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Responses of fruit flies (Diptera: Tephritidae) to protein bait: Effects of tree canopy, species, physiological state and weather

Msizi R. Ramaoka [a], Aruna Manrakhan [b,c], Christopher W. Weldon [a,*]

[a] Forestry and Agricultural Biotechnology Institute (FABI), Department of Zoology and Entomology, University of Pretoria, Hatfield, 0028, Pretoria, South Africa
[b] Citrus Research International, Nelspruit, 1200, South Africa
[c] Department of Conservation Ecology and Entomology, Stellenbosch University, Matieland, 7602, Stellenbosch, South Africa

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ABSTRACT

The application of protein baits forms the core of fruit fly (Diptera: Tephritidae) control measures in many orchard environments. Protein baits target adult fruit flies, which need protein for reproductive maturation. A proper understanding of the factors influencing fruit fly responses to protein is required to optimise control outcomes. In this study, responses to protein bait by three fruit fly species, *Ceratitis capitata* (Wiedemann), *C. cosyra* (Walker), and *Bactrocera dorsalis* (Hendel), were investigated. This was done in two field cages erected over lemon trees within an orchard. The response of each species to protein bait presented as a station and positioned at different heights within the tree canopy was evaluated. For each species, effects of fly age, sex, and nutritional status were evaluated under conditions of varying temperature and relative humidity. The highest response occurred when protein baits were placed in the middle to upper tree canopy. Species, nutritional status, sex and age also affected protein foraging. *Ceratitis capitata* responded more to bait compared to *B. dorsalis* and *C. cosyra*. The lowest response to protein bait was by one-day-old protein fed flies, while 10-day-old protein-deprived flies were most responsive. Protein deprived females were more attracted to protein than males fed the same diet. Flies responded to bait when temperatures were between 20 and 30 °C. Based on the results, the success of protein baits in controlling fruit flies in a tree canopy is height dependent and can be lower in areas with alternative protein sources.

1. Introduction

Insects, like many other animals, need to forage for food, shelter, mates, and oviposition sites to maximise their fitness. The type and intensity of foraging for these resources vary according to location and time (Bell, 1990), and depend on extrinsic and intrinsic factors (Gadenne et al., 2016; Bell, 1990). Extrinsic factors influencing insect activity may include abiotic and biotic environmental conditions (Balagawi et al., 2014). Abiotic factors, such as temperature, have a major effect on insect activity and behaviour, with extremely high or low temperatures negatively impacting development rate, distribution, reproduction, migration, and adaptation in all ectotherms (Weldon et al., 2022; Sehgal et al., 2006; Balagawi et al., 2014). The intrinsic factors that affect foraging activities in insects include sex, age, nutritional state, reproductive maturity and mating status (Gadenne et al., 2016).

Manipulation of the foraging behaviour of insects is exploited for control of pests in agricultural crops (Roitberg, 2007). This is the case for many fruit fly (Diptera: Tephritidae) pests, which affect fruit production worldwide (White and Elson-Harris, 1992). Female fruit flies oviposit within host fruit where the hatched larvae feed and develop, making the fruit unmarketable (Reddy et al., 2020). For many fruit fly pests, the foraging behaviour of adults for protein is frequently exploited as a key strategy in their management. Protein baits are often used in detection surveys of these pests since they are not sex or species specific (Epsky et al., 2014). Furthermore, behavioural manipulation tactics such as the use of semiochemicals for attracting and killing adult fruit fly pests are widely used for their control. There are two attract and kill techniques used for fruit fly pests: male annihilation technique (MAT) and bait application technique (BAT) (Epsky et al., 2014). MAT is specific to males and is highly effective against fruit flies when they exhibit strong responses to a lure (Tan et al., 2014). BAT involves the use of protein-based attractants (Mangan, 2014). Protein-based attractants can be mixed with an insecticide and applied as either foliar sprays (coarse droplets on trees) or bait stations (devices set at specific densities in an

* Corresponding author.
E-mail address: cwweldon@zoology.up.ac.za (C.W. Weldon).

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area) (Mangan, 2014; Piñero et al., 2014). Protein baits can also be used in traps – a technique referred to as mass trapping (Navarro-Llopis and Vacas, 2014).

In South Africa, well established programmes are in place to control a diverse suite of fruit fly pests that affect major commercial fruit crops. These pests include the oriental fruit fly, *Bactrocera dorsalis* (Hendel), the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), and the marula fly, *Ceratitis cosyra* (Walker) (Prinsloo and Uys, 2015; Annecke and Moran, 1982). In South Africa, *C. capitata* is a prominent pest that attacks commercial fruit such as citrus and deciduous fruit (Prinsloo and Uys, 2015), both of which have high export values. *Bactrocera dorsalis* has been declared established in the northern parts of South Africa since 2013 (Manrakhan et al., 2015b). *Bactrocera dorsalis* still has a restricted distribution in South Africa and surveys conducted in the north in the years following its establishment showed that it had a limited host range, with mangos being infested more than other fruit types (Theron et al., 2017; Grové et al., 2017). *Ceratitis cosyra* is an important pest of subtropical fruit in South Africa (Grové et al., 2016, De Villiers et al., 2013). *Ceratitis cosyra* is associated with the marula tree, which is more prevalent throughout the northern and eastern regions of the country in the Mpumalanga, Eastern Cape, Limpopo, and KwaZulu-Natal provinces but this range also overlaps with the production of mangos, which is its main commercially-grown host (De Villiers et al., 2013).

Protein-based attractants in the form of protein hydrolysates are commercially available in South Africa for fruit fly control (Barnes, 2000; Manrakhan and Kotze, 2011). These protein hydrolysates are mixed with toxicants such as malathion, trichlorfon and cyantraniliprole and applied as foliar sprays (CRI, 2005). Ready-to-use products containing a protein-based attractant combined with an insecticide are also commercially available in South Africa. These formulations require only dilution with water before being applied as foliar sprays. One example is GF-120 Naturalyte. GF-120 Naturalyte incorporates an insecticide called Spinosad (Dow AgroSciences LLC, Indianapolis, IN, USA) (Mangan et al., 2006). The M3 fruit fly bait station (Ware et al., 2003) is also commercially available in South Africa and contains protein hydrolysate, a proprietary combination of hydrolysed vegetable proteins, or yeast proteins (e.g., HymLure, LokLure and Buminal), and a toxicant (e.g., alpha-cypermethrin) (River Bioscience [Pty] Ltd., Humewood, South Africa) (CRI, 2005; Manrakhan and Daneel, 2013). M3 bait stations are highly effective in suppressing fruit fly populations (i.e., *Ceratitis capitata* and *Ceratitis rosa* (Karsch)) in citrus orchards (Ware et al., 2003), with densities that range from 300 to 400 units per hectare (Manrakhan and Kotze, 2011; Ware et al., 2003). The majority of crops need M3 bait stations to be applied only once per season because the devices remain efficient for at least three months (Manrakhan and Kotze, 2011). However, protein baits have distinct odour profiles, and the attraction to protein odours varies across fruit fly species, as demonstrated by varying olfactory responses to protein-based odours among different species (Manrakhan and Kotze, 2011; Barry et al., 2006). For instance, Manrakhan and Kotze (2011) showed that higher numbers of *C. capitata* responded to baits (HymLure, GF-120 Naturalyte and M3 bait) when compared with *Ceratitis cosyra* (Walker) and *C. rosa*. Intrinsic factors such as age, diet history, and sexual maturity also influence responses to protein baits (Manrakhan and Lux, 2008; Kendra et al., 2005).

