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Alkaline hydrolyzed torula yeast as an attractant for *Anastrepha obliqua* (Diptera: Tephritidae)

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Abstract

Acid hydrolyzed proteins with borax and torula yeast pellets are attractants commonly used for monitoring the West Indian fruit fly, *Anastrepha obliqua* (Macquart). Alkaline hydrolysis of proteins has been poorly studied as a source of attractants for tephritids, despite it increasing the production of ammonia, a known attractant for *A. obliqua* and other tephritid flies. Pairwise laboratory experiments revealed a significantly greater response of flies to torula yeast suspension alkalized with NaOH at pH 10.3, 11.3, 12.3 and 13.3 compared to torula yeast + borax suspension at pH 9.15. Traps baited with alkalized torula yeast at pH 13.3 captured significantly higher numbers of flies than torula yeast at pH 10.3, 11.3 or 12.3. The emission of ammonia from these traps was positively correlated with pH. *Anastrepha obliqua* was less attracted to torula yeast at pH 13.3 when alkalized with KOH than with NaOH, although no significant differences were detected in the emission of ammonia. In mango orchards in Mexico, the mean number of *A. obliqua* flies per trap per day was higher for traps baited with torula yeast at pH 13.3 than for acid hydrolyzed protein + borax or torula yeast pellets containing borax. While ammonia emissions from torula yeast at pH 13.3 and acid hydrolyzed protein + borax were reduced after one week in the field, the ammonia emission of torula yeast pellets increased after one week to levels similar to those of the other attractants. Alkaline hydrolysis of torula yeast at pH 13.3 increased the release of ammonia, reduced the surface tension of the liquid and proved more effective at capturing *A. obliqua* flies in laboratory cages and mango orchards when compared with standard attractants. This preparation is simple, cheap, and stable and could find application in monitoring programs targeted at *A. obliqua*.

Keywords: food attractants, hydrolyzed protein, pest monitoring, ammonia, pH

1 INTRODUCTION

Anastrepha fruit flies are major agricultural pests in the Americas (White & Elson-Harris, 1992). The West Indian fruit fly, *Anastrepha obliqua* (Macquart) (Diptera: Tephritidae), is an important pest of mango, *Mangifera indica* L., that threatens the commercialization and export of those fruits (Hernández-Ortiz & Aluja, 1993). It is also considered a potential threat to crops in tropical and subtropical regions elsewhere, such as southern Asia and northeastern Australia (Flu et al., 2014).

Food attractants have been used in surveillance programs targeted at *Anastrepha* fruit flies for over eight decades (Epsky, Kendra & Schnell, 2014). Food lures, however, are considered to be weak attractants and elicit highly variable fly responses, depending on the type and age of the attractant and also vary across tephritid species, crops, and ambient conditions (Malo, 1992; Epsky et al., 1993; Epsky, Kendra & Heath, 2004). Nonetheless,

food lures are the only effective attractants for *Anastrepha* spp. because efficient host-based attractants or pheromonal attractants have not yet been developed (Epsky et al., 2014).

Acid hydrolyzed proteins are cheap, widely available from different sources in the food industry and have been standardized for use in traps to monitor *Anastrepha* spp. (Epsky et al., 2014). Borax (sodium tetraborate) is mixed with hydrolyzed protein to increase the stability of the mixture, avoid the decomposition of the captured flies, and reduce the attraction of non-target insects (López & Becerril, 1967). The addition of borax also increases the pH of the mixture resulting in an increased release of ammonia (Bateman & Morton, 1981; Mazor, Gothilf, & Galun, 1987; Flath, Matsumoto, Binder, Cunningham, & Mon 1989) and an increased capture of some tephritid species (Epsky et al., 1993; Heath et al., 1994).

Ammonia is considered a key attractant for tephritids, but several studies have revealed that attraction to protein-based lures of alkaline pH is not attributable solely to the release of ammonia (Bateman & Morton, 1981; Mazor et al., 1987; Flath et al., 1989; Matsumoto, Buttery, Flath, Mon, & Teranishi, 1985). Indeed, other volatile compounds released following the alkalization of amino acids, peptides or proteins appear to interact with low concentrations of ammonia, resulting in mixtures that are highly attractive to adult tephritids (Piñero, Souder, & Vargas, 2020; Lasa & Williams, 2021).

Torula yeast, *Candida utilis*, is a by-product of lignocellulosic wastes from the paper industry. It is rich in protein with a balanced amino acid composition that makes it a cheap dietary supplement for animal feed (Reed & Nagodawithana, 1991). Early studies revealed that mixtures of torula yeast and borax increased trap captures of the Caribbean fruit fly, *Anastrepha suspensa* Loew, compared to traps baited with mixtures of borax and enzymatically hydrolyzed proteins from maize or cotton seed (López, Steiner, & Holbrook, 1971), even when pelletized (López, Spishakoff, & Hernández, 1968). Since then, pelleted combinations of torula yeast + borax (pH ~9.15) have been widely used as an attractant for several tephritid species, often with similar or higher captures than observed with acid hydrolyzed proteins (Epsky et al., 1993). Consequently, pellets of autolyzed torula yeast + borax, acid hydrolyzed proteins + borax and some enzymatically hydrolyzed proteins are now widely used and recommended by international agencies for surveillance programs targeted at *Anastrepha* spp. (Burditt, 1982; IAEA, 2003; Epsky et al., 2004; IAEA, 2007).

Despite the wide use of torula yeast + borax pellets, and evidence that increased pH improves the capture of *A. obliqua* (Lasa & Williams, 2021), a systematic evaluation of the effects of alkaline hydrolysis of torula yeast on tephritid attraction has not been performed. Increased alkalization of torula yeast could potentially lead to the release of higher concentrations of ammonia and other products of hydrolysis that may favor the attraction of *Anastrepha* spp.

The present study compared the attraction and capture of *A. obliqua* flies of both sexes to traps baited with torula yeast at different pHs using borax, sodium hydroxide and potassium hydroxide as alkalization agents under laboratory conditions. Field experiments were then performed to compare captures of *A. obliqua* in traps baited with alkalized torula yeast, acid hydrolyzed protein + borax or torula yeast pellets. Fly responses to traps and the release of ammonia by these attractants were also compared in both laboratory and field conditions.

2 MATERIALS AND METHODS

2.1 Attractants and traps

Inactive powdered torula yeast (Lake States type B, Lallemand Inc., Montreal, Canada), (49% protein) was used for experiments. This type of torula yeast is used in Mexico to mass-rear *Anastrepha* spp. for pest control programs involving the sterile insect technique. Its amino acid composition has been described previously (Hernández et al., 2016).

