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Isotopic discrimination and persistence of the ^{13}C marker in adults of *Anastrepha fraterculus* (Diptera: Tephritidae) Brazilian-1 morphotype

Victor Wilson Botteon[1], Maria de Lourdes Zamboni Costa[1], Luis Anselmo Lopes[1], Adalecio Kovaleski[2], Luiz Antonio Martinelli[1], and Thiago Mastrangelo[1,]*

Abstract

Stable carbon isotope ratios can be used to discriminate between wild and sterile insects that are caught in surveillance traps when 2 isotopically distinct dietary sources are available for the immature or adult stages. Artificial diets containing naturally ^{13}C-labelled sugar can isotopically mark the adults of some tephritids, but when sexually mature flies are released in the field, their food source usually changes from a C_4 to a C_3 plant. Consequently, the isotopic composition of flies can change toward the isotopic signatures associated with the new diet. For isotope labelling to be more meaningful in a pest management program that integrates the sterile insect technique, it is important to know the persistence of the carbon isotope marker in field-release sterile adults. Therefore, this study was intended to assess the degree of isotopic differentiation between wild samples of *Anastrepha fraterculus* (Wiedemann) (Diptera: Tephritidae) and flies reared on different artificial diets, and to estimate the turnover of carbon in flies after shifts to different adult diets. The whole bodies of flies reflected the overall isotopic composition of their larval diets immediately after emergence. When the adult diet was switched, the $\delta^{13}C$ signatures of flies changed rapidly for 6 to 8 d, then reached an isotopic equilibrium with the final diet. Depletions up to −5.6‰ (parts per thousand) were observed in the signatures of flies switched from a C_4 to a C_3-based diet. However, appropriate feeding on diets with C_4 sources ensured that the isotopic composition of larval diets was fixed in body structures of adult *A. fraterculus*, maintaining measurable ^{13}C signals distinct from wild flies for 15 d after diet switching. No differences were found between males and females when they fed on different pre-release diets or after diet change. The ^{13}C stable isotope proved to be a reliable marker for differentiating wild and laboratory-reared *A. fraterculus* flies.

Key Words: Sterile Insect Technique; stable isotope analysis; monitoring; South American fruit fly

Resumo

A razão entre os isótopos estáveis do carbono pode ser utilizada na distinção entre insetos selvagens e estéreis que são capturados em armadilhas de monitoramento, quando duas fontes de dieta isotopicamente distintas são disponíveis para os estágios imaturos ou adultos. Dietas artificiais contendo açúcar naturalmente marcado com ^{13}C podem marcar isotopicamente os adultos de algumas espécies de tefritídeos, mas quando moscas sexualmente maduras são liberadas no campo, sua fonte alimentar geralmente muda de uma planta C_4 para outra com metabolismo C_3. Consequentemente, a composição isotópica das moscas pode cambiar em direção às assinaturas isotópicas associadas com a dieta mais recente. Para que a marcação isotópica tenha sentido em um programa de manejo de pragas que integra a Técnica do Inseto Estéril, é importante saber a persistência do marcador isotópico de carbono nos adultos estéreis liberados no campo. Este estudo objetivou, portanto, avaliar o grau de diferenciação entre amostras de *Anastrepha fraterculus* (Wiedemann) (Diptera: Tephritidae) selvagem e moscas criadas em diferentes dietas artificiais, e estimar o turnover do carbono nas moscas após a mudança das dietas de adulto. Os corpos das moscas refletiram a composição isotópica geral de suas dietas larvais logo após a emergência. Quando a dieta de adulto foi mudada, as assinaturas de $\delta^{13}C$ das moscas mudaram rapidamente por 6 a 8 dias e logo alcançaram um equilíbrio isotópico com a dieta final. Depleções de até −5.6‰ puderam ser observadas nas assinaturas das moscas que mudaram de uma dieta C_4 para uma C_3. Entretanto, uma alimentação adequada nas dietas com fontes C_4 permitiu que a composição isotópica das dietas larvais fosse fixada nas estruturas corporais de *A. fraterculus*, mantendo sinais de ^{13}C mensuráveis e distintos das moscas selvagens por 15 dias após a troca de dietas. Não foram encontradas diferenças entre machos e fêmeas quando elas se alimentaram em diferentes dietas pré-liberação ou após a troca de dieta. O isótopo estável ^{13}C provou ser um marcador confiável para a diferenciação de moscas selvagens e criadas em laboratório de *A. fraterculus*.

Palavras Chave: Técnica do Inseto Estéril; análise de isótopos estáveis; monitoramento; mosca-das-frutas Sul-americana

The sterile insect technique (SIT) is a genetic control method that relies on area-wide inundative releases of sterilized mass-reared insects to reduce the fertility of populations of the same species (Knipling 1968). It has been applied in many parts of the world to control tephritid populations or prevent their establishment (Dyck et al. 2005; Vreysen et al. 2007). Because the success of the technique relies on the transfer of sperm containing dominant lethal mutations to females in the field (Bakri et al. 2005), the released sterile males should be sexu-

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ally mature. The release of sterile males prior to their sexual maturity is avoided in most sterile insect technique programs to prevent high mortality of the insects by predation, starvation, or other causes (Hendrichs et al. 2007; Dor et al. 2014). However, many fruit fly species have long pre-reproductive periods, and the adults must be kept in containers or rooms at emergence centers (Dowell et al. 2005). During this holding period, shortly after emergence, the flies must be provided with a water source and adult food, which is usually sugar- or yeast-enriched diets (Yuval et al. 2007; Perez-Staples et al. 2009; Liedo et al. 2013; Teal et al. 2013; Utgés et al. 2013).

To estimate sterile to wild ratios in the field, it is necessary to perform an accurate distinction between the sterile and wild flies caught in surveillance traps. Before being shipped to emergence facilities, fly pupae usually are marked with fluorescent dyes and then irradiated (Arredondo et al. 2017). Dye particles adhere to the ptilinum of the adults as they emerge, and are incorporated into the head capsule as the ptilinum withdraws into the fly's head. Upon capture, marks in the adult heads are sought under an ultraviolet lamp or epifluorescence microscopy (Enkerlin et al. 1996; Guillen-Aguilar et al. 2016). An alternative to the fluorescent dye marking method may be the use of carbon stable isotopes as biomarkers (IAEA 2009).

The utility of carbon isotope markers is based on the fact that plants with C_3 or C_4 metabolism possess different $^{13}C/^{12}C$ ratios due to fractioning during photosynthetic carbon fixation (O'Leary 1988). The isotopic signature of C_3 plants is in the range of −25 to −35‰ (average of −27‰) relative to Vienna PeeDee Belemnite (VPDB), whereas C_4 plants have signatures between −7 and −18‰ (average at −11‰) (Dawson & Brooks 2001). Many studies have demonstrated that carbon isotopic signatures can be used for insects that have C_3 plants as hosts in the field but that are reared on C_4-based diets in mass-rearing facilities (Hood-Nowotny et al. 2011, 2016; Opiyo et al. 2016). Hood-Nowotny et al. (2009) demonstrated that the $^{13}C/^{12}C$ ratio can be used to distinguish adults of the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), reared on diets containing C_4 sugar from wild flies with > 95% confidence.