Protein baits when applied as foliar sprays may lead to phytoxicity on some fruit types, for example mandarin, if bait droplets land accidently on fruit (Manrakhan et al., 2015a). To circumvent the phytotoxic effects of baits on fruit, some growers have considered applying baits on the ground below the tree canopy in citrus orchards, (A. Manrakhan, pers. comm.). Therefore, it is important to determine if flies respond to protein bait placed on the ground rather than on the tree canopy. It is also important to establish the optimal placement of the baits at different canopy heights of the tree to optimise bait application and increase fly response. This is not just a matter needing attention in the citrus industry because phytotoxic effects of protein bait sprays have also been reported on mangoes (Vayssieres et al., 2009), cherries (DeLury et al., 2009), cucurbits (Vijaysegaran, 1985), apples (Baronio et al., 2019), and grapes (Baronio et al., 2018).

The objective of this study was to establish the response of three pest fruit flies, *B. dorsalis*, *C. capitata,* and *C. cosyra*, to protein baits on a fruit tree with regard to bait location, fly age, sex, and nutritional status. We predicted that more flies would visit protein baits placed in a tree canopy than when placed on the ground. Young flies were expected to visit protein baits more often than older flies, as they undergo sexual maturation. We also predicted that flies deprived of essential nutrients for development or with lower nutritional reserves would visit protein baits more, and visitation would increase with environmental temperature due to its effect on fly activity.

2. Materials and methods

2.1. Field cage experiments

The study was undertaken in two hexagonal 40 % white shade cloth field cages at the University of Pretoria Innovation Africa campus (S 25° 45′ 09″ E 28° 15′ 05″), located in Pretoria, Gauteng province, South Africa. Experiments were conducted between May 2022 and March 2023 in two hexagonal field cages (3 m at the widest point and 3 m in height) without a floor and based on those recommended for sterile fruit fly quality control tests (FAO/IAEA/USDA, 2014).

The two field cages were erected over established, planted lemon trees located 60 m apart to avoid any interference between the cages. The lemon trees were located within a small (approx. 0.35 ha) experimental citrus orchard that was irrigated but received no other management interventions. Each lemon tree was pruned to approximately 2.5 m tall with a canopy diameter of 2 m. The lemon trees were used as representative fruit trees, and their inclusion does not imply their host status with respect to the tested fruit flies. A prior study conducted in South Africa demonstrated that commercially grown, export-grade lemons are non-hosts for the three fruit fly species targeted in this study (Manrakhan et al., 2018).

A small, 3D-printed Stevenson screen fitted with a temperature and relative humidity data logger (DS1923, iButton Maxim Integrated, San Jose, CA, USA) was placed in each tree canopy at a height of 1.5 m above the ground and 0.5 m within the canopy to record temperature and relative humidity during the tests described below.

2.2. Fruit fly rearing and handling

Experimental flies were obtained as pupae from *B. dorsalis*, *C. capitata,* and *C. cosyra* cultures maintained in the Department of Zoology and Entomology, University of Pretoria, South Africa, during the period of the study. The culture had been maintained for approximately twelve generations with 300 adults in each generation. Adults were kept in ventilated laboratory cages (30 × 30 × 30 cm) in a climate room kept at a constant temperature of 24 ± 1 °C with relative humidity buffered at 50–60 % and a 12 h light: 12 h dark photoperiod together with simulated dusk and dawn in the first and last hour of the light phase. This was done to promote male and female mating for all three tested species since *C. capitata* mate during the day, while *B. dorsalis* and *C. cosyra* begin mating at dusk (Manrakhan, 2016). Lights were turned on at 06h00 and turned off at 18h00 (South African Standard Time; GMT+2).

Gravid female flies were provided with oviposition substrates after 10-days old following adult emergence. For each species, eggs were collected on a weekly basis for experiments and to continue the culture. The oviposition substrate comprised a 100 mL plastic cup containing a water-soaked tissue paper that had been moistened with distilled water to increase egg viability and 3 mL of guava juice concentrate (Halls; Tiger Consumer Brands Limited, Bryanston, South Africa) or a piece of guava fruit purchased from a supermarket, which was used for the collection of eggs for all the three species. Guava is an important host plant for *B. dorsalis* (Mahmoud et al., 2020), *C. capitata*, and *C. cosyra* (De Meyer et al., 2002). The egg cup was sealed with a double layer of laboratory film (Parafilm M, Bemis Flexible Packaging, Neenah, WI, USA), which was pierced multiple times using an insect pin to enable female flies to detect the stimulus of the fruit. Female flies were given two-to-three hours to oviposit, and distilled water was thereafter used to wash off eggs from the laboratory film into a small plastic container where they were allowed to settle. Eggs were sucked up with a 3 mL disposable plastic Pasteur pipette and again left to settle at the tip of the pipette. A single drop full of eggs (approximately 300 eggs) was then transferred to 100 g of each prepared larval diet. The larval diet was a carrot-based medium supplied by Citrus Research International, South Africa. A plastic lid was loosely placed over the inoculated larval diets (to prevent eggs from drying out: (Howie, 2008)) before they were placed on a 2 cm layer of dry sand in individual 2 L plastic containers (15 × 21 × 8 cm) that were fitted with a ventilated lid to prevent light exposure and fungal growth. Eighteen days after eggs were inoculated, the container was removed and sieved daily to collect fly pupae. Retrieved pupae were placed inside cylindrical plastic containers (94-mm inner diameter, 3-mm thickness), which were subsequently placed inside insect rearing cages (BugDorm, MegaView Science Co., Ltd., Talchung, Taiwan) for adult eclosion. Upon eclosion of young adults, individuals from each species were provided with either granulated sucrose and hydrolysed yeast (HG000BX6.500, Merck, Wadesville, South Africa) (Sugar + YH), or granulated sucrose only (Sugar only). At the ages of one and 10 days after emergence, female and male flies that could fly out of the cylindrical plastic containers were selected, separated by sex, and placed in 100 mL plastic cup cages (depth 6.5 cm, diameter 5.4 cm) using an aspirator. Flies were then supplied with the same adult diet as they were given after adult eclosion and water. In each cup, adult diet was contained in microcentrifuge tube lids and water was dispensed from 200 μL pipette tips which were slightly covered with a putty-like adhesive (Bostik) (Bostik Pty Ltd, France).

2.3. Bait

A protein hydrolysate solution (HymLure, MIC Landboudienste (Pty) Ltd, Centurion, South Africa), commercially used for fruit fly control in South Africa, was prepared and tested as bait stations for the experiment. To produce a bait station, a 60 mm filter paper (Catalog no. WHA1441060) was soaked in a 2 % HymLure solution in a 60-mm disposable plastic Petri dish (Manrakhan and Kotze, 2011). The Petri dish with the filter paper was placed in a fume hood for 3 h in order to dry out. A clip was then attached to the filter paper to act as a hook for hanging the bait station. The tree canopy was marked at different heights and the bait station was randomly placed in one of four different canopy heights. Four height categories were defined: ground = 0, lower canopy = 0.5 m, middle canopy = 1.5 m and upper canopy = 2 m. Wire was used to hang the bait station from a branch by threading it through the clip. The bait station on the ground comprised of a filter paper still fitted within the Petri dish used for its production.

