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The use of stable isotope as tracers of *Anastrepha fraterculus* (Wiedemann, 1830) (Diptera: Tephritidae)

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“For all the good times

For every moment

That we forever spent

There was always sun on all our fun

When we go, I hope that you will know

That it means a lot to me

I hope that you can see

I'm having a good day

And that's all I have to say

I'm having fun playing,

And I'm hoping you're listening

To what I'm saying

I love you all

Hoping that you will recall

All the good

Hoping that you would never get rid

Of all that we did

For all the good times...”

Dedicated to my family

With all my love

Author: Jen A.

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"I wanted to contribute to agriculture in ways other than pulling a cotton sack down the row… I don't see any biological or technical barriers to accomplishing control of most of the major pests. Nature has given us the tools. We just need to shape them and use them in the proper manner."

- Edward F. Knipling, in a 1995 interview.

ABSTRACT

BOTTEON, V. W. **The use of stable isotope as tracers of *Anastrepha fraterculus* (Wiedemann, 1830) (Diptera: Tephritidae).** 2018. 100 p. Dissertação (Mestrado) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2018.

Aiming to suppress the South American fruit fly, *Anastrepha fraterculus* (Diptera: Tephritidae), an Area-Wide Integrated Pest Management (AW-IPM) program that integrates the Sterile Insect Technique (SIT) will be implemented on apple growing areas in Southern Brazil. An accurate distinction between the sterile-released flies and the wild fertile flies is essential, since false detections could trigger unnecessary and costly control actions over the surveyed area. In this context, the use of Stable Isotopes Analysis (SIA) emerges as a potential tool for discriminating the origin of the insects. The present study demonstrated whether it is possible to adequately identify laboratory-reared *A. fraterculus* flies, besides evaluating the influence of attractive and preservative substances on flies' isotopic composition over time. This study also assessed for the first time the trophic discrimination factor (TDF) and the turnover rate of *A. fraterculus* after diet switching, once the lack of these species-and element-specific laboratory-derived parameters could limit the SIA application in understanding the ecological patterns of this fruit fly. The $\delta^{13}C$ and $\delta^{15}N$ signals of the larval and adult diets, laboratory-flies, wild flies captured in Southern Brazil (Vacaria), attractive and preservative substances and the flies immersed in the substances were performed by CF-IRMS at CENA/USP. The isotopic compositions from males and females, either from laboratory and wild flies, did not differ significantly. The *A. fraterculus* flies reared on larval diets presented different $\delta^{13}C$ values compared to wild flies and those reared on fruits (C_3-based diets). The values of $\delta^{15}N$ were not conclusive for flies differentiation, and traceability could not depend solely on $\delta^{15}N$ values. In relation to the capture and preservation methods tested (CeraTrap™, grape juice and absolute ethanol), it was observed that, depending on the substance, at least one of the isotopic values can suffer alteration compared to the controls. Despite the fact that isotopic compositions of the laboratory-flies could be affected by attractive and preservative substances and by the time that the flies remained immersed in the trap, it was still possible to distinguish flies reared on artificial diet from wild flies. After the diet switching experiment, the $\delta^{13}C$ signals of flies began to change, reflecting their recent diet as a result of metabolic turnover, expressed in half-life ($t_{1/2}$).

All treatments showed significant difference in $\delta^{13}C$ values over time. The $\delta^{15}N$ showed values fewer conclusive when compared to the $\delta^{13}C$ values, because the variation of the diet sources was based on the stable isotopes of carbon. Despite of these results, after 15 days of diet shift, the isotopic compositions of laboratory-flies in all treatments were statistically different from the isotopic composition of wild flies. The C_4-based diets in the larval and adult stages can be considered suitable tracers of *A. fraterculus* in an SIT program.

Keywords: Sterile Insect Technique. Artificial diet. Trophic discrimination factor. Isotopic turnover.

RESUMO

BOTTEON, V. W. **Utilização de isótopos estáveis como traçadores de *Anastrepha fraterculus* (Wiedemann, 1830) (Diptera: Tephritidae).** 2018. 100 p. Dissertação (Mestrado) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2018.

Objetivando a supressão da mosca-da-fruta sul-americana, *Anastrepha fraterculus* (Diptera: Tephritidae), um programa de Manejo Integrado de Pragas (MIP) integrando a Técnica de Insetos Estéril (TIE) será implementado em áreas de cultivo de maçã na região sul do Brasil. Para isso, é necessária uma distinção precisa entre as moscas estéreis liberadas e as moscas férteis selvagens, uma vez que detecções falsas podem desencadear ações de controle desnecessárias e onerosas sobre a área de interesse. Neste contexto, o uso da Análise de Isótopos Estáveis (AIE) surge como uma ferramenta potencial para discriminar a origem dos insetos. O presente estudo demonstrou se é possível identificar adequadamente as moscas criadas em laboratório para programas de TIE, além de avaliar a influência de substâncias atrativas e preservativas na composição isotópica das moscas ao longo do tempo. Este estudo também determinou, pela primeira vez para *A. fraterculus*, o fator de discriminação isotópica e a taxa de *turnover* após a troca de dietas, uma vez que a ausência desses parâmetros de laboratório, específicos para a espécie e elemento, poderia limitar a aplicação da AIE no entendimento dos padrões ecológicos dessa mosca-das-frutas. Os sinais de $\delta^{13}C$ e $\delta^{15}N$ das dietas larvais e de adultos, moscas de laboratório, moscas selvagens capturadas no sul do Brasil (Vacaria), substâncias atrativas e preservativas, e as moscas imersas nessas substâncias foram adquiridos por CF-IRMS no CENA/USP. As composições isotópicas de machos e fêmeas, tanto de laboratório quanto selvagens, não diferiram significativamente entre si. As moscas *A. fraterculus* criadas em dietas larvais apresentaram diferentes valores de $\delta^{13}C$ em comparação com moscas selvagens e criadas em frutos (dietas à base de fontes C_3). As assinaturas de $\delta^{15}N$ não foram conclusivas para a diferenciação das moscas, e a rastreabilidade não pode depender apenas desses valores. Em relação aos métodos de captura e preservação testados (CeraTrap®, suco de uva e etanol absoluto), observou-se que, dependendo da substância, pode existir alteração de pelo menos um dos valores isotópicos quando comparados aos controles. Apesar das composições isotópicas das moscas de laboratório terem sido afetadas pelo tempo e substâncias nas quais as moscas permaneceram imersas na armadilha, ainda foi possível distinguir as moscas criadas em dietas artificiais das moscas selvagens. Após o experimento de mudança de dieta, os sinais de $\delta^{13}C$ das moscas começaram

a mudar, refletindo a dieta mais recente como resultado do *turnover* metabólico, expresso em meia-vida ($t_{1/2}$). Todos os tratamentos mostraram diferença significativa nos valores de $\delta^{13}C$ ao longo do tempo. Valores de $\delta^{15}N$ apresentaram resultados menos conclusivos quando comparados aos valores $\delta^{13}C$, porque a variação das fontes da dieta foi baseada nos isótopos estáveis do elemento carbono. Apesar destes resultados, após 15 dias de mudança de dieta, a composição isotópica das moscas em todos os tratamentos foi estatisticamente diferente da composição isotópica das moscas selvagens. As dietas baseadas em fonte C_4 na fase larval e na fase adulta podem ser consideradas como traçadores adequados para *A. fraterculus* em um programa de TIE.

Palavras-chave: Técnica do Inseto Estéril. Dieta artificial. Fator de discriminação isotópica. Taxa de *turnover*.

Summary

1 INTRODUCTION

The South American fruit fly, *Anastrepha fraterculus* (Wiedemann, 1830) (Diptera: Tephritidae) is a polyphagous key pest of fruit orchards, with great economic and quarantine importance for fresh fruits exportation. South region of Brazil concentrates 99% of the apple growing areas, with a harvest of more than 1.1 million tons of fruits and a supply chain moving up to USD 1.9 billion annually (BRAZILIAN APPLE YEAR BOOK, 2017). Despite the occurrence of several species of fruit flies in the region, the South American fruit fly represents almost 99% of the flies captured in commercial apple orchards, being considered the main species of economic importance (KOVALESKI; SUGAYAMA; MALAVASI, 2000; KOVALESKI; RIBEIRO, 2002; KOVALESKI, 2004; SCOZ; BOTTON; GARCIA, 2004; RIBEIRO, 2010; SANTOS et al., 2017). For example, the gross value of yield losses associated with the chemical control of this pest was estimated at more than USD 7.9 million per year (KOVALESKI; RIBEIRO, 2003).

The necessity to develop alternative control measures against *A. fraterculus* is urgent, mainly in Southern Brazil where great damage is caused to apple growing areas, and the need to replace conventional chemical methods has stimulated the application of the Sterile Insect Technique (SIT) and the use of parasitoids against fruit flies (COSTA et al., 2016). In the Center for Nuclear Energy in Agriculture (University of São Paulo), the SIT against *A. fraterculus* has been investigated in the past recent years (NUNES et al., 2013; WALDER et al., 2014; COSTA et al., 2016). To ensure the success of the SIT, efforts need to be allocated to obtain good quality flies in the mass-rearing process, to determine the adequate dose to induce sterility and appropriate field release methods (ALLINGHI et al., 2007a; 2007b; MASTRANGELO et al., 2010; CLADERA et al., 2014).

In addition to the research bases for the implementation and to ensure the success of any SIT program, an adequate monitoring is crucial. In general, reared pupae are sterilized and dusted with fluorescent dyes to be distinguished from wild flies when recaptured in traps in the release area (SCOLARI et al., 2008). However, this type of monitoring is not 100% secure, since the paint can be detached from the insect body, been necessary to use other tools to discriminate the origin of the insects (HAGLER; JACKSON, 2001; SCOLARI et al., 2008). In this context, the use of the Stable Isotope Analysis (SIA) emerges as a potential tool to discriminate the geographic origin of the fly and to trace the individuals that are reared in laboratory conditions and released in the field (HOOD-NOWOTNY et al., 2009).

The use of SIA consists in the fact that the isotopic composition of the animal tissues reflects the isotopic composition of its food, discounting any isotopic fractionation in metabolic process of acquiring, digesting and assimilation of elements from the diet that are incorporated in a particular tissue of the consumer (DENIRO et al., 1978; 1981; DUCATTI et al., 2002). Isotopic composition assimilated into the organisms' tissues is promoted by the discrimination of heavier isotopes during certain biological processes. Stable isotopes are considered easy to apply, safe and non-toxic labels that do not affect the insect behavior (HOOD-NOWOTNY; KNOLS, 2007; HYODO, 2015).

Stable isotopes are important tools as biological intrinsic-markers and for studying invertebrate food webs (LAVANDERO et al., 2004; HOOD-NOWOTNY; KNOLS, 2007; BOECKLEN et al., 2011; HYODO, 2015). The isotopic composition of the flies' diet reflects different forms of elements fixation in the ecosystem (*i.e.*, basic composition of primary producers) (HOOD-NOWOTNY et al., 2009). The distinct isotopic ratio of sugar (or other components) present in artificial diets for flies originates mostly from C_4-type plants (*e.g.* sugarcane), and may help to distinguish sterile-released flies from the wild ones in the field, which have hosts with different isotopic signatures (mostly C_3-type plants) (ALUJA; BIRKE, 1993; JÁCOME; ALUJA; LIEDO, 1999; HOOD-NOWOTNY et al., 2009).

Beside the discrimination of the laboratory-flies from wild ones by SIA, it is imperative to know if the attractive substances and preservation methodologies used for captured wild flies could affect their isotopic composition, since aggregation of isotopes from other sources could result in improper interpretation of the isotopic signatures (PONSARD; AMLOU, 1999). Population levels of wild *A. fraterculus* are usually monitored by traps and several studies have evaluated the efficiency of attractive substances for the capture of fruit flies in Southern Brazil (GARCIA; CAMPOS; CORSEUIL, 1999; 2003; RAGA et al., 2006; SCOZ et al., 2006; MONTEIRO et al., 2007; TEIXEIRA et al., 2010), being the 25% grape juice the attractive substance most used to capture *A. fraterculus* flies in apple orchards (KOVALESKI, 2004; RIBEIRO, 2010; ROSA et al., 2017). The wild flies captured need to be taken to the laboratory to determine their origin, and the rapid processing is usually necessary to avoid decomposition of the sample and subsequent alteration of the results from SIA (JESUS et al., 2015).

Once the flies reared on artificial larval diets may feed on fruits after release in the field, until when would it be possible to discriminate laboratory *A. fraterculus* flies from the wild ones after a field release of sterile flies? To answer this important question in the monitoring context, we need to understand the trophic discrimination factor (TDF) and turnover rate of the flies. Howsoever, species-and element-specific laboratory-studies assessing these parameters for *A. fraterculus* are nonexistent.

To use SIA for diet inference and discrimination of an insect's origin, it is necessary to understand the TDF that occurs in consumer tissues in relation to their diet and the isotopic turnover rate for adequate interpretations (CAUT; ANGULO; COURCHAMP, 2009; HOLÁ et al., 2015). The TDF, also called trophic enrichment, is the difference between isotopic ratios of the diet and a specific animal's tissue, typically expressed as $\Delta^{13}C$ for *C* and $\Delta^{15}N$ for *N* (TIESZEN et al., 1983; CAUT; ANGULO; COURCHAMP, 2009). In its turn, isotopic turnover is the continuous renewal of the chemical elements and their isotopes in a body tissue, providing records of animals' dietary histories integrated over time (TIESZEN et al., 1983). According to Ducatti et al. (2002), the period over which the animal isotopic composition reflects the isotopic composition of a particular diet will depend on the turnover rate of the body (or tissue) and physiological processes (synthesis and catabolism) of each tissue component. Thus, when the fly changes its diet, it will tend to reflect a mix of food sources, initially, and then it will tend to reflect the isotopic composition of the new food over time, if the diet remains the same (TIESZEN et al., 1983; HOOD-NOWOTNY et al., 2009).

Determination of species-specific TDF and turnover rate are essential for using SIA in prey-consumer relationship and several ecological studies, once the precise values allow reliable estimates of diet contribution and inaccurate values can lead to misinterpretation of field data (DUCATTI et al., 2002; CAUT; ANGULO; COURCHAMP, 2009; BOECKLEN et al., 2011). However, despite the potential applicability of SIA, Hood-Nowotny and Knols (2007) cited the scarcity of entomological studies in which these methodological tools are used. In an unique study, Vander Zanden et al. (2015) elaborated a dataset including 486 isotopic turnover estimates for 86 species, mainly from fishes, mammals and birds, taken from 85 separate peer-reviewed studies (1982-2014), being only 44 species of invertebrates using whole body for SIA. As already mentioned, there have been no experimentally derived estimates of TDF and turnover rates for *A. fraterculus*.

For the first time, our study provides a foundation for future application of stable isotopes as tracers for the South American fruit fly, assessing the feasibility of using SIA as a diet tracer approach to distinguish over time *A. fraterculus* flies reared in laboratory from wild flies of native populations that are target of a SIT project in Brazil. Therefore, this study aimed to determine the isotopic compositions ($\delta^{13}C$ and $\delta^{15}N$) of larval diets (artificial and natural), fruit fly individuals (reared on artificial diets and wild flies), to evaluate the influence of attractive and preservative substances commonly used on traps or for sample preservation on the flies' isotopic signatures, and to determine the trophic discrimination factor and the turnover rate of *A. fraterculus* flies after diet switching. Our results can serve as a baseline for interpreting isotopic patterns of *A. fraterculus*.

2. LITERATURE REVIEW

2.1 *Anastrepha fraterculus* flies

Fruit flies species (Diptera: Tephritidae) in Brazil belong to four genera: *Anastrepha*, *Bactrocera*, *Ceratitis* and *Rhagoletis* (ZUCCHI, 2000). These pests cause great damage to the world fruit production and *Anastrepha* Schiner (1868) is the largest and one of the most economically important genus of the Tephritidae family in the Americas, with around 197 species described (MALAVASI; ZUCCHI, 2000; HERNÁNDEZ-ORTIZ, 2003; NORRBOM; KORYTKOWSKI, 2011). In Brazil, 115 species of *Anastrepha* are known, highlighting the species complex of the South American fruit fly, *A. fraterculus* (Wiedemann, 1830) (MALAVASI; ZUCCHI, 2000; ZUCCHI, 2008; NORRBOM; KORYTKOWSKI, 2011; GARIOU-PAPALEXIOU et al., 2016).

In several fruit producing areas of Argentina, Brazil, Peru and Uruguay, the only species of economic and quarantine importance are *Ceratitis capitata* (Wiedemann, 1824) and *A. fraterculus* (Wiedemann, 1830) (MANSO; LIFCHITZ, 1992). The South American fruit fly is polyphagous, feeding on more than 100 species of fruits (DA SILVA et al., 1996; STECK, 1999; ZUCCHI, 2008), and presenting a geographic distribution from Texas (USA.) to Argentina (DA SILVA et al., 1996; STECK, 1999; ZUCCHI, 2008).

In general, fruit flies are holometabolous insects, completing their biological cycle through egg, larva, pupa and adult. Development periods may vary according to intrinsic characteristics of each species and several abiotic variables, such as temperature, relative humidity and photoperiod. At 25 ºC, *A. fraterculus* presents incubation period ranging from 1 to 3 days; larval stage of 12-14 days; pupal period of 11 to 21 days; with fecundity reaching 40 eggs/female/day (mean of 25.2 eggs/female/day), and 979 eggs can be laid during life (KAMIYA, 2010).

In Brazil, 116 hosts of *A. fraterculus* are known (SALLES, 1995; ZUCCHI, 2008; GARCIA; NORRBOM, 2011; NUNES et al., 2012) and the studies on the relationship between the fruit flies and their hosts focus on species of economic importance, mainly species of the families Myrtaceae and Rosaceae (ZUCCHI, 2007; 2008). In the Rio Grande do Sul State (RS), besides apple orchards (*Malus domestica*), sources of infestation are observed in commercial peach orchards (*Prunus persica*), citrus (*Citrus* spp.) and grapevine (*Vitis vinifera*) (SALLES, 1995; ZUCCHI, 2008; ZART; BOTTON; FERNANDES, 2011; NUNES et al., 2012; SAVARIS et al., 2013). Cultivation of new fruits, such as blackberry

(*Rubus* spp.), blueberry (*Vaccinium ashei*) and Brazilian cherry (*Eugenia uniflora*), may also become sources of infestation for *A. fraterculus* (BISOGNIN et al., 2013).

The damage caused by these flies occurs directly in the fruit by the adult female in the epidermis perforation during oviposition, and by the larvae, which can totally damage the pulp of the fruits, rendering them useless for consumption (MALAVASI; ZUCCHI, 2000; SILVA et al., 2006). Fruit flies oviposit in the fruit by puncturing the epidermis with the aculeus (ovipositor), causing rotting and darkening of nearby tissues. The oviposition wounds may also serve as a gateway to pathogens. Regarding indirect damages, it is worth mentioning the cost of the regulatory measures required to export fresh fruit to countries that present fruit flies species as quarantine pests and the adaptation to the requirements of the consumer markets (PARANHOS et al., 2008).