The isotopic signature that flies obtain during the larval period is reflective of their diet, but it may not remain immutable throughout their adult life. In sterile insect technique programs to control fruit flies, the sterile flies are held for several d at emergence facilities on pre-release diets, and once in the field, they feed on natural food sources for the rest of their lives (Hendrichs et al. 1991; Liedo et al. 2013). Through respiration and feeding on new diets with different carbon signatures, the released flies could lose their characteristic signal due to the turnover of carbon isotopes of C_4 origin. Isotopic turnover is the continuous renewal of chemical elements and their isotopes in body tissues, usually described as isotopic half-life in d (i.e., half-life is the time required to reach 50% isotopic equilibrium with the diet) (Vander Zanden et al. 2015). However, animals in general do not incorporate the isotopic values of diets instantaneously, but rather the rate at which they trade elements determines how fast the isotopic signal of a novel food resource is acquired into animals' tissues (Martínez del Rio & Carleton 2012). Because isotopic incorporation rates differ among species (Hyodo 2015), their determination is necessary for the correct interpretation of field isotopic data. Until now, only Hood-Nowotny et al. (2009) have estimated the turnover time for a tephritid by shifting the carbon isotopic composition of the diets of laboratory-reared medflies.

With all the knowledge gathered on the artificial rearing, sterilization, and genetics of *Anastrepha fraterculus* (Wiedemann) (Diptera: Tephritidae) (Cladera et al. 2014), a sterile insect technique project called MOSCASUL has begun trials in southern Brazil to suppress populations of this pest from commercial apple orchards using parasitoids and sterile flies (Costa et al. 2016; Mastrangelo et al. 2018). Intrinsic isotope labelling could be a useful complementary strategy to conventional dye marking to identify sterile flies that had been poorly marked and released in the field. A preliminary study conducted in Brazil revealed that *A. fraterculus* adults can be labelled by stable isotopes from larval diets, providing sufficient separation in carbon isotopic signals to determine whether the captured fly was wild or laboratory-reared (e.g., the $\delta^{13}C$ signatures of flies reared on C_4-based diets were about −15.0 ± 1.0‰, whereas the $\delta^{13}C$ of wild flies from apple orchards was about −25.5 ± 1.5‰) (Botteon & Mastrangelo 2017).

The length of time over which the carbon isotope marker can be retained in *A. fraterculus* flies when the adult diet changes is still unknown. Generally, *A. fraterculus* males present a long precopulatory period, starting pheromone calling and mating in 5 to 7 d after emergence (Liendo et al. 2013; Segura et al. 2013). Before the field release of sexually mature males, they must be held at emergence facilities on a pre-release dietary regime, which may include C_4 sugar-based diets (Liendo et al. 2013).

For $\delta^{13}C$ signatures from *A. fraterculus* samples to be more meaningful in a sterile insect technique context, it is important to know the persistence of the carbon isotope marker in adult life, and the turnover times of carbon when the adult diet changes from C_4 to C_3, or vice versa. Therefore, the aim of this study was to (1) assess the degree of isotopic differentiation between wild *A. fraterculus* and flies reared on different diets, and (2) estimate the turnover of ^{13}C in flies after shifts on isotopically distinct adult diets, also verifying if the turnover would be influenced by sex.

Materials and Methods

SAMPLING OF WILD *ANASTREPHA FRATERCULUS* FLIES

The wild *A. fraterculus* flies were captured in commercial apple orchards located in the municipality of Vacaria (28.525044°S, 50.815244°W), Rio Grande do Sul State, Brazil, in early 2017 with McPhail traps containing Ceratrap™ (Bioibérica, Barcelona, Spain).

The traps were inspected at 24 h intervals. All manipulations of flies in the field were carried out using gloves. After collection, the flies were washed with distilled water, dried for 6 h on tissue paper, and placed individually in Eppendorf tubes. For the stable isotope analysis at the Center for Nuclear Energy in Agriculture, 60 flies were sampled and identified (Hernández-Ortiz et al. 2012).

REARING *ANASTREPHA FRATERCULUS* ON DIFFERENT LARVAL DIETS

A colony of *A. fraterculus* Brazilian-1 morphotype, originally established from larvae collected in *Acca sellowiana* (Berg) Burret (Myrtaceae) at Vacaria (Dias et al. 2016), provided the eggs to be seeded in artificial diets and the adults to oviposit in papaya fruits. This colony has been maintained in laboratory for 2 yr following the procedures described by Walder et al. (2014). Eggs collected from ovipositing cages were bubbled in a water bath at 25 °C for 72 h, then aliquots of 2 mL of eggs were seeded in trays containing 1 L of artificial diet, with the larvae remaining there for 8 to 9 d at 25 °C (Walder et al. 2014).

Two different artificial diets were used for larval rearing: (a) Diet I, consisting of 50 g of brewer's yeast Brewcell™ (Biorigin, Lençois Paulista, São Paulo, Brazil), 30 g of sugar (sugar cane source) Carave-

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las™ (Usina Colombo, Ariranha, São Paulo, Brazil), 300 g of corn bran Yoki™(General Mill Alimentos, Barueri, São Paulo, Brazil), 2 mL of Nipagin, 2 g of sodium benzoate, 6 g of citric acid, and 1,000 mL of distilled water; and (b) Diet II, a gelled diet adapted from Salles (1992), consisting of 3 g of agar, 60 g of corn bran Yoki™ (General Mill Alimentos), 60 g of sugar Caravelas™ (Usina Colombo), 60 g of brewer's yeast Brewcell™ (Biorigin), 1 g of sodium benzoate, 6 mL of hydrochloric acid, 8 mL of Nipagin fungicide, and 900 mL of distilled water. The $\delta^{13}C$ values of these artificial diets were constant throughout the course of the tests and characteristic of C_4 plants.

To obtain adult flies from an alternative host (C_3-based diet), larvae were reared on papaya fruits (*Carica papaya* L. cv. 'Golden') (Caricaceae) (Machota et al. 2010). Adults from the mother-colony were kept in screened cages (45 × 45 × 45 cm; about 600 couples per cage) with a mixture of sugar and hydrolyzed brewer's yeast *Bionis* YE MF™ (Biorigin) at a 3:1 rate and water ad libitum (Nunes et al. 2013) under laboratory conditions (24 ± 1 °C and 70 ± 10% RH) for the infestation of fruits.

The papaya fruits were inserted in each cage separately when flies were 7 d old. After 24 h, the fruits were removed from cages and conditioned in plastic boxes (40 × 20 × 15 cm) for future collection of larvae. Third instar larvae were washed from the artificial diets and transferred to plastic cups (500 mL) with moist vermiculite for pupation in a dark room. When larvae began crawling from fruits, they were collected twice a d, and left to pupate in plastic cups (500 mL) with vermiculite. The experimental units were distributed randomly, and 5 replicates were performed for each treatment.

Diet Switching Experiments

Emerged adult flies that originated from the diets previously described were kept separately in screened cages (30 × 30 × 30 cm; about 600 couples per cage) at 24 °C, 65% RH, and a 12:12 h (L:D) photoperiod. Flies that came from Diets I and II were divided into 3 different groups, each containing 1 type of adult diet and water ad libitum. The most common pre-release diets used in operational tests conducted by the Center for Nuclear Energy in Agriculture staff were: (a) 100% refined cane sugar *Caravelas*™ (Usina Colombo); (b) a mixture of cane sugar and hydrolyzed yeast *Bionis* YE MF™ (Biorigin) at a 3:1 rate; and (c) the Gainesville diet (formulated with 1.58 g of agar, 0.05 g of ascorbic acid, 0.005 g of sodium benzoate, 100 mL of citrus honey, and 100 mL of water) (Garcia & Ricalde 2013).