2.4. Spatial and temporal foraging experiments

Ten flies of the same species, sex, diet, and age were released in a field cage, and the time of release was recorded. Flies were released between 08h00 and 17h00 when temperatures were predicted by a smart phone weather application (local weather: weather forecast) to reach 20 °C. The release would happen 1 h before the test in order to allow flies to explore and settle in the cage and tree canopy. A protein bait station was then introduced in one of the four different locations to observe the number of flies responding to the bait in that specific location. The different bait locations were tested separately. The observation of flies was conducted for 1 h during which flies were counted and removed from the cage using an aspirator, as soon as they landed on the bait station. An extra 15 min after the test was given to each cage to locate and collect non-responding flies on the cage wall and tree canopy after the 1-h test period.

Two field cages were used simultaneously so that an observation period in one cage coincided with the acclimatisation of flies in the other. Both responsive and non-responsive flies were placed on ice in separate microcentrifuge tubes, and then stored individually in a freezer at −80 °C for later determination of body protein and lipid content using the vanillin colorimetric assay described in Weldon et al. (2019).

The study followed a full factorial design which combined three species, two sexes, two age groups, two dietary regimes and four bait locations, all of which resulted in a total of 96 interactions. The procedure was repeated with sex, age, diet, and bait station location being randomised. Fly availability determined when species was tested in the field cages but tests of each species were distributed haphazardly across the entire duration of the experiment. A sample of 9600 flies were used in total and a total number of 10 replicates were performed for each species, sex, diet, age, and bait location combination, which was equivalent to 960 tests. Replicates were carried out sequentially based on insect availability over a period of 11 months. This allowed the observation of fly response to protein baits over a wide range of temperature and relative humidity conditions.

2.5. Fly composition

A subsample of 226 flies (aiming for two responders and two non-responders per replicate) were selected from the 9600 flies tested in the field cages using a stratified random sampling approach from those collected during the spatial and temporal foraging experiments for each species, sex, and diet combination. This was done to determine if fly protein and lipid content had an influence on the response to protein baits applied in the field using established methods by Weldon et al. (2019) (see Supplementary material). Following field cage results, which showed the response by large proportions of flies to protein baits placed in the mid to upper canopy heights, the variable of canopy height was excluded when determining the lipid and protein content of flies. In addition, the one-day old age group was also excluded due to a low response regardless of species.

2.6. Data analysis

Statistical analyses and data visualisation were performed using R software (version 4.3.1). Exploratory graphical analyses were conducted to assess the distribution of fly counts relative to the factors (species, age, sex, diet and bait location) and covariates (temperature, relative humidity). A generalised linear model (GLM), with a negative binomial error distribution and log-link to predictor variables, was applied to explain the magnitude of fly response to protein bait. The predictor variables were the main effects and interactions of species, age, sex, diet, and protein bait location, and the response variable was the number of flies responding to protein baits. A negative binomial distribution was chosen to account for over-dispersion of fly counts. It was also the model with the lowest Akaike's information criterion (AIC) relative to other candidate models. Thereafter, the minimum adequate model was determined using the 'step' function, which sequentially removed the least significant terms in the model until there was no further improvement in the AIC. When significant effects were detected, Tukey's HSD tests were used to perform post-hoc pairwise comparisons to establish which categories differed from each other.

Analyses of the effect of temperature and relative humidity on the number of flies responding to bait were run separately for each species using a subset of the data: 10 day-old protein deprived females as they had the highest responses. Canopy height was excluded since it was analysed earlier for the full dataset. This removed a large number of zero values from the data. To analyse temperature and relative humidity effects on each species response, the initial linear model included the linear effects of temperature, relative humidity, and temperature x relative humidity, and the nonlinear, quadratic effects of temperature (temperature^2) and relative humidity (relative_humidity^2). The nonlinear effects were included to account for potential declines in response at temperature and relative humidity values above an optimum (Huey and Stevenson, 1979).

Generalised linear mixed effects models with gamma family and log link were used to explain the body weight, protein and lipid content of a fly. For body weight, the predictors were the main effects and interactions of species, sex and diet. Body weight was added as a covariate for the protein and lipid composition models to account for fly size. Canopy height was excluded from analysis following field cage results which showed an increased response to baits located in the mid to upper canopy heights. In addition, the one-day-old age group was also excluded due to low responses across all species. To analyse the effect of body composition on the likelihood of response by flies to bait stations, GLMs with binomial family were applied. A binomial error structure was needed for this analysis because the response variable was categorical (i.e., response or non-response), recalling that body weight, lipid and protein content were measured from flies responding to protein bait in the field cages and those that did not. Responders were scored a value of 1 whereas the non-responders were scored a value of 0. The values from the body composition assays were used as covariates. Stepwise deletion was again used to remove least significant variables based on AIC to obtain the minimal adequate models. The differences between groups were detected using type III sums of squares in order to summarise the effect of each factor in the minimal adequate model. Tukey's tests were used to perform pairwise comparisons to assess where categories differ from each other if a main significant effect or interaction term was detected.

3. Results

3.1. Effects of species, age, sex, diet, and protein bait location on the magnitude of response

Bait location significantly affected fruit fly response (Table 1). However, there were also significant interactions between species and bait location, bait location and age, and species, age and diet (Table 1). Overall, a higher number of flies responded to baits located in the middle (1.00 ± 0.119) and upper canopy (1.32 ± 0.149) compared with baits located at the lower (0.258 ± 0.047) and ground level (0.054 ± 0.017) (Fig. 1). Despite these overall effects, a higher number of *C. capitata* responded to protein baits than *B. dorsalis* and *C. cosyra* in the lower, middle and upper canopy, with *C. capitata* and *B. dorsalis* not differing significantly at these canopy heights (Fig. 1). There were no significant differences in the number of *B. dorsalis, C. capitata* or *C. cosyra* responding to bait when it was placed on the ground (Fig. 1). Both 1- and 10 day-old flies responded significantly more to the bait when placed in the mid- and upper canopy than the lower canopy.

Table 1
Analysis of deviance table (type III tests) for the minimal adequate model explaining the effects of species, age, sex, diet and bait location on the magnitude of response by fruit flies to protein bait stations in a tree canopy under field cage conditions.

Effect	Likelihood ratio χ^2	df	P
Species	4.435	2	0.109
Bait location	46.116	3	**<0.001**
Age	9.847	1	**0.002**
Diet	15.584	1	**<0.001**
Sex	15.708	1	**<0.001**
Species × Bait location	16.361	6	**0.012**
Species × Age	17.352	2	**<0.001**
Species × Diet	16.091	2	**<0.001**
Bait location × Age	14.412	3	**0.002**
Age × Diet	0.235	1	0.628
Species × Age × Diet	10.907	2	**0.004**

However, this effect was larger in the 10 day-old flies (Tukey test; middle canopy: estimate = −3.9688, $P < 0.001$; upper canopy: estimate = −4.265, $P < 0.001$) than in the 1 day-old ones (middle canopy: estimate = - 1.499, $P < 0.001$; upper canopy: estimate = −1.589, $P < 0.001$).