2.2 *Anastrepha fraterculus* and pomiculture in Brazil

Brazil stands out in the world production of apples (FACHINELLO et al., 2011; BRAZILIAN APPLE YEAR BOOK, 2017), but faces several problems with fruit flies (Diptera: Tephritidae) (KOVALESKI; SUGAYAMA; MALAVASI, 2000; KOVALESKI; RIBEIRO, 2002; KOVALESKI, 2004; RIBEIRO, 2010; SANTOS et al., 2017). Pomiculture was introduced in Southern Brazil in the early 1970's and now this region concentrates 99% of the apple growing areas, with a harvest of more than 1.1 million tons of fruits and a supply chain moving up to USD 1.9 billion annually (BRAZILIAN APPLE YEAR BOOK, 2017).

Unfortunately, there has been an increase in the occurrence of the South American fruit fly in apple production areas of Southern Brazil in recent years. Although other species of fruit flies occur in Southern Brazil, *Anastrepha fraterculus* represents almost 99% of the total capture in apple orchards, and most control strategies should target this species as, under high infestations, losses may reach 2-3% in commercial orchards (SUGAYAMA et al., 1997; SCOZ; BOTTON; GARCIA, 2004; SANTOS et al., 2017). In a recent study performed in Rio Grande do Sul State, besides apple orchards infestation, Marsaro Júnior (2014) observed the predominance of *A. fraterculus* infesting fruits of *Annona rugulosa*, *Acca sellowiana*, *Campomanesia guazumifolia*, *Campomanesia xanthocarpa*, *Diospyros kaki*, *Eugenia involucrata*, *Eugenia pyriformis*, *Eugenia uniflora*, *Psidium cattleianum*, *Psidium guajava* and *Prunus persica*. In Vacaria-RS, larger infestations of this pest can be observed in *Campomanesia xanthocarpa*, *Eugenia involucrata* and *Feijoa sellowiana* (DE SOUZA; KOVALESKI, 2012).

The fruit production losses caused by *A. fraterculus* are reflected both in the national market, with lesser fruits available for commercialization, and in the international market, due to the exports decrease and quarantine restrictions (DUARTE; MALAVASI, 2000).

2.3 *Anastrepha fraterculus* management

In some regions, fruit flies can compromise 100% of fruit production (KORYTKOWSKY; OJEDA, 1969; ORLANDO; SAMPAIO, 1973). The International Atomic Energy Agency (IAEA), for example, found that the economic damage caused by *C. capitata* in the Maghreb region (North Africa) was in the range of USD 60-90 million/year, with insecticides amounting to USD 7-10 million/year (IAEA, 1995). According to Malavasi (2001), the carambola fly, *Bactrocera carambolae* (DREW; HANCOCK, 1994), could cause annual losses of production estimated at approximately USD 60 million in the Brazilian territory.

Currently, pre-harvest control is done almost exclusively by chemical methods, mainly by organophosphate and other insecticides, which are gradually being withdrawn from the market and residue tolerance levels have been drastically reduced for exported fruits (SCOZ; BOTTON; GARCIA, 2004; PIGNATI et al., 2017). Organophosphate insecticides are characterized by high toxicity, low selectivity to natural enemies and a long persistent period in the environment (SCOZ; BOTTON; GARCIA, 2004; NAVA; BOTTON, 2010).

Indiscriminate use of insecticides increases the selection pressure for the emergence of resistant insect populations, may lead to the emergence of new insect pests, resurgence of secondary pests and present toxicity to the environment and non-target organisms (BRATTSTEN et al., 1986). The need to replace conventional chemical methods has stimulated the search for environmentally friendly methods for fruit flies management.

Integrated Pest Management (IPM) consists in the integration of different control tactics in ways that facilitate the suppression of pest populations, either at field-by-field or at area-wide (AW) (VREYSEN et al., 2007). IPM programs in fruit farming can promote the use of several control methods, and a feasible alternative to promote the suppression of *A. fraterculus* in AW could be through the use of sterile males, obtained by the Sterile Insect Technique (SIT), not generating toxic residues in the fruits and not promoting resistance of the target flies. SIT can be defined as a "pest control method that uses inundative releases of

sterile insects in area-wide to reduce the fertility of a wild population of the same species" (FAO, 2005), whose success depends on the mating competitiveness of sterile-released males against wild males (ALLINGHI et al., 2007a, 2007b; MASTRANGELO et al., 2010). According to Lux et al. (2002) mating decisions are made by direct males competition and the effectiveness of SIT programs is determined by the ability of sterile-released males to successfully mate and inseminate wild females (KNIPLING, 1955; 1959).

Basically, it can be said that the SIT is composed of 3 stages: (1) target species mass-rearing, (2) insect sterilization and (3) field release. By transferring sperm containing dominant lethal mutations to wild-type females, the sterile male promotes a gradual decline in the wild population, reducing the reproductive potential and rendering the next generation unfeasible, even culminating in pest eradication (KNIPLING, 1955; 1959).

The SIT has been used for many decades against fruit flies in several countries (HENDRICHS et al., 2002) and it is a promising alternative for *A. fraterculus* management. The expansion of the use of this technique has helped to protect fruit growing areas against fruit fly infestation, preventing embargos of billions of dollars in export programs and meeting current requirements of consumer markets and fruit importing countries (HENDRICHS et al., 2002). Aiming to suppress *A. fraterculus* populations, an Area-Wide Integrated Pest Management (AW-IPM) program that integrates the SIT, called MOSCASUL, will be implemented on apple growing areas in Southern Brazil in the coming years (COSTA et al., 2016).

2.4 Sterile Insect Technique programs against fruit flies

Currently, 22 AW-management programs in the world integrate the SIT for the control of fruit flies (ENKERLIN, 2005; IDIDAS, 2017). Strategies for suppression and eradication of fruit flies through the SIT have been adapted from the *Cochylyomia hominivorax* (Coquerel, 1858) (Diptera: Calliphoridae) eradication program in the 1950's (KNIPLING, 1955; 1959; FAY, 1989). It is in Mexico that the largest number of suppression campaigns can be observed for several species of the *Anastrepha* genus. The direct damage caused by the four major economically important flies in that country (*Anastrepha ludens* Loew, *Anastrepha obliqua* Macquart, *Anastrepha striata* Schiner and *Anastrepha serpentina* Wiedemann) was estimated at over USD 230 million, with total losses estimated at USD 710 million per year (REYES et al., 1991; 2000).

Through the National Campaign against Fruit Flies initiated in 1993, the world's largest bio-industrial complex for the production of sterile flies (part of the MOSCAFRUT program) was created in Metapa de Dominguez, Mexico, which produces more than 1 billion insects per week (700 million *C. capitata*, 300 million *A. ludens*, 30 million *A. obliqua*, and 50 million parasitoid *Diachasmimorpha longicaudata* Ashmead) (RULL et al., 1996). As a result, these flies have already been eradicated from more than 35,000 ha of citrus, mango, apple and pear from the Northwest region (SAGARPA, 2001). In the Northeast region of Mexico, populations of fruit flies have been suppressed to low-prevalence levels in more than 30,000 ha of citrus. In the first four years after 1997, when Northwest eradication was officially declared, direct benefits totaled USD 25 million. In addition, in the same period, the gains with the price differentiated by import markets and the economy with quarantine treatments summed almost USD 35 million (SAGAR/IICA, 2001). In the specific case of the State of Sonora, where there are 10,000 ha of citrus declared free of fruit flies, the total exported in 6 years was over 130,000 t (an estimated value of USD 10.3 million) (DGSV, 2002). Recently, the maintenance of fly-free zones in the Northwest region of Mexico allowed the expansion of fruit cultivation by 50,000 ha (ENKERLIN, 2005).

In Argentina, the regions of Patagonia and part of Cuyo (Vale de Uco), specialized in temperate fruits, were considered free of fruit flies. However, in the Northeast (provinces of Corrientes and Entre Rios, with 56,200 ha of citrus), damages by *C. capitata* and *A. fraterculus* reached 13%, approximately 143,000 t/year (ca. USD 37 million/year). Recently, the *Servicio Nacional de Sanidad y Calidad Agroalimentaria* (SENASA) planned to expand the national fruit fly control program (PROCEM), aiming to reduce the previous damage level to 0.5% by the 4th year of the program, and to maintain the status of free and low prevalence areas. Currently, the liberation of *C. capitata* sterile males is made in 160,000 ha. The expanded program should incorporate 90,000 ha, which will require an increase from the current 140 to 300 million sterile males/week. The total cost of this expansion was estimated at approximately USD 57 million for the five years of implementation (half financed by the *Secretaría de Agricultura, Ganadería, Pesca y Alimentos* (SAGPyA), half by the federal and state governments and the private sector). In the 4th year of the project, an increase in commercialization of 116,000 t of fruit (approximately USD 7 million/year in current prices) and a reduction of 67,900 L/year of Malathion are expected (GUILLÉN; SANCHEZ, 2007). Studies for the implementation of SIT against *A. fraterculus* are also underway (CLADERA et al., 2014).

In Brazil, AW-management integrating the SIT was only adopted in 2006 by the *Biofábrica Moscamed* Brazil, with the purpose of suppressing the populations of *C. capitata* in the semi-arid irrigated areas of the San Francisco River Valley. The program was in operation with a production of two to three million sterile males per week (PARANHOS, 2007). On the other hand, no program had been carried out in Brazil for *A. fraterculus*. To solve this, the MOSCASUL Biological Control Center was installed at the *Estação Experimental de Vacaria of Embrapa Uva & Vinho*, after an investment of USD 600,000 from the Ministry of Agriculture, Livestock and Food Supply (*Ministério da Agricultura, Pecuária e Abastecimento* - MAPA) at the end of 2014. This project aims to establish a mass-rearing facility to produce millions of sterile flies and parasitoids for the biological control of *A. fraterculus* in Southern Brazil (COSTA et al., 2016).

2.5 Fruit fly monitoring for Sterile Insect Technique programs

The application of the SIT against populations of the South American fruit fly is a promising alternative and the successful management depends on the mating competitiveness of released sterile males against wild males (KNIPLING, 1955; 1959; LUX et al., 2002). However, according to Cladera et al. (2014), several aspects related to the SIT against *A. fraterculus* are still lacking, such as those related to the number of sterile insects that should be released, flies' dispersion and how to identify them in the field.

After the flies have been released, monitoring becomes a crucial part of the program, once it may allow the detection of outbreaks, to take emergency control actions and to reduce management costs (ENKERLIN; LOPEZ; CELEDONIO, 1996). Monitoring is the basis of an IPM program, and McPhail traps have been widely used for monitoring fruit flies in various parts of the world, especially for species of *Anastrepha* (ALUJA et al., 1989). Population levels of wild *A. fraterculus* are usually monitored by traps, and several studies have evaluated the efficiency of attractive substances for the capture of fruit flies in Southern Brazil (GARCIA; CAMPOS; CORSEUIL, 1999; GARCIA; CAMPOS; CORSEUIL, 2003; RAGA et al., 2006; SCOZ et al., 2006; MONTEIRO et al., 2007; TEIXEIRA et al., 2010), being the 25% grape juice the attractive substance most used to capture *A. fraterculus* flies in apple orchards (KOVALESKI, 2004; RIBEIRO, 2010; ROSA et al., 2017).

For SIT program monitoring, an accurate distinction between sterile-released flies and wild fertile flies is crucial after their capture in commercial orchards, since false detections could trigger unnecessary and costly control actions over the surveyed area.

In the context of field release, the monitoring of the wild population is necessary for the implementation and evaluation of a SIT program (VREYSEN, 2005).

Initially, the marking method for tephritid fruit flies consisted of externally marking sterilized pupae with fluorescent dye powder and to observe the captured flies under an UV light lamp (NORRIS, 1957; STEINER, 1965; SCHROEDER et al., 1972; SCHROEDER; MITCHELL, 1981; ENKERLIN; LOPEZ; CELEDONIO, 1996). Posteriorly, a system based on fluorescent microscopy to better identify marked flies with fluorescent dye powder was developed (ENKERLIN; LOPEZ; CELEDONIO, 1996). These methods are still widely used in SIT programs, but they are not 100% secure, since the paint can be detached from the insect body and a few flies can escape from being marked (HAGLER; JACKSON, 2001; PARKER, 2005). In its turn, oil–based dyes can have detrimental effects (SCHROEDER; MITCHELL; MIYABARA, 1974).

Besides the fluorescent dye powder and oil–based dyes, the ovarian dissection technique could be used as a confirmation of sterility of trapped females (GUILLEN-AGUILAR et al., 2016). Bartolucci et al. (2008) showed differences in ovary sizes of *A. fraterculus* from the 4th day after emergence that can be used to differentiate sterile flies from non-irradiated ones. Considering *A. fraterculus* males, these authors observed no differences on testis length and width from irradiated and fertile flies. Besides onerous, dissection of genitalia is not always appropriate for the identification of sterile-released males that were not adequately marked by dye powder (GUILLEN, 1983; CLADERA et al., 2014).

Research efforts to evaluate other monitoring systems have been performed. Niyazi et al. (2005) isolated a dominant mutation for *Ceratitis capitata* (Diptera: Tephritidae), which affects the third stripe on the abdomen, allowing to distinguish flies reared on laboratory from the wild flies. Besides, it was identified a sperm marking system based on the use of the *C. capitata* spermatogenesis-specific b2t promoter driving a fluorescent marker (SCOLARI et al., 2008).

In this context, the use of Stable Isotopes Analysis (SIA) emerges as a potential tool for discriminating the origin of insects and assisting the monitoring system of an operational SIT program (McKECHNIE, 2004; HOOD-NOWOTNY; KNOLS, 2007; HOOD-NOWOTNY et al., 2009). According to Hood-Nowotny et al. (2009; 2016) the SIA proved to be effective in tracing the medfly (*Ceratitis capitata*) and seven moth species (Lepidoptera), indicating the potential of identification of insects released in SIT programs.

Thus, the SIA provides an effective methodology of insect tracer without restrictions imposed by traditional marker methods such as fluorescent powder dyes or colored substances incorporated into the artificial diet (HAGLER; JACKSON, 2001; HOOD-NOWOTNY et al., 2009; 2016).

2.6 Stable Isotope Analysis (SIA)

For the traceability of an insect, a simple method of analysis such as the Stable Isotope Analysis (SIA) is required, which could be applied both in laboratory studies and in the field (HAGLER; JACKSON, 2001; HOOD-NOWOTNY; KNOLS, 2007). Among the stable isotopes, carbon (C) and nitrogen (N) are the most frequently used in ecology studies (POST, 2002), being used as biological intrinsic-tracers to determine larval food based on differences in isotopic composition of hosts (HOOD-NOWOTNY et al., 2009; BOECKLEN et al., 2011). Therefore, the stable isotopes of these two elements have been considered important tools for studying invertebrate food webs (LAVANDERO et al., 2004; HOOD-NOWOTNY; KNOLS, 2007; BOECKLEN et al., 2011; HYODO, 2015).

Stable isotopes naturally occur in the environment, consisting on atoms of the same chemical element (same number of protons), but differing in the number of neutrons and mass, consequently. Thus, isotopes present the same chemical characteristics but different physical properties and do not decay, making them an attractive non-invasive marker for biological systems (DAWSON; BROOKS, 2001). Their assessment can be performed by mass spectrometers, which ionize gaseous molecules and separate the ion beams according to the mass/charge ratio, by electric and magnetic fields (BRENNA et al., 1997).

The isotopic composition is mostly expressed in terms of δ (delta), which represent parts per thousand (‰) of the isotope difference of the sample in relation to the standard: $\delta X = [(R_{sample}/R_{standard}) - 1] \times 1{,}000$, where R is the ratio between less and more abundant isotope. The ^{13}C:^{12}C ratio (expressed as $\delta^{13}C$) and ^{15}N:^{14}N ratio (expressed as $\delta^{15}N$) are widely used to investigate dietary habits of animals, which reflects a balance between ingestion of these isotopes through diet and loss through respiration and excretion (POST, 2002; WEHI; HICKS, 2010). Different forms of *C* fixation during the photosynthetic process lead to different proportions of isotopes (^{13}C and ^{12}C) in plant tissues (*i.e.*, isotopic composition) and, consequently, in phytophagous insects' tissues, with distinctions in the isotopic values for consumers of C_3- or C_4-based diets (BOECKLEN et al., 2011). In the biochemical route of carbohydrates in plants, when the first organic compound synthesized is a sugar with

3 atoms of *C*, these plants present the C_3-photosynthetic cycle; when the first sugar formed have 4 *C* atoms, these plants present the C_4-photosynthetic cycle (HATCH; SLACK, 1968). A third mechanism of CO_2 concentration at the rubisco site is found in crassulacean acid metabolism (CAM), similar to C_4-photosynthetic cycle in many aspects (LÜTTGE, 2004).

Isotopic values for the carbon element, expressed as $\delta^{13}C$ per thousand (‰), are in the range of about -34‰ to -24‰ (mean of -27‰) for C_3-type photosynthesis plants, and from -16‰ to -9‰ for plants of C_4-type photosynthesis (mean of -13‰), compared to the Vienna Peedee Belemnite (0‰) (VPDB standard, the international *C* isotope standard for stable isotopes ^{13}C and ^{12}C) (SMITH; EPSTEIN, 1971; DEINES, 1980; O'LEARY, 1988; FARQUHAR; EHLERINGER; HUBICK, 1989). The negative value indicates that the sample is lighter than the standard (more ^{12}C than ^{13}C). Due to these variations, the dietary source of the animals can be evaluated by isotopic determination of their tissues (HOOD-NOWOTNY; KNOLS, 2007; HYODO; KOHZU; TAYASU, 2010).

Isotopic variation of N may also help to elucidate the trophic relationships in food webs, where consumers reflect isotopic compositions of primary producers, which reflect environmental characteristics (PEREIRA; BENEDITO, 2008). The isotopic variation of plants that cannot fix atmospheric nitrogen is generally dependent on soil isotopic abundance and fertilization variations (SHEARER; KOHL; COMMONER, 1974; CHOI et al., 2002). The *N* isotope values tend to increase with trophic levels, increasing the concentration of 3‰ to 5‰ of $\delta^{15}N$ value (MINAGAWA; WADA, 1984). This progressive enrichment demonstrated in animal tissues is due to the catabolic pathways that favor the release of the lighter isotope. In this way, the food source will be determinant in the composition of the tissues analyzed.