The acid hydrolyzed protein, Captor 300 (Promotora Agropecuaria Universal, Mexico City, Mexico), was included for comparison as this hydrolyzed protein is also widely used to monitor *Anastrepha* spp. (Lasa & Cruz, 2014). Captor was prepared by mixing 10 ml of Captor 300 with 5 g of borax (J. T. Baker, Mexico City) and 235 ml of water as indicated by the Mexican phytosanitary authorities for this type of fruit fly lure (Anonymous 1999). Adjustments of pH were performed using sodium hydroxide pellets (98% NaOH, Golden Bell Products Inc., Orange, CA, USA) or potassium hydroxide pellets (98% KOH, Consorcio Químico Industrial, Ecatepec de Morelos, Mexico). To reduce variability, all experimental suspensions were prepared using bottled drinking water (pH 7.1 - 7.3) (Xallapan, Grupo Doso SA de CV, Xalapa, Mexico). The pH was measured for at least seven samples of each attractant.

For experiments, bottle-shaped MS2® traps of 600 ml capacity were used (Fitozoosanitaria SA de CV, Texcoco, Mexico). Traps consisted of a yellow base cup (250 ml) with a transparent upper section that had three 11 mm diameter circular holes spaced 5 cm apart at two-thirds of the height of the upper section, through which flies enter the trap.

2.2 Laboratory rearing of *Anastrepha obliqua*

A colony of *A. obliqua* was reared at the Instituto de Ecología AC, Xalapa, Veracruz, Mexico. The colony was started in 2019 using pupae collected from tropical plum, *Spondias mombim* L., close to Tuzamapan de Galeana, Veracruz (19°25'4" N; 96°52'18" W, 957 m altitude). Twice weekly, groups of 200 pupae were placed in plastic cups (200 ml capacity) containing vermiculite moistened with 0.3% (w/v) sodium benzoate. Adults of both sexes emerged inside 60 x 60 x 90 cm mesh-covered cages maintained at 24 ± 1 °C, 60 ± 10 % relative humidity (RH) and 12:12 h (L:D) photoperiod. When flies were 2-5

weeks old, groups of mated females (~40 individuals) together with ~10 males were placed in acrylic cages of 30 x 30 x 30 cm containing four green mangoes var. Manila for oviposition. Following larval development, pupae were collected for the following cycle of insect production. For experiments, 7-10 day-old flies of both sexes were collected from emergence cages and were assumed to be sexually mature but unmated. All adults were provided with continuous access to torula yeast, sugar, and water before their use in experiments.

2.3 Attraction of *A. obliqua* to torula yeast at different pHs

In a first set of experiments, five pairwise comparisons were performed to assess the attraction of *A. obliqua* flies to torula yeast hydrolyzed with NaOH or borax. A reference mixture was prepared comprising 2.75% (wt/vol) torula yeast + 2.25% (wt/vol) borax, i.e. the composition that results from the dilution of three torula yeast pellets (each 5 g in weight) in 300 ml water following manufacturer guidelines (https://www.betterworldmanufacturing.com/atrayentes). The pH of this preparation was approximately 9.15 (range 9.12-9.19).

A similar concentration of torula yeast (2.75% wt/vol) was alkalized using NaOH pellets to one of five different pHs: 9.15, 10.3, 11.3, 12.3 and 13.3, measured using a calibrated laboratory pH meter (Hanna HI 2211 benchtop pH meter, Hanna Instruments Ltd., Leighton Buzzard, UK). All torula yeast mixtures were prepared in the laboratory and hydrolysis occurred during agitation on a magnetic stirrer (Stable Temp, Cole-Parmer, IL, USA) at 320 rpm for 20-24 h prior to use. The pH of torula yeast + borax remained stable (<1% variation) after 20-24 h of agitation, whereas the suspension of torula yeast + NaOH (pH 9.15) fell to less than pH 7 after 24 h of agitation, so the pH of that preparation was adjusted back to pH 9.15 by the addition of NaOH prior to use in cage experiments. Little variation (1-3%) in pH was observed following 24 h of agitation for the torula yeast + NaOH mixtures at pH 10.3, 11.3, 12.3 or 13.3, so these preparations were not adjusted prior to use. These preparations were then used immediately in cage experiments.

The attraction of flies to traps was evaluated in pairwise comparisons using an MS2 trap loaded with a 250 ml suspension of torula yeast + NaOH at a specific pH, and a similar trap baited with a 250 ml suspension of torula yeast + borax (pH 9.15) as the reference treatment. Traps were randomly assigned to opposite sides (50 cm distance between traps) of a mesh cage of 90 x 60 x 60 cm and hung at a height of 60 cm i.e., 30 cm below the top of the cage. The position of traps was subsequently switched for each new replicate. A group of 20 female and 20 male non-starved flies of 7-10 days old was released inside the cage. All tests were performed under the same conditions used to rear the laboratory insect colony. Illumination was provided by two 10 w LED strips (Miled10 6500k, Megamex

SAPI de CV, Guadalajara, Mexico) placed at 2 cm above the cage. The light intensity in the center of the cage was 940-980 lux measured using a light meter (YK-10LX, Luton, Taipei, Taiwan). Flies captured in traps were collected 23 h later, counted and sorted by sex. Uncaptured flies inside the cage were discarded. Traps were used once and then washed and reloaded for the subsequent replicate. Each test was performed simultaneously in four independent cages and traps were evaluated at both positions within each cage, giving a total of eight replicates for each pairwise comparison.

In a second experiment, the capture of *A. obliqua* flies in traps baited with torula yeast alkalized to one of four different pHs (10.3, 11.3, 12.3 and 13.3) using NaOH was compared in a caged choice experiment. Torula yeast suspensions at each pH were prepared the day before and hydrolyzed by agitation for 20-24 h, as mentioned in the first experiment. Four MS2 traps loaded with 250 ml torula yeast suspension (2.75% wt/vol) + NaOH at each pH value were assigned to random positions at each corner (50 cm distance between traps) of a 90 x 60 x 60 cm mesh cage and hung at a height of 60 cm. A group of 20 female and 20 male non-starved 7-10-day-old flies was released inside each cage. All tests were performed under the same laboratory conditions as the first experiment. Flies captured in traps were collected 23 h later, counted and sorted by sex. Uncaptured flies inside the cage were discarded. Traps were used once and then washed and reloaded for the subsequent replicate. The position of each trap was switched to the next position for each new replicate. The experiment was performed simultaneously in four independent cages and traps were evaluated at each of the four positions within each cage, giving sixteen replicates in total.