The number of flies responding to the bait differed significantly between species, age and diet (Fig. 2). Overall, 10 day-old flies responded significantly more (1.09 ± 0.094) to protein bait than 1 day-old flies (0.229 ± 0.036). For both *B. dorsalis* and *C. capitata*, 1 and 10 day-old flies fed a sugar only diet were more likely to respond to the bait compared to those fed on a sugar + YH diet (Tukey test; *B. dorsalis* immature: estimate = 2.829, $P = 0.007$; mature: estimate = 2.339, $P < 0.001$; Fig. 2A; *C. capitata* immature; estimate = 0.637, $P = 0.032$; mature; estimate = 1.613, $P < 0.001$; Fig. 2B). One day-old *C. cosyra* fed on a sugar only diet responded slightly more than those of the same age category fed sugar + YH (Fig. 2C), but this difference was not significant. However, 10 day-old *C. cosyra* flies fed sugar only were significantly more responsive to the bait than those of the same age category fed sugar + YH (mature: estimate = 1.496, $P < 0.001$; Fig. 2C).

The species with the greatest increase in response to protein bait with age when fed a sugar only diet was *B. dorsalis* (estimate = −3.096, $P < 0.001$), followed by *C. capitata* (estimate = −1.802, $P < 0.001$) and *C. cosyra* (estimate = −1.439, $P = 0.002$), but *B. dorsalis* and *C. capitata* did not differ in their response when 10 days-old and fed sugar only. There was also a significant effect of sex in relation to fruit fly response to protein bait (Table 1). Females (0.802 ± 0.083) responded more to protein bait than males (0.519 ± 0.062) (Fig. 2) regardless of species, age or diet.

3.2. Effects of temperature and relative humidity on magnitude of bait response

The number of flies responding to the protein bait increased between 20 °C and 30 °C and between 40 % and 80 % relative humidity (Fig. 3). For *B. dorsalis*, there was a significant effect of relative humidity and significant interaction between temperature and relative humidity on the magnitude of response to the protein bait (Table 2). The responses of *B. dorsalis* to protein bait decreased with an increase in relative humidity (Fig. 3A: coefficient = −0.236, $P = 0.020$), but this was more pronounced at temperatures around 20 °C. The responses also increased as temperature and humidity increased together (Fig. 3A: coefficient = 0.006, $P = 0.010$). There was no significant linear or quadratic effect of temperature on the number of *B. dorsalis* responding to the protein bait.

There was a significant interaction between the linear effects of temperature and relative humidity on the response of *C. capitata* to the protein bait, with the linear effect of temperature, and the linear and quadratic effects of relative humidity also being significant (Table 2). An increase in temperature and relative humidity led to a linear decline in *C. capitata* response (temperature: coefficient = −0.237, $P = 0.002$; relative humidity: coefficient = −0.229, $P = 0.024$). However, the interaction of the linear effects of temperature and relative humidity indicated that the strength of the negative effect of temperature on response is dependent on relative humidity (Fig. 3B; coefficient = 0.006, $P = 0.003$). In this case, it appears that response is lowest at low temperature and high relative humidity, or high temperature and low relative humidity (Fig. 3B). There was a significant non-linear effect of relative humidity on the number of *C. capitata* responses to the protein bait (coefficient = 0.001, $P = 0.037$), with this species predicted to not respond below 20 % relative humidity and above 80 % relative humidity. *Ceratitis capitata* was predicted to respond optimally to protein baits at relative humidity levels varying between 40 % and 80 %.

For *C. cosyra*, there was no significant effect of temperature, relative humidity or their interaction (Table 2; Fig. 3C).

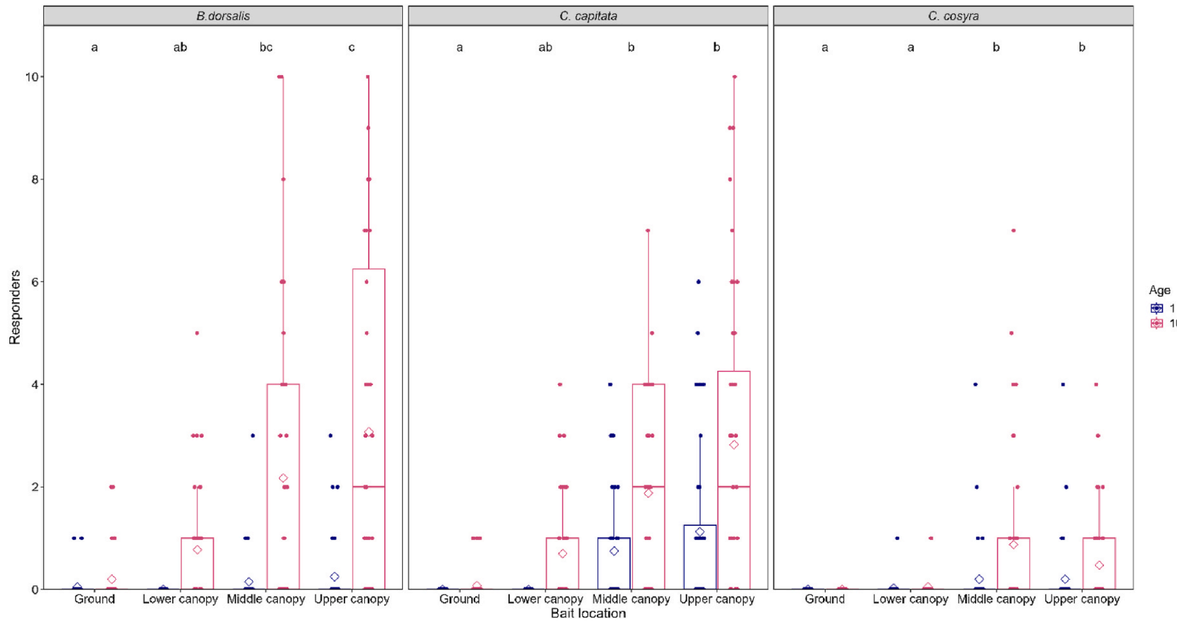

Fig. 1. Box plots showing patterns of responses by immature (day-1) and mature (day-10) *B. dorsalis*, *C. capitata*, and *C. cosyra* to protein bait applied at four different levels within a canopy of a lemon tree. The diamond shapes indicate mean responses. Box plots with different letters indicate significant differences between bait locations within the canopy in relation to response.

3.3. Fly body composition

Adult fresh body weight varied between species, sex and diet (Table 3). There were also significant interactions that occurred between species and sex, and species and diet (Table 3). Overall, post-hoc Tukeys tests showed that *B. dorsalis* was significantly heavier (17.221 ± 0.298) than both *C. capitata* (7.799 ± 0.125) and *C. cosyra* (8.919 ± 0.140), while *C. cosyra* weighed heavier than *C. capitata* (Fig. 4). In general, females of *B. dorsalis* (17.858 ± 0.402), *C. capitata* (8.413 ± 0.139) and *C. cosyra* (9.514 ± 0.203) were significantly heavier than males (Fig. 4). Adult fly body weight increased significantly when *B. dorsalis* (19.075 ± 0.334), *C. capitata* (8.275 ± 0.176) and *C. cosyra* (9.527 ± 0.217) were fed sugar + YH than when fed sugar only (Fig. 4).