Differences between atmospheric CO_2 and plants were first investigated by Nier and Gulbransen (1939). Then, the use of environmental isotopes was performed in the early 1950's involving their application in studies of the element cycles (CLARK; FRITZ, 1997). In the 1980's, it was possible to the ecologists to apply this technique to understand the natural composition of stable isotopes in animal and plant organisms (PETERSON; FRY, 1987). The SIA can now be used in many environmental studies (POST, 2002; HOOD-NOWOTNY; KNOLS, 2007). In Brazil, the studies are relative recent in this area, with most of the research groups focusing on the understand of energy flows and ecological interactions in ecosystems; metabolism and animal nutrition; biogeochemical markers; paleo-environmental studies; tracing physiological mechanisms in organisms; traceability of cycling pathways in agronomics, biomedical and geochemical sciences; or even traceability and

analysis of the authenticity of food products; among other studies (PESSENDA et al., 1996; MARTINELLI et al., 1999; ROSSI et al., 1999; LEITE et al., 2002; CARRIJO et al., 2006; VIDOTTO et al., 2007; ANA FILHO et al., 2008; SLEIMAN et al., 2008; COSTA et al., 2013; DUCATTI et al., 2016). However, entomological studies related to IPM and SIT are scarce and inexistent in Brazil (PRASIFKA; HEINZ, 2004; HOOD-NOWOTNY; KNOLS, 2007).

Since the insect's isotopic composition depends on what it feeds, Hood-Nowotny et al. (2009; 2015) have demonstrated that certain food components can serve as intrinsic markers of mass-reared insects. These authors have shown that especially the $\delta^{13}C$ signatures can be used for the identification of species that are reared on diets that apply C_4-sugar in their making, but that have C_3-hosts under natural conditions.

2.7 *Anastrepha fraterculus* rearing

Laboratory insect populations can be reared on artificial or natural diets, but the diet supplied must contain the nutrients necessary to meet the nutritional requirements of them, nutrients must be digested, assimilated and incorporated into tissues, provide high viability and to originate adults with high reproductive capacity, among other characteristics aiming the reproduction of colonies comparable to the wild insects found in nature (SINGH, 1977; SINGH; MOORE, 1985; PARRA, 2001; 2009). Artificial diets allow insect rearing under laboratory conditions, since, when balanced nutritional deficiencies are met, they make feasible to control pathogens, can reduce production costs and mass-rearing can be enabled (SINGH, 1977; SINGH; MOORE 1985; PARRA, 2009).

Development of insect rearing techniques in the last decades has allowed great advances in Entomology, especially the implementation of mass-rearing programs for pest control. Research advances in the area of insect nutrition have made possible to create artificial diets, facilitating the multiplication of populations to perform basic studies and to be applied in the most varied areas of Entomology (PARRA, 2000; 2001; 2009). Mass-rearing can be defined as “a systematic activity, automated, in integrated facilities, with the objective of producing a relative large supply of insects for distribution” (LEPPLA; ADAMS, 1987).

Few attempts have been made to establish *A. fraterculus* mass-rearing in South America, which has become viable only in the recent years (ALANA, 1999; NUÑEZ; GUZMAN, 1999; SALLES, 1999; JALDO; GRAMAJO; WILLINK, 2001; WALDER; COSTA; MASTRANGELO, 2006; VERA et al., 2007; WALDER et al., 2014).

Salles (1999) described the rearing procedures adopted in Pelotas (Brazil) and Jaldo et al. (2001) described a potential mass-rearing method that was used by Vera et al. (2007) later in Argentina. Vera et al. (2007) presented demographic parameters and quality control obtained from 2002 to 2004 for a bisexual strain of *A. fraterculus* successfully established at Tucumán (Argentina), following Jaldo et al. (2001) with some methodological modifications.

At CENA/USP in Brazil, the first colony of *A. fraterculus* was established in 2006 by Walder et al. (2006), with larvae collected from *Eugenia pyriformis*. The first three generations were reared on papaya fruits and then an artificial larval diet proposed by Salles (1992) was used, obtaining low insect production and viability in the beginning. Currently, the rearing of *A. fraterculus* at CENA/USP follows the procedures described by Walder et al. (2014), which allow a rapid build up of colonies.

Artificial diet is probably the most important component in the mass-rearing of sterile insects, allowing the production of sufficient numbers of high quality flies for maintenance of mother-colonies and field operations (PARRA, 1991; FAO, 2003; 2014; PARKER, 2005; WALDER et al., 2014; RULL et al., 2012). For the production of almost 300 million sterile flies per week, the Moscafrut facility from Mexico, for example, prepares 8,965 t of diet per day (OROZCO-DAVILA et al., 2017). Inside a mass-rearing facility, the larvae and flies feed only on the artificial diets offered to them.

As the isotope ratios of consumers reflect consumer's dietary patterns (DENIRO et al., 1978; 1981), the $\delta^{13}C$ and $\delta^{15}N$ signals can provide useful information in several ecological studies, what could be extremely helpful for monitoring systems (POST, 2002; HOOD-NOWOTNY; KNOLS, 2007; HOOD-NOWOTNY et al., 2009) and the implementation of SIT against the South American fruit fly in Brazil. Therefore, large natural differences in the isotopic compositions of artificial diets could be explored by novel labeling technologies in the mass-rearing of fruit flies (HOOD-NOWOTNY et al., 2009).

2.8 SIA for fruit flies monitoring

The use of SIA consists in the fact that the isotopic composition of the animal tissues reflects the isotopic composition of their food (DENIRO et al., 1978; 1981). The isotopic composition of the flies' diet reflects different forms of elements fixation in the ecosystem (*i.e.*, basic composition of primary producers) (HOOD-NOWOTNY et al., 2009).

Animals are usually a reflection of what they consume and, in the case of insects, the diet is essential in mass-rearing since it directly influences many aspects of their biology (PARRA, 1991).

According to Hagler and Jackson (2001), stable isotopes need to meet the following criteria for application as tracers for entomological studies: body retention; cause no effect on insect behavior; durability; non-toxicity; ease of application and identification, and low cost. According to Hood-Nowotny et al. (2011), it is possible to rear isotopically labeled flies. Stable isotopes, therefore, can be useful in complementary marking techniques, being also important tools in diets reconstruction, characterization of trophic relationships, elucidating patterns of resource allocation, building food webs, and tracing pest origins in quarantine areas (ROUNICK; WINTERBOURN, 1986; SPENCE; ROSENHEIM, 2005; NEWSOME et al., 2007; CAUT; ANGULO; COURCHAMP, 2009; HYODO; KOHZU; TAYASU, 2010; WEHI; HICKS, 2010; BOECKLEN et al., 2011; CHIKARAISHI et al., 2011; HOLDER et al., 2014; HYODO, 2015; BERGEN et al., 2016).

Hood-Nowotny et al. (2009; 2016) proved that SIA was effective in tracing the medfly (*Ceratitis capitata*) and seven moth species, also being an effective insect tracer method of mosquitoes and tsetse flies (HOOD-NOWOTNY; MAYR; KNOLS, 2006; HELINSKI et al., 2007; HELINSKI; HOOD-NOWOTNY; KNOLS, 2008; HOOD-NOWOTNY et al., 2011; HAMER et al., 2012). In another recent study, effective marking of *Anopheles gambiae* and *Aedes* spp. mosquitoes derived from enriched ^{13}C and ^{15}N larval habitats was accomplished (OPIYO et al., 2016).

Isotopic composition of consumers can be predicted with certain accuracy if the isotopic signatures of the diet are well known (PETERSON; FRY, 1987; POST, 2002). However, the SIA is only useful for SIT in situations where two isotopically distinct dietary sources are available (HOOD-NOWOTNY et al., 2009; 2016). More distinct, the better. Carbon isotopic ratio of consumers is usually similar to that of their diets, while the isotopic ratio of nitrogen is commonly used to estimate trophic positions (DENIRO; EPSTEIN, 1978; 1981; MINAGAWA; WADA, 1984). C_4-based diets could be used as markers to distinguish mass-reared flies from wild ones, since stable isotopes presented in flies' body are derived from the isotopes of larval diet (HOBSON; CLARK, 1992; HOOD-NOWOTNY et al., 2009). Some studies have showing that the distinct isotopic ratio of C_4-based diets can be used to distinguish flies reared on laboratory from wild flies, which have hosts with different isotopic composition (C_3-based diets), presenting significantly difference in terms of $\delta^{13}C$ values (HOOD-NOWOTNY et al., 2009; 2016).

Dietary inference of the $\delta^{13}C$ values ($^{13}C/^{12}C$) is based on plant foliar $\delta^{13}C$ values due to the divergent photosynthetic pathways of C_3 (BASSHAM et al., 1953) and C_4-plants (HATCH; SLACK, 1968). Natural isotopic abundance of C can be used to identify consumer diet from primary producers with different photosynthetic pathways. Depending on the photosynthetic mode, stable carbon isotope values range from about -35 to -22‰ in C_3-type photosynthesis and from -16 to -9‰ in the C_4-type photosynthesis (DEINES, 1980; SMITH; EPSTEIN, 1971; FARQUHAR; EHLERINGER; HUBICK, 1989). The distinct isotopic ratio of sugar (or other components) present in artificial diets for fruit flies originates mainly from C_4-type plants (*e.g.*, sugarcane), and may help to distinguish sterile-released insects from the wild ones in the field, which most commonly exploit C_3-host plants.

In contrast, the dietary inferences of the $\delta^{15}N$ values ($^{15}N/^{14}N$) are more complex, varying with many factors (AMBROSE, 1991). Isotopic variation of *N* can help to elucidate the trophic relationships in food webs, where consumers reflect isotopic compositions of primary producers, which reflect environmental characteristics (CHOI et al., 2002; PEREIRA; BENEDITO, 2008). The *N* isotope values tend to increase with trophic levels, gradually increasing the concentration of 3‰ to 5‰ of $\delta^{15}N$ value (MINAGAWA; WADA, 1984).

Therefore, SIA can provide an effective traceability of insects without restrictions imposed by traditional marker methods such as fluorescent dyes or colored substances incorporated into the artificial diet (HOOD-NOWOTNY et al., 2009; 2016). However, despite its importance, SIA still is an unusual technique employed in IPM (PRASIFKA; HEINZ, 2004; GIRARD et al., 2011).

2.9 Trophic Discrimination Factor (TDF) and Isotopic Turnover

The isotopic composition assimilated into the organisms' tissues is promoted by the discrimination of heavier isotopes during certain biological processes (DUCATTI et al., 2002). In order to estimate trophic level of consumers through SIA, it is necessary to take into account assumptions like: the type of diet and the time for nutrients to be incorporated into the consumer tissues; the rearing conditions must be constant during the experiment and the individuals sampled having proximal age; and the comparison of trophic discrimination factors (TDFs) should be done between the same types of tissues (PHILIPPSEN; BENEDITO, 2013).

Determination of species-specific TDF and turnover rate are essential for using SIA in prey-consumer relationship and several ecological studies, once the precise values allow reliable estimates of diet contribution and inaccurate values can lead to misinterpretation of field data (DUCATTI et al., 2002; CAUT; ANGULO; COURCHAMP, 2009; BOECKLEN et al., 2011). Ratios of stable isotopes can switch between diet and consumer due to metabolic processes and differential digestion or fractionation process during nutrients assimilation (TIESZEN et al., 1983; DUCATTI et al., 2002; HOLÁ et al., 2015). This process of isotope discrimination can be characterized by the TDF, consisting in an enrichment or depletion of the heavier isotope of the sample in chemical reactions (GALIMOV, 1985; EHLERING, 1991; MARTÍNEZ DEL RIO et al., 2009; HOLÁ et al., 2015). Variations in metabolic rate of different tissues of an animal can influence the TDF and turnover rate (TIESZEN et al., 1983; HOBSON; CLARK, 1992; VANDER ZANDEN et al., 2015), being also subject to diet quality, consumer's nutritional status, lipid extraction and other factors (MINAGAWA; WADA, 1984; VANDERKLIFT; PONSARD, 2003).

TDF is essential to the use of SIA in studies of animal diets and trophic levels, depending on the metabolic processes and the fractionation of the diet in the animal tissues, the type of diet consumed and the turnover rate (*i.e.*, cellular renewal rate) of each tissue. The quantification of the discrimination and the biochemical renewal rate of each tissue are essential for the precise estimation of trophic levels in order to understand the interactions in the ecosystem (VANDER ZANDEN; RASMUSSEN, 2001; POST, 2002; McCUTCHAN et al., 2003; VANDERKLIFT; PONSARD, 2003; SPENCE; ROSENHEIM, 2005; CAUT; ANGULO; COURCHAMP, 2009; BERGEN et al., 2016). In a study involving 22 terrestrial herbivorous arthropods feeding on 18 different host plants, Spence and Rosenheim (2005) observed a range from -3.5‰ to 1.9‰ for $\delta^{13}C$ enrichments across plants to herbivores (mean of -0.5 ± 0.3‰) and -0.2‰ to 6.6‰ for $\delta^{15}N$ (mean of 1.9 ± 0.8‰).

In its turn, isotopic turnover is the continuous renewal of the chemical elements and their isotopes in a body tissue, providing records of animals' dietary histories integrated over time (TIESZEN et al., 1983). Turnover results are usually expressed in half-life (HL), defined as the time required to the animal reach the balance of 50% ($t_{1/2}$) with its diet, reconstructing assimilated dietary sources over time (TIESZEN et al., 1983; VANDER ZANDEN et al., 2015). According to Ducatti et al. (2002), the period over which the animal isotopic composition reflects the isotopic composition of a particular diet will depend on the turnover rate of the body (or tissue) and physiological processes (synthesis and catabolism)

of each tissue component. Knowledge of the turnover rates and transition phase from laboratory to field provides a basis for rational monitoring planning in a SIT program.

Either discrimination factors or the turnover rates of isotopes in insects tissues are poorly know. When the *C* and *N* of the diet are incorporated into a particular consumer's tissue, a specific fractionation in the isotope ratio is expected and it needs to be evaluated for correct data interpretation (KENNED; KROUSE, 1990; CAUT; ANGULO; COURCHAMP, 2009). Determining the turnover rates of *A. fraterculus* flies will help to elucidate until when it is possible to discriminate laboratory-flies from wild ones.

3 MATERIALS AND METHODS

3.1 Collection of wild *Anastrepha fraterculus* flies

The capture of wild *Anastrepha fraterculus* flies was carried out in early 2017 with McPhail traps containing Ceratrap™ (Bioibérica, Barcelona, Spain). All collections were performed in apple orchards located at the municipality of Vacaria, Rio Grande do Sul State, Brazil. Vacaria (28º30'S, 50º54' W) is located in the north-eastern region of the State, 950 m above sea level (Figure 1). The flies were captured by technicians of Empresa Brasileira de Pesquisa Agropecuária (Embrapa Uva & Vinho) in three different locations (Figure 2):

A) Apple orchard on private property (28°30'59.26" S; 50°52'16.80" W);

B) Experimental field area of Embrapa Uva & Vinho (28°30'58.98 S; 50°53'0.97" W);

C) Commercial area of Schio LTDA (28°32'9.30" S; 50°49'18.30" W).

Figure 1 - State map of Rio Grande do Sul, locating the city of Vacaria (Embrapa Uva & Vinho)

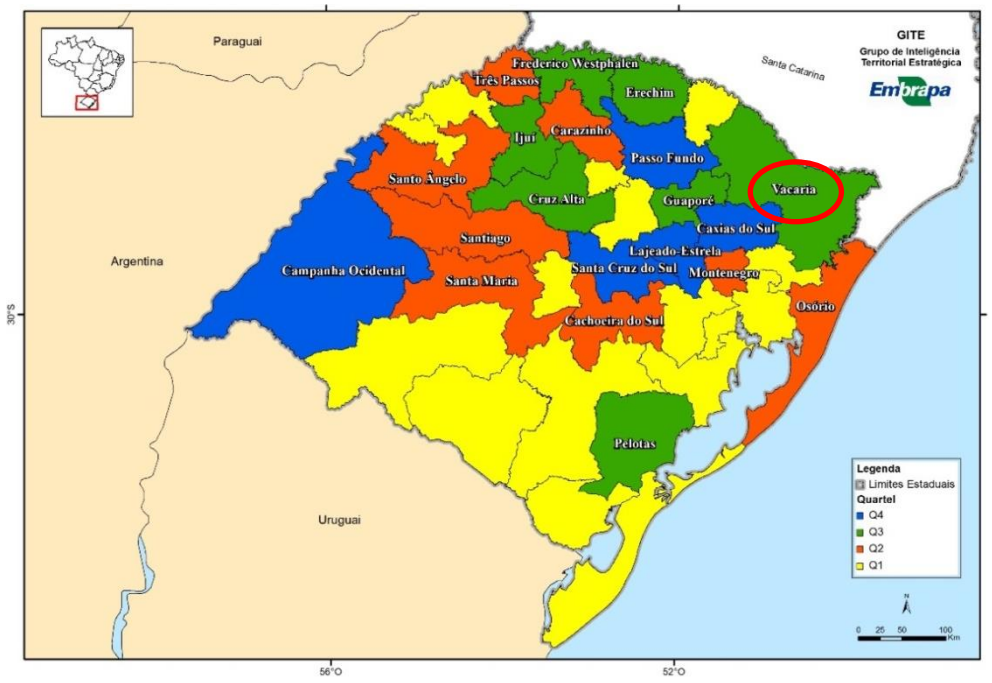


Figure 2 - Map of Vacaria (Google Earth) and collection sites A, B and C (date of the image: 10/29/2016; elevation of 912 m; altitude of the point of view: 23.10 km)

The traps were inspected at 8 h intervals. For the stable isotope analyses (SIA), 20 flies (10 ♂ and 10 ♀) from each sampling site were used. Species identification was done with the aid of a specialist from Embrapa Uva & Vinho (Dr. Adalécio Kovaleski), following Hernández-Ortiz et al. (2012). No endangered or protected species were involved in the study and no specific permits were required for the described field studies or for the import of material.

All flies manipulations in the field were carried out using gloves to avoid contaminations. After collection, the flies were gently washed with distilled water and dried for 6 h on paper tissue. The samples were individualized and conditioned in Eppendorf tubes, then sent to Laboratório de Irradiação de Alimentos e Radioentomologia (LIARE) of CENA/USP at Piracicaba-SP, Brazil, where they were kept in controlled environment room (25±1 ºC and 65±10% relative humidity).

The mean values of isotopic compositions (δ^{13}C and δ^{15}N) from males and females of the three apple orchards were compared by the Student's *t*-test (α= 0.01). For the mean values of δ^{13}C and δ^{15}N from the wild flies of the three collection sites, the one-way analysis of variance *F*-test was applied at the 1% of significance (ANOVA) and, when significant differences were detected, the Tukey's honestly significance difference (HSD)

test (α=0.01) was applied to compare the means. Homogeneity of variances and normality of model residuals were checked in all instances (BARTLETT, 1937; SHAPIRO; WILK, 1965). The analyses were performed by the statistical program SAS 9.4 (SAS INSTITUTE, 2013).

3.2 Labelling *Anastrepha fraterculus* flies with different larval diets

To verify if *A. fraterculus* adult flies could be labelled by the naturally occurring isotopes from larval diets, experiments with different diets and fruit hosts were conducted at the LIARE from CENA/USP. The eggs to be seeded in artificial diets and the adults to oviposit in fruits were obtained from a bisexual strain of *A. fraterculus*, originally established with wild populations from Vacaria (RULL et al., 2013; DIAS et al., 2016). The *A. fraterculus* mother-colonies have been maintained following the procedures described by Walder et al. (2014) in controlled environment rooms (25 ± 1 ºC, 65 ± 10% RH, and photoperiod of 12:12 [L:D] h).