2.4 Comparison of torula yeast hydrolyzed with NaOH or KOH

Due to the high attraction of flies to alkalized torula yeast in the previous experiments, a pairwise comparison was performed for 2.75% (wt/vol) torula yeast suspension adjusted to pH 13.3 using NaOH or KOH pellets. Each suspension was hydrolyzed for a 24 h period prior to use. Two MS2 traps loaded with 250 ml of torula yeast suspension + NaOH or torula yeast suspension + KOH, were then randomly assigned to opposite sides (50 cm distance) of a mesh cage (90 x 60 x 60 cm) and hung at a height of 60 cm. The position of each trap was subsequently switched for each new replicate. A group of 20 females and 20 males non-starved 7-10-day-old flies was released inside each cage. All tests were performed under the same conditions and same collection regime as the previous experiments. Each test was performed simultaneously in four independent cages and traps were evaluated at both positions within each cage, giving sixteen replicates in total.

2.5 Determination of ammonia released by traps

The release of ammonia from attractants was determined using the equipment and methods described previously (Lasa & Williams, 2021). The apparatus comprised a plastic bottle (500 ml capacity, with three 11 mm diam. holes at two-thirds the height of the bottle) placed inside a 5-liter opaque glass jar (255 mm height, 140 mm diam.). The bottle was loaded with 250 ml of each attractant. Air, at a flow rate of 150 ml/min, passed through the glass jar to collect ammonia gas which was then dissolved in a glass water trap containing 10 ml distilled water. The concentration of ammonium (NH^{4+}) ions in the water trap was quantified by reaction with potassium tetraiodomercurate (II) (Nessler's reagent) This reaction results in the production of a bright yellow compound the intensity of which was quantified using an ammonia medium range photometer (Hanna Instruments Inc., Woonsocket, RI, USA). Ammonia quantification was performed under laboratory conditions at 24 ± 1 °C, following the manufacturer's recommendations. The duration of the capture time ranged from 1 to 2 h, which resulted in concentrations between 0.3 and 3 mg/l of ammonia in the distilled water trap that fell within the accuracy range of the ammonia photometer. The final quantity of ammonia was calculated in terms of micrograms of ammonia per hour (µg/h). Five replicate collections and ammonia determinations were performed for each attractant.

2.6 Attraction to alkalized torula yeast under field conditions

Field experiments with three different attractants were performed between June and July 2021, in mango orchards close to the village of Jalcomulco, Veracruz, Mexico. The orchard used for experiment 1 (19°19’40.57” N; 96°45’27.77” W, altitude 335 m) was a distance of 2.5 km from the orchard used for experiment 2 (19°20’43.65” N; 96°46’16.51” W, altitude 422 m). Both orchards comprised large (7-8 m height) mature mango trees. MS2 traps were loaded with one of three different attractants: i) captor + borax, ii) torula yeast pellets containing borax (three pellets in 300 ml water, Better World Manufacturing Mexico SA de CV, Mexico City) and iii) 2.75% torula yeast suspension hydrolyzed with NaOH to pH 13.3 for 24 h prior to use. The captor + borax mixture and torula yeast pellets were prepared 2-3 h before use in the field. Samples of these attractants were taken and tested for ammonia release at 24 h after preparation. Traps were baited with 250 ml of each attractant and were hung at a height of 3-4 m on mango trees separated by a distance of 8-10 m between traps. Four blocks of approximately 25 x 25 m, at least 15-20 m apart, were considered within a 2 Ha area of each orchard. Traps were randomly assigned to initial positions within each block and were rotated clockwise at weekly intervals to control for positional effects. At each weekly sample, insects captured in each trap were placed in 70% alcohol and taken to the laboratory. Weekly samples of captured flies were counted, identified to species, and sorted by sex in the laboratory. The experiment lasted six weeks

and all the traps were sampled twice at each position within each block. At each weekly sample the attractant was replaced with fresh attractant. In the first experiment, just after flies were recovered, the liquid of each attractant within a block was collected from each trap independently and taken to the laboratory in plastic bottles to quantify ammonia emissions. Measurements of ammonia emission were performed on a total of six replicates of each attractant following 7 d of deployment in the field. The mean daily temperature and relative humidity of orchard area was registered with a data logger (Hobo Onset UX100) that was placed in a mango tree at 3 m height near the center of the orchard of experiment 1.

2.7 Surface tension of attractants

During laboratory experiments it was observed that flies trapped in alkalinized torula yeast at pH 13.3 sank to the bottom of the trap, whereas flies in liquids at other pH values tended to float. To explore this phenomenon the surface tension of attractants was determined using Traube's stalagmometer technique (Lasa & Williams, 2017). Samples of 500 ml of water and liquid protein attractants were prepared and measured in relation to distilled water at 20 °C that has a surface tension of 72.8 dyne/cm (Findlay, 1945). A Traube's stalagmometer (Alamo, Madrid, Spain) was mounted vertically, filled with the sample liquid and the number of drops released between two marked positions was counted. Surface tension was obtained following the formula: surface tension of protein lure, ST1 = ST2 ($n2/n1$) × (d1/d2), where ST2 is the surface tension of distilled water at 20 °C, $n1$ and $n2$ are the number of drops produced by protein lure and distilled water, respectively, and d1 and d2 are the densities at 20 °C of protein lure and distilled water, respectively. The density of five replicate samples of 1 ml of each protein lure was measured using a micropipette and a precision electronic balance (Ohaus Explorer, Ohaus, New Jersey, USA) to an accuracy of 0.1 mg. The mean surface tension of each protein attractant was then calculated.

2.8 Statistical analysis

Mean numbers of *A. obliqua* flies and the percentage of females captured in traps loaded with torula yeast + borax or torula yeast + NaOH at different pHs in the laboratory were compared by paired t-test. Under laboratory choice experiments, the numbers of *A. obliqua* flies of each sex were $\sqrt{(x + 0.5)}$ transformed to stabilize variance and compared by analysis of covariance with pH as a continuous covariable. Fly captures were then pooled across sexes, $\sqrt{(x + 0.5)}$ transformed and subjected to linear regression of numbers of trapped flies with torula yeast + NaOH at different pH values. The mean numbers of trapped *A. obliqua* flies and percentage of females captured in traps containing torula yeast alkalized with

NaOH or KOH were compared by paired t-test. The numbers of *A. obliqua* flies captured per trap per day (FTD) in orchard experiments were compared among attractants by fitting a generalized lineal model (GLM) with a binomial error distribution (selected based on the Akaike information criterion value), followed by Bonferroni mean comparisons. Percentages of females captured were not normally distributed and were compared by non-parametric Kruskal-Wallis test. A log-linear regression was used to model ammonia releases from torula yeast alkalized at different pH values. Ammonia releases from torula yeast alkalized with NaOH or KOH were compared by t-test. Ammonia production from attractants at different evaluation times (24 h or 7 days), were compared by fitting a generalized linear model (GLM) with a quasi-Poisson error distribution to account for overdispersion in the data. The surface tension of attractants was analyzed by one-way analysis of variance followed by Tukey test. All analyses were performed using the R-based package Jamovi v.1.2.27.0 (Jamovi, 2020).