There was a significant interaction of species with body weight on body protein content (Table 3), where the slope of the positive relationship was steeper in *C. cosyra* than *B. dorsalis* (coefficient = 0.126 ± 0.045, $P = 0.005$). The slope of the relationship between body weight and protein content for *C. capitata* did not differ from that of *B. dorsalis* (coefficient = 0.079 ± 0.044, $P = 0.077$). There was also a significant interaction of species, sex and diet (Table 3). When *B. dorsalis* females were fed sugar + YH, they had higher protein content than *C. capitata* and *C. cosyra* females fed the same diet (Fig. 5). Similarly, *B. dorsalis* males of the same nutritional status had a higher protein content compared to the other species (Fig. 5). With *C. capitata*, females that were fed sugar only had significantly higher protein content than those fed sugar + YH (Tukey test; estimate = −0.369 ± 0.098, $P < 0.001$). Similarly, *C. cosyra* males fed sugar only had significantly high levels of protein compared to those fed sugar + YH (Tukey test; estimate = −0.229 ± 0.105, $P = 0.030$). Generally, there was no effect of sex on protein content but in *C. cosyra* fed sugar only, males had significantly more protein content than females (Tukey test; estimate = −0.362 ± 0.100, $P < 0.001$).

The body lipid content varied between species with *C. cosyra* generally having significantly higher lipid content compared with other species (Fig. 6). Body lipid content was significantly affected by the interaction of species and body weight (Table 3). For *C. cosyra,* as body weight increased, the lipid body content increased more compared to *B. dorsalis* (coefficient = 0.210 ± 0.079, $P = 0.008$). There was a significant interaction between species and sex on body lipid content (Table 3). *Ceratitis cosyra* females and males had significantly higher lipid content than those of *B. dorsalis* (Tukey test; Females: estimate = −0.893 ± 0.293, $P = 0.007$: Males: estimate = −1.236 ± 0.334, $P = 0.001$). *Ceratitis cosyra* males had a significantly higher lipid content than those of *C. capitata* (Tukey test; estimate = −1.047 ± 0.401, $P < 0.026$). Sex had minimal impact on lipid content in *B. dorsalis* and *C. capitata*, but the effect of sex on lipid content was significant in *C. cosyra (*Fig. 6*)*. *C. cosyra* males had a significantly higher lipid content than females (Tukey test; estimate = −0.441 ± 0.173, $P < 0.011$). Sex and diet also interacted to significantly affect body lipid content (Table 3). In general, males fed sugar + YH diet had a significantly higher lipid content than females of the same nutritional status (Tukey test; estimate = −0.265 ± 0.130, $P = 0.043$). While, females fed sugar + YH had a significantly lower body lipid content compared to those fed sugar only diet (Tukey test; estimate = −0.301 ± 0.128, $P = 0.019$).

3.4. Effect of protein and lipid content on the likelihood of response

The likelihood of response to protein bait was significantly affected by diet, body protein content and body lipid content (Table 4). There was also a significant interaction between diet and body protein content on the likelihood of response to protein (Table 4), where the slope of the negative relationship between protein and bait response was significantly steeper in flies fed on sugar only than those fed on a sugar + YH diet (Fig. S1; coefficient = −0.003 ± 0.001, $P = 0.002$). There was a significant negative relationship between body lipid content and response (Fig. S2; coefficient = −0.004 ± 0.001, $P < 0.001$).

4. Discussion

The location of protein baits with respect to the tree canopy strongly affected foraging for protein baits by *B. dorsalis*, *C. capitata* and *C. cosyra*. There were higher responses to bait placed in the middle to upper canopy heights, compared to bait placed either in the lower canopy or ground level. Responses of another fruit fly species, *Bactrocera tryoni* (Froggatt), to protein bait was also found to be affected by tree canopy height (Balagawi et al., 2012), with more flies responding to protein bait in the mid-to upper canopy compared to lower canopy. Food foraging (Lux et al., 2017), location of mating partners (Collins et al., 2008), and

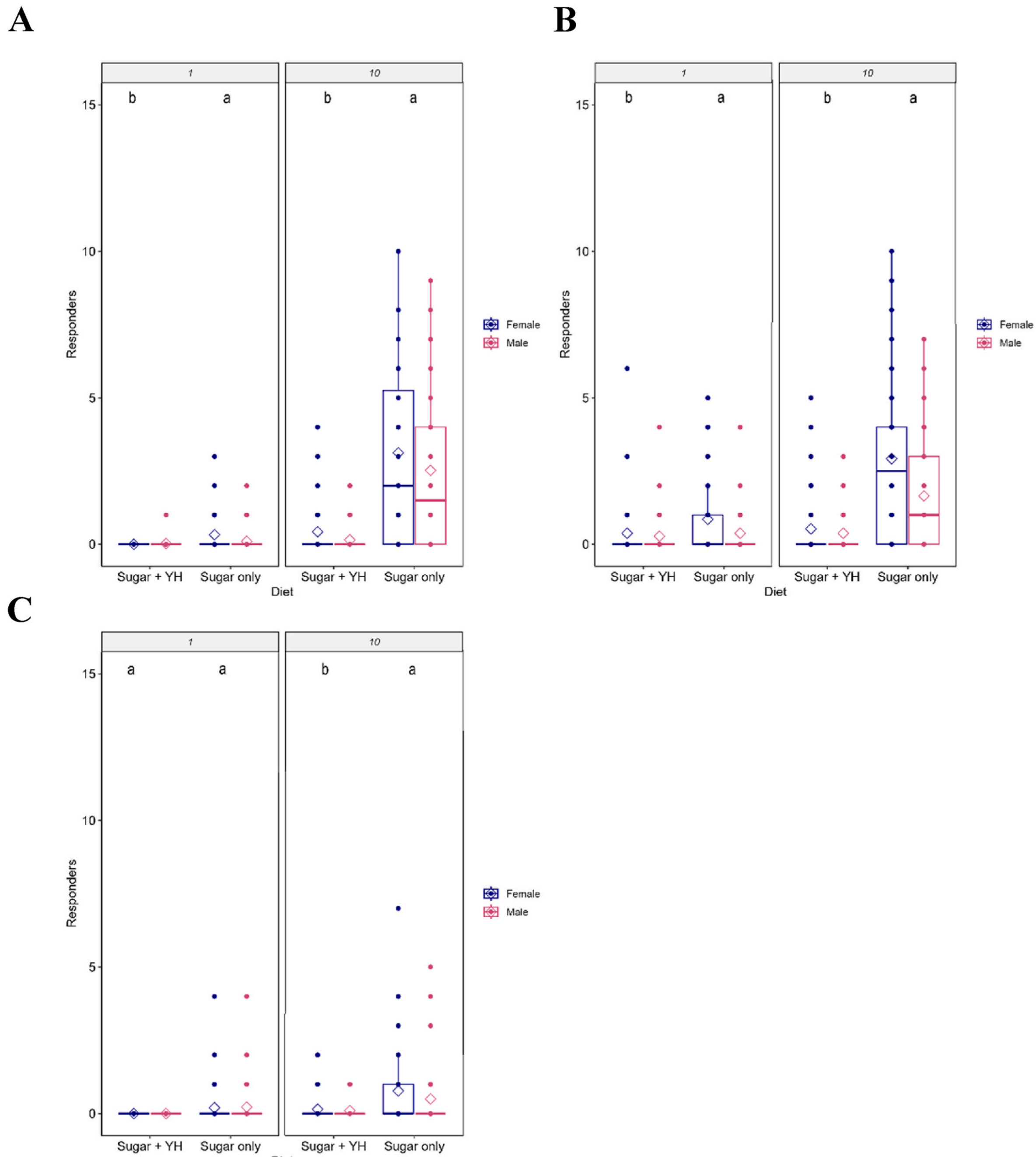

Fig. 2. Box plots showing patterns of responses to protein bait by immature (day-1) and mature (day-10) males and females of (A) *B. dorsalis*, (B) *C. capitata*, and (C) *C. cosyra* fed one of two adult diets. The diamond shapes indicate mean responses. Box plots with different letters indicate significant differences between diet groups within the same age in relation to response.

host foraging strategies (Balagawi et al., 2012) take place within the tree canopy and these could be the reason as to why we also found higher responses to protein bait in these positions. While *C. cosyra* showed similar trends in protein bait response with canopy height as *B. dorsalis* and *C. capitata*, it responded less than the other two species. The low response to protein in *C. cosyra* is likely linked to their lower reproductive output and associated protein requirements (Roets et al., 2018; Pogue et al., 2022).