The adults in ovipositing cages were provided with a mixture of sugar and hydrolyzed brewer's yeast *Bionis* YE MF™ (Biorigin, Lençois Paulista, Brazil) at 3:1 rate and water *ad libitum* (JALDO; GRAMAJO; WILLINK, 2001; NUNES et al., 2013). Eggs collected were bubbled in water bath at 24 ºC for 48 h. Aliquots of 2 mL of eggs were seeded in trays containing 1 L of artificial diet, with the larvae remaining there for 10-12 days at 24 ºC (Figure 3).

Figure 3 - *Anastrepha fraterculus* larvae reared on artificial diet

The larvae were reared on two distinct artificial diets:

I) Diet I was formulated with 50 g of brewer's yeast *Brewcell*™ (Biorigin, Brazil), 30 g of sugar (sugarcane source) *Caravelas*™ (Usina Colombo, Brazil), 300 g of corn bran Yoki™ (General Mill Alimentos, Brazil), 2 mL of Nipagin, 2 g of sodium benzoate, 6 g of citric acid and 1,000 mL of distilled water.

II) Diet II was a gelled diet adapted from Salles (1992), formulated with 3 g of agar (algae), 60 g of corn bran Yoki™ (General Mill Alimentos, Brazil), 60 g of sugar *Caravelas*™ (Usina Colombo SA, Brazil), 60 g of brewer's yeast *Brewcell*™ (Biorigin, Brazil), 1 g of sodium benzoate, 6 mL of hydrochloric acid, 8 mL of Nipagin fungicide and 900 mL of distilled water.

To obtain adult flies from a natural and an alternative host (C_3-based diets), larvae were reared on apples (*Malus domestica* Borkh. cv. 'Gala') (SUGAYAMA et al., 1997) and papaya fruits (*Carica papaya* L. cv 'Golden') (MACHOTA et al., 2010), respectively. The fruits were purchased at the market and well cleaned with detergent and water. Adults from the mother-colony of the *A. fraterculus* bisexual strain were kept in screened cages (30 x 30 x 30 cm; ≈ 600 couples/cage) with water and the adult diet previously described under laboratory conditions (25±1 ºC and 65±10% RH) for the infestation of the fruits. When the flies were 7 days old, the fruits were inserted in each cage separately. After 24 h, the fruits were removed and conditioned in boxes (40 x 20 x 15 cm) with lid for the larval development (Figure 4). The experimental units were distributed randomly and five replicates were performed for the treatments.

Figure 4 - *Anastrepha fraterculus* larvae reared on papaya fruit

After the larval development period, the third instar larvae were separated from the artificial diets by washing and then transferred to 500 mL plastic cups with moist vermiculite for pupation under laboratory conditions in dark room (24 ± 1 ºC, 65 ± 10% RH) (Figure 5).

Figure 5 – Collection of *Anastrepha fraterculus* larvae from the fruits for later pupation

After 5 days from the oviposition, the fruits were placed on Styrofoam plates, and the bottom of the boxes was filled with water to receive the larvae from the fruits. When the larvae started crawling of the fruits, they were collected twice a day and left to pupate in 500 mL plastic cups with vermiculite (Figure 6).

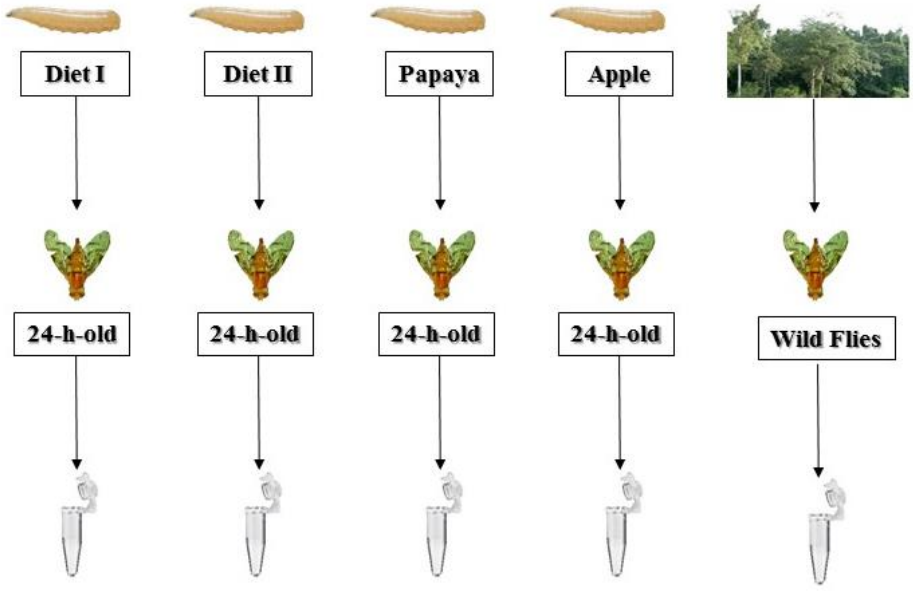
Figure 6 - *Anastrepha fraterculus* third instar larvae in 500-mL plastic cups with moist vermiculite for pupation

One day before emergence, samples of ~100 mL of pupae from each treatment were placed in screened cages (30 x 30 x 30 cm) for adult emergence, with only water provided by vials with cotton wicks (Figure 7).

Figure 7 – Emergence of *Anastrepha fraterculus* adults from the different larval diets in screened cages

After emergence, the 24-h-old flies were frozen to death, the individuals were conditioned in Eppendorf microtubes and then put to dry in a ventilated stove at 50 ºC for 72h for future stable isotope analysis (whole body of the flies were used for SIA). The methodology of the test can be observed in Figure 8. Ten flies were analyzed per treatment (five males and five females were randomly collected per treatment).

Figure 8 - Scheme of the methodology presented in '3.2 Labelling *Anastrepha fraterculus* flies with different larval diets'. Groups of ten adults of *A. fraterculus* obtained from different larval diets and fruits were prepared for stable isotopes analysis, as well as wild flies from Vacaria



The mean values of isotopic compositions (δ^{13}C and δ^{15}N) from males and females of each larval diet and fruit were compared by the Student's *t*-test (α= 0.01). For the mean values of δ^{13}C and δ^{15}N from the larval diets, fruits, adults obtained from the diets, and wild flies (overall mean values from the three collection sites), the one-way analysis of variance *F*-test was applied at the 1% of significance (ANOVA) and, when significant differences were detected, the Tukey's honestly significance difference (HSD) test (α=0.01) was applied to compare the means. Homogeneity of variances and normality of model residuals were checked in all instances (BARTLETT, 1937; SHAPIRO; WILK, 1965). The analyses were performed by the statistical program SAS 9.4 (SAS INSTITUTE, 2013).

3.3 Trophic Discrimination Factors of stable carbon and nitrogen isotopes of *Anastrepha fraterculus* flies

Trophic discrimination factors (TDFs) of carbon (Δ^{13}C) and nitrogen (Δ^{15}N) were calculated by subtracting the mean δ^{13}C or δ^{15}N values of the whole-body of the 24-h-old flies from the mean δ^{13}C or δ^{15}N values of the diet (MARTÍNEZ DEL RIO et al., 2009; HOLÁ et al., 2015):

$$\Delta X = \delta X_{\text{fly' whole-body}} - \delta X_{\text{diet}} \quad (1)$$

where Δ represents the trophic discrimination factor and X is the chemical element being used, represented by the heaviest stable isotope.

3.4 Influence of attractive and preservative substances on the isotopic composition of *Anastrepha fraterculus* flies

This experiment was carried out to evaluate the influence of capture substances usually applied to attract fruit flies and a preservative (ethanol) on the isotopic composition (δ^{13}C and δ^{15}N) of *A. fraterculus* flies. Three substances were tested: concentrated CeraTrap™ (Bioibérica, Barcelona, Spain), concentrated grape juice (Cooperativa Vinícola Garibaldi, Garibaldi, Brazil) and absolute ethanol (Química Moderna Indústria & Comércio, Barueri, Brazil). They were placed separately in McPhail traps (200 mL of each substance per trap), which were distributed randomly at acclimatized room (25 ± 1 ºC and 65 ± 10% RH) (Figure 9). For the control group, the McPhail traps were filled with distilled water only.

Figure 9 – Adults of *Anastrepha fraterculus* immersed in attractive substance (CeraTrap™) in a McPhail trap

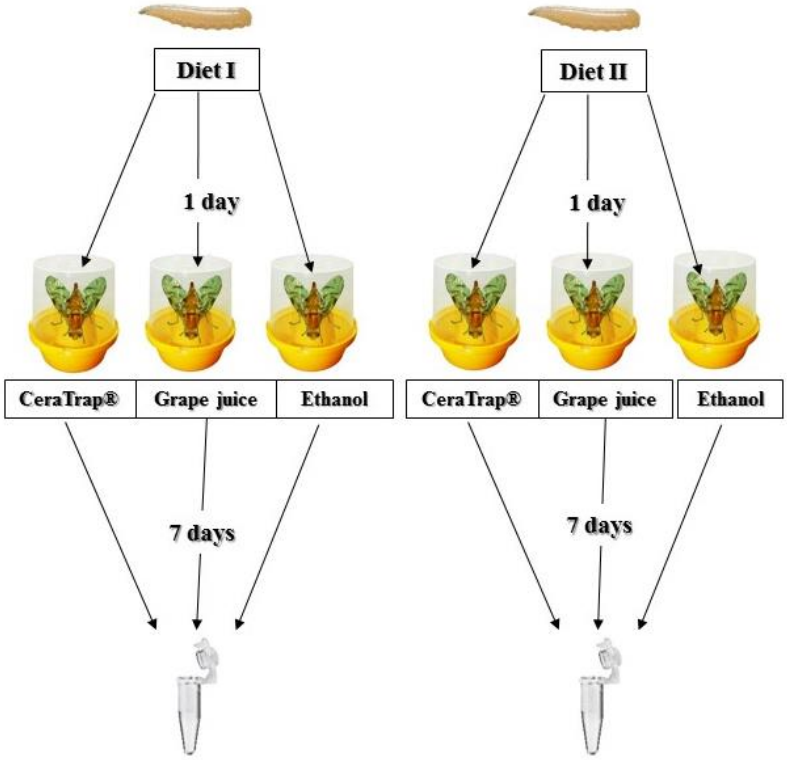
About 100 flies (24-h-old) whose larvae were reared on Diet I and Diet II were separated to be anesthetized by cold (10 min. at -20ºC) and then immersed in McPhail traps containing each one of the treatments (distilled water, CeraTrap™, grape juice or absolute ethanol). The flies were randomly collected 1 day and 7 days after the immersion (Table 1) (Figure 10), washed with distilled water and dried for 6 h on paper tissue. After that, the individualized samples were conditioned in Eppendorf tubes and placed to dry in a ventilated stove at 50 ºC for 72 hours for future isotopic analysis. Six replicates were performed for all treatments.

Table 1 – Treatments presented in "2.2.3 Influence of attractive and preservative substances on the isotopic composition of *Anastrepha fraterculus* flies"

Diet I	Diet II
Water I*	Water II
Absolute ethanol (ET I)	Absolute ethanol (ET II)
Ceratrap™ (CT I)	Ceratrap™ (CT II)
Grape Juice (GJ I)	Grape Juice (GJ II)
Ceratrap™ to Ethanol (CTET I)	Ceratrap™ to Ethanol (CTET II)

*I= Diet I; II= Diet II; ET= ethanol; CT= CeraTrap™; GJ= grape juice; CTET= CeraTrap™ for 7 days and then immersed in absolute ethanol for 7 days.

Figure 10 - Scheme of the methodology presented in '3.4 Influence of attractive and preservative substances on the isotopic composition of *Anastrepha fraterculus* flies'. Adults of *A. fraterculus*, reared on different artificial diets were immersed in substances (CeraTrap™, grape juice or absolute ethanol) in a McPhail trap and later submitted to stable isotopes analysis



To verify the effect of both attractive and preservative substances on the isotopic compositions of the flies (CeraTrap™ and ethanol), a group of flies from Diets I and II that had been immersed in CeraTrap™ for 7 days was cleaned and washed with distilled water, and then immersed in absolute ethanol for one week (Table 1). Six replicates were performed for each treatment.

For the isotopic compositions (δ^{13}C and δ^{15}N) of the wild flies (overall mean values from the three collection sites) and from the different treatments (Table 1) and times (immersion for 1 or 7 days), a two-way analysis of variance *F*-test was applied at a 5% level of significance, considering time and treatment (factorial scheme 2 by 11) as factors. Homogeneity of variances and normality of model residuals were checked in all instances (BARTLETT, 1937; SHAPIRO; WILK, 1965). The analyses were performed by the statistical program SAS 9.4 (SAS INSTITUTE, 2013).

3.5 Bioassay of turnover rate (C_3-C_4-based diet switching)

To verify the turnover rate of *A. fraterculus* flies, C_3-C_4-based diet switching experiments were conducted at the LIARE/CENA. The larvae were reared on either a C_4-based diet (Diets I and II) or a C_3-based diet (papaya) in controlled environment room (25 ± 1 ºC, 65 ± 10% RH and photoperiod of 12:12 [L:D] h), following the methodology previously described.

After emergence, the adults were kept in screened cages (30 x 30 x 30 cm; up to 600 couples/cage) and the groups of flies that came from C_4-based artificial diets were maintained with water *ad libitum* by vials with cotton wicks and with three different types of adult diets separately for 5 days, corresponding to the adult diets usually used in the laboratory colonies of LIARE:

a) 100% refined cane sugar *Caravelas*™ (Usina Colombo, Brazil);

b) Mixture of cane sugar and hydrolyzed yeast *Bionis* YE MF™ (Biorigin, Brazil) at 3:1 rate;

c) Gainesville diet (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).

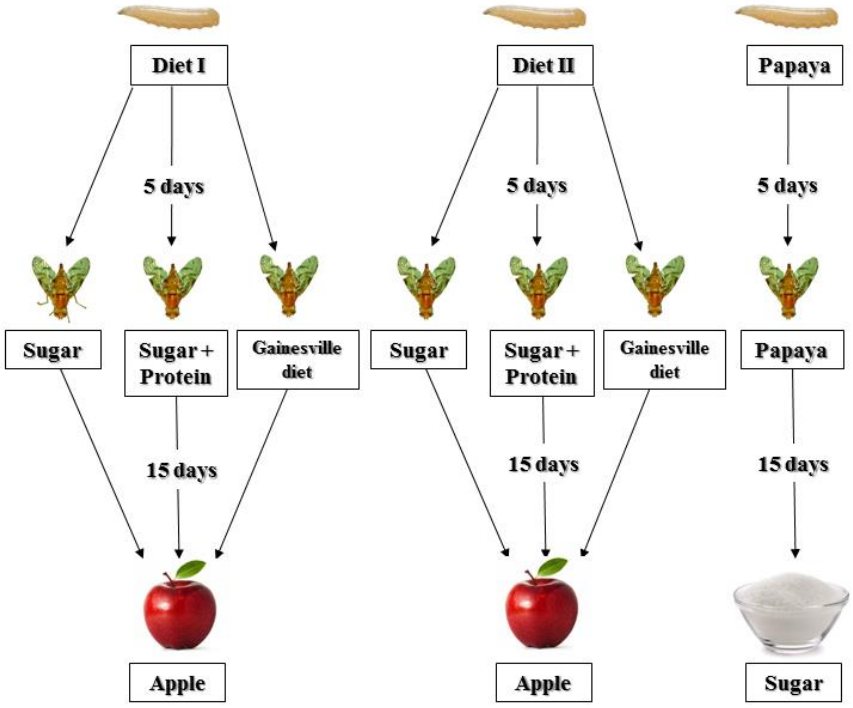
After 5 days, the adult diet of these flies was switched to apple (C_3-based diet), simulating the transition from a C_4-based diet to a field C_3-based diet. Meanwhile, flies whose larvae were reared on papaya fruit (C_3-based diet) were maintained with water *ad libitum* and papaya slices for 5 days. After this period, the adult diet of these flies was switched to 100% refined cane sugar.

Due to the long pre-copulatory period, the *A. fraterculus* males have to be maintained in laboratory for 5-7 days before field release (SEGURA et al., 2013). During this period, the adults have to be fed on a diet like the ones tested in this study. After the release, the flies usually switch their dietary source for natural ones. As release-recapture experiments in apple orchards have shown that more than 90% of recaptures occur during the 17-20 days after release (KOVALESKI; SUGAYAMA; MALAVASI, 1999), the flies were allowed to feed on apple slices or sugar for 15 days in our tests.

Five males and five females from each treatment were randomly sampled at 0, 1, 2, 5, 7, 12 and 15 days after the diet change, where the time 0 is the time of diet shift (or when the flies were 5-d-old). The fly samples were frozen to death, individualized in

Eppendorf tubes and then put to dry in a ventilated stove at 50 ºC for 72 hours for future stable isotope analyses (whole-body of the flies were used for SIA). The scheme of the methodology of the test can be observed in Figure 11.

Figure 11 - Scheme of the methodology presented in '3.5 Bioassay of turnover rate (C_3-C_4-based diet switching)'. Adults of *A. fraterculus* were fed on different types of diets for 5 days and, after this period, the diet was switched to a different source of diet. Samples were sent for stable isotopes analyses in 0, 1, 2, 5, 7, 12 and 15 days after diet shift



A two-source mixing model was performed to calculate the proportion of structural carbon in the *A. fraterculus* using two dietary sources (*i.e.*, isotopic data of the flies derived from papaya and fed on sugar after diet switching), following Hood-Nowotny et al. (2009):

Proportion (% C) derived from papaya source =

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

Flies from papaya were used for the assessment of the proportion of structural carbon due to the maintenance of the dietary source from larval stage after emergence. One larval diet and two different adult diets were used in the other treatments, making the model more complex, increasing variability and uncertainty in estimates of source contributions (PHILLIPS; NEWSOME; GREGG, 2005).

The Boltzmann Sigmoid Model was applied in order to measure the carbon isotopic turnover in a specific period of time, explaining the sigmoidal form of the experimental results, expressed by the function:

$$Y = A_2 + [A_1 - A_2/(1 + e^{(x - x_0)/dx})] \quad (2)$$

or

$$\delta^{13}C_{(t)} = \delta^{13}C_{(f)} + [\delta^{13}C_{(i)} - \delta^{13}C_{(f)}/(1 + e^{(t - x_0)/dx})], \text{ in isotopic form;} \quad (3)$$

Where:

Y / $\delta^{13}C_{(t)}$: flies' relative enrichment at any time (t);

A_1 / $\delta^{13}C_{(i)}$: initial relative enrichment;

A_2 / $\delta^{13}C_{(f)}$: final relative enrichment;

X_0: inflection point of the sigmoid. It represents the carbon half-life (HL= $t_{1/2}$). Expressed in unit of time (day);

dx: time constant. Expressed in unit of time;

t: experimental time. Expressed in unit time (day).