3 RESULTS

3.1 Attraction of *A. obliqua* to torula yeast + NaOH at different pHs

Under laboratory cage conditions, torula yeast + borax (pH 9.15) and torula yeast + NaOH at pH 9.15, captured similar mean numbers of flies, whereas significantly higher numbers of flies were captured using torula yeast + NaOH at pH 10.3, 11.3, 12.3 and 13.3 (p <0.02), compared to traps baited with torula yeast + borax (pH 9.15) (Figure 1). Similar percentages of females, between 39% and 67%, were captured in traps containing torula yeast + borax at pH 9.15 or torula yeast + NaOH at pH 9.15, 10.3, 11.3 and 13.3 (p >0.05). However, a significantly higher percentage of females (mean ± SE, 60 ± 5%) was caught in traps containing torula yeast + NaOH at pH 12.3 compared to the torula yeast + borax (pH 9.15) reference treatment (40 ± 5%) (p=0.014).

Under caged choice conditions (Figure 2A), trapped *A. obliqua* flies of both sexes were pooled as numbers captured at different pH values did not vary between sexes (F=0.014, df=1,125, p=0.906). Linear regression revealed that the number of trapped *A. obliqua* flies increased significantly with the pH of the preparation (F=28.6, df=1,62, p<0.001). The highest numbers captured were observed in traps baited with torula yeast + NaOH pH 13.3, and captures decreased steadily as the pH was reduced to pH 10.3. Considerable variation was present in fly captures and the linear regression model explained one-third of the observed variation (adjusted R^2 = 0.304) (Figure 2A).

The quantities of ammonia released from torula yeast increased exponentially with increasing pH (F=117, df=1,18, p<0.001), from 3.5 ± 0.5 µg/h at pH 10.3 to 28.2 ± 1.7 µg/h at pH 13.3 (Figure 2B). In comparison, the release of ammonia by 2.75% (wt/vol) torula yeast + 2.25% (wt/vol) borax mixture (pH 9.15) and torula yeast pellets (pH 9.15) was

similar, at 1.9 ± 0.3 µg/h and 1.8 ± 0.4 µg/h, respectively (t=0.245, df=8, *p*=0.813), both lower values than when torula yeast was alkalized with NaOH.

3.2 Comparison of torula yeast hydrolyzed with NaOH or KOH

Fly captures in traps containing torula yeast + NaOH at pH 13.3 were significantly higher than when torula yeast was alkalized using KOH at the same pH (*p*=0.004) (Figure 3A). The mean percentage of females captured was similar in each treatment (46-53% females; t=1.06, df=15, *p*=0.307). Despite differences in the capture of flies, the mean (±SE) quantities of ammonia released by torula yeast alkalized with KOH (23.6 ± 2.8 µg/h) and NaOH (28.2 ± 1.7 µg/h) were similar (*p*=0.199) (Figure 3B).

3.3 Attraction to alkalized torula yeast under field conditions

A total of 2953 *Anastrepha* spp. flies were collected in the first field experiment. Of these, 2493 flies (84.4%) were *A. obliqua* (1471 females and 1022 males), 451 individuals were of *A. serpentina* and 9 of *A. ludens*. Only *A. obliqua* flies were included in the analysis. The mean number of flies trapped per day (FTD) differed significantly among attractants (GLM: χ^2=31.73, df=2, *p*<0.001). The highest captures were registered in traps baited with torula yeast + NaOH at pH 13.3 with 38-58% fewer captures in traps baited with torula yeast pellets or Captor + borax (Figure 4A). The mean (±SE) percentage of females captured was significantly lower for torula yeast + NaOH at pH 13.3 with 56.7 ± 1.7% than for Captor + borax that captured 66.5 ± 2.6 % of females (Kruskal-Wallis H=9.73, df=2, *p*=0.008). Torula yeast pellets captured an intermediate percentage, 58.0 ± 2.3%, and did not differ significantly from either of the other attractants.

In the second field experiment, a total of 2670 *Anastrepha* spp. flies were trapped, of which 2539 flies (95.1%) were *A. obliqua* (1689 females and 850 males), 121 were *A. serpentina* and 10 were *A. ludens*. Only *A. obliqua* flies were included in the analysis. The mean number of flies per trap per day varied significantly among attractants (GLM: χ^2=7.53, df=2, *p*=0.023), with the highest FTD value in traps baited torula yeast + NaOH at pH 13.3, the lowest value in traps containing Captor + borax and an intermediate value in traps containing torula yeast pellets (Figure 4B). The mean percentage of females captured varied between 68.2% and 75.1% and did not vary significantly among attractants (Kruskal-Wallis H=5.45, df=2, *p*=0.066).

Significant differences were observed among the attractants of experiment 1 in the mean release of ammonia (GLM: χ^2=77.46, df=2, *p*<0.001) and the interaction of attractant*evaluation time (24 h and 7 d) (GLM: χ^2 =35.08, df=2, *p*<0.001) (Figure 4C). The quantities of ammonia released from the Captor + borax and torula yeast + NaOH pH 13.3

were reduced after a week of deployment in the field, whereas torula yeast pellets increased ammonia releases to a level similar to that of the other attractants (white boxes in Figure 4C).

The pH of torula yeast preparations was fairly stable with only slight reductions in the pH values of all attractants after 7 d in the field. Traps baited with captor + borax had an initial pH of 9.07 ± 0.02 that changed to 8.68 ± 0.05 after 7 days. The initial pH of the suspension of torula yeast pellets was 9.15 ± 0.02 which fell to 9.05 ± 0.02 after 7 days. The torula yeast + NaOH pH 13.3 suspension fell from 13.30 ± 0.02 to 13.19 ± 0.11 after 7 d in the field.

The mean temperature and relative humidity in the experimental area during the evaluation period was 25.7 ± 4.0 °C (daily temperature range 21 – 30 °C) and 83 ± 18% relative humidity. Rainfall occurred on 16 days during the six weeks of the experiments.

3.4 Surface tension of attractants

The surface tension of the suspension of torula yeast + NaOH at pH 13.3 was significantly lower than for any of the other attractants ($F=10.0$, $df=6,28$, $p<0.001$) (Table 1). The surface tension was similar for captor + borax, torula yeast pellets and torula yeast alkalized with NaOH at pHs of 9.15 to 12.3.