Contrary to our prediction, older flies visited baits more than the younger ones regardless of the species, sex and diet. Other studies have found high protein intake increasing with higher protein requirements with increasing age as flies mature sexually (Kouloussis et al., 2017). Our findings match those of Vargas et al. (2009) where older females of *Z. cucurbitae* were reported to visit protein baits more than young adult flies. The reduced response by young flies could have occurred due to reduced fly manoeuvrability in young flies as a result of wing muscles not fully developed (Chen et al., 2015). A study carried out by Makumbe et al. (2020) found the effect of age on the total distance covered, with 3-day old young flies flying shorter distances than older age groups. In a similar way, a laboratory based study by Sharp et al. (1975) found that tethered flight increased with age in both females and males of *B. dorsaliş C. capitata* and *Z. cucurbitae*. In addition, Lux et al. (2017) found that 14-day old adult female flies of *Rhagoletis cerasi* (Linnaeus) to be more active than 5-day-old young adults.

Flies fed sugar only responded more to the applied protein baits compared to those fed sugar + YH, regardless of age, sex, and species.

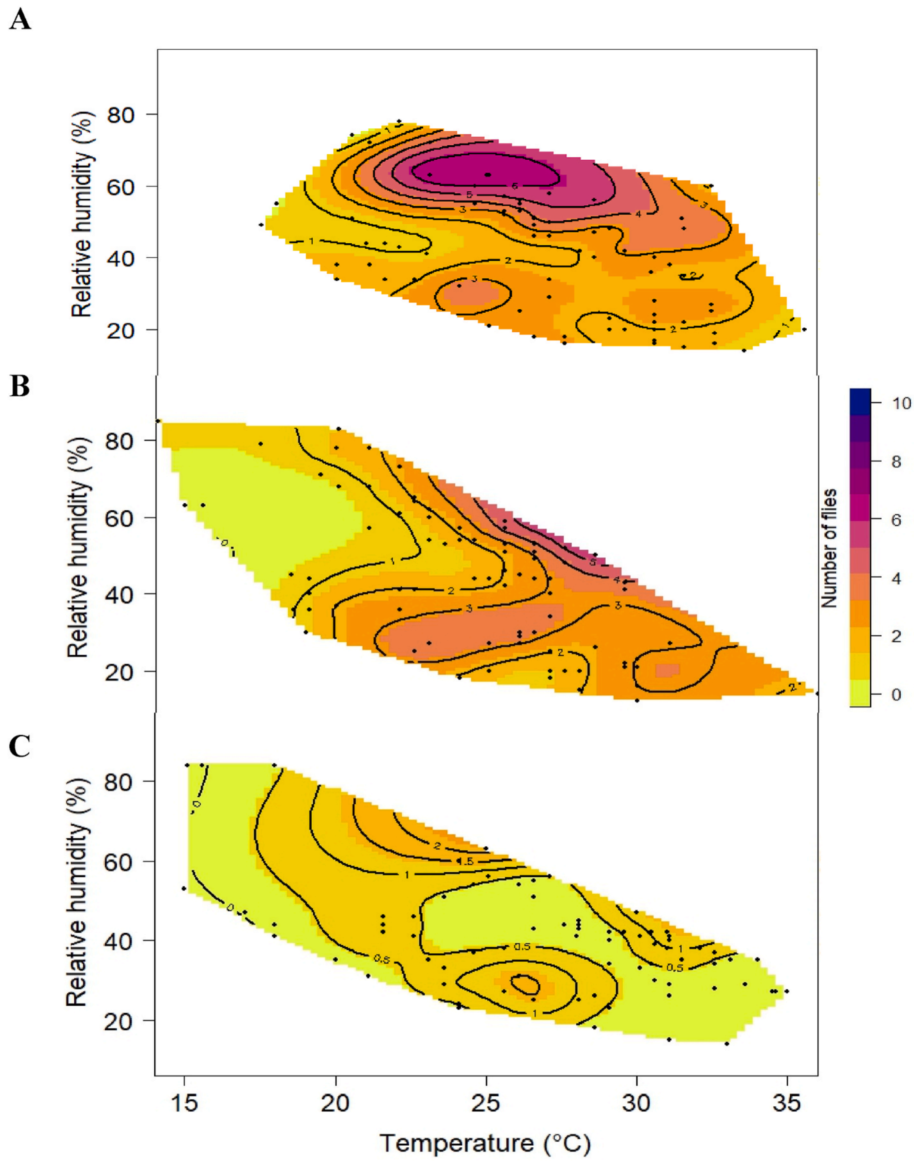

Fig. 3. Relationship between responses of (A) *B. dorsalis*, (B) *C. capitata*, and (C) *C. cosyra* to protein bait and fluctuations in both temperature and relative humidity. The forecasted temperatures and actual temperatures during the study were different and some tests were carried out at temperatures lower than 20 °C.

Table 2
Summary of minimal adequate generalised linear models evaluating the effect of temperature and relative humidity on the response of three fruit fly species to a protein bait.

Species	Effect	*F*	*df*	*P*
B. dorsalis	Temperature	3.629	1	0.058
	Relative humidity	5.116	1	**0.024**
	I(Relative humidity^2)	0.006	1	0.062
	Temperature × Relative humidity	6.780	1	**0.010**
C. capitata	Temperature	9.071	1	**0.002**
	Relative humidity	7.969	1	**0.005**
	I(Relative humidity^2)	4.377	1	**0.037**
	Temperature × Relative humidity	9.238	1	**0.003**
C. cosyra	Temperature	2.161	1	0.142
	Relative humidity	2.277	1	0.132
	Temperature × Relative humidity	2.942	1	0.087

Ceratitis capitata and *C. cosyra* with a high body protein had a lower probability of response to protein baits, while *B. dorsalis* with high protein content in the body still had a higher likelihood of response. Previous studies have shown that fly responsiveness to bait decreases when fed food with nutrients available in baits (Vargas et al., 2002; Yee and Chapman, 2009; Diaz-Fleischer et al., 2009), however the response was not positively related to fly body protein content. In tephritids, both sexes but particularly females require a meal containing protein to enhance reproductive success, which drives protein foraging behaviours and consumption. When *C. cosyra*, *C. fasciventris* (Bezzi), and *C. capitata* were starved of protein, the discrimination threshold for finding proteins was decreased (Manrakhan and Lux, 2009), therefore leading to protein foraging and consumption in order to make up for nutritional deficiencies. The higher likelihood of response to protein bait in *B. dorsalis* that had high body protein was thus unexpected. This may indicate different metabolic processes between *B. dorsalis* and *Ceratitis* species, particularly with regard to utilisation of amino acids for energetic needs during metamorphosis and also in early adult stages. Nestel et al. (2003) reported a decrease in the levels of protein during *C. capitata* pre-pupal stage which occurred due to high energetic

Table 3
Analysis of deviance tables for generalised linear models evaluating the effect of species, sex, diet, and their interactions on the fresh body weight and effect of species, sex, diet, body weight and their interactions, on the body protein and lipid content of *B. dorsalis*, *C. capitata*, and *C. cosyra*.