Value of Y ($\delta^{13}C_{(t)}$) in X_0 is the half of the distance between the two limit values A_1 and A_2: $Y(X_0) = (A_1 + A_2)/2$. The value of Y changes according to variations of X. The magnitude of these variations is approximately dx (DA SILVA; DUCATTI; COSTA, 2007).

The turnover rate is typically expressed in terms of half-life (HL), referring to the amount of time required for the stable isotope of the consumer reaches the mean value balance observed between the original diet and the new diet. To determine the 50% HL ($t_{1/2}$) of carbon atoms, X_0 values were used.

The mean values of isotopic compositions ($\delta^{13}C$ and $\delta^{15}N$) from males and females of all diet switching treatments at the first (t= 0 day) and last period (t= 15 days) of evaluation were compared by the Student's t-test (α= 0.01). For the mean values of $\delta^{13}C$ and $\delta^{15}N$ from the adults 1-d-old obtained from the diets and fed on different diets for five days, and for the

isotopic composition of the adult diets, the one-way analysis of variance *F*-test was applied at the 1% of significance (ANOVA) and, when significant differences were detected, the Tukey's honestly significance difference (HSD) test (α= 0.01) was applied to compare the means.

The analysis of $\delta^{13}C$ curve values for turnover rate obtained for flies after diet switching (*i.e.* adults ageing between 5 and 20-d-old) were obtained from methods of regression for non-linear functions and fitted statistically by the Origin® 8.0 Professional (MOTULSKY; RANSNAS, 1987; MICROCAL SOFWARE, 1999).

The possible differences in $\delta^{13}C$ and $\delta^{15}N$ values in flies collected at the last sampling period after diet switching (day 15) and the isotopic values of wild flies (overall mean values from the three collection sites) were also verified 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 analyses were performed by the statistical program SAS 9.4 (SAS INSTITUTE, 2013).

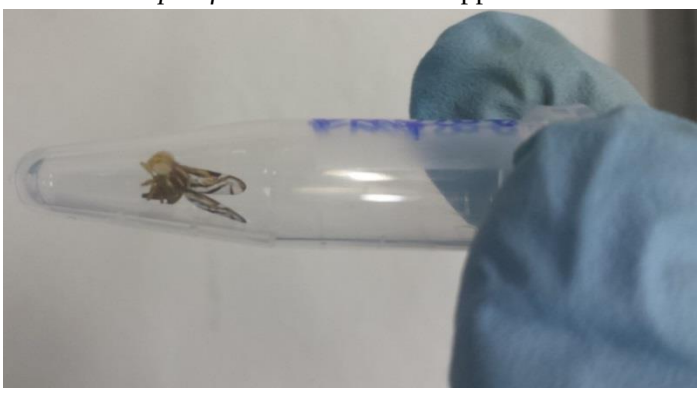
3.6 Stable Isotope Analyses ($\delta^{13}C$ and $\delta^{15}N$)

The manipulation of the flies, both from field and laboratory, were carried out using gloves to avoid contaminations. Analyses of ^{13}C:^{12}C and ^{15}N:^{14}N ratios were performed in order to determine the isotopic compositions of the artificial diets and fruits, laboratory 24-h-old flies, wild flies collected in Vacaria - RS, attractive and preservative substances, fly samples immersed in different substances, and flies from the diet switching experiments. Stable isotope ratios were reported as delta (δ) notation.

The δ was calculated by $\delta X=[(R_{sample}/R_{standard}) -1]$, where δX refer to $\delta^{13}C$ or $\delta^{15}N$, and R is 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 per thousand (‰) relative difference to the ratio of international reference standards ($R_{standard}$) which are Vienna PeeDee Belemnite (VPDB) and atmospheric nitrogen (N_2) for *C* and *N*, respectively.

After fly collections on each treatment of the experiments, the individualized samples were conditioned in Eppendorf tubes and placed to dry in a ventilated laboratory stove at 50 ºC for 72 hours for future isotopic analysis (Figure 12). Then the dried samples were macerated (whole body) until reaching constant masses.

Figure 12 - Dried *Anastrepha fraterculus* adult in Eppendorf tube before maceration



All dried material were weighed (0.3 - 0.5 mg for artificial diets, 0.8 - 1.0 mg for fly samples, and 2.5 - 2.8 mg for fruit samples) in a precision analytical balance ME 36S (Sartorius, Göttingen, Germany) and enclosed in tin capsules (Elemental Analysis 5 x 3.5 mm) (Figure 13 and 14). For the ethanol and attractive substances, about 1 mL was used for the analyses. The enclosed samples were analyzed in an elemental analyzer (CHN 1110 – CE Instruments, Rodano, Italy), coupled with a Continuous Flow Isotope Ratio Mass Spectrometry (CF-IRMS) (Delta Plus – Thermo Scientific, Bremen, Germany), located in the *Laboratório de Ecologia Isotópica* (LEI) of CENA/USP.

Figure 13 - Preparation for Stable Isotopes Analysis with dried material weighed in a precision analytical balance

Figure 14 - Samples enclosed in tin capsules for Stable Isotopes Analysis in Continuous Flow Isotope Ratio Mass Spectrometry

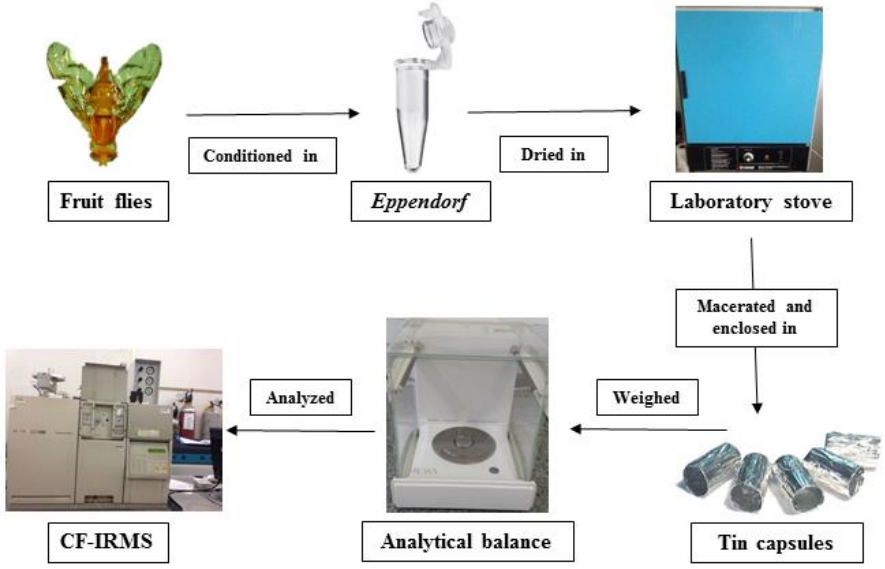
Samples were fired in an oxygen atmosphere at approximately 1700 °C and the resulting N_2 and CO_2 passed through a series of scrubbers to remove the impurities and waste water in the elemental analyzer through a chromatographic separation column in ultrapure helium carrier. The CO_2 and the N_2 peaks were evaluated in the CF-IRMS to determine the isotopic ratios (Figure 15).

Figure 15 - Stable Isotopes Analysis performed in Continuous Flow Isotope Ratio Mass Spectrometry (CF-IRMS)

The results were normalized to the international standards through the use of secondary reference materials (NBS-19, NBS-22, IAEA-N1, IAEA-N2) (EHLERINGER; RUNDEL, 1989; COPLEN, 1994; GRONING, 2004). An in-house working standard (*i.e.*, sugarcane leaf) was used for quality control every 11 samples in each run, from where the precision was evaluated as better than 0.15‰ for both elements.

Each sample was analyzed twice to obtain the mean values with precision of 0.3‰. The analytical error of the isotopic measurements was estimated at 0.3‰ for $\delta^{13}C$ and 0.5‰ for $\delta^{15}N$ by means of repeated measurements of the internal standard (*i.e.*, sugarcane leaf) (Figure 16).

Figure 16 - Scheme of the methodology presented in '3.6 Stable Isotope Analyses ($\delta^{13}C$ and $\delta^{15}N$)'



4 RESULTS

4.1 Isotopic compositions of wild *Anastrepha fraterculus* flies

A total of 60 wild flies collected in three different apple orchards from Vacaria were analyzed for δ^{13}C and δ^{15}N. The isotopic compositions of wild males did not differ from the compositions of females within each of the apple orchards (sites A, B and C) ($P > 0.01$) (Table 2).

Table 2 - Mean isotopic composition of males and females of wild *Anastrepha fraterculus* flies from three different apple orchards

Orchard	Sex	δ^{13}C ‰	δ^{15}N ‰
A	♂	-25.5 ± 0.2a**	5.9 ± 0.6a
	♀	-25 ± 0.17a	5.3 ± 0.3a
	ANOVA*	$F_{1,19}$= 6.22; C.V.= 1.98%; P= 0.026	$F_{1,19}$= 1.11; C.V.= 20%; P= 0.306
B	♂	-25.8 ± 0.2a	4.3 ± 0.6a
	♀	-25.6 ± 0.3a	4.24 ± 0.7a
	ANOVA	$F_{1,19}$= 0.47; C.V.= 2.31%; P= 0.503	$F_{1,19}$= 0.04; C.V.= 23.42%; P= 0.837
C	♂	-26 ± 0.2a	5.8 ± 0.7a
	♀	-26.2 ± 0.2a	6.6 ± 0.6a
	ANOVA	$F_{1,19}$= 1.0; C.V.= 2.15%; P= 0.329	$F_{1,19}$= 1.72; C.V.= 23.74%; P= 0.206

*ANOVA = analysis of variance; C.V.= coefficient of variation.
**Means (± SE) followed by the same letters in the columns do not differ significantly by the Student's *t*-test (α= 0.01).

The δ^{13}C and δ^{15}N mean values of the flies collected at site A were -25.3 ± 0.1‰ and 5.6 ± 0.3‰, respectively; site B were -25.7 ± 0.1‰ and 4.3 ± 0.3‰, respectively, and at site C were -26.1 ± 0.1 ‰ for δ^{13}C and 6.2 ± 0.3‰ for δ^{15}N.

The overall mean values of all flies captured (n= 60) were -25.7 ± 0.1‰ for δ^{13}C and 5.4 ± 0.2‰ for δ^{15}N.

The dispersion of the $\delta^{13}C$ and $\delta^{15}N$ values of the wild flies are presented in Figures 17 and 18. The $\delta^{13}C$ values ranged from -27 to -24‰ (Figure 17) and the $\delta^{15}N$ values ranged from 2.6 to 9.6‰ (Figure 18).

Figure 17 - Dispersion of the $\delta^{13}C$ values of wild *Anastrepha fraterculus* flies (n= 60)

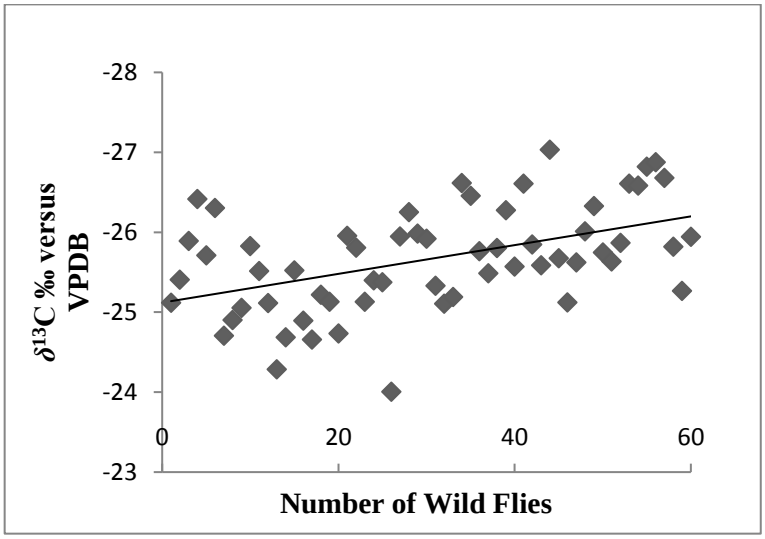
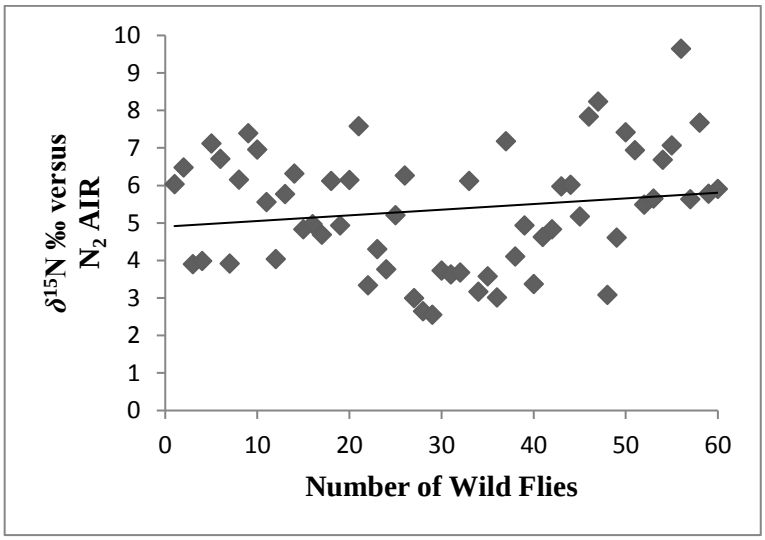


Figure 18 - Dispersion of the $\delta^{15}N$ values of wild *Anastrepha fraterculus* flies (n= 60)



As the isotopic compositions of the sexes within each collection site did not differ significantly, the $\delta^{13}C$ and $\delta^{15}N$ values from the wild flies of each site were compared among them. Significant differences were found between the apple orchards (Table 3).

Table 3 - Mean isotopic composition of wild *Anastrepha fraterculus* flies from 3 different apple orchards

Orchard	**δ^{13}C ‰**	**δ^{15}N ‰**
A	-25.3 ± 0.1a**	5.6 ± 0.3a
B	-25.7 ± 0.1ab	4.3 ± 0.3b
C	-26.1 ± 0.1b	6.2 ± 0.3a
ANOVA*	$F_{2,59}$= 10.58; C.V.= 2.22%; P< 10^{-3}	$F_{2,59}$= 10.49; C.V.= 25.75%; P< 10^{-3}

*ANOVA= analysis of variance; C.V.= coefficient of variation.
**Means (± SE) followed by the same letters in the columns do not differ significantly by the Tukey's test (α= 0.01).

Considering the δ^{13}C values, the flies from site A differed significantly from the flies from site C, and the δ^{15}N values of the flies from site B differed from both sites A and C, possible reflecting differences in the management of the areas and evidence of resource partitioning or even distinct habits to exploit food resources in the agroecosystem (OULHOTE et al., 2011).

4.2 Influence of larval diets on the isotopic compositions of *Anastrepha fraterculus* flies

The comparisons between the isotopic compositions of male and female of flies whose larvae were reared on larval diets (Diet I and Diet II) and fruits (papaya and apple) showed no significant differences (P> 0.01) (Table 4).

Table 4 - Mean isotopic composition of males and females of *Anastrepha fraterculus* flies reared on different larval diets and fruits

TREATMENT	SEX	δ^{13}C ‰	δ^{15}N ‰
Flies reared on Diet I	♂	-15.5 ± 0.1a**	3.9 ± 0.2a
	♀	-15.2 ± 0.2a	4 ± 0.1a
	ANOVA*	$F_{1,8}$= 7.97; C.V.= 1.02%; P= 0.025	$F_{1,8}$= 0.23; C.V.= 4.66%; P= 0.645
Flies reared on Diet II	♂	-16 ± 0.2a	4.3 ± 0.1a
	♀	-16.1 ± 0.1a	4 ± 0.3a
	ANOVA	$F_{1,9}$= 0.07; C.V.= 2.64%; P= 0.80	$F_{1,9}$= 0.58; C.V.= 12.4%; P= 0.467
Flies reared on papaya	♂	-27.1 ± 0.2a	6.9 ± 0.4a
	♀	-26.9 ± 0.4a	6.8 ± 0.4a
	ANOVA	$F_{1,9}$= 0.28; C.V.= 2.23%; P= 0.615	$F_{1,8}$= 0.04; C.V.= 12.97%; P= 0.838
Flies reared on apple	♂	-25.7 ± 0.1a	2.9 ± 0.1a
	♀	-26 ± 0.1a	2.9 ± 0.4a
	ANOVA	$F_{1,8}$= 5.64; C.V.= 0.87%; P= 0.049	$F_{1,9}$= 0.01; C.V.= 24.83%; P= 0.942

*ANOVA= analysis of variance; C.V.= coefficient of variation.
**Means (± SE) followed by the same letters in the columns do not differ significantly by the Student's *t*-test (α= 0.01).

The δ^{13}C and δ^{15}N mean values and comparisons among the larval diets, fruits, laboratory and wild flies (mean values from the three different apple orchards) are expressed in Table 5. The larval Diet I differed significantly from the δ^{13}C values of the Diet II and the fruits (C_3-based diets). The apple fruits presented the lowest δ^{13}C value (-28.4‰), whilst Diet I presented the highest value (-12.6‰) (Table 5).

The results indicated that the δ^{13}C values of the *A. fraterculus* adults reared in laboratory were significantly influenced by the artificial diets or fruits in which they developed. The δ^{13}C mean values of *A. fraterculus* reared on artificial Diet I and Diet II reflected carbon sources of C_4-based diet, differing from the δ^{13}C mean values observed for flies reared on fruits and the wild flies (C_3-based diets). The δ^{13}C mean values of wild flies (-25.7 ± 0.1‰) did not differ from the flies reared on apple (-25.9 ± 0.1‰) and the papaya fruits (-26 ± 0.1‰), reflecting carbon sources of C_3-based diets. The flies whose larvae were

reared on Diet I presented the highest δ^{13}C value (-15.3‰), while the flies whose larvae were reared on papaya presented the lowest δ^{13}C mean value (-27‰) (Table 5).

Considering the δ^{15}N values, the papaya differed significantly from the larval diets and apple. Diet I showed some proximity to the Diet II, and Diet II to apple. The papaya presented the highest δ^{15}N mean value (5.5‰), differing significantly from the δ^{15}N values of the others diets, and apple presented the lowest value (2‰). The δ^{15}N of *A. fraterculus* reared on larval diets and papaya did not differ from the δ^{15}N values of the wild flies. The flies whose larvae were reared on apple presented the lowest δ^{15}N mean value (2.9‰), differing significantly from the δ^{15}N values of other flies (including the δ^{15}N of wild flies), whilst the flies whose larvae were reared on papaya presented the highest δ^{15}N mean value (6.8‰), showing no difference to the δ^{15}N of wild flies (Table 5).