After 5 d, the adult diets from these groups of flies were switched to apple (*Malus domestica* Borkh. cv. 'Gala') (Rosaceae), a C_3-based diet. Meanwhile, flies whose larvae were reared on papaya fruits were maintained in cages (30 × 30 × 30 cm; up to 600 couples per cage) containing water and papaya slices ad libitum for 5 d, at which point the papaya slices were switched to 100% refined cane sugar. The timing of diet switch (5 d) was chosen to reflect the release time for flies of the bisexual strain used (Segura et al. 2013; Dias et al. 2016). To serve as C_4 and C_3 controls, an additional group of adults from each of the larval diets remained feeding on sugar for 15 d, whereas another group with flies from papaya remained feeding on papaya slices, respectively.

Five males and 5 females of each treatment were randomly sampled at 24 h after emergence, and at 0, 1, 2, 5, 7, 12, and 15 d after the diet switch, where time 0 is the d of the diet switch (i.e., when adults were 5 d old). A schematic view of the diet switching experiments is shown in Figure 1.

As release-recapture experiments in apple orchards have shown, more than 90% of recaptures occur within 17 d after release (Kovaleski et al. 1999), and the flies were allowed to feed on apple slices or sugar for 15 d in our tests. Fly samples from each d were freeze killed (15–20 min at −20 °C) and placed in individual Eppendorf tubes.

A 2-source mixing model was used to calculate the proportion of structural carbon in *A. fraterculus* fed on 2 dietary sources (i.e., flies derived from papaya, and fed on sugar after diet switching), following Hood-Nowotny et al. (2009): Proportion (% C) derived from the papaya diet = $[(\delta^{13}C_{fly} - \Delta^{13}C_{papaya}) - \delta^{13}C_{sugar}] / (\delta^{13}C_{papaya} - \delta^{13}C_{sugar})$; where $\Delta^{13}C_{papaya}$ is the trophic discrimination factor ($\Delta^{13}C_{papaya} = \delta^{13}C_{fly} - \delta^{13}C_{papaya}$). Flies from papaya were used to assess the proportion of structural carbon, due to the maintenance of the dietary source from larval stage upon emergence. One larval diet and 2 different adult diets were used in the other treatments, which would increase the complexity of the model, because of the variability and uncertainty in estimates of source contributions (Phillips et al. 2005).

The carbon isotopic data from each treatment were analyzed by the Boltzmann sigmoidal regression model, according to the following equation: $\delta^{13}C_{(t)} = \delta^{13}C_{(f)} + \{(\delta^{13}C_{(i)} - \delta^{13}C_{(f)}) / [1 + e^{(t - x_0)/dx}]\}$, where $\delta^{13}C_{(t)}$ is the flies' relative signature at any given time (t), $\delta^{13}C_{(f)}$ is the isotopic signature at time 't', $\delta^{13}C_{(i)}$ is the initial isotopic signature, X_0 is the inflection point of the sigmoid curve expressed in d (it also represents carbon half-life; HL = $t_{1/2}$), and *dx* is the time constant expressed in d. The shift in isotopic signature was perceived as complete from the moment in which the $\delta^{13}C$ signature reached a linear plateau, becoming almost constant (Ducatti et al. 2014).

Stable Isotope Analyses

Analyses of ^{13}C:^{12}C and ^{15}N:^{14}N ratios were performed to determine the isotopic compositions of the artificial diets and papaya fruits, wild flies collected in Vacaria, and laboratory-reared flies sampled at different d after the diet switch. The δ was calculated by $\delta X = [(R_{sample}/R_{standard}) - 1]$, where δX refers to $\delta^{13}C$ or $\delta^{15}N$, and R is the molar ratio of rare to abundant isotopes (^{13}C:^{12}C or ^{15}N:^{14}N) of the sample (R_{sample}) and standard ($R_{standard}$). Isotope ratios are expressed in relative difference per thousand (‰) to the ratio of international reference standards (Vienna PeeDee Belemnite [VPDB] and atmospheric nitrogen [N_2] for C and N, respectively) (IAEA 2009).

Individual fly samples were placed to dry in a ventilated stove at 50 °C for 72 h, then macerated until reaching constant weight. The whole body of flies was used for stable isotope analysis, because analysis of total carbon from small organisms provide accurate measures of dietary exposures (DeNiro & Epstein 1978). All resulting dried materials were weighed (0.5 mg for artificial diets, 0.8 to 1.0 mg for fly samples, and 2.8 mg for fruit samples) in a precision analytical balance (Model ME 36S, Sartorius, Göttingen, Germany), and enclosed in 5 × 3.5 mm tin capsules (Elemental Analysis Ltd., Okehampton, Cornwall, United Kingdom).

The samples were analyzed in a Carlo Erba CHN-1110 elemental analyzer (CE Instruments, Rodano, Italy), coupled to a Continuous Flow Isotope Ratio Mass Spectrometer (CF-IRMS) (Delta Plus-Thermo Scientific, Bremen, Germany). Samples were combusted in an oxygen atmosphere at approximately 1,700 °C, and the resulting CO_2 and N_2 were purified in the elemental analyzer through a chromatographic separation column in an ultrapure helium carrier, and sequentially admitted to the mass spectrometer with an interface (Conflo II, Thermo Scientific, Bremen, Germany). The CO_2 and the N_2 peaks were evaluated in the CF-IRMS to determine isotopic ratios.

The results were normalized to international standards using secondary reference materials (NBS-19, NBS-22, IAEA-N1, IAEA-N2) (Groning 2004). An internal standard (i.e., sugar cane leaf) was used for quality control in every 11 samples in each run, in which the precision was evaluated as ± 0.15‰ for both elements. Each sample was analyzed twice to obtain the mean values with precision of 0.15‰. The analytical error of the isotopic measurements was estimated at ± 0.15‰