Body content	Effect	Likelihood ratio χ^2	df	*P*
Weight	Species	928.65	2	**<0.001**
	Sex	8.21	1	**0.004**
	Diet	72.87	1	**<0.001**
	Species × Sex	8.08	2	**0.018**
	Species × Diet	8.20	2	**0.017**
Protein	Species	7.753	2	**0.021**
	Sex	0.089	1	0.766
	Diet	2.909	1	0.088
	Weight	0.267	1	0.605
	Species × Sex	1.068	2	0.586
	Species × Diet	15.342	2	**<0.001**
	Sex × Diet	0.213	1	0.645
	Species × Weight	9.147	2	**0.010**
	Species × Sex × Diet	8.002	2	**0.018**
Lipid	Species	2.961	2	0.227
	Sex	2.721	1	0.099
	Diet	5.509	1	**0.019**
	Weight	3.393	1	0.065
	Species × Sex	9.419	2	**0.009**
	Sex × Diet	4.701	1	**0.030**
	Species × Weight	7.766	2	**0.021**

demands causing amino acids to be oxidised and integrated into other energetic metabolites. Similar studies should be conducted to better understand the role of protein in energy reserves for *B. dorsalis*. An increase in body lipid content in flies resulted in a decreased likelihood of response to protein bait. A low response with increased body lipid levels may result from the prioritisation of energy storage and investment in reproduction over other activities (i.e., protein foraging) after obtaining sufficient lipid body content, causing a trade-off with foraging behaviour (Nasiri Moghadam et al., 2015; Arrese and Soulages, 2010). The increased response to protein in flies with lower lipid levels may be attributed to their greater energy for flight, which likely enhanced their ability to forage for resources. For instance, Nasiri Moghadam et al. (2015) reported that multiple processes in insects use fats as a primary source of energy at the same time, which lead to predictable intricate trade-offs across various attributes. In other studies lipid body composition has been shown to reflect energetic status, with a decrease observed when flies are in high energy demands (Nestel et al., 1985, 2004). Additional research is required to better understand the role played by fly nutrition in influencing the response to odour-based lures applied in the field. Contrary to some of our results, other studies have shown that flies fed a protein-rich diet tend to have high levels of protein (Kaspi et al., 2002; Aluja et al., 2011), while those fed sucrose only having higher lipid levels (Nestel et al., 2005). For instance, Kaspi et al. (2002) showed that protein-fed flies had more protein reserves compared to flies that were protein-starved during their development. On the other hand, Nestel et al. (2005) reported that a diet containing only sugar significantly increased the lipid content of female *C. capitata*. In this study, flies were fed on a protein-rich carrot based larval diet during their larval stage, which might have led to an accumulation of protein in flies despite having been fed sugar only during their adult stage. For instance, Nestel and Nemny-Lavy (2008) and Weldon et al. (2019) reported protein body content to be largely influenced by larval feeding during the larval stage since the larval stage also contributes to metabolic reserves found in adults.

Our results indicate that moderate temperatures and moderately humid conditions are favourable for fruit fly attraction to protein baits. Temperature influenced fruit fly response to the protein baits in a non-linear manner, with flies exhibiting a higher response during the warmer part of the day at temperatures ranging between 20 and 30 °C, at a relative humidity that ranged between 40 and 80 %. However, this was species dependent because a lower number of *B. dorsalis* and *C. capitata* were still responsive at temperatures greater than 30 °C and 80 % relative humidity. Similar patterns have been observed in *Anastrepha* species, which were more responsive to synthetic lures during hot and dry seasons at moderately low relative humidity and higher temperatures (Canal et al., 2007). These results may be a consequence of flies

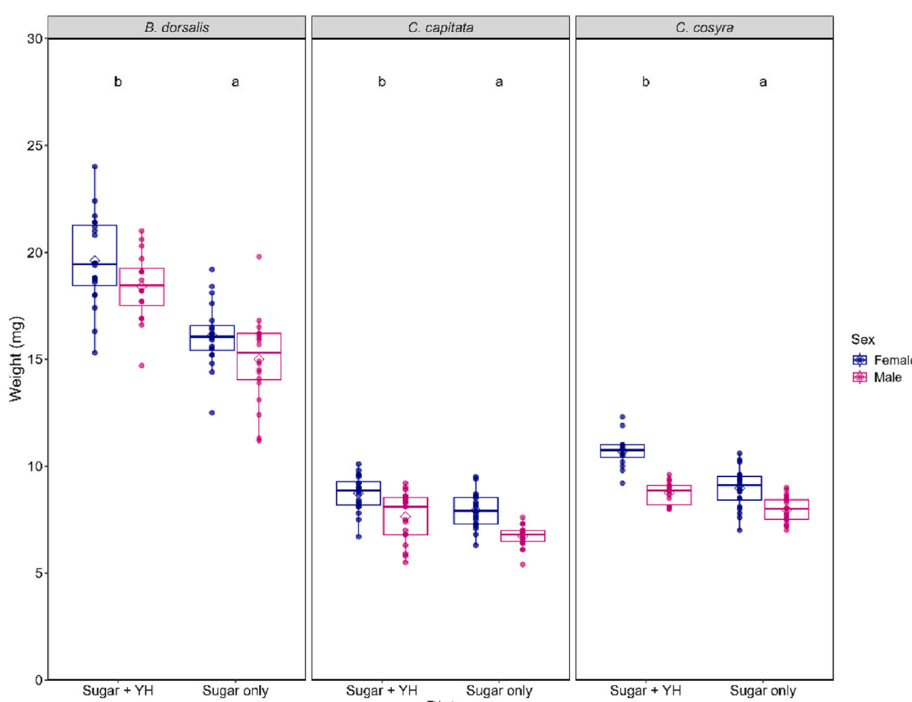

Fig. 4. Fresh body weight of 10-day old females and males of *B. dorsalis*, *C. capitata*, and *C. cosyra* fed on sugar only and sugar plus hydrolysed yeast diet. The diamond shapes indicate mean responses. Box plots with different letters indicate significant differences between diet groups across species in relation to fresh body weight.