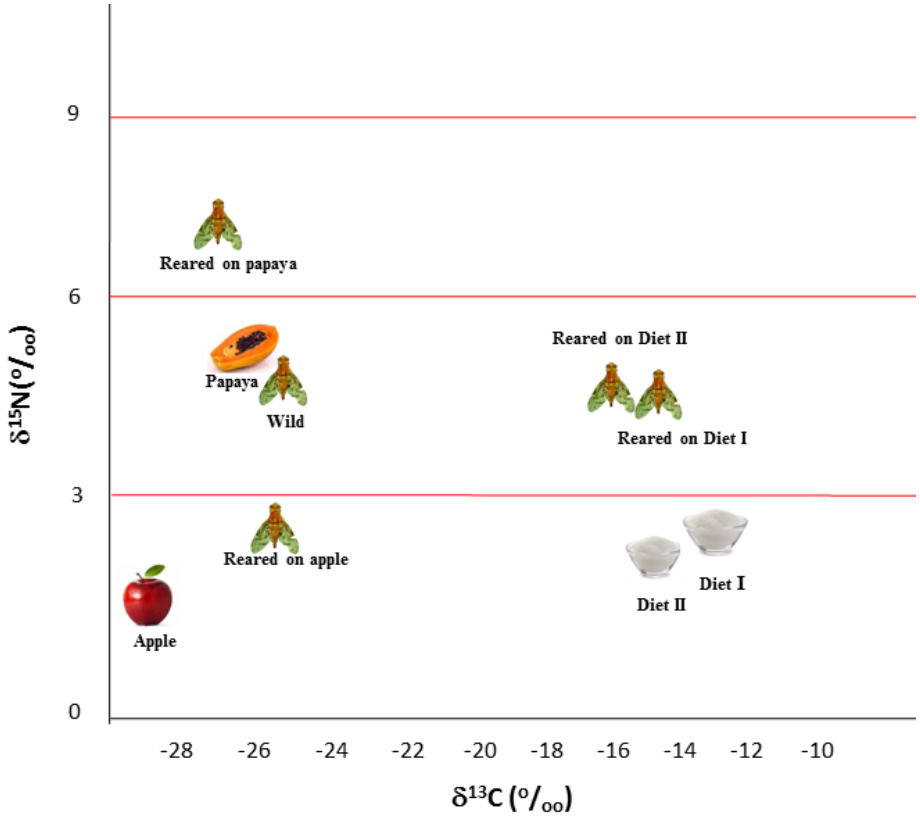
Table 5 - Mean isotopic composition of larval diets, fruits, laboratory and wild *Anastrepha fraterculus* flies

TREATMENT	**δ^{13}C ‰**	**δ^{15}N ‰**
Artificial Diet I	-12.6 ± 0.1a**	2.9 ± 0.1 d
Artificial Diet II	-14.3 ± 0.1b	2.4 ± 0.1 de
Papaya	-26 ± 0.1 e	5.5 ± 0.2 ab
Apple	-28.4 ± 0.1 g	2 ± 0.2 e
Flies reared on Diet I	-15.3 ± 0.1 c	4 ± 0.1 c
Flies reared on Diet II	-16 ± 0.1 d	4.1 ± 0.2 bc
Flies reared on papaya	-27 ± 0.2 f	6.8 ± 0.3 a
Flies reared on apple	-25.9 ± 0.1 e	2.9 ± 0.2 d
Wild flies* **	-25.7 ± 0.1 e	5.4 ± 0.3 abc
ANOVA*	$F_{8,63}$= 2303.30; C.V.= 0.53%; P< 10^{-3}	$F_{8,64}$= 48.60; C.V.= 10.76%; P< 10^{-3}

*ANOVA= analysis of variance; C.V. = coefficient of variation.
**Means (± SE) followed by the same letters in the columns do not differ significantly by the Tukey's test (α= 0.01).
***The mean values of the wild flies from the three different apple orchards were considered.

The dispersion of the mean values of $\delta^{13}C$ and $\delta^{15}N$ of larval diets, fruits, wild flies and *A. fraterculus* reared on laboratory are showed in Figure 19.

Figure 19 - Dispersion of the mean values of $\delta^{13}C$ and $\delta^{15}N$ of larval diets, fruits, wild flies and *Anastrepha fraterculus* reared in laboratory



4.3 Trophic Discrimination Factors of stable carbon and nitrogen isotopes of *Anastrepha fraterculus* flies

The TDFs (Δ) for $\delta^{13}C$ and $\delta^{15}N$ in *A. fraterculus* flies are indicated in Table 6. The $\Delta^{13}C$ ranged from -2.7 to 2.5‰, while $\Delta^{15}N$ varied from 0.9 to 2.1‰.

In comparison to the respective diets, the flies reared on larval Diets I and II had a decrease in $\delta^{13}C$ value of 2.7‰ and 1.7‰, respectively. A decrease of 1‰ in $\delta^{13}C$ value of the flies whose larvae were reared on papaya and an increase of 2.5‰ in $\delta^{13}C$ value of the flies whose larvae were reared on apple were observed.

In relation to the $\delta^{15}N$ values, the flies reared on larval Diets I and II had an increase of 2.1‰ and 1.8‰, respectively. Increases in $\delta^{15}N$ values of the flies whose larvae were reared on papaya (1.3‰) and apple (0.9‰) were also observed (Table 6).

Table 6 - Mean carbon (δ^{13}C) and nitrogen (δ^{15}N) isotope values of the larval diets and *Anastrepha fraterculus* flies, and their mean trophic discrimination factors

TREATMENT		δ^{13}C ‰	δ^{15}N ‰
Diet I	**Diet**	-12.6 ± 0.1	2.9 ± 0.1
	Fly	-15.3 ± 0.1	4 ± 0.1
	TDF*	**Δ^{13}C= -2.7**	**Δ^{15}N= 2.1**
Diet II	**Diet**	-14.3 ± 0.1	2.4 ± 0.1
	Fly	-16 ± 0.1	4.1 ± 0.2
	TDF	**Δ^{13}C= -1.7**	**Δ^{15}N= 1.8**
Apple	**Diet**	-28.4 ± 0.1	2 ± 0.2
	Fly	-25.9 ± 0.1	2.9 ± 0.2
	TDF	**Δ^{13}C= 2.5**	**Δ^{15}N= 0.9**
Papaya	**Diet**	-26 ± 0.1	5.5 ± 0.2
	Fly	-27 ± 0.2	6.8 ± 0.3
	TDF	**Δ^{13}C= -1**	**Δ^{15}N= 1.3**

*TDF= Trophic Discrimination Factor (Δ). All values of δ^{13}C and δ^{15}N are presented in ‰.

4.4 Influence of capture and preservative substances on the isotopic composition of *Anastrepha fraterculus* flies

The mean δ^{13}C and δ^{15}N values of CeraTrap™ were -25 ± 0.1‰ and 3.4 ± 0.2‰, respectively. For grape juice, the means were -26.3 ± 0.1‰ and 2.8 ± 0.1‰, respectively; while the values in absolute ethanol were -12.1 ± 0.5‰ for δ^{13}C and 1.7‰ for δ^{15}N. For wild flies and the ones from control treatments (Water I and II), the isotopic composition values were kept without noticeable variation until the 7th day of evaluation.

The immersion treatment in different substances had significant effects over the δ^{13}C values of the flies (F= 271.83, $P< 10^{-3}$) as the time of exposure (1 or 7 days) in traps (F= 17.49, $P< 10^{-3}$). However, no significant difference was found in the interaction of the factors time and treatments on the δ^{13}C values of the flies (F= 1.90, P= 0.051).

In the first day of evaluation, the δ^{13}C value of *A. fraterculus* reared on larval Diet I and immersed in water (control= Water I) differed significantly from the treatments in which flies were immersed in grape juice and in Ceratrap™ followed by ethanol for 7 days (CTET I). However, the flies from both artificial diets immersed in Ceratrap™ for a single day did not differ from the controls (Water I and II). The δ^{13}C value of flies reared on Diet II and kept in water (control=Water II) showed significant difference in comparison to the ET II, GJ II and CTET II treatments (Table 7).

In the second moment of evaluation (7 days), the δ^{13}C values of *A. fraterculus* reared on Diet I and left in water, absolute ethanol, Ceratrap™ and Ceratrap™ followed by ethanol for 7 days did not differ significantly. The δ^{13}C values of *A. fraterculus* reared on Diet II in water (Water II) differed only from the GJ II treatment. The flies immersed in grape juice for 7 days differed from both controls. Independently of the treatment, the δ^{13}C values of the flies reared on Diets I and II differed significantly from the values of wild flies in the two time periods considered (Table 7).

Table 7 - Mean $\delta^{13}C$ values of wild and laboratory *Anastrepha fraterculus* flies that were immersed in attractive and preservative substances for 1 day and 7 days

Treatment	$\delta^{13}C$ ‰ (1 day)	$\delta^{13}C$ ‰ (7 days)	ANOVA*
Wild flies**	-25.7 ± 0.1 A	-25.7 ± 0.1A	-
Water I	-15.4 ± 0.1 DE	-15.4 ± 0.1DE	-
Water II	-15.9 ± 0.1 CD	-15.9 ± 0.1CDE	-
ET I	-15 ± 0.2 Ea***	-15 ± 0.2 Ea	$F_{1,11}$= 0.03; C.V.= 3.51%; P= 0.856
ET II	-16.8 ± 0.1 Ba	-17.1 ± 0.1 BCDb	$F_{1,11}$= 6.53; C.V.= 1.44%; P= 0.028
CT I	-15.2 ± 0.2 DEa	-16.3 ± 0.3 CDEb	$F_{1,11}$= 11.14; C.V.= 3.88%; P= 0.007
CT II	-15.6 ± 0.2 CDEa	-16.8 ± 0.2 BCDb	$F_{1,11}$= 13.82; C.V.= 3.32%; P= 0.004
GJ I	-14.8 ± 0.1 BCa	-17.3 ± 0.8 BCa	$F_{1,11}$= 1.39; C.V.= 8.49%; P= 0.266
GJ II	-16.4 ± 0.1 Ba	-18.1 ± 0.9 Ba	$F_{1,11}$= 2.47; C.V.= 8.62%; P= 0.147
CTET I	-16.3 ± 0.3 BCa	-16.3 ± 0.1 CDEa	$F_{1,11}$= 0; C.V.= 2.99%; P= 0.963
CTET II	-16.8 ± 0.2 Ba	-17.6 ± 0.2 BCb	$F_{1,11}$= 6.2; C.V.= 3.07%; P= 0.03
ANOVA	$F_{10,68}$= 399.13; C.V.= 2.51%; P< 10^{-3}	$F_{10,68}$= 80.03; C.V.= 5.24%; P< 10^{-3}	

*ANOVA= analysis of variance; C.V.= coefficient of variation. I= Diet I; II= Diet II; ET= ethanol; CT= CeraTrap™; GJ= grape juice; CTET= CeraTrap™ + ethanol for seven days.

** The mean values of the wild flies from the three different apple orchards were considered.

***Means (± SE) followed by the same uppercase letter in the columns or lowercase letter in the lines do not differ significantly by the Tukey's test (α= 0.05).

After 1 day of immersion, most of the treatments resulted in slightly enrichment of $\delta^{13}C$ in comparison to the isotopic composition of the flies of the Water I control (ET I= +0.4‰; CT I= +0.2‰; GJ I= +0.6‰). In comparison to the Water II control, enrichment of $\delta^{13}C$ in the CT II (+0.3‰) and depletion in the GJ II (-0.5‰) and ET II (-0.9‰) were observed.

After 7 days of immersion, most of the treatments resulted in depletion of $\delta^{13}C$ comparing to the Water I control (CT I= -0.9‰; GJ I= -1.9‰), with enrichment observed only in ET I (+0.4‰). Comparing to the Water II control, depletion was observed in all treatments (ET II= -1.2‰; CT II= -0.9‰; GJ II= -1.9‰). The $\delta^{13}C$ mean value of the CTET I treatment did not differ from the mean value of the CT I treatment, and depletion of 0.8‰ was detected in $\delta^{13}C$ of CTET II compared to the CT II treatment. The immersion in grape juice for 7 days caused the most of $\delta^{13}C$ depletion (1.9‰ in flies reared on Diet I and 2.1‰ in flies reared on Diet II).

Considering the factor time within each of the treatments, significant differences were found for the ET II, CT I, CT II, and CTET II treatments ($P < 0.05$) (Table 7). On the 7th day of evaluation, the $\delta^{13}C$ values of the flies from these four treatments suffered depletions of 0.3‰, 1.1‰, 1.2‰, and 0.8‰, respectively.

The immersion treatment in different substances had significant effects over the $\delta^{15}N$ values of the flies (F= 11.93, $P < 10^{-3}$), whilst the effects of immersion time (1 or 7 days) in traps were not significant for most cases (F= 1.81, P= 0.18). However, the interaction of the factors time and treatments on the $\delta^{15}N$ value of the flies was significant (F= 4.96, $P< 10^{-3}$).

In the first day of evaluation, the $\delta^{15}N$ mean value of *A. fraterculus* reared on larval Diets I and II and immersed in water (controls= Water I and II) differed significantly only from the values of the treatments in which the flies were immersed in Ceratrap™ followed by ethanol for 7 days (CTET I and II). Considering the mean $\delta^{15}N$ values, the flies from the different treatments (except the controls) did not differ significantly from the wild flies (Table 8).

In the second moment of evaluation (7 days), the mean $\delta^{15}N$ mean values of flies from Water I control differed significantly from the treatments in which the flies were immersed in Ceratrap™ and Ceratrap™ followed by ethanol for 7 days. The mean $\delta^{15}N$ value of flies from Water II control differed significantly from values of the CT I, CT II and CTET I treatments. The mean $\delta^{15}N$ values only from flies of the water controls and GJ I treatment differed from the $\delta^{15}N$ values of wild flies (Table 8).

Table 8 - Mean δ^{15}N values of wild and laboratory *Anastrepha fraterculus* flies that were immersed in attractive and preservative substances

Treatment	δ^{15}N ‰ (1 day)	δ^{15}N ‰ (7 days)	ANOVA*
Wild flies**	5.4 ± 0.3 AB	5.4 ± 0.3 AB	-
Water I	4 ± 0.1 C	4 ± 0.1 C	-
Water II	4.1 ± 0.3 C	4.1 ± 0.3 C	-
ET I	4.4 ± 0.1 BCa***	4.5 ± 0.1 BCa	$F_{1,11}$= 0.28; C.V.= 4.77%; P= 0.608
ET II	4.6 ± 0.1 BCa	4.9 ± 0.1 BCb	$F_{1,11}$= 15.25; C.V.= 3.20%; P= 0.002
CT I	4.5 ± 0.2 BCa	6.3 ± 0.4 Ab	$F_{1,11}$= 18.35; C.V.= 13.11%; P= 0.001
CT II	4.4 ± 0.2 BCa	5.5 ± 0.2 ABb	$F_{1,11}$= 13.59; C.V.= 10.44%; P= 0.004
GJ I	4.8 ± 0.3 BCa	4.2 ± 0.4 Ca	$F_{1,11}$= 1.71; C.V.= 17.65%; P= 0.22
GJ II	4.6 ± 0.1 BCa	4.8 ± 0.1 BCa	$F_{1,11}$= 0.99; C.V.= 6.10%; P= 0.343
CTET I	6.3 ± 0.4 Aa	5.4 ± 0.1 ABb	$F_{1,11}$= 5.86; C.V.= 11.2%; P= 0.036
CTET II	5.5 ± 0.2 ABa	5 ± 0.2 BCa	$F_{1,11}$= 2.01; C.V.= 10.07%; P= 0.187
ANOVA	$F_{10,68}$= 8.54; C.V.= 11.93%; P< 10^{-3}	$F_{10,68}$= 8.35; C.V.= 12.01%; P< 10^{-3}	

*ANOVA= analysis of variance; C.V.= coefficient of variation. I= Diet I; II= Diet II; ET= ethanol; CT= CeraTrap™; GJ= grape juice; CTET= CeraTrap™ + ethanol for seven days.

** The mean values of the wild flies from the three different apple orchards were considered.

***Means (± SE) followed by the same uppercase letter in the columns or lowercase letter in the lines do not differ significantly by the Tukey's test (α= 0.05).

After 1 day, all the treatments resulted in enrichment of δ^{15}N in relation to the isotopic composition of the flies of the Water I control (ET I= +0.4‰; CT I= +0.5‰; GJ I= +0.8‰). In comparison to the Water II control, enrichment of δ^{15}N in all treatments (ET II= +0.5‰; CT II= +0.3‰; GJ II= +0.5‰) was observed.

Considering 7 days of immersion, enrichment of δ^{15}N was observed in flies from all treatments in comparison to the Water I (ET I= +0.5‰; CT I= +2.3‰; GJ I= +0.1‰) and Water II controls (ET II= +0.8‰; CT II= +1.4‰; GJ II= +0.7‰). For the flies of the CTET I treatment, it was observed a depletion of 0.9‰ compared to the CT I, while a depletion of 0.5‰ was detected in flies of CTET II compared to flies from the CT II treatment. The CeraTrap™ treatment for 7 days had the most significantly impact in δ^{15}N values, with enrichment of 2.3‰ in δ^{15}N of flies reared on larval Diet I and 1.4‰ in flies reared on larval Diet II.

Considering the factor time within each of the treatments, significant differences were found in the ET II, CT I, CT II, and CTET I treatments ($P < 0.05$) (Table 8). On the 7th day of evaluation, the δ^{15}N values of the flies from the treatments ET II, CT I and CT II were enriched in 0.3‰, 1.8‰ and 1.1‰, respectively, while the flies from the CTET I treatment suffered a depletion of 0.9‰.

4.5 Turnover rate of *Anastrepha fraterculus* flies

The isotopic compositions of laboratory-males did not differ from the compositions of females within each of the diet switching treatments at the first period (t= 0 day) of evaluation (P > 0.01) (Table 9).

Table 9 - Mean isotopic composition of males and females of *Anastrepha fraterculus* flies fed on different adult diets (after emergence) at the first period of evaluation (t= 0 day)

TREATMENT	SEX	δ^{13}C ‰	δ^{15}N ‰
Flies fed on papaya for 5 days	♂	-26.1 ± 0.2 a**	6.8 ± 0.4 a
	♀	-25.5 ± 0.3 a	6.8 ± 0.2 a
	ANOVA*	$F_{1,9}$= 2.84; C.V.= 2.31%; P= 0.13	$F_{1,9}$= 0; C.V.= 9.65%; P= 0.996
Flies I fed on sugar for 5 days	♂	-13.7 ± 0.1 a	4.2 ± 0.1 a
	♀	-13.3 ± 0.3 a	4.3 ± 0.1 a
	ANOVA	$F_{1,9}$= 1.03; C.V.= 3.85%; P= 0.339	$F_{1,9}$= 0.13; C.V.= 6.47%; P= 0.73

to be continued

			conclusion
Flies I fed on sugar and hydrolyzed yeast for 5 days	♂	-13.6 ± 0.2 a	3.6 ± 0.2 a
	♀	-13 ± 0.1 a	2.8 ± 0.2 a
	ANOVA	$F_{1,9}$= 6.39; C.V.= 2.82%; P= 0.035	$F_{1,9}$= 7.7; C.V.= 14.71%; P= 0.024
Flies I fed on Gainesville diet for 5 days	♂	-20.4 ± 0.3 a	4.2 ± 0.1 a
	♀	-20 ± 0.2 a	4.4 ± 0.1 a
Flies II fed on sugar for 5 days	♂	-14 ± 0.2 a	4.3 ± 0.2 a
	♀	-14.4 ± 0.3 a	4.5 ± 0.1 a
	ANOVA	$F_{1,9}$= 0.54; C.V.= 4.39%; P= 0.483	$F_{1,9}$= 1.87; C.V.= 7.35%; P= 0.209
Flies II fed on sugar and hydrolyzed yeast for 5 days	♂	-13.7 ± 0.4 a	3.5 ± 0.5 a
	♀	-13.6 ± 0.4 a	3.4 ± 0.4 a
	ANOVA	$F_{1,9}$= 0.03; C.V.= 6.18%; P= 0.858	$F_{1,9}$= 0.03; C.V.= 28.78%; P= 0.87
Flies II fed on Gainesville diet for 5 days	♂	-20.8 ± 0.3 a	4.5 ± 0.2 a
	♀	-20.4 ± 0.4 a	4.6 ± 0.1 a
	ANOVA	$F_{1,9}$= 0.5; C.V.= 3.8%; P= 0.498	$F_{1,9}$= 0.42; C.V.= 7.31%; P= 0.535

*ANOVA= analysis of variance; C.V.= coefficient of variation; I= flies whose larvae were reared on artificial Diet I; II= flies whose larvae were reared on artificial Diet II.
**Means (± SE) followed by the same letters in the columns do not differ significantly by the Student's *t*-test (α= 0.01).

