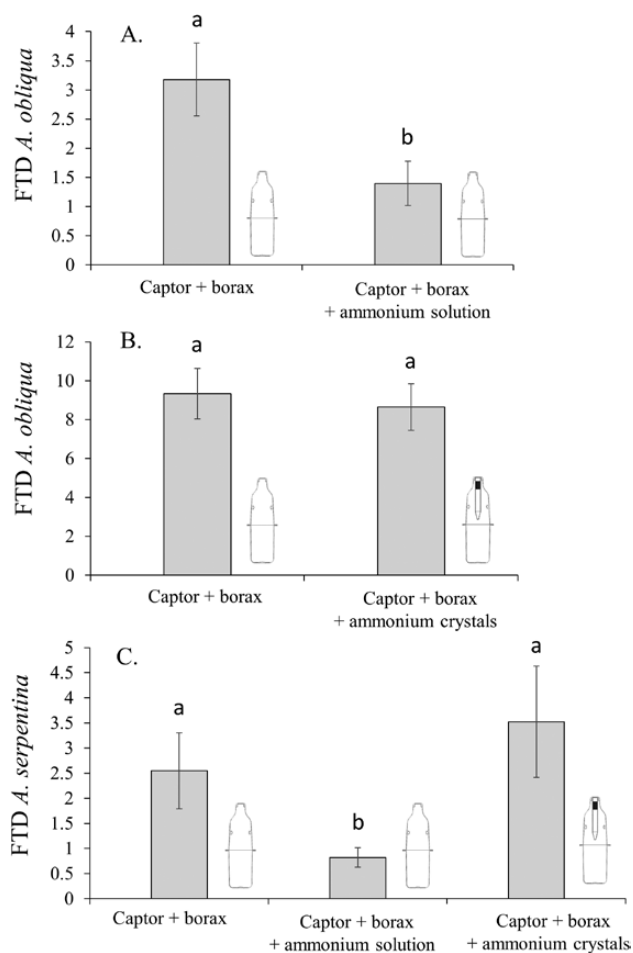
## Results

#### Attraction to Captor + Borax with Ammonium Acetate

In total 488 *Anastrepha* flies were collected in experiment 1, of which 90% were *A. obliqua* and small numbers of *A. serpentina* (9%) and *Anastrepha ludens* (1%) that were not included in the analyses. Captor + borax captured significantly more *A. obliqua* flies than Captor + borax plus 2.5 g of ammonium acetate in solution ( $t = 2.51$ ;  $df = 23$ ;  $P = 0.019$ ) (Fig. 1A). The mean percentage of females varied between 48% and 56% and was similar for both attractants ( $t = 0.599$ ;  $df = 16$ ;  $P = 0.558$ ).

In experiment 2, a total of 2,076 *Anastrepha* flies were collected, of which 97% *A. obliqua* and 3% were *A. serpentina* that were not included in the analyses. FTD values of *A. obliqua* flies were similar for Captor + borax and Captor + borax plus 2.5 g of ammonium acetate crystals in the interior tube ( $t = 0.440$ ;  $df = 15$ ;  $P = 0.666$ ) (Fig. 1B). The mean percentage of females varied between 69% and 72% and was similar for both lures ( $t = 0.887$ ;  $df = 15$ ;  $P = 0.389$ ).

A total of 709 *Anastrepha* flies were captured in experiment 3, of which 81.7% were *A. serpentina* and lower numbers of *A. obliqua* (17.6%) and *A. ludens* (0.7%) that were not included in the analyses. FTD values varied significantly among treatments (Kruskal-Wallis  $H = 7.22$ ;  $df = 2$ ;  $P = 0.027$ ) (Fig. 1C). Traps baited with Captor + borax captured similar numbers of *A. serpentina* flies as traps containing Captor + borax plus 2.5 g of ammonium acetate crystals in the interior tube, whereas FTD values were significantly lower in the traps containing Captor + borax plus 2.5 g of ammonium acetate in solution. The mean percentage of females (46–64%) was similar among all attractants (Kruskal-Wallis  $H = 3.15$ ;  $df = 2$ ;  $P = 0.207$ ).



**Fig. 1.** Mean FTD ( $\pm$  SE) values of (A) *Anastrepha obliqua* captured in traps baited with Captor + borax or Captor + borax with the addition of ammonium acetate in the drowning solution in experiment 1 and (B) Captor + borax with the addition of ammonium acetate crystals in an interior ventilated tube in experiment 2. (C) mean FTD ( $\pm$  SE) values of *Anastrepha serpentina* captured in traps baited with Captor + borax alone or with the addition of ammonium acetate in solution or as crystals inside a ventilated tube in experiment 3.

### pH and Emission of Ammonia Under Laboratory Conditions

The pH of attractants was pH 6.7 for Captor alone which increased to pH 9.0 by the addition of borax or pH 8.5 in mixtures with borax + 2.5 g of ammonium acetate in solution (Table 1).

Traps containing Captor alone released trace quantities of ammonia (0.6–3.7  $\mu\text{g}/\text{h}$ ) and were not included in statistical analyses (Table 1). The quantities of ammonia released from traps containing Captor + borax or Captor + borax with ammonium acetate in solution or as crystals, differed significantly ( $F = 98.3$ ;  $df = 2, 18$ ;  $P < 0.001$ ). Traps containing Captor + borax released an average of  $26.8 \pm 1.2 \mu\text{g}/\text{h}$  of ammonia at 24 h after preparation and this increased 4.5-fold and 1.6-fold by the addition of ammonium acetate in solution or as crystals in the interior tube, respectively (Table 1). After ageing for 7 d in the laboratory the release of ammonia had fallen significantly in all treatments ( $F = 45.9$ ;  $df = 1, 18$ ;  $P < 0.001$ ). Traps containing Captor + borax released an average of  $19.4 \pm 1.4 \mu\text{g}/\text{h}$  of ammonia after 7 d whereas traps with added ammonium acetate added in solution or as separate crystals released 2.8-fold and 1.6-fold higher quantities of ammonia, respectively, after 7 d (Table 1).