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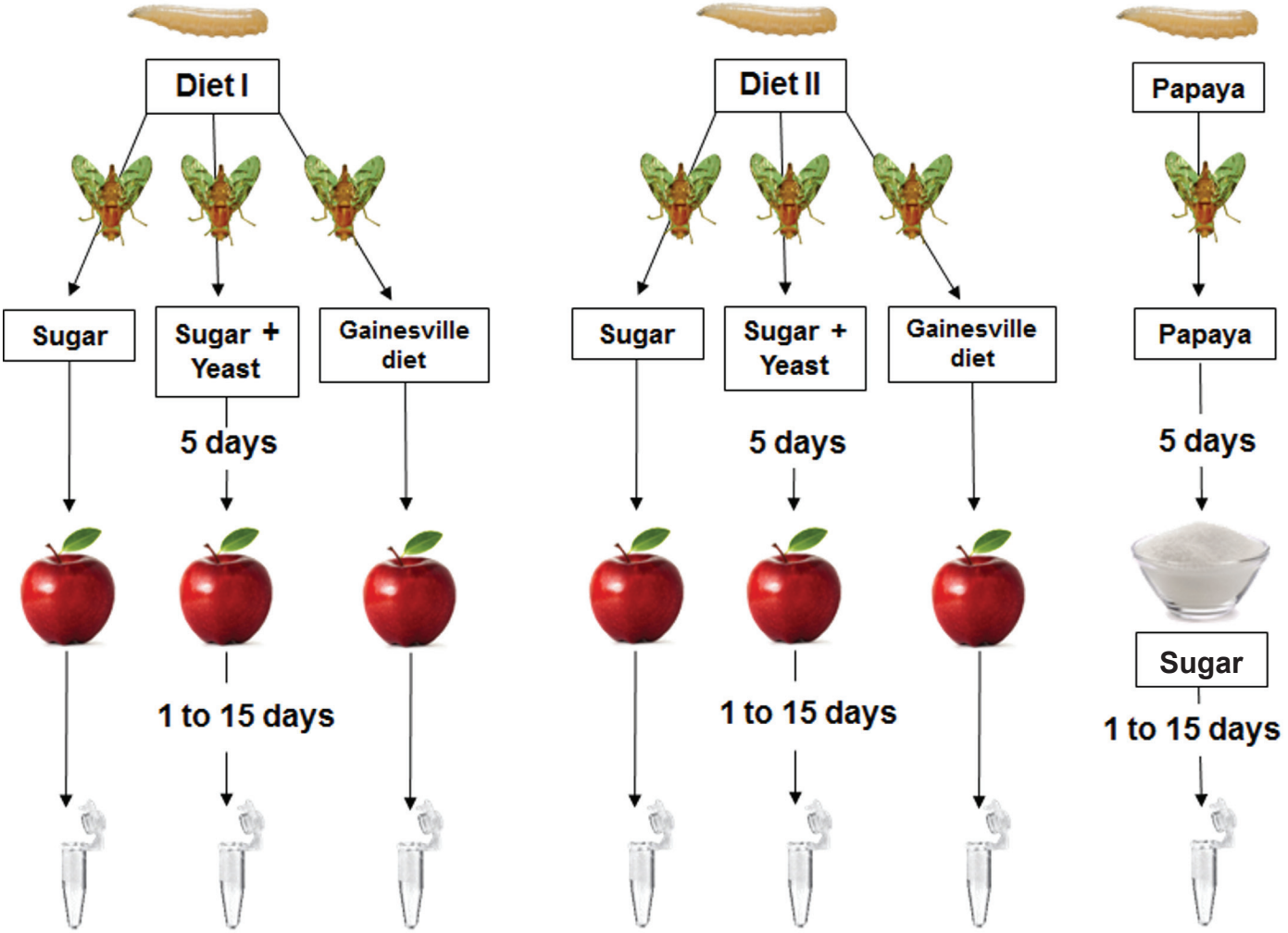


Fig. 1. Schematic view of the diet switching experiments.

for $\delta^{13}C$ and $\delta^{15}N$ by means of repeated measurements of the internal standard.

Statistical Analyses

The mean values of isotopic signatures ($\delta^{13}C$ and $\delta^{15}N$) from males and females of all diet switching treatments on the first (t = 0 d) and the last d (t = 15 d) of the assay were compared using the Student's *t*-test (α = 0.01). For the mean values of $\delta^{13}C$ and $\delta^{15}N$ from the 1-d-old adults obtained from larval diets and fed on different diets for 5 d, and for the isotopic composition of the adult diets, the 1-way analysis of variance (ANOVA) *F*-test was applied at the 1% of significance, and when significant differences were detected, the Tukey's honestly significance difference (HSD) test (α = 0.01) was applied to compare the means.

The $\delta^{13}C$ values of flies from diet switching tests (i.e., adults between 5 and 20 d old) were fitted with regression methods for nonlinear functions by the software Origin 8.1® (OriginLab 2009). The possible differences in $\delta^{13}C$ and $\delta^{15}N$ values between flies collected on the last sampling period after diet switching (d 15) and wild flies also were assessed by the Tukey's honestly significance difference (HSD) test (α = 0.01). Homogeneity of variances and normality of model residuals were checked in all instances (Bartlett 1937; Shapiro & Wilk 1965). The ANOVA analyses and comparison of means tests were performed by the statistical program SAS 9.4 (SAS 2013).

Results

ISOTOPIC SIGNATURES OF DIETS AND *ANASTREPHA FRATERCULUS* FLIES

Wild flies (n = 60) gave $\delta^{13}C$ and $\delta^{15}N$ signatures of −25.7 ± 0.2‰ and 5.4 ± 0.6‰, respectively, which reflects the C_3 metabolism of their host plant species. The mean $\delta^{13}C$ and $\delta^{15}N$ values from larval and adult diets, wild and laboratory-reared flies (1 and 5 d old) are presented in Table 1. The mean $\delta^{13}C$ signatures of the adult diets (100% sugar, mixture of sugar plus hydrolyzed yeast, and the Gainesville diet) were significantly different from larval diets (papaya, Diets I and II) ($P < 0.01$). The $\delta^{13}C$ signals of sugar only and the mixture of sugar plus hydrolyzed yeast did not differ significantly ($P > 0.01$). The $\delta^{15}N$ signatures from the 2 adult diets containing sugar differed significantly from the values of larval diets (Table 1).

No significant differences were observed between the $\delta^{13}C$ signatures of 1-d-old flies that came from Diets I and II, nor between flies from these diets that were fed on adult diets for 5 d containing cane sugar (Table 1). However, the $\delta^{13}C$ of flies fed on the Gainesville diet differed from all others ($P < 0.01$). Newly emerged flies from papaya differed from wild flies in $\delta^{13}C$, but this difference disappeared when those flies were fed on papaya slices for 5 d. With the exception of the

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Table 1. Isotopic signatures (mean ± SE) of larval diets, adult diets and *Anastrepha fraterculus* flies (1 and 5 d old).

Treatment	δ^{13}C ‰	ANOVA	δ^{15}N ‰	ANOVA
Larval diet I	−12.6 ± 0.1 b**		2.9 ± 0.1 bc	
Larval diet II	−14.3 ± 0.1 c		2.4 ± 0.1 c	
Papaya	−26 ± 0.1 e		5.5 ± 0.2 a	
Apple	−28.4 ± 0.1 f	$F_{6,32}$ = 709.53; $P < 10^{-3}$	2 ± 0.2 c	$F_{6,32}$ = 43.9; $P < 10^{-3}$
Sugar diet	−11.9 ± 0.1 a		—	
Sugar and hydrolyzed yeast diet	−12 ± 0.1 a		−1.1 ± 0.1 d	
Gainesville diet	−24 ± 0.1 d		4.5 ± 0.6 ab	
Flies reared on diet I (1 d old)	−15.3 ± 0.1 b		4.0 ± 0.1 cde	
Flies I* fed on sugar	−13.5 ± 0.2 a		4.2 ± 0.1 cd	
Flies I fed on sugar and hydrolyzed yeast	−13.3 ± 0.1 a		3.2 ± 0.2 e	
Flies I fed on Gainesville diet	−20.1 ± 0.2 c		4.3 ± 0.1 cd	
Flies reared on diet II (1 d old)	−16.0 ± 0.1 b		4.1 ± 0.2 cde	
Flies II* fed on sugar	−14.2 ± 0.2 a	$F_{10,106}$ = 859.71; $P < 10^{-3}$	4.4 ± 0.1 bcd	$F_{10,106}$ = 40.58; $P < 10^{-3}$
Flies II fed on sugar and hydrolyzed yeast	−13.6 ± 0.2 a		3.5 ± 0.3 de	
Flies II fed on Gainesville diet	−20.6 ± 0.2 c		4.5 ± 0.1 bc	
Flies reared on papaya (1 d old)	−27.0 ± 0.2 e		6.8 ± 0.3 a	
Flies from papaya fed on papaya	−25.8 ± 0.2 d		6.8 ± 0.2 a	
Wild flies	−25.7 ± 0.2 d		5.4 ± 0.6 b	

*I/II = flies whose larvae were reared on artificial Diet I or II and fed on an adult diet for 5 d after emergence. **Means (± SE) within columns followed by the same letter do not differ significantly at the 1% probability level by the Tukey's test.

latter group of flies, the δ^{13}C of laboratory-reared *A. fraterculus* flies differed from the δ^{13}C of wild flies (Table 1).