The isotopic compositions of laboratory-males did not differ from the compositions of females within each of the diet switching treatments at the last period (t= 15 days) of evaluation (P > 0.01) (Table 10).

Table 10 - Mean isotopic composition of males and females of *Anastrepha fraterculus* flies fed on different adult diets at the last period of evaluation (t= 15 days)

TREATMENT	SEX	δ^{13}C ‰	δ^{15}N ‰
Flies from papaya switched for sugar for 15 days	♂	-20.9 ± 0.6 a	7.3 ± 0.1 a
	♀	-20.9 ± 0.6 a	7.5 ± 0.1 a
	ANOVA	$F_{1,9}$= 1.96; C.V.= 4.82%; P= 0.198	$F_{1,9}$= 0.71; C.V.= 3.95%; P= 0.423
Flies I from sugar switched for apple for 15 days	♂	-16.3 ± 0.1 a	4.5 ± 0.1 a
	♀	-16.8 ± 0.3 a	4.3 ± 0.1 a
	ANOVA	$F_{1,9}$= 2.82; C.V.= 2.84%; P= 0.131	$F_{1,9}$= 1.92; C.V.= 4.24%; P= 0.203
Flies I from sugar and hydrolyzed yeast switched for apple for 15 days	♂	-18.1 ± 0.4 a	3 ± 0.1 a
	♀	-19.6 ± 0.3 a	3.5 ± 0.2 a
	ANOVA	$F_{1,9}$= 7.83; C.V.= 4.41%; P= 0.023	$F_{1,9}$= 3.28; C.V.= 11.35%; P= 0.107
Flies I from Gainesville diet switched for apple for 15 days	♂	-19.3 ± 0.2 a	4.3 ± 0.1 a
	♀	-19.6 ± 0.5 a	4.4 ± 0.2 a
	ANOVA	$F_{1,9}$= 0.29; C.V.= 4.77%; P= 0.607	$F_{1,9}$= 0.11; C.V.= 8.54%; P= 0.743
Flies II from sugar switched for apple for 15 days	♂	-17.6 ± 0.2 a	4.4 ± 0.1 a
	♀	-18 ± 0.3 a	4.2 ± 0.1 a
	ANOVA	$F_{1,9}$= 1.43; C.V.= 3.04%; P= 0.266	$F_{1,9}$= 1.79; C.V.= 5.23%; P= 0.217
Flies II from sugar and hydrolyzed yeast switched for apple for 15 days	♂	-17.5 ± 0.1 a	3.3 ± 0.1 a
	♀	-17.7 ± 0.4 a	3.2 ± 0.3 a
	ANOVA	$F_{1,9}$= 0.35; C.V.= 4.22%; P= 0.571	$F_{1,9}$= 0.12; C.V.= 16.5%; P= 0.735
Flies II from Gainesville diet switched for apple for 15 days	♂	-20.7 ± 0.2 a	4.3 ± 0.1 a
	♀	-21.6 ± 0.2 a	3.9 ± 0.2 a
	ANOVA	$F_{1,9}$= 7.44; C.V.= 2.52%; P= 0.025	$F_{1,9}$= 1.99; C.V.= 8.69%; P= 0.196

*ANOVA= analysis of variance; C.V.= coefficient of variation; I= flies whose larvae were reared on artificial Diet I; II= flies whose larvae were reared on artificial Diet II.

**Means (± SE) followed by the same letters in the columns do not differ significantly by the Student's *t*-test (α= 0.01).

The mean $\delta^{13}C$ and $\delta^{15}N$ values from the larval diets, adult diets, and laboratory flies of 1-day-old and 5-d-old (flies fed on adult diet different than the previous larval diets) are expressed in Table 11. The mean $\delta^{13}C$ and $\delta^{15}N$ values of the adult diets (100% sugar, mixture of sugar plus hydrolyzed yeast, and the Gainesville diet) presented significant differences in comparison to the larval diets (papaya, Diets I and II). Only the $\delta^{13}C$ signals of sugar diet and the mixture of sugar plus hydrolyzed yeast did not differ significantly (Table 11). As the isotopic compositions of the sexes within each diet treatment did not differ significantly (Tables 9 and 10), the $\delta^{13}C$ and $\delta^{15}N$ values from the flies one and 5-days-old fed on different adult diets were compared among them (Table 11).

Table 11 - Mean isotopic composition of larval diets, adult diets and laboratory *Anastrepha fraterculus* flies (1 and 5 day old)

TREATMENT	$\delta^{13}C$ ‰	$\delta^{15}N$ ‰
Larval Diet I	-12.6 ± 0.1 ab***	2.9 ± 0.1 ef
Larval Diet II	-14.3 ± 0.1 cd	2.4 ± 0.1 f
Papaya fruit	-26 ± 0.1 hi	5.5 ± 0.2 b
Sugar diet	-11.9 ± 0.1 a	-
Sugar and hydrolyzed yeast diet	-12 ± 0.1 a	-1.1 ± 0.1 g
Gainesville Diet	-24 ± 0.1 g	4.5 ± 0.6 bc
Flies reared on Diet I	-15.3 ± 0.1 de	4 ± 0.1 cde
Flies I* fed on sugar	-13.5 ± 0.2 bc	4.2 ± 0.1 cd
Flies I fed on sugar and hydrolyzed yeast	-13.3 ± 0.1 bc	3.2 ± 0.2 def
Flies I fed on Gainesville diet	-20.1 ± 0.2 f	4.3 ± 0.1 bcd
Flies reared on Diet II	-16 ± 0.1 e	4.1 ± 0.2 cd
Flies II* fed on sugar	-14.2 ± 0.2 cd	4.4 ± 0.1 bcd
Flies II fed on sugar and hydrolyzed yeast	-13.6 ± 0.2 bc	3.5 ± 0.3 cdef
Flies II fed on Gainesville diet	-20.6 ± 0.2 f	4.5 ± 0.1 bc
Flies reared on papaya	-27 ± 0.2 i	6.8 ± 0.3 a
Flies from papaya fed on papaya	-25.8 ± 0.2 h	6.8 ± 0.2 a
ANOVA**	$F_{15,126}$= 785.4; C.V.= 3.01%; P< 10^{-3}	$F_{14,121}$= 51.47; C.V.= 6.87%; P< 10^{-3}

*I= flies whose larvae were reared on artificial Diet I and fed on different adult diets after emergence for 5 days; II= flies whose larvae were reared on artificial Diet II and reared on different adult diets after emergence for 5 days.

**ANOVA= analysis of variance; C.V.= coefficient of variation.

***Means (± SE) followed by the same letters in the columns do not differ significantly by the Tukey's test (α= 0.01).

The results indicated that the $\delta^{13}C$ values of the *A. fraterculus* adults reared in laboratory were significantly influenced by the diets in which they were fed. The $\delta^{13}C$ mean values of *A. fraterculus* reared on artificial larval Diet I and Diet II, and fed on adult diet of sugar and mixture of sugar plus hydrolyzed yeast reflected C_4-carbon sources, differing from the $\delta^{13}C$ mean values observed for flies reared on papaya (C_3-based diets) and the Gainesville diet (mixture of C_4-C_3-based diets). Considering the $\delta^{13}C$ values, the flies whose larvae were reared on Diets I and II and fed on sugar or the mixture of sugar plus hydrolyzed yeast after emergence for 5 days did not differ significantly. The flies whose larvae were reared on Diet I and fed on the adult diet of mixture of sugar plus hydrolyzed yeast presented the highest $\delta^{13}C$ value (-13.3‰), while the flies whose larvae were reared on papaya presented the lowest $\delta^{13}C$ value (-27‰) (Table 11).

After 5 days of emergence, the $\delta^{13}C$ values of the flies whose larvae were reared on Diet I were enriched in 1.8‰ when fed on sugar diet and 2‰ when fed on sugar plus hydrolyzed yeast, while the flies from the Gainesville diet suffered a depletion of 4.8‰. Flies whose larvae were reared on Diet II presented the same behavior in relation to $\delta^{13}C$ values: enrichment of 1.8‰ when fed on sugar diet and 2.4‰ when fed on sugar and hydrolyzed yeast diet, while the flies from the Gainesville diet suffered a depletion of 7‰. Flies whose larvae were reared on papaya and remained on the same diet after emergence suffered an enrichment of 1.2‰ in the $\delta^{13}C$ signal. Therefore, feeding the flies for 5 days with different adult diets was sufficient to induce significant changes in their original $\delta^{13}C$ signatures.

Considering the $\delta^{15}N$ values, no significant differences were found among flies from larval Diets I and II and fed on different adult diets, except for the flies reared on Diet I and fed on sugar plus hydrolyzed yeast which differed from the flies reared on Diet II that were fed on the Gainesville diet. Flies whose larvae were reared on papaya and that continued feeding on papaya presented the highest $\delta^{15}N$ mean value (6.8‰), and flies from larval Diet I and fed on sugar and hydrolyzed yeast diet presented the lowest value (3.2‰).

After 5 days of emergence, the $\delta^{15}N$ values of the flies whose larvae were reared on Diet I were enriched in 0.2-0.3 ‰ when fed on sugar diet and Gainesville diet, while the flies from sugar plus hydrolyzed yeast suffered a depletion of 0.8‰. Flies whose larvae were reared on Diet II presented the same behavior in relation to the $\delta^{15}N$ values: enrichment of 0.3‰ when fed on sugar diet and 0.4‰ when fed on the Gainesville diet, while the flies from the sugar and hydrolyzed yeast diet suffered a depletion of 0.6‰. Flies whose larvae were reared on papaya and remained on the same diet after emergence did not suffer nitrogen ratio fractionation and sustained the $\delta^{15}N$ signal.

Proportion of structural *C* was determined using a simple two-source mixing model equation from flies derived from papaya and sugar diet switching experiment, where δ^{13}Cfly= -20.5‰ (isotopic composition in the last period of evaluation after diet switching); $\Delta^{13}C_{papaya}$= -1‰; $\delta^{13}C_{papaya}$= -26‰ (first dietary source); $\delta^{13}C_{sugar}$= -11.9‰ (second dietary source):

$$[(\delta^{13}C_{fly} - \Delta^{13}C_{papaya}) - \delta^{13}C_{sugar}] / (\delta^{13}C_{papaya} - \delta^{13}C_{sugar}) =$$

$$= [\{-20.5 – (-1)\} – (-11.9)] / [-26 – (-11.9)] = 0.54$$

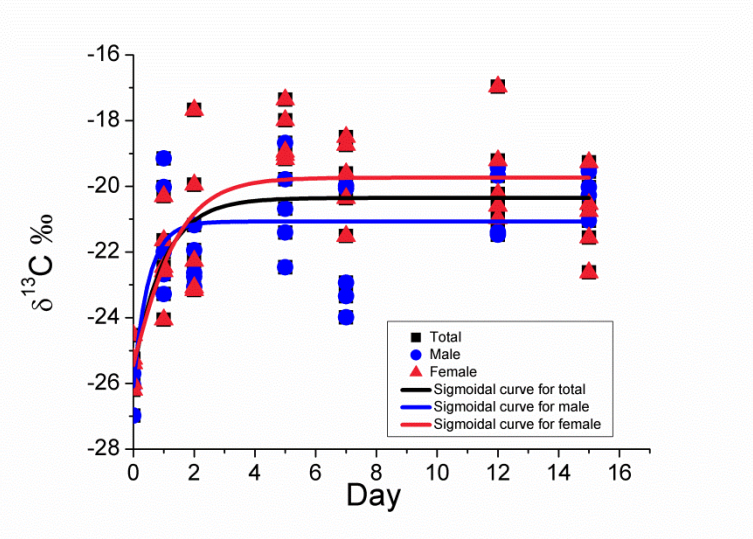
This equation showed that ≈ 50% of the carbon in the South American fruit fly is structural and 50% is metabolically active.

The isotopic turnover rates of δ^{13}C resultant from the diet switching experiment are expressed from Figure 20 to Figure 26, plotted in standard isotopical curves. The Boltzmann Sigmoidal Model explained the isotopical results with accuracy in most of treatments.

The mean δ^{13}C signals of flies reared on papaya diet (C_3-based diet) and switched to 100% refined sugar (C_4-based diet) moved significantly from -25.8 ± 0.2‰ in t= 0 to -20.5 ± 0.3‰ in t= 15 days (Figure 20). It took 7.8 days to reach the equilibrium level according to the Boltzmann Model (r^2= 0.58, δ^{13}C= [-16550.626+20.35481/ (1 + $e^{(t+7.8365)/0.97551)}$]-20.35481), which is the time needed to incorporate 50% of the shifted diet (*i.e.*, half-life ; HL= $t_{1/2}$).

Considering only the male flies, $t_{1/2}$ for δ^{13}C was 6.4 days to reach the equilibrium level (r^2= 0.75, δ^{13}C= [-27689.44+20.68146/(1 + $e^{(t+6.41451)/0.75)}$]- 20.68146), while for female flies the $t_{1/2}$ was 9.9 days (r^2= 0.98, δ^{13}C= [-8575+19.6855/(1 + $e^{(t+9.91342)/1.35671)}$]- 19.6855) (Figure 20).

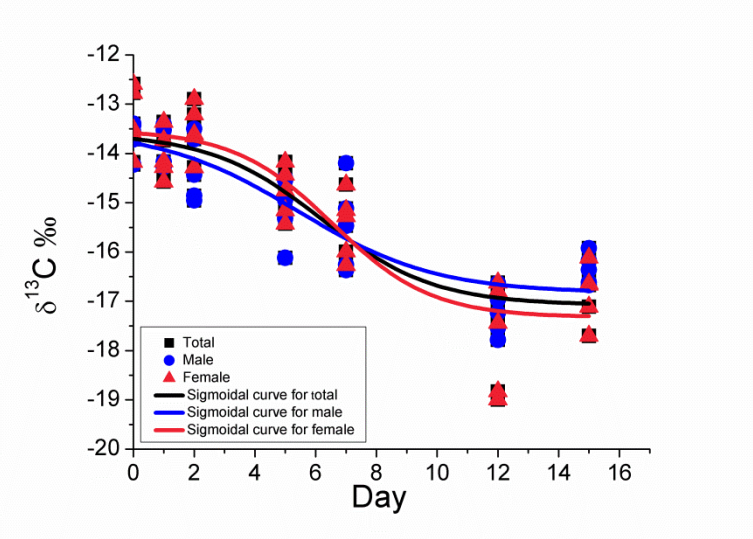
Figure 20 - $\delta^{13}C$ composition of *Anastrepha fraterculus* switched from papaya (C_3-based diet) to sugar (C_4-based diet) over time



Mean $\delta^{13}C$ signals of the flies whose larvae were reared on Diet I and then fed on 100% refined sugar diet for 5 days after emergence and switched to apple after this period (C_4- to C_3-based diet) was altered significantly from -13.5 ± 0.2‰ in t= 0 to -16.6 ± 0.2‰ in t= 15 days (Figure 21). The equilibrium level was reached in 6.2 days (r^2= 0.79, $\delta^{13}C$= [-13.58842+17.07525/(1 + $e^{(t-6.19838)/1.84459)}$]-17.07525).

Considering only the male flies, $t_{1/2}$ for $\delta^{13}C$ was 5.4 days (r^2= 0.78, $\delta^{13}C$= [-13.51513+16.83065/(1 + $e^{(t-5.42796)/2.25961)}$]-16.83065), while for female flies the $t_{1/2}$ was 6.5 days (r^2= 0.8, $\delta^{13}C$= [-13.52068+17.32455/(1 + $e^{(t-6.55231)/1.6259)}$]-17.32455) (Figure 21).

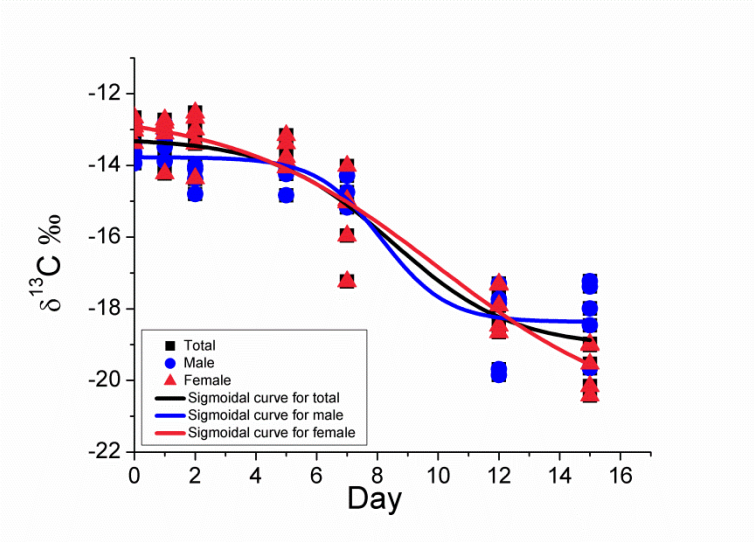
Figure 21 - $\delta^{13}C$ composition of *Anastrepha fraterculus* switched from sugar (C_4-based diet) to apple (C_3-based diet) over time



Mean $\delta^{13}C$ signals of the flies whose larvae were reared on Diet I and then fed on a mixture of sugar and hydrolyzed yeast diet for 5 days after emergence and switched to apple after this period (C_4- to C_3-based diet) was altered significantly from -13.3 ± 0.2‰ in t= 0 to -18.9 ± 0.3‰ in t= 15 days (Figure 22). The HL was 8.5 days (r^2= 0.89, $\delta^{13}C= [-13.24803+19.08428/(1 + e^{(t-8.516)/1.97944)}]-19.08428$).