**Table 1.** Attractant pH of the drowning solution and mean ( $\pm$  SE) release rate of ammonia from individual traps baited with Captor, Captor + borax with ammonium acetate in solution, or as separate crystals in an interior ventilated tube

Captor mixture	pH	Ammonia release rate ( $\mu\text{g}/\text{h}$ )	
		24 h	7 d
Captor alone	6.7	$0.6 \pm 0.2$	$3.7 \pm 0.8$
Captor + borax	9.0	$26.8 \pm 1.2$ a	$19.4 \pm 1.4$ a
Captor + borax + ammonium solution	8.5	$122.4 \pm 9.9$ c	$55.0 \pm 8.6$ b
Captor + borax + ammonium crystals	9.0	$44.2 \pm 3.2$ b	$31.1 \pm 0.9$ c

Traps were evaluated after 24 h or 7 d in the laboratory. Means in columns followed by a different letter were significantly different (Tukey test,  $P > 0.05$ ). Captor alone was not included in the statistical analysis.

### Discussion

The release of semiochemicals from foods plays a pivotal role in the location and utilization of nutrition resources by tephritid flies. Ammonia is considered to be a key component of semiochemical profiles for these insects (Mazor 1987, Flath et al. 1989, Bateman and Morton 1981, Epsky et al. 2014). However, the interaction of ammonia with other attractant constituents is poorly understood, even in the case of the hydrolyzed protein-borax mixtures that are widely used to trap these pests (Lasa and Williams 2021).

The capture of *A. obliqua* and *A. serpentina* flies using Captor + borax, with or without the addition of ammonium acetate, was not related to the quantity of ammonia released by the traps. However, the capture of flies differed depending on the form in which ammonium acetate was present in the trap. Fewer flies of both species were captured in Captor + borax baited traps in which ammonium acetate was added to the drowning solution, although this resulted in a 4.5-fold increase in the quantity of ammonia released at 24 h, which averaged  $122.4 \pm 9.9 \mu\text{g}/\text{h}$ . This concentration of ammonia was previously found to be highly attractive to *A. obliqua* under laboratory conditions in the absence of food-based attractants, although different responses were observed in field experiments (Lasa and Williams 2021), a finding that stimulated the present study.

In contrast, when ammonium acetate was present as crystals inside the trap, no significant differences were observed in the capture of *A. obliqua* and *A. serpentina* flies compared to Captor + borax alone, despite that traps containing ammonium acetate crystals released ~1.6-fold more ammonia than the Captor + borax treatment. In previous studies, a similar mean capture of *A. obliqua* was observed in traps baited with the ammonium acetate-based attractant Biolure 2C than in Captor + borax baited traps (Lasa and Cruz 2014), but not so for *A. serpentina*, which was more frequently captured in traps containing Biolure 2C than in Captor + borax baited traps (Rodríguez et al. 2015). In the present study, traps containing ammonium acetate crystals with Captor + borax had slightly increased captures of *A. serpentina* (Fig. 1C) compared to Captor + borax alone, but this was not statistically significant, probably due to the variable response of flies to both types of attractants.

The attraction of *Ceratitis*, *Bactrocera*, and *Zeugodacus* species to proteinaceous and non-proteinaceous substances was studied by Piñero et al. (2020), with and without the addition of ammonium acetate. These authors observed that ammonia tended to be less attractive to tephritids when protein-derived odors were also present. Their results may not be directly comparable with ours, however, as they offered the mixture in

droplets placed on plastic lids that were hung in laboratory cages for a short period (20 min), a methodology quite different from our use of traps. The release of elevated quantities of ammonia from traps was repulsive for *C. capitata* (Mazor et al. 2002). Indeed, a previous study noted that pure ammonia gas released from traps was attractive for *A. obliqua* in the absence of protein-derived odors, but this attraction was not observed in the presence of a range of food-based odor sources, including torula yeast, a selection of hydrolyzed protein products, and several commercial attractants (Lasa and Williams 2021). The findings of the present study, therefore, suggest that protein-based odors eliminate or overwhelm fly attraction to ammonia, at least in the case of *A. obliqua* and *A. serpentina*. The results of the present study also suggest that the addition of ammonium acetate to the drowning solution can significantly reduce fly captures, possibly due to an unknown chemical interaction with borax or hydrolyzed proteins that has not been previously reported. Similarly, the presence of ammonium acetate crystals inside Captor + borax baited traps did not improve fly captures for either *A. obliqua* or *A. serpentina*. To our knowledge, this is the first study to evaluate the incorporation of ammonium acetate into protein-borax mixtures used in monitoring traps. Although attraction appears to vary across species, these findings reinforce previous observations that have indicated that the amount of ammonia released by food-based substances is less influential on the attraction of tephritid flies when other protein-derived odors are also present (Piñero et al. 2020; Lasa and Williams 2021). Additional studies on protein-derived semiochemicals and their interaction with ammonia could lead to improved attractants for trap-based monitoring of tephritid pests.

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## Author Contributions

Conceptualization, R.L. and T.W.; methodology, R.L.; formal analysis, R.L.; investigation, R.L. and T.W.; data curation, R.L.; writing—original draft, R.L.; writing—review and editing, T.W. and R.L.; project administration, R.L.; funding acquisition, R.L. Authors have read and agreed to the published version of the manuscript.

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