4 DISCUSSION

Laboratory and field experiments revealed that alkaline hydrolysis of torula yeast using NaOH at high pH was highly attractive to *A. obliqua* and resulted in higher numbers of captured flies than torula yeast hydrolyzed at lower pHs or standard torula yeast + borax pellets. Despite the many studies on food attractants for tephritid monitoring programs, many countries continue to use pellets of torula yeast + borax that have a pH of approximately 9.15 (FAO, 2006). These pellets were developed in the 1970's (López et al., 1971). The torula pellets have often been used for comparative studies of fruit fly attractants and have proved to be as attractive or more attractive than other food-based lures for species such as *A. obliqua* (Piñero, Aluja, Vázquez, Equihua, & Varón, 2003; Thomas et al., 2008), *A. suspensa* (Burditt 1982; Epsky et al., 1993), *Anastrepha ludens* Loew (Heath et al., 1994; Thomas & Robacker, 2006; Conway & Forrester, 2007; Mangan & Thomas, 2014), *Anastrepha serpentina* Wiedemann (Piñero et al., 2003), *Ceratitis capitata* (Heath et al., 1994; Shelly, Kurashima, & Fezza 2016), *Zeugodacus cucurbitae* (Coquillet) (Shelly et al., 2016) and *Bactrocera dorsalis* (Hendel) (Shelly & Kurashima, 2018).

As far as we are aware, there are no previously published studies on alkaline hydrolysis of torula yeast. An early study reported favorable results using high quantities of NaOH with proteins from different sources, but did not include torula yeast (McPhail, 1939). Alkalinized proteins such as casein, gelatin, animal blood and cow hair, were highly

attractive to *A. striata*, but not to *A. ludens* (McPhail, 1939). A later experiment reported a positive response of *A. suspensa* to alkaline hydrolyzed casein using NaOH in gels up to pH 11.1, although higher pHs were not tested (Sharp, 1987). In the present study, higher attraction of *A. obliqua* to torula yeast + NaOH was observed at pHs of 10.3 to 13.3 compared to the pH of 9.15 that is characteristic of torula yeast pellets. Subsequent choice experiments revealed a higher capture of flies at the highest pH 13.3. The amount of ammonia released was positively correlated with the pH of the hydrolyzed yeast preparations. Sex-specific differences in responses to these preparations were not observed in cage experiments, with the exception of torula yeast at pH 12.3 which should be further investigated. Ammonia is considered a key attractant component for tephritid flies (Epsky et al., 1993; Bateman & Morton 1981; Mazor et al., 1987; Flath et al., 1989; Lasa & Williams, 2021), but other volatile compounds produced by alkaline hydrolysis may interact with low concentrations of ammonia improving the attraction of several tephritid species (Bateman & Morton 1981; Flath et al., 1989; Piñero et al., 2020; Lasa & Williams, 2021). Certain compounds not measured in this study, such as pyrazines (2,3-, 2,5- and 2,6-dimethylpyrazines, 2-ethyl-3-methylpirazine, 2,5-dimethyl-3-ethylpirazine and trimethylpyrazine, among others), were produced following the alkaline hydrolysis of maize proteins using KOH (pH 8.7) but were not detected in acid hydrolyzed preparations (Flath et al., 1989). Given variation in the attraction to ammonia when additional protein-derived odors are present, future studies on attractants for tephritid pest should focus on identifying key semiochemicals resulting from the breakdown of proteins and their potential interactions with ammonia.

A seven-fold higher amount of ammonia was released by torula yeast hydrolyzed at pH 13.3 than at pH 10.3. Ammonia released by torula yeast at pH 13.3 (28.2 ± 1.7 µg/h) was similar to that of acid hydrolyzed proteins plus borax and sachet lures like Biolure® (14-56 µg/h), and was much lower than the ammonium hydroxide solution (295 µg/h) that we previously reported to be highly attractive for *A. obliqua* under cage conditions (Lasa & Williams, 2021). Interestingly, the alkalization of torula yeast using KOH was significantly less attractive to *A. obliqua* flies in the laboratory, despite a similar rate of ammonia production after 24 h. The reason for these differences in fly responses is unclear but may be related to the stronger base characteristics of KOH over NaOH, because the first ionization energy of sodium is higher (496 KJ/mol) than that of potassium (419 KJ/mol). As such, the interaction of these alkalis with compounds resulting from hydrolysis, or those originating from the saponification of yeast-derived lipids, likely differed in preparations treated with sodium or potassium hydroxide, possibly resulting in the production of different volatile compound profiles.

The response to food volatiles may be attenuated if flies have previously consumed proteins (Robacker and Moreno 1995). However, previously fed flies are commonly used in the screening of attractants before field tests. This is based on the assumption that attractants are likely to be effective if laboratory-reared flies respond even after recently ingesting proteins. Thus, our laboratory cage findings were corroborated in two field experiments. Under field conditions, traps baited with torula yeast + NaOH pH 13.3 captured higher numbers of *A. obliqua* than the acid hydrolyzed protein Captor + borax or torula yeast pellets. Field captures were female biased, and a lower percentage of females was captured in torula yeast + NaOH pH 13.3 than in traps baited with Captor + borax in one experiment but not in the other. The release of ammonia differed markedly among attractants following 7 d exposure to warm field temperatures (average 23-27 °C). After 7 days, emissions of ammonia by torula yeast + NaOH pH 13.3 and Captor + borax had fallen by 48% and 28% of the initial emission, whereas torula yeast pellets, that started with a very low emission of ammonia (1.8 µg/h), increased ammonia emissions by five-fold to reach a similar level to that of the other two attractants. Ammonia releases could be related to ongoing protein hydrolysis as considerable amounts of ammonia originate from the partial deamidation of amino acids and from the structural breakdown of the protein during alkaline hydrolysis (Warner & Cannan, 1942).

The effect of borax on amino acids and short peptides present in acid hydrolyzed proteins such as Captor is likely to differ from its effect on torula yeast that comprises intact proteins. The hydrolysis process likely proceeds more slowly in mixtures with borax at pH 9.15 compared to the rapid hydrolysis of yeast proteins at pH 13.3 and the resulting release of ammonia. In this respect, we observed that when mixed with water, the proteins in torula yeast pellets precipitated and sedimented on the bottom of the trap quickly (within ~30 mins) a process that was much slower in torula yeast + NaOH pH 13.3 (~28-30 h). No precipitate or sedimentation was observed in preparations of Captor + borax, consistent with the idea that acid hydrolysis of Captor had eliminated most long chain polypeptides.

Early studies on protein attractants also reported rapid proliferation of the microbiota (Gow, 1954), that resulted in the loss of lure attractiveness to fruit flies (Green, Beroza, & Hall, 1960). The bacteriostatic effect of borax and its buffer capacity maintains a relatively steady pH, reduces the rate of decomposition by microorganisms, and preserves the bodies of captured flies for subsequent identification (López & Becerril, 1967). In contrast, hydrolyzed maize protein + NaOH at pH 8-9 was not stable over time and this compromised its potential use under field conditions (Heath, Vazquez, Schnell, & Epsky, 2009).