The δ^{15}N results were less conclusive than δ^{13}C, with no significant differences between wild flies and some 5-d-old flies from Diet II (Table 1). The flies that fed on papaya slices presented the highest δ^{15}N value (6.8‰), whereas the lowest value (3.2‰) was observed for adults that came from Diet II, and that were fed on the mixture of sugar and hydrolyzed yeast.

No significant differences were found between males and females when they fed on different pre-release diets for 5 d, or after shifting the adult diets for 15 d ($P > 0.01$) (Tables 2 & 3).

ISOTOPIC TURNOVER IN *ANASTREPHA FRATERCULUS* FLIES

The changes in δ^{13}C values of flies versus time from diet switching experiments are shown in Figures 2 and 3, whereas the regression equations and estimated isotopic half-lives for males and females are presented in Table 4. The δ^{13}C of flies from C_4 and C_3 controls remained practically unchanged (i.e., at −14.0 ± 0.5‰ and −25.5 ± 0.3‰, respectively) until d 15 of evaluation (data not shown).

The stable carbon isotope values of flies reared on papaya shifted toward more positive values over the 15 d of the experiment, varying from −25.8 ± 0.2‰ to −20.5 ± 0.3‰ (Tables 1 & 5). This shift was consistent with the incorporation in the adults of isotopically enriched carbon from C_4 sugar. After changing the flies from C_4-based diets (sugar or mix) to apple, isotope ratios of the adults shifted in the direction of the δ^{13}C value of apple (−28.4 ± 0.1‰) (Fig. 3). In those 5 experiments, the rate at which carbon derived from papaya or pre-release diets was replaced by carbon derived from the new diet was adequately described by the Boltzmann sigmoidal model (Table 4).

The flies reared on Diets I or II that were fed on the Gainesville diet presented lower δ^{13}C values (e.g., −20.5 ± 0.2‰ and −20.6 ± 0.2‰,

Table 2. Isotopic composition (mean ± SE) of males and females of *Anastrepha fraterculus* that fed on different adult diets for 5 d after emergence.

Treatment		Sex	δ^{13}C ‰	ANOVA*	δ^{15}N ‰	ANOVA
Flies from papaya	Papaya slices	♂	−26.1 ± 0.2*	$F_{1,9}$ = 2.84	6.8 ± 0.4	$F_{1,9}$ = 0.01;
		♀	−25.5 ± 0.3	P = 0.13	6.8 ± 0.2	P = 0.99
Flies reared on Diet I	Sugar	♂	−13.7 ± 0.1	$F_{1,9}$ = 1.03;	4.2 ± 0.1	$F_{1,9}$ = 0.13;
		♀	−13.3 ± 0.3	P = 0.34	4.3 ± 0.1	P = 0.73
	Sugar and hydrolyzed yeast	♂	−13.6 ± 0.2	$F_{1,9}$ = 6.39;	3.6 ± 0.2	$F_{1,9}$ = 7.7;
		♀	−13.0 ± 0.1	P = 0.035	2.8 ± 0.2	P = 0.02
	Gainesville diet	♂	−20.4 ± 0.3	$F_{1,9}$ = 1.25;	4.2 ± 0.1	$F_{1,9}$ = 0.65;
		♀	−20.0 ± 0.2	P = 0.29	4.4 ± 0.1	P = 0.44
Flies reared on Diet II	Sugar	♂	−14.0 ± 0.2	$F_{1,9}$ = 0.54;	4.3 ± 0.2	$F_{1,9}$ = 1.87;
		♀	−14.4 ± 0.3	P = 0.483	4.5 ± 0.1	P = 0.21
	Sugar and hydrolyzed yeast	♂	−13.7 ± 0.4	$F_{1,9}$ = 0.03;	3.5 ± 0.5	$F_{1,9}$ = 0.03;
		♀	−13.6 ± 0.4	P = 0.85	3.4 ± 0.4	P = 0.87
	Gainesville diet	♂	−20.8 ± 0.3	$F_{1,9}$ = 0.5;	4.5 ± 0.2	$F_{1,9}$ = 0.42;
		♀	−20.4 ± 0.4	P = 0.49	4.6 ± 0.1	P = 0.53

*Means (± SE) within columns did not differ significantly at the 1% probability level by the Student's *t*-test.

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Table 3. Isotopic composition (mean ± SE) of males and females of *Anastrepha fraterculus* that fed on different adult diets for 15 d after diet switching.

	Treatment	Sex	$\delta^{13}C$ ‰	ANOVA	$\delta^{15}N$ ‰	ANOVA
Flies from papaya	Papaya slices	♂	−20.9 ± 0.6*	$F_{1,9}$ = 1.96;	7.3 ± 0.1	$F_{1,9}$ = 0.71;
		♀	−20.9 ± 0.6	P = 0.198	7.5 ± 0.1	P = 0.423
Flies reared on Diet I	Sugar	♂	−16.3 ± 0.1	$F_{1,9}$ = 2.82;	4.5 ± 0.1	$F_{1,9}$ = 1.92;
		♀	−16.8 ± 0.3	P = 0.131	4.3 ± 0.1	P = 0.203
	Sugar and hydrolyzed yeast	♂	−18.1 ± 0.4	$F_{1,9}$ = 7.83;	3 ± 0.1	$F_{1,9}$ = 3.28;
		♀	−19.6 ± 0.3	P = 0.023	3.5 ± 0.2	P = 0.107
	Gainesville diet	♂	−19.3 ± 0.2	$F_{1,9}$ = 0.29;	4.3 ± 0.1	$F_{1,9}$ = 0.11;
		♀	−19.6 ± 0.5	P = 0.607	4.4 ± 0.2	P = 0.743
Flies reared on Diet II	Sugar	♂	−17.6 ± 0.2	$F_{1,9}$ = 1.43;	4.4 ± 0.1	$F_{1,9}$ = 1.79;
		♀	−18 ± 0.3	P = 0.266	4.2 ± 0.1	P = 0.217
	Sugar and hydrolyzed yeast	♂	−17.5 ± 0.1	$F_{1,9}$ = 0.35;	3.3 ± 0.1	$F_{1,9}$ = 0.12;
		♀	−17.7 ± 0.4	P = 0.571	3.2 ± 0.3	P = 0.735
	Gainesville diet	♂	−20.7 ± 0.2	$F_{1,9}$ = 7.44;	4.3 ± 0.1	$F_{1,9}$ = 1.99;
		♀	−21.6 ± 0.2	P = 0.025	3.9 ± 0.2	P = 0.196

*Means (± SE) within columns did not differ significantly at the 1% probability level by the Student's *t*-test.

respectively) at the time when the adult diet was switched to apple. The $\delta^{13}C$ signatures of these flies varied little until the end of the test (final values of −19.4 ± 0.3‰ and −21.1 ± 0.2‰, respectively), and were more closely related to C_3-based diets. Due to the little variation in $\delta^{13}C$ of this group of flies, the Boltzmann model could not provide a good fit for the data, and equations of the form $Y = a + b/\chi^2$ were used. In this equation, Y is the $\delta^{13}C$ of fly, X is the considered time, 'a' is the intercept, and 'b' is the angular coefficient. Nevertheless, poor coefficients of determination were obtained (r^2 < 30%) and the regression model was not significant for any of the treatments containing the Gainesville diet (P > 0.05) (Table 4).