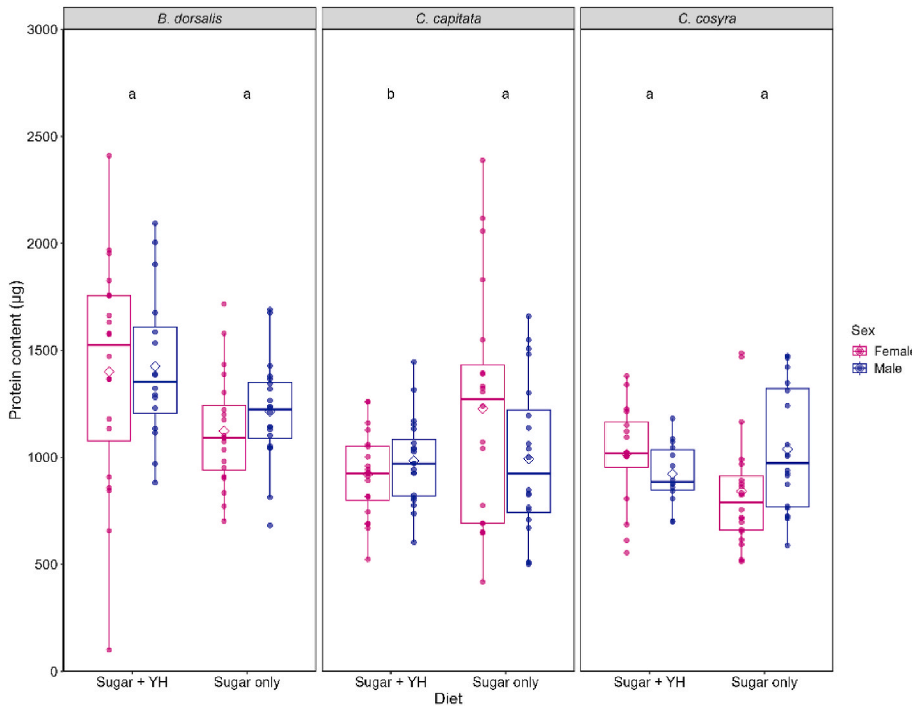

Fig. 5. Protein content in the body of 10-day old females and males of *B. dorsalis*, *C. capitata*, and *C. cosyra* fed on sugar and sugar plus hydrolysed yeast diet. The diamond shapes indicate mean responses. Box plots with different letters indicate significant differences between diet groups across species in relation to protein content.

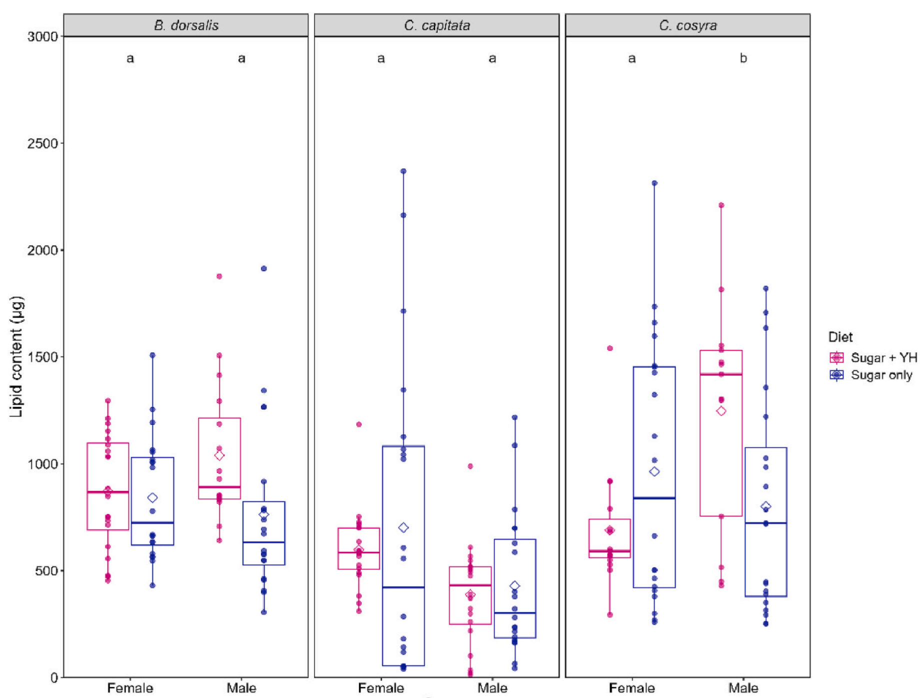

Fig. 6. Lipid content in the body of 10-day old females and males of *B. dorsalis*, *C. capitata*, and *C. cosyra* fed on sugar and sugar plus hydrolysed yeast diet. The diamond shapes indicate mean responses. Box plots with different letters indicate significant differences between sex groups across species in relation to lipid content.

seeking cooler, sheltered microclimates to avoid thermal stress when it is very hot. For example, *B. tryoni* walk and rest in the lower levels of a tree canopy, where relative humidity was high and light intensity was low, during the hottest times of the day (Inskeep et al., 2021). Thermal limits for flight behaviour also probably contributed to the observed effects of temperature on fruit fly responses to protein baits. Insect flight is an energetically expensive behaviour and the energy needed is released by temperature-dependent biochemical reactions (Reinhold, 1999). Makumbe et al. (2020) found that tethered flight performance by *B. dorsalis* was optimal between 20 and 24 °C, and dropping to low levels at 12 °C. All muscular functions are lost by *B. dorsalis* at temperatures below 9.1 °C and above 46.2 °C (Motswagole et al., 2019).

Table 4
Analysis of deviance tables for generalised linear models evaluating the effect of species, diet, fly protein or body lipid content, and their interactions, on the likelihood of response by *B. dorsalis*, *C. capitata*, and *C. cosyra*. Flies were categorised as either responders (1) or non-responders (0) based on their response to a protein bait, so a binomial distribution was used to fit the data.

Effect	Likelihood ratio χ^2	df	*P*
Species	0.844	2	0.656
Diet	10.292	1	**0.001**
Protein	10.881	1	**0.001**
Lipid	20.913	1	**<0.001**
Protein × Diet	6.819	1	**0.009**
Diet × Species	4.006	2	0.135
Diet × Lipid	2.258	1	0.133

The practical implications of this study are that: (1) protein bait should be applied on the canopy (at least 1.5m above the ground) for it to be effective in control, (2) application of protein bait should be adjusted based on availability of natural food sources (honeydew, pollen, extra floral nectaries, plant exudates fruit juice from fallen and rotten fruits etc.) in their ecosystem (Christenson and Foote, 1960), which if present may lead to a reduced response, and (3) protein bait should be applied when temperatures are above 20 °C for increased fly response. Our findings also show that there is variability of response amongst fruit fly species. Therefore, it should not be assumed that baits will be equally effective for all tephritid species if there are numerous fruit fly pests present in an area. Hence, it is crucial that species composition in an area is identified as well as the effectiveness of baits used against each species present, before the implementation of a fruit fly control program. It is important to note that fruit fly species tested in the present study were released sequentially, which is not entirely representative of natural environments where several fruit fly species may possibly interact and impact protein bait response. Furthermore, the study did not evaluate the duration of bait attractiveness. Future research should quantify the attractiveness of natural protein sources in comparison to artificial protein baits, which would entail observing fruit fly activity on natural food sources and then applying protein baits to investigate the effectiveness of protein baits in the presence of natural protein sources.

CRediT authorship contribution statement

Msizi R. Ramaoka: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Aruna Manrakhan:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Christopher W. Weldon:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Christopher W. Weldon reports financial support was provided by Citrus Research International Pty Ltd. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cropro.2025.107331.

Data availability

The data supporting the findings of this study are openly accessible and can be found here: https://doi.org/10.25403/UPresearchdata.27900741.v2.

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