We did not observe signs of microbial growth or decomposition in torula yeast + NaOH pH 13.3 even when contaminated by numerous trapped flies after 7 days of field

use. Also, this alkaline hydrolyzed yeast preparation did not acquire a putrid smell or cloudy appearance typical of microbial decomposition. Alkaline media effectively inhibit microbial growth, especially at pH values above pH 10 (Irwin, 2020). Moreover, even at pH 13.3, captured flies were recovered intact from traps after 7 days, and despite a slight darkening of the cuticle, were readily identified to species as characteristic features of the wings, thorax and ovipositor were not modified by exposure to high pH. The abdomen of these trapped flies became semi-transparent after storage in 70% alcohol, but sexual maturation of dissected females could be clearly observed though the established characteristics of ovarian development. Spermathecae were also isolated and visualized with sperm under a compound microscope at x400 for captured mated females. Ovarian development and the insemination status of females are generally not important for studies of population dynamics in pest-infested areas but are important if used for detection in monitoring surveys performed in pest quarantine areas (Enkerlin et al., 2015). The markedly reduced surface tension of torula yeast + NaOH pH 13.3 may also have the benefit of increasing fly retention once flies have entered the trap, as contact with the liquid surface is more likely to result in drowning of the insect (Lasa & Williams, 2017).

Sodium hydroxide is cheaper than borax and a pH of 13.3 can be attained with a low concentration of NaOH (~1%), compared to the 2-10% of borax that is commonly used with acid hydrolyzed proteins. Alkalized torula yeast + NaOH is also cheaper than ammonium salts. Sachets containing a mixture of torula yeast + NaOH pellets or granules, at a dose suitable for a 250 – 300 ml trap, could be a simple and cheap system for *A. obliqua* monitoring - a proposal that merits validation through field testing.

Future experiments should focus on alkaline hydrolysis of other proteins and their attractiveness to species of tephritids such as *Anastrepha*, *Ceratitis* and *Rhagoletis* that are commonly monitored with food attractants. It would also be useful to identify differences in the volatile compounds produced through acid and alkaline hydrolysis of proteins at different pHs to gain insights into the key volatiles that elicit tephritid attraction to traps baited with hydrolyzed proteins. Due to its low cost, simplicity, and efficacy, alkaline hydrolyzed torula yeast may also find applications in mass trapping strategies to control *A. obliqua* and other pestiferous fruit flies.

CONFLICT OF INTEREST

The authors declare no conflict of interest with any aspect of this study.

AUTHOR CONTRIBUTION

RL and TW conceived research. RL conducted experiments. RL and TW contributed material. RL and TW analyzed data and conducted statistical analyses. RL and TW wrote the manuscript. RL secured funding. Both authors have read and approved the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings are available from zenodo: https://doi.org/10.5281/zenodo.6142420

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Figure Legends

FIGURE 1**.** Mean number of *A. obliqua* males and females capture in traps baited with torula yeast + borax at pH 9.15 and torula yeast alkalinized with NaOH to different pH values. Total flies captured were analyzed by paired t-test (NS p>0.05, *p<0.05, **p<0.01). Statistical comparisons of mean numbers of flies captured by torula yeast + borax at pH 9.15 and torula yeast + NaOH at pH 9.15 (t=1.90, df=7, p=0.100), pH 10.3 (t=3.37, df=7, p=0.012), pH 11.3 (t=3.21, df=7, p=0.015), pH 12.3 (t=3.55, df=7, p=0.009) and pH 13.3 (t=2.99, df=7, p=0.020). Comparisons of mean percentages of female flies for torula yeast + borax at pH 9.15 and torula yeast + NaOH at pH 9.15 (t=1.19, df=6, p=0.277), pH 10.3 (t=1.65, df=7, p=0.143), pH 11.3 (t=1.45, df=7, p=0.663), pH 12.3 (t=3.26, df=7, p=0.014) and pH 13.3 (t=1.34, df=7, p=0.223).

FIGURE 2**.** Captures of flies and ammonia production in laboratory traps baited with torula yeast following alkaline hydrolysis. (A) Linear regression of number of *A. obliqua* males and females captured in traps baited with torula yeast + NaOH at different pH values. Linear regression: $y = 0.576x - 4.432$ (adjusted R^2=0.304). (B) Quantities of ammonia released by torula yeast + NaOH at different pH values. Log-linear regression: $y = e^{0.727x-6.35}$ (adjusted R^2=0.860). Non-transformed values are represented in both (A) and (B).

FIGURE 3**.** Captures of flies and ammonia production in laboratory traps in which torula yeast was subjected to hydrolysis using different alkalis. (A) Mean number of *A. obliqua* males and females capture in traps baited with torula yeast + NaOH pH 13.3 or torula yeast + KOH pH 13.3. (B) Mean quantities of ammonia released by torula yeast + NaOH or torula yeast + KOH. Total flies captured (males + females) (A) and mean quantity of ammonia (B) labeled with the same letter do not differ significantly (t-test, p >0.05). Statistical values for comparison of mean numbers of flies captured (t=3.36, df=15, p=0.004) and quantity of ammonia released from traps (t=1.40, df=8, p=0.199).

FIGURE 4**.** Mean *A. obliqua* flies per trap (FTD) captured in traps baited with Captor + borax, torula yeast pellets or torula yeast + NaOH at pH 13.3 in (A) field experiment 1 and (B) field experiment 2. (C) Mean quantities of ammonia released by each of the attractants at 24 h after preparation (grey circles) and after one week deployed in the field (white squares). FTD values in (A) and (B), and the release of ammonia (C) labeled with the same letter do not differ significantly (GLM, Bonferroni, p >0.05).

Fig 1.