In treatments in which the C_4 pre-release diet was switched to a C_3-based diet, the overall half-life for carbon ranged from 6.2 to 8.5 d, with half-life intervals of males and females overlapping (5.4–8.1 d and 6.5–10 d, respectively) (Table 4). Adult males that came from larval Diet I showed a more rapid turnover than females, reaching the linear plateau about 1 to 2 d earlier (Fig. 3).

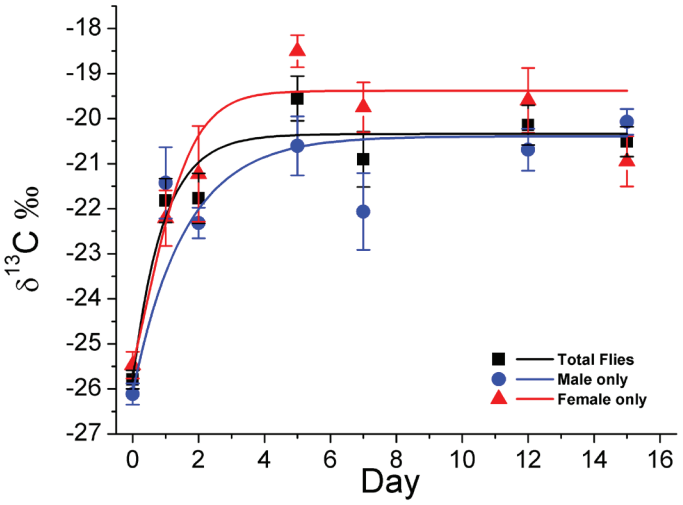


Fig. 2. Stable carbon isotopic signature of *Anastrepha fraterculus* adults switched from papaya slices (C_3-based adult diet) to sugar (C_4-based adult diet) over time.

When the diet was switched from papaya slices to sugar, the estimated half-life in males was shorter (6.4 d) than in females (9.9 d), but the overall rate at which carbon derived from the new diet was incorporated (7.8 d) was within the range observed for other treatments (Table 4). In general, the results indicated a rapid turnover of dietary carbon in *A. fraterculus* flies, with mean half-lives between 6 and 8 d (Table 4).

Regardless of treatment diet, none of the switched flies suffered a complete turnover of carbon until 20 d of age. Approximately 50% of carbon in *A. fraterculus* adults was structural, and 50% was metabolically active. This proportion of structural carbon was estimated using a simple 2-source mixing model for flies from the papaya and sugar diet switching treatment:

$$[(\delta^{13}C_{fly} - \Delta^{13}C_{papaya}) - \delta^{13}C_{sugar}] / [(\delta^{13}C_{papaya} - \delta^{13}C_{sugar})] = \{[(-20.5) - (-1)] - (-11.9)\} / [(-26) - (-11.9)] = 0.54$$

Fifteen d after the diet switch, it was still possible to distinguish all the laboratory-reared flies from the wild ones with 99% confidence despite the changes observed on both $\delta^{13}C$ and $\delta^{15}N$ signatures (Table 5). Overall, compared to the isotopic composition of 5-d-old flies, $\delta^{13}C$ values of flies switched from a C_4-based diet to apple decreased, with the highest depletion (−5.6‰) observed in flies from Diet I that were fed on the mixture of sugar and hydrolyzed yeast. On d 15 of evaluation, the $\delta^{15}N$ values were depleted up to −0.4‰ only in flies for which larvae were reared on Diet II. Other treatments presented a slight enrichment of $\delta^{15}N$, with the highest enrichment observed for flies from papaya (+ 0.6‰).

Discussion

The isotopic signature of insects is reflective of their diets (Hyodo 2015); therefore, carbon stable isotope ratios have been tested to track the origin of sterile released insects. This proved to be an accurate means of distinguishing wild from mass-reared insects, based primarily on the isotopic differences between wild host plants species and the larval diets used in mass-rearing facilities (Hood-Nowotny et al. 2009, 2011, 2016; Opiyo et al. 2016). To avoid high mortality before reaching the mating age, the sterile males of species with a long pre-copulatory period should

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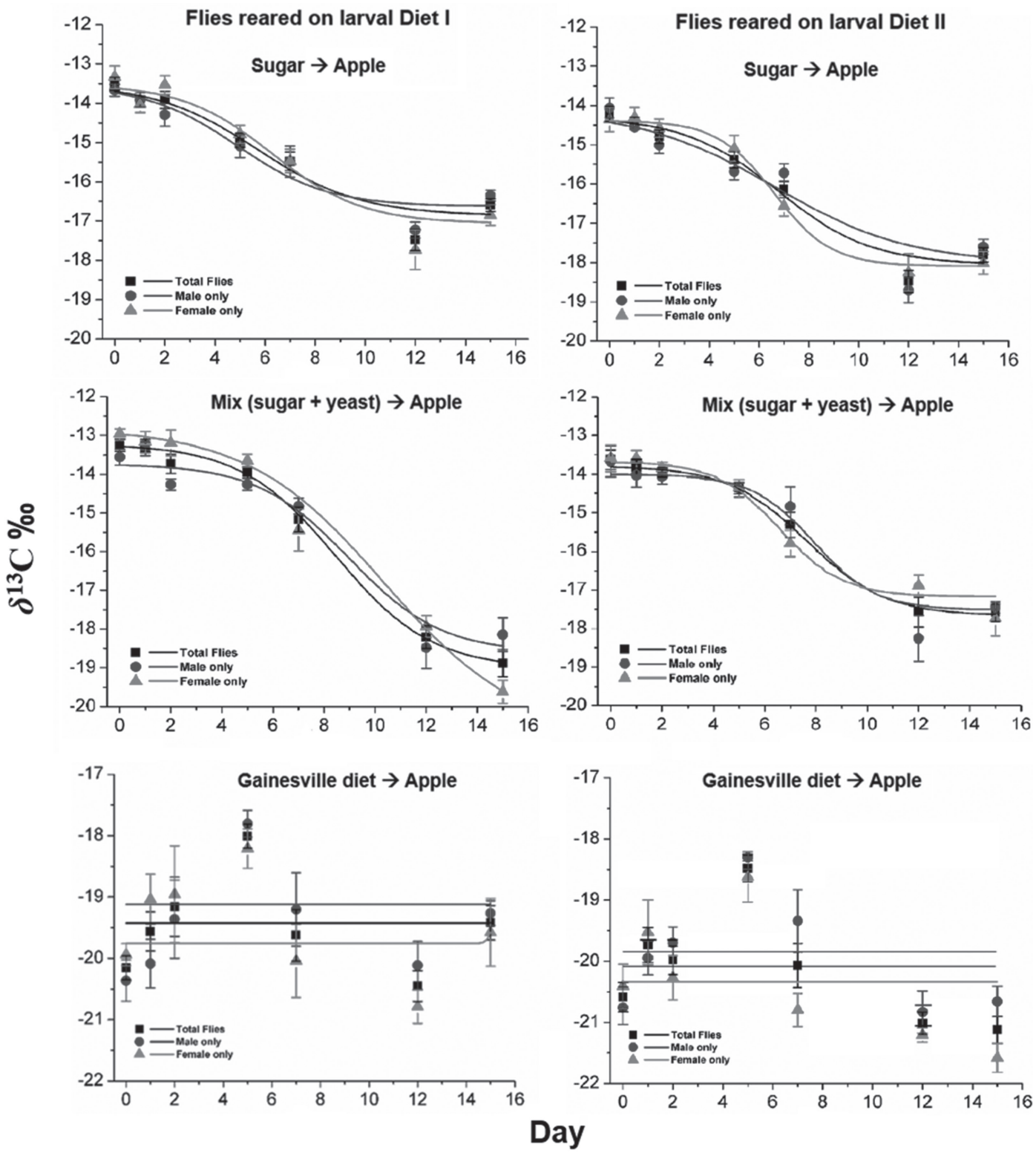