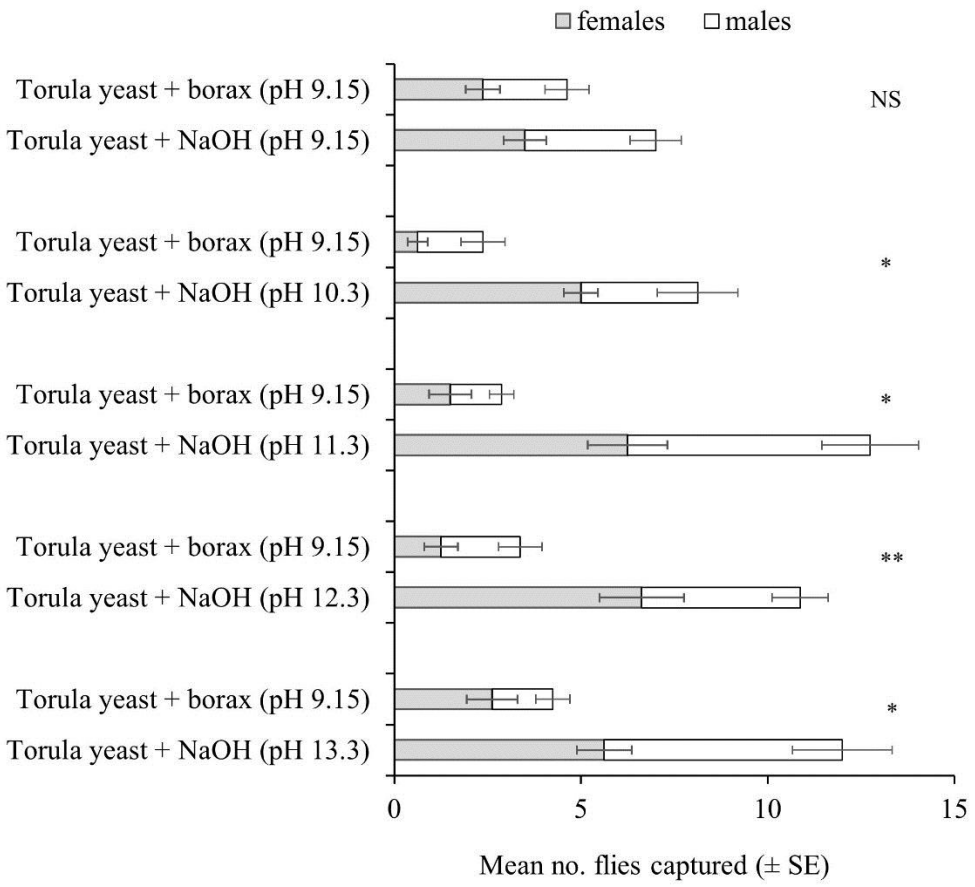

females
males
Torula yeast + borax (pH 9.15)
Torula yeast + NaOH (pH 9.15)
NS
Torula yeast + borax (pH 9.15)
Torula yeast + NaOH (pH 10.3)
*
Torula yeast + borax (pH 9.15)
Torula yeast + NaOH (pH 11.3)
*
Torula yeast + borax (pH 9.15)
Torula yeast + NaOH (pH 12.3)
**
Torula yeast + borax (pH 9.15)
Torula yeast + NaOH (pH 13.3)
*
0
5
10
15
Mean no. flies captured (± SE)

Fig. 2

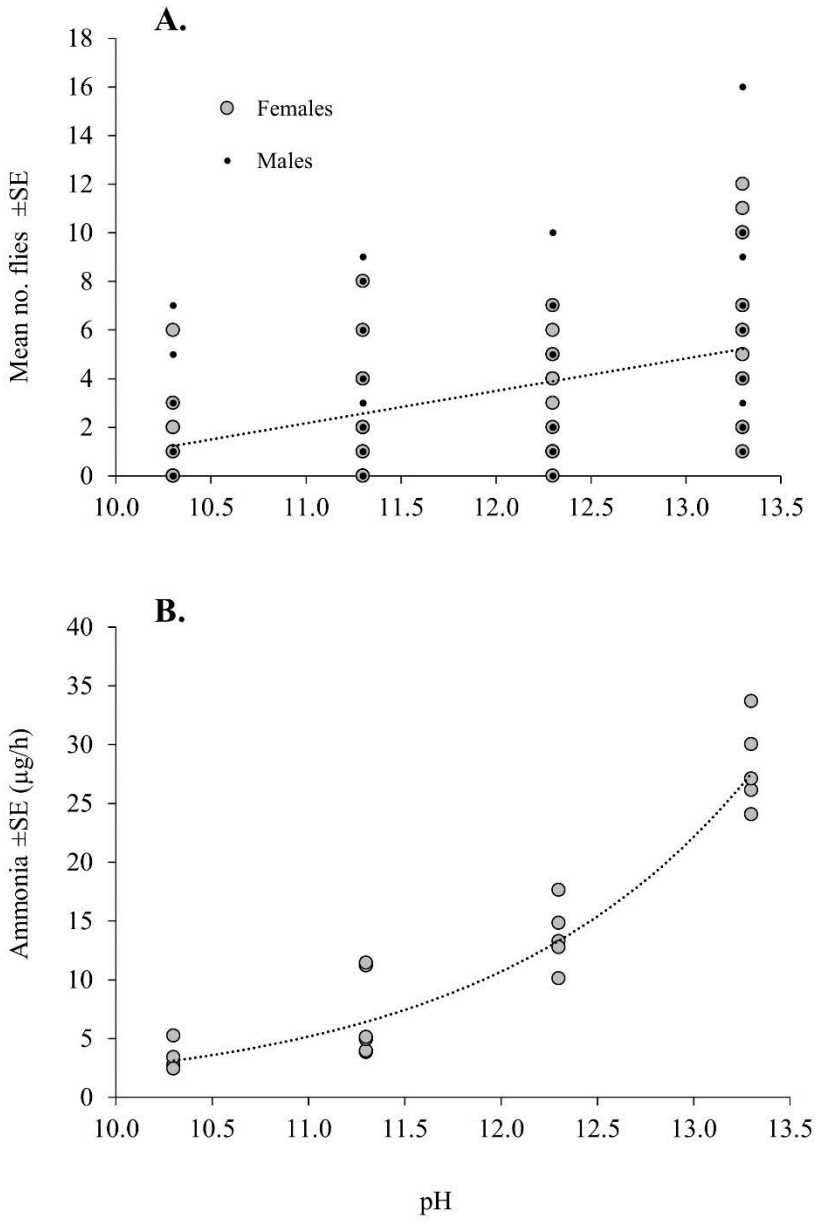

A.
Females
Males
Mean no. flies ±SE
0
2
4
6
8
10
12
14
16
18
10.0
10.5
11.0
11.5
12.0
12.5
13.0
13.5
B.
Ammonia ±SE (µg/h)
0
5
10
15
20
25
30
35
40
pH