Fig. 3. Stable carbon isotopic signature of *Anastrepha fraterculus* adults switched from different adult diets (sugar, mixture of sugar plus hydrolyzed yeast, or Gainesville diet) to apple (C_3-based diet) over time.

be kept at emergence facilities on a pre-release dietary regime (Liendo et al. 2013; Teal et al. 2013). Once in the field, the sterile insects have access to different sources of adult food (Hendrichs et al. 1991; Aluja et al. 2011, 2012). When the food source changes from a C_4 to C_3 plant, the isotopic composition of the insect may gradually move toward the signatures associated with the new food sources (Vander Zanden et al. 2015; Hood-Nowotny 2017). Knowing how adult diet switching affects the isotope signature of the species is very important, not only to understand the isotopic data for the sterile insects of that particular species in the field, but also to allow implementation of the stable isotope analyses

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Table 4. Regression equations and half-life for carbon in *Anastrepha fraterculus* flies from diet switching experiments.

Treatments		Sex	Equation	R^2	Half-life (d)
Larval diet	Diet switch				
Papaya	Papaya → Sugar	♂	$\delta^{13}C = \{(-27689.4 + 20.7)/[1 + e^{(t + 6.4)/0.75}]\} - 20.7$	0.75	6.4
		♀	$\delta^{13}C = \{(-8575 + 19.7)/ [1 + e^{(t + 9.9)/1.4}]\} - 19.7$	0.98	9.9
		Overall	$\delta^{13}C = \{(-16550.6 + 20.4)/[1 + e^{(t + 7.84)/0.98}]\} - 20.4$	0.58	7.8
Diet I	Sugar → Apple	♂	$\delta^{13}C = \{(-13.5 + 16.8)/[1 + e^{(t - 5.4)/2.3}]\} - 16.8$	0.78	5.4
		♀	$\delta^{13}C = \{(-13.5 + 17.3)/[1 + e^{(t - 6.6)/1.63}]\} - 17.3$	0.80	6.5
		Overall	$\delta^{13}C = \{(-13.6 + 17.1)/[1 + e^{(t - 6.19)/1.8}]\} - 17.1$	0.79	6.2
	Mix (sugar + yeast) → Apple	♂	$\delta^{13}C = \{(-13.8 + 18.4)/[1 + e^{(t - 8.15)/1.08}]\} - 18.4$	0.88	8.1
		♀	$\delta^{13}C = \{(-12.5 + 21.1)/[1 + e^{(t - 9.96)/3.4}]\} - 21.1$	0.92	10.0
		Overall	$\delta^{13}C = \{(-13.2 + 19.08)/[1 + e^{(t - 8.52)/1.9}]\} - 19.08$	0.89	8.5
	Gainesville diet → Apple	♂	$\delta^{13}C = -19.3 + [(-1.1 \times 10^{-50})/t^2]$ ($F_{1,34} = 1.37$; $P = 0.29$)	0.22	—
		♀	$\delta^{13}C = -19.6 + [(-5.03 \times 10^{-51})/t^2]$ ($F_{1,34} = 0.27$; $P = 0.63$)	0.05	—
		Overall	$\delta^{13}C = -19.4 + [(-7.8 \times 10^{-51})/t^2]$ ($F_{1,34} = 0.82$; $P = 0.41$)	0.14	—
Diet II	Sugar → Apple	♂	$\delta^{13}C = \{(-14.4 + 18.23)/(1 + e^{(t - 7.27)/1.82})\} -18.23$	0.82	7.3
		♀	$\delta^{13}C = \{-14.4 + 18.15/[1 + e^{(t - 6.64)/1.12}]\} -18.2$	0.84	6.6
		Overall	$\delta^{13}C = \{(-14.4 + 18.2)/ [1 + e^{(t - 6.95)/1.63}]\} -18.2$	0.83	6.9
	Mix (sugar + yeast) → Apple	♂	$\delta^{13}C = \{(-13.9 + 17.9)/[1 + e^{(t - 7.99)/0.82}]\} -17.9$	0.80	8.0
		♀	$\delta^{13}C = \{(-13.8 + 17.9)/[1 + e^{(t - 7.13)/2.9}]\} -17.1$	0.84	7.1
		Overall	$\delta^{13}C = \{(-13.7 + 17.7)/[1 + e^{(t - 7.56)/1.5}]\} -17.7$	0.81	7.6
	Gainesville diet → Apple	♂	$\delta^{13}C = -19.8 + [(-9.6 \times 10^{-51})/t^2]$ ($F_{1,34} = 0.93$; $P = 0.38$)	0.16	—
		♀	$\delta^{13}C = -20.3 + [(-7.5 \times 10^{-52})/t^2]$ ($F_{1,34} = 0.003$; $P = 0.96$)	0.007	—
		Overall	$\delta^{13}C = -20.1 + [(-5.2 \times 10^{-51})/t^2]$ ($F_{1,34} = 0.25$; $P = 0.64$)	0.05	—

in a sterile insect technique program (IAEA 2009). This study is the first one dealing with the behavior of the stable isotope ^{13}C in *A. fraterculus* adults during sexual maturation and after diet change.

The whole bodies of flies reflected the overall isotopic composition of their larval diets immediately after emergence (Table 1). Due to the presence of cane sugar and corn flour in larval Diets I and II, the $\delta^{13}C$ of adults from those diets was closer to the signatures of C_4 plants. This made it possible to distinguish between wild flies and 20-d-old flies based on the carbon signature alone, regardless of the adult food source (Tables 1 & 5). Flies reared on papaya gave ^{13}C signals characteristic of C_3 plants, not differing from the values of wild flies when papaya slices were kept as adult food (Table 1). The native host fruits of *A. fraterculus*, most of them from the families Rosaceae and Myrtaceae (Zucchi 2007), follow the C_3 photosynthetic pathway, and although some variability in the carbon isotopic signature of flies developing on different hosts may exist, their $\delta^{13}C$ signatures will be lower than the values of flies reared on C_4-based diets, as demonstrated in this study (Tables 1 & 5).

No significant differences were found between sexes of 5-d-old or 20-d-old flies presented with different adult diet regimes (Tables 2 & 3), demonstrating that when *A. fraterculus* adults are derived from naturally ^{13}C labelled diets, they can be sent indistinctly to stable isotope analyses. This is consistent with the results of previous studies with other insect species. No major within-species differences in $\delta^{13}C$ were seen between the sexes in *Drosophila* spp. (Lauxaniidae) (Markow et al. 2000), *Aedes* and *Anopheles* (both Diptera: Culicidae) mosquitoes (Opiyo et al. 2016), cactus moth (Lepidoptera: Pyralidae) (Hood-Nowotny et al. 2016), and in wild medflies (Hood-Nowotny et al. 2009). Balagawi et al. (2014) found no differences in total body nitrogen or carbon for both sexes of wild *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae). In contrast, some researchers have observed sex-associated variations in $\delta^{13}C$ signatures, especially when females present a higher storage of lipids, which are generally depleted in ^{13}C relative to bulk biomass (Hayes 2001; Hood-Nowotny et al. 2009; Sato & Azuma 2016).

This is the first attempt to observe the turnover of carbon in *A. fraterculus* adults by switching the carbon stable isotope composition

Table 5. Isotopic composition of wild and laboratory-reared *Anastrepha fraterculus* flies after 15 d of the diet switch.

Treatment	$\delta^{13}C$ ‰	$\delta^{15}N$ ‰
Wild flies	−25.7 ± 0.2 a**	5.4 ± 0.6 b
Flies I* from sugar to apple	−16.6 ± 0.2 f	4.4 ± 0.1 c
Flies I from sugar and hydrolyzed yeast to apple	−18.9 ± 0.3 de	3.3 ± 0.1 d
Flies I from Gainesville diet to apple	−19.4 ± 0.3 cd	4.4 ± 0.1 c
Flies II from sugar to apple	−17.8 ± 0.2 ef	4.3 ± 0.1 c
Flies II from sugar and hydrolyzed yeast to apple	−17.6 ± 0.2 ef	3.2 ± 0.1 d
Flies II* from Gainesville diet to apple	−21.1 ± 0.2 b	4.1 ± 0.1 c
Flies from papaya to sugar	−20.5 ± 0.3 bc	7.4 ± 0.1 a
ANOVA	$F_{7,78} = 124.66$; $P < 10^{-3}$	$F_{7,78} = 88.11$; $P < 10^{-3}$

*I = flies whose larvae were reared on artificial Diet I or II fed on different adult diets.
** Means (± SE) within columns followed by the same letter do not differ significantly at the 1% probability level by the Tukey's test.

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of diets. In most treatments, the $\delta^{13}C$ signatures changed quickly when the adult diet was switched for 5-d-old flies. Fifteen d after switching from a C_4- to a C_3-based diet, depletions in $\delta^{13}C$ by up to −5.6‰ were observed compared to the signals of 5-d-old flies (Figs. 2, 3). The change in carbon isotope ratios followed the same pattern in males and females (Fig. 3). The drop in ^{13}C signal was fast for 6 to 8 d, which reflects the elimination of C_4 carbon through respiration or excretion, and the uptake of C_3 carbon from apple, and then it slowed down as flies' tissues reached isotopic equilibrium with the new diet. This new equilibrium consisted of an isotopic mixture of nonstructural metabolic carbon, which was completely replaced by C_3 carbon from the new diet, and structural carbon that retains most of the original C_4 signature (Martínez del Rio & Carleton 2012).

Likewise, the $\delta^{13}C$ of flies reared on papaya changed rapidly in the first d after switching, tending toward the more positive values of flies from C_4-based diets, followed by a lower rate of change until the final d of evaluation (Fig. 2). Although refined sugar is unlikely to be found under natural conditions, and reports of *A. fraterculus* feeding on natural C_4 plants are not known, tephritid fruit flies have a clear preference for high-energy food sources such as sucrose when available, because they are readily digestible and lead quickly to a satiation stage ("junk food") (Cangussu & Zucoloto 1992; Jacome et al. 1999). Therefore, it is important to understand how fast the isotopic incorporation in the C_3 to C_4 switching situation occurs. The flies' pattern of change of $\delta^{13}C$ signature was similar (average half-life of 6–8 d) when they shift from a C_3 to C_4 diet and vice versa (Table 4). Isotopic turnover is expected to be short in tissues of small insects (Vander Zanden et al. 2015).

The turnover of carbon after switching from a C_4 to C_3 diet and from a C_3 to C_4 diet was successfully described by the Boltzman model (Table 4). This model has been used to describe isotopic turnover in several tissue types and animal species (Balesdent & Mariotti 1996; Silva et al. 2007; Martínez del Rio et al. 2009; Ducatti et al. 2014; Sandre et al. 2016), but it did not fit the data from treatments with the Gainesville diet. Because the $\delta^{13}C$ values of flies fed on this diet remained between −19‰ and −22‰ even after diet switching, the half-life could not be estimated. The honey used to prepare the Gainesville diet had citrus plants (*Citrus sinensis* [L.] Obseck cv. 'Hamlin') (Rutaceae) as a monofloral source, giving a $\delta^{13}C$ signal (−24.0 ± 0.1‰) close to the signals of C_3 plants like apple (−28.4 ± 0.1‰). Nonetheless, the signatures of 20-d-old flies from this diet treatment were still different from those of wild flies (Table 5).

Until the end of the experiments, complete turnover of carbon was not verified in flies' bodies, with the results demonstrating that about 50% of the body carbon is structural and does not turnover within the average lifespan of *A. fraterculus* adults under laboratory conditions (21–29 d) (Cardoso et al. 2002; Gómez-Cendra et al. 2007; Walder et al. 2014). Assuming that growth is absent in *A. fraterculus* adults (Nascimento & Oliveira 1997), the observed turnover of carbon could be attributed to metabolic processes. Appropriate feeding on diets with C_4 sources ensured that the isotopic composition of larval diets was fixed into body structures of *A. fraterculus*, maintaining measurable ^{13}C signals distinct from signals of wild flies 15 d after diet switching. Our estimate of structural carbon is in accordance with previous results obtained for mosquitoes (Hood-Nowotny et al. 2006; Hamer et al. 2012) and the medfly (Hood-Nowotny et al. 2009).

The only study of switching diets based on $\delta^{13}C$ signature conducted with fruit flies was performed by Hood-Nowotny et al. (2009). The authors demonstrated that mass-reared medflies slowly shift their carbon signal (i.e., drop from −19‰ to −23‰), approaching the new isotopic steady state at 10 to 12 d after the diet change. Similar to our findings, a sufficient degree of isotopic differentiation between wild and medflies reared on C_4-based diets was maintained during the testing period. However, those authors only performed a 1-way shifting test (i.e., from C_4 to C_3), whereas we evaluated both diet shifts.

In conclusion, switching the adult food source leads to a rapid change in $\delta^{13}C$ signatures of *A. fraterculus* flies, regardless of the initial and final food source. Even so, $\delta^{13}C$ traces from larval diet remains for a sufficient time, helping to determine the origin of the fly and to track shifts between C_4 and C_3 diets for at least 15 d after diet change. Therefore, it is feasible to mark *A. fraterculus* adults by rearing them in larval diets with sugar from C_4 sources, and the analysis of ^{13}C stable isotope can be a useful complementary method to conventional dye marking.

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