Fig. 3

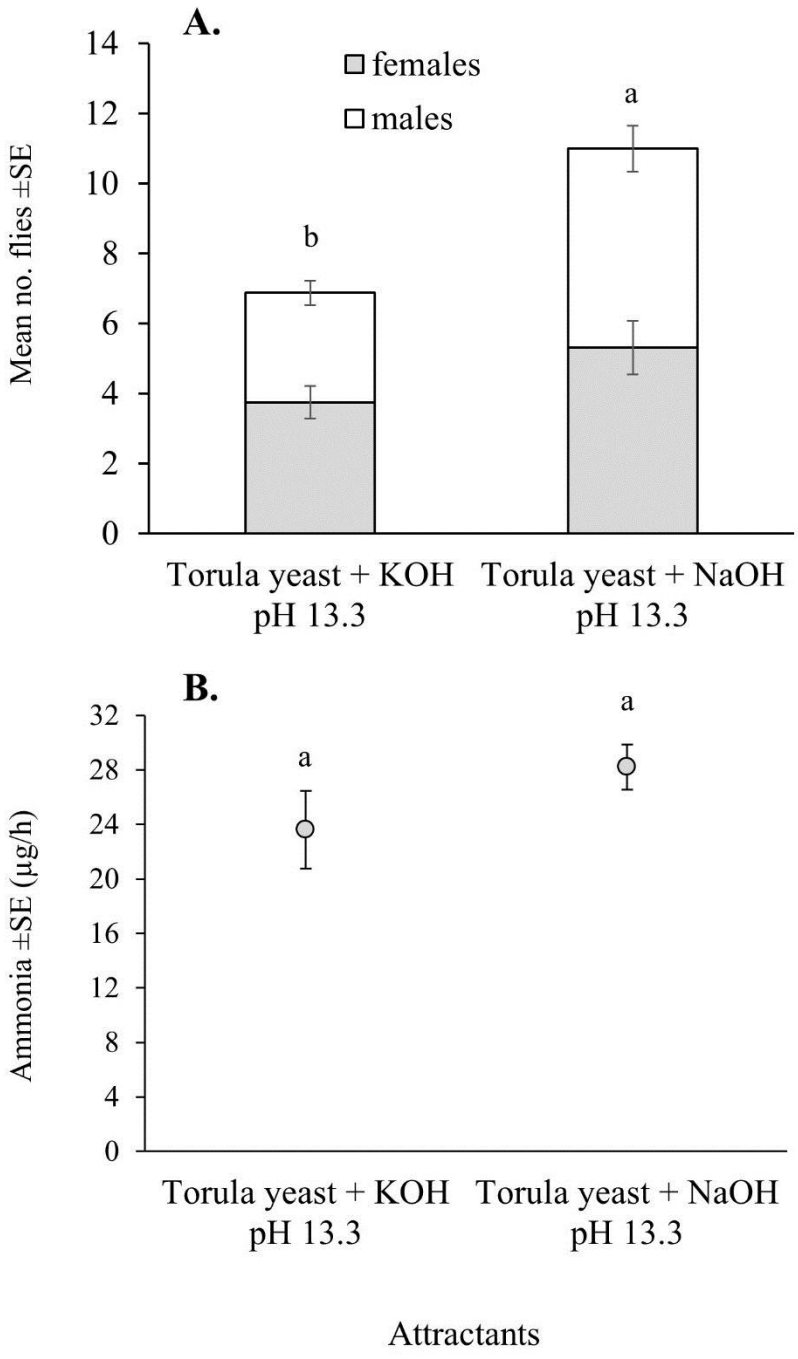
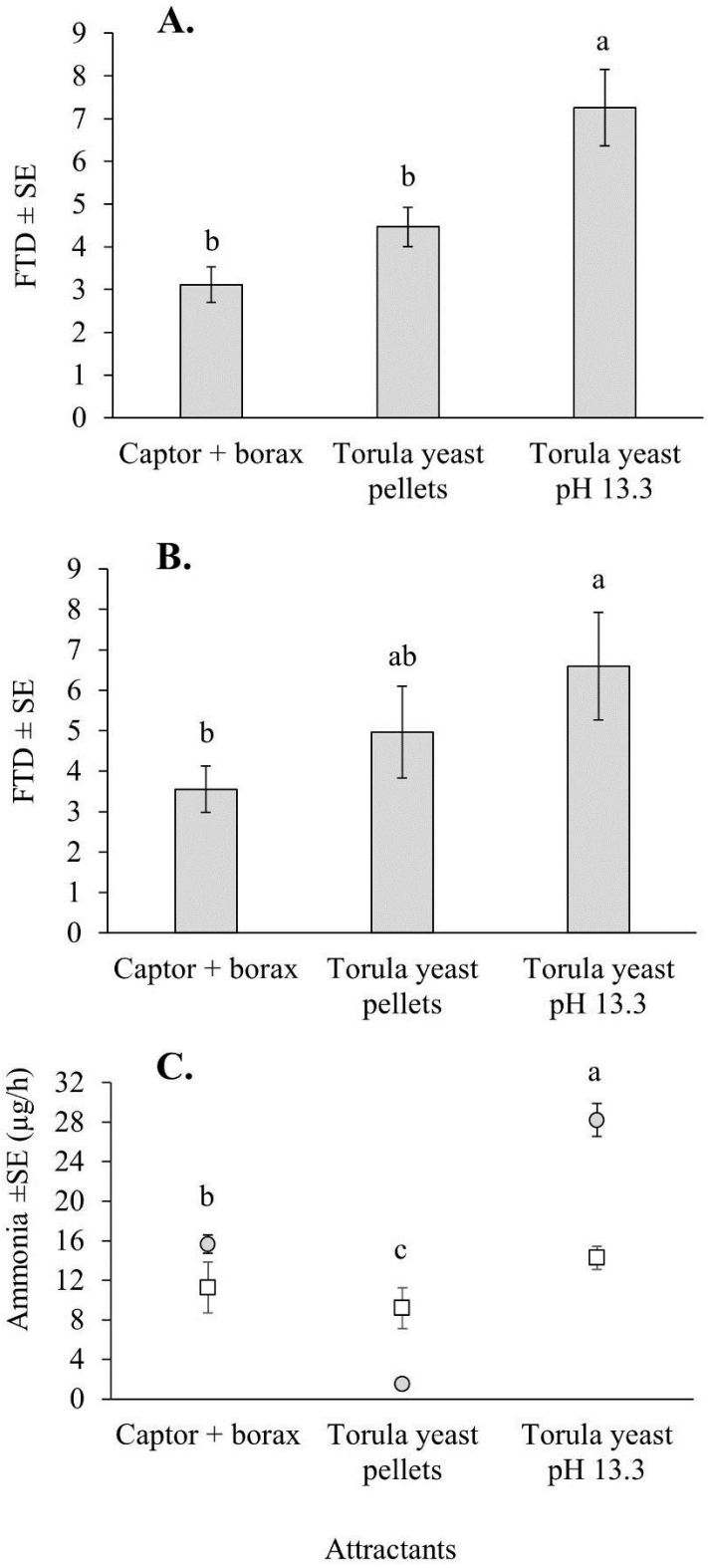

A.
females
males
Mean no. flies ±SE
14
12
10
8
6
4
2
0
a
b
Torula yeast + KOH pH 13.3
Torula yeast + NaOH pH 13.3
B.
Ammonia ±SE (µg/h)
32
28
24
20
16
12
8
4
0
a
a
Torula yeast + KOH pH 13.3
Torula yeast + NaOH pH 13.3
Attractants

Fig. 4


A.
FTD ± SE
0
1
2
3
4
5
6
7
8
9
b
b
a
Captor + borax
Torula yeast pellets
Torula yeast pH 13.3
B.
FTD ± SE
0
1
2
3
4
5
6
7
8
9
b
ab
a
Captor + borax
Torula yeast pellets
Torula yeast pH 13.3
C.
Ammonia ±SE (µg/h)
0
4
8
12
16
20
24
28
32
b
c
a
Captor + borax
Torula yeast pellets
Torula yeast pH 13.3
Attractants