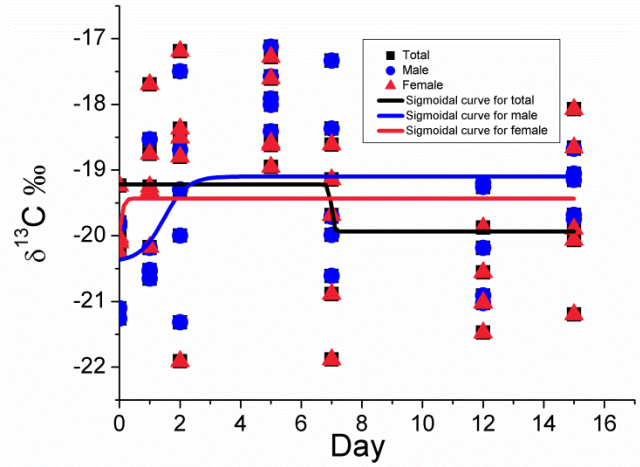
Considering only the male flies, $t_{1/2}$ for $\delta^{13}C$ was 8.1 days (r^2= 0.88, $\delta^{13}C= [-13.77195+18.37089/(1 + e^{(t-8.151)/1.07666)}]-18.37089$), while for female flies the $t_{1/2}$ was 10 days (r^2= 0.92, $\delta^{13}C= [-12.49748+21.12938/(1 + e^{(t-9.96187)/3.35349)}]-21.12938$) (Figure 22).

Figure 22 - $\delta^{13}C$ composition of *Anastrepha fraterculus* switched from sugar and hydrolyzed yeast at 3:1 rate (C_4-based diet) to apple (C_3-based diet) over time



Mean $\delta^{13}C$ signals of the flies whose larvae were reared on Diet I and then fed on the Gainesville diet (honey and agar) for 5 days after emergence and switched to apple after this period (C_3- to C_3-based diet) was altered from -20.15 ± 0.19‰ in t= 0 to -19.42 ± 0.28‰ in t= 15 days (Figure 23). The very low coefficients of determination obtained (r^2 < 10% in all cases) indicated inadequate fitness of the turnover rate curves for the Boltzmann Model. The overall $t_{1/2}$ was 7 days (r^2= 0.02, $\delta^{13}C= [-19.22028+19.93612/(1 + e^{(t-6.99039)/0.03858)}]-19.93612$), while the $t_{1/2}$ was 1.5 days for males (r^2= 0.1, $\delta^{13}C= [-20.39339+19.09716/(1 + e^{(t-1.46111)/0.3892)}]-19.09716$) and 0.05 days for female (r^2= 0, $\delta^{13}C= [-20.14007+19.43535/(1 + e^{(t-0.05)/0.057)}]-19.43535$).

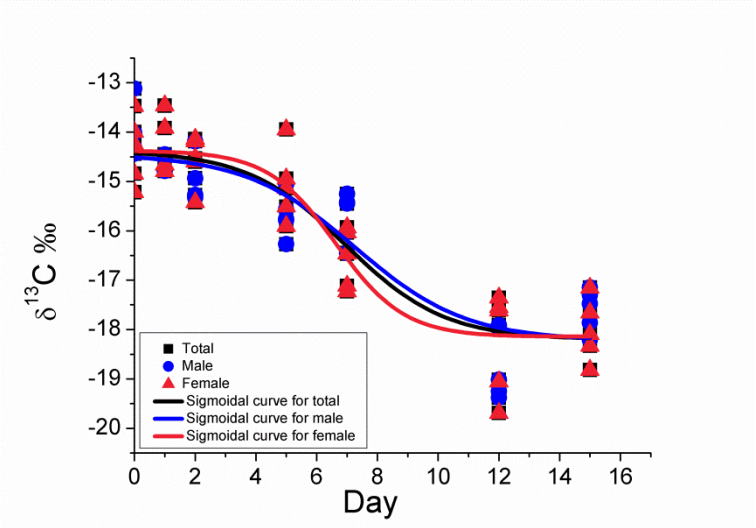
Figure 23 - $\delta^{13}C$ composition of *Anastrepha fraterculus* switched from the Gainesville diet (C_3-based diet) to apple (C_3-based diet) over time



Mean $\delta^{13}C$ signals of the flies whose larvae were reared on Diet II and then fed on 100 % refined sugar diet for 5 days after emergence and switched to apple after this period (C_4- to C_3-based diet) were altered significantly from -14.2 ± 0.2‰ in t= 0 to -17.8 ± 0.2‰ in t= 15 days (Figure 24). The HL was 6.9 days (r^2= 0.83, $\delta^{13}C$= [-14.37406+18.20355/ $(1 + e^{(t-6.95371)/1.63238)}]$-18.20355).

Considering only the male flies, it took 7.3 days to reach the equilibrium level (r^2= 0.82, $\delta^{13}C$= [-14.44525+18.22615/$(1 + e^{(t-7.26828)/1.81614)}]$-18.22615), while for female flies the $t_{1/2}$ was 6.6 days (r^2= 0.84, $\delta^{13}C$= [-14.37559+18.14747/$(1 + e^{(t-6.63597)/1.12)}]$-18.14747) (Figure 24).

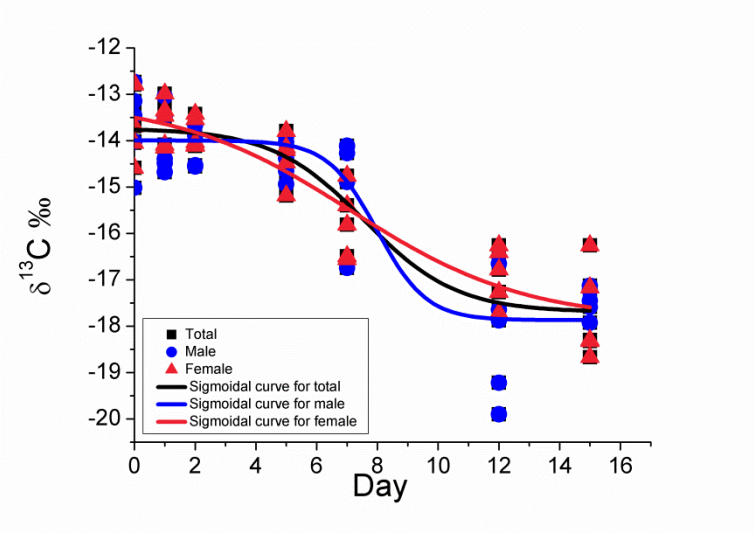
Figure 24 - $\delta^{13}C$ composition of *Anastrepha fraterculus* switched from sugar (C_4-based diet) to apple (C_3-based diet) over time



Mean $\delta^{13}C$ signals of the flies whose larvae were reared on Diet II and then fed on a mixture of sugar and hydrolyzed yeast for 5 days after emergence and switched to apple after this period (C_4- to C_3-based diet) were altered significantly from -13.6 ± 0.2‰ in t= 0 to -17.6 ± 0.2‰ in t= 15 days (Figure 25). Following the Boltzmann Model, $t_{1/2}$ was 7.6 days (r^2= 0.81, $\delta^{13}C= [-13.73718+17.69812/(1 + e^{(t-7.56907)/1.5159)}]-17.69812$).

Considering only the male flies, $t_{1/2}$ for $\delta^{13}C$ was 8 days (r^2= 0.8, $\delta^{13}C= [-13.99603+17.87171/(1 + e^{(t-7.98829)/0.81513)}]-17.87171$), while for female flies the $t_{1/2}$ was 7.1 days (r^2= 0.84, $\delta^{13}C= [-13.813+17.88069/(1 + e^{(t-7.13013)/2.87267)}]-17.13013$) (Figure 25).

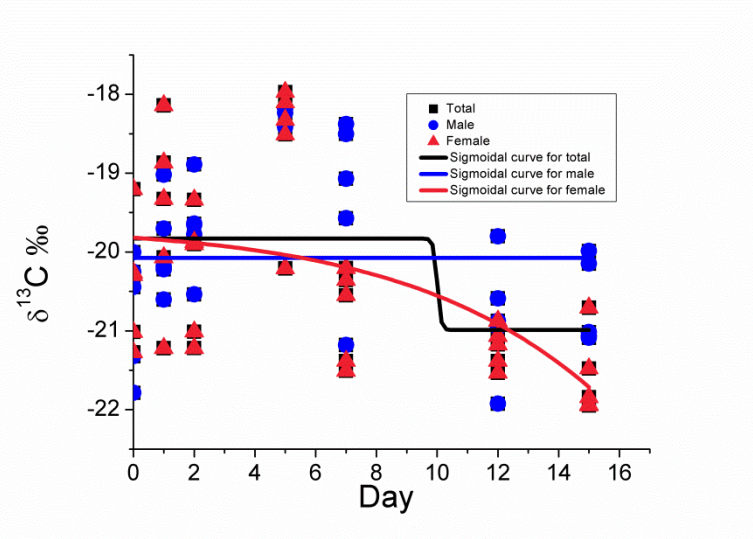
Figure 25 - $\delta^{13}C$ composition of *Anastrepha fraterculus* switched from a mixture of sugar and hydrolyzed yeast at 3:1 rate (C_4-based diet) to apple (C_3-based diet) over time



Mean $\delta^{13}C$ signals of the flies whose larvae were reared on Diet II and then fed on the Gainesville diet (honey and agar) for 5 days after emergence and switched to apple after this period (C_3- to C_3-based diet) were altered from -20.6 ± 0.2‰ in t= 0 to -21.1 ± 0.2‰ in t= 15 days (Figure 26). The very low coefficients of determination obtained (r^2 < 30% in all cases) indicated inadequate fitness of the turnover rate curves for the Boltzmann Model. The HL was estimated in 10 days according to the Boltzmann Model (r^2= 0.23, $\delta^{13}C= [-19.82942+20.98947/(1 + e^{(t-10.00795)/0.06063)}]-20.98947$).

Considering only the male flies, $t_{1/2}$ for $\delta^{13}C$ 28.4 days in Boltzmann Model (r^2= 0, $\delta^{13}C= [-19.86897+20.07623/(1 + e^{(t-28.40899)/-0.408)}]-20.07623$), while for female flies the $t_{1/2}$ was 27.3 days (r^2= 0.25, $\delta^{13}C= [-19.66395+40.34052/(1 + e^{(t-27.33456)/5.5895)}]-40.34052$) (Figure 26). The values of $t_{1/2}$ were estimated, although, with low accuracy in this treatment.

Figure 26 - $\delta^{13}C$ composition of *Anastrepha fraterculus* switched from the Gainesville diet (C_3-based diet) to apple (C_3-based diet) over time



The $\delta^{13}C$ values of the flies at the final evaluation after diet switching were enriched only in flies from larval Diet I and switched from the Gainesville diet to apple (Δ= 0.7 ‰), and flies from papaya to sugar (Δ= 5.2‰). Usually, shifting a C_4-diet to apple (C_3-diet) tended to decrease the $\delta^{13}C$ values. Considering the $\delta^{13}C$ values, the highest depletion was observed in flies from larval Diet I and switched from sugar plus hydrolyzed yeast to apple (Δ= -5.6‰).

The $\delta^{15}N$ values at the final evaluation of the flies after diet switching were depleted in flies whose larvae were reared on Diet II. Other treatments showed slightly enrichment in the $\delta^{15}N$ values, with the highest enrichment observed in flies switched from papaya to sugar diet (Δ= 0.6‰) and the highest depletion was observed in flies from larval Diet II and switched from the Gainesville diet to apple (Δ = -0.4‰).

After 15 days of diet switching, $\delta^{13}C$ and $\delta^{15}N$ signals of flies from all treatments were still statistically different ($P< 0.01$) from the wild flies' isotopic composition (Table 12). In comparison to the wild flies, the most distant $\delta^{13}C$ signal was observed in flies from larval Diet I and switched from sugar to apple, and the closest signal was detected in flies from larval Diet II and switched from the Gainesville diet to apple. In its turn, the most distant $\delta^{15}N$ signal was observed in flies from larval Diet II and switched from sugar plus hydrolyzed yeast to apple, and the closest signals were detected in flies from larval Diet I and switched from sugar to apple and switched from the Gainesville diet to apple.

Table 12 - Mean isotopic composition of laboratory *Anastrepha fraterculus* flies after diet switching for 15 days in comparison to wild *Anastrepha fraterculus* flies

TREATMENT	δ^{13}C ‰	δ^{15}N ‰
Wild flies*	-25.7 ± 0.1 a***	5.4 ± 0.3 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; C.V.= 3.97%; P< 10^{-3}	$F_{7,78}$= 88.11; C.V.= 9.78%; P< 10^{-3}

*The mean values of the wild flies from the three different apple orchards were considered.
**I= flies whose larvae were reared on artificial Diet I, reared on different adult diet after emergence for 5 days and switched to another source of diet for 15 days; II= flies whose larvae were reared on artificial Diet II, reared on different adult diet after emergence for 5 days and switched to another source of diet for 15 days. ANOVA= analysis of variance; C.V.= coefficient of variation.
***Means (± SE) followed by the same letters in the columns do not differ significantly by the Tukey's test (α= 0.01).

5 DISCUSSION

The present study allowed us to interpret the natural variation of isotopic composition of *Anastrepha fraterculus* in order to discriminate laboratory-flies from wild fertile ones, what is extremely relevant for monitoring purposes. Monitoring is an essential component for the implementation and evaluation of integrated pest management (IPM) and sterile insect technique (SIT) programs, and pre-release marking with fluorescent powder has been the most used technique to differentiate factory-reared flies from wild individuals (HAGLER; JACKSON, 2001). The stable isotope composition of organisms, caused by discrimination against the heavier isotopes during biological processes, can serve as an intrinsic marker of insects and be useful to discriminate both groups of flies (HOOD-NOWOTNY et al., 2009). Dietary inference of the $\delta^{13}C$ signals ($^{13}C/^{12}C$) is based on plant foliar $\delta^{13}C$ values due to the divergent photosynthetic pathways of C_3 (BASSHAM et al., 1953) and C_4-plants (HATCH; SLACK, 1968). In contrast, the dietary inferences of the $\delta^{15}N$ values ($^{15}N/^{14}N$) are more complex, varying with many factors (AMBROSE, 1991).

Despite the close proximity of the collection sites, the isotopic signatures of the wild flies from the three apple orchards differed significantly (Table 3). In Brazil, 116 hosts of *A. fraterculus* are known (SALLES, 1995; ZUCCHI, 2008; GARCIA; NORRBOM, 2011; NUNES et al., 2012) and the studies on the relationship between the fruit flies and their hosts focus on species of economic importance, mainly species of the families Myrtaceae and Rosaceae (ZUCCHI, 2007; 2008). In the Rio Grande do Sul State, besides apple orchards (*Malus domestica*), sources of infestation are observed in commercial peach orchards (*Prunus persica*), citrus (*Citrus* spp.) and grapevine (*Vitis vinifera*) (SALLES, 1995; ZUCCHI, 2008; ZART; BOTTON; FERNANDES, 2011; NUNES et al., 2012; SAVARIS et al., 2013). Cultivation of new fruits, such as blackberry (*Rubus* spp.), blueberry (*Vaccinium ashei*) and Brazilian cherry (*Eugenia uniflora*), may also become sources of infestation for *A. fraterculus* (BISOGNIN et al., 2013). In a recent study performed in this State, Marsaro Júnior (2014) observed the predominance of *A. fraterculus* infesting fruits of *Annona rugulosa*, *Acca sellowiana*, *Campomanesia guazumifolia*, *Campomanesia xanthocarpa*, *Diospyros kaki*, *Eugenia involucrata*, *Eugenia pyriformis*, *Eugenia uniflora*, *Psidium cattleianum*, *Psidium guajava* and *Prunus persica*. In Vacaria, larger infestations of this pest can be observed in *Campomanesia xanthocarpa*, *Eugenia involucrata* and *Feijoa sellowiana* (DE SOUZA; KOVALESKI, 2012). Future determinations of the isotopic signatures of these hosts' fruits and *in loco* studies using stable isotope analyses (SIA) could help to elucidate those

differences observed between wild flies from different sites and to improve our understanding of ecological issues such as the interaction between *A. fraterculus* and different hosts from within a region.

In the present study, the $\delta^{13}C$ signatures of *A. fraterculus* reared on larval Diet I and Diet II tended to reflect carbon sources of C_4-based diet (-15.3‰ and -16‰, respectively), differing to the mean $\delta^{13}C$ values observed for flies whose larvae were reared on fruits (apple and papaya) and from wild flies (Table 5). Comparisons showed similarity between the mean $\delta^{13}C$ values of wild flies (-25.7‰) and the flies reared on apple (-25.9‰) and papaya (-27‰), reflecting carbon sources of C_3-based diets. In comparison to the respective diets, the flies reared on larval Diets I and II had a decrease in $\delta^{13}C$ value of 2.7‰ and 1.7‰, respectively. A decrease of 1‰ in $\delta^{13}C$ value of the flies whose larvae were reared on papaya and an increase of 2.5‰ in $\delta^{13}C$ value of the flies whose larvae were reared on apple were also observed.

In contrast, the $\delta^{15}N$ showed values less conclusive when compared to $\delta^{13}C$ values, demonstrating no significant differences among some treatments, making not possible to distinguish the laboratory-flies from wild ones. In comparison to the respective diets, the flies reared on larval Diets I and II had an increase in $\delta^{15}N$ values of 2.1‰ and 1.8‰, respectively. An increase in $\delta^{15}N$ value of the flies whose larvae were reared on papaya (1.3‰) and apple (0.9‰) was also observed. According to Hood-Nowotny et al. (2009), the sources for the $\delta^{15}N$ of food resources are variable and depend on the nitrogen status of the different hosts. Flies reared on diets with known nitrogen compositions can produce protein that is enriched in ^{15}N relative to the food source, presenting higher $\delta^{15}N$ values in comparison to its diets (MINAGAWA; WADA, 1984). The fractionation of stable isotopes of nitrogen is more unpredictable than the stable isotopes of carbon, and, therefore, traceability could not depend solely on $\delta^{15}N$ values, unless the artificial diet be enriched with *N* sources, which could increase the costs.

In relation to the sex-associated variations in isotopic signals, the $\delta^{13}C$ and $\delta^{15}N$ values from the males did not differ from the values of the females, neither in flies reared on different larval diets nor in the wild flies collected in different sites (Tables 2 and 4). Hood-Nowotny et al. (2016) also found no significant differences in isotope signatures between males and females of the cactus moth. In contrast, Hood-Nowotny et al. (2009) observed differences in the $\delta^{13}C$ signals between males and females of *C. capitata* reared on artificial larval diets, but no differences were detected between sexes of wild medflies. These authors attributed the differences in the $\delta^{13}C$ values possibly to the fact that female medfly could route

an excess of assimilated carbon to lipid storage. As lipids are generally depleted in ^{13}C relative to bulk biomass (HAYES, 2001), female medflies could present a more negative $\delta^{13}C$.

In a recent study, Sato and Azuma (2016) detected significant differences in whole-adult weights and in $\delta^{13}C$ values between male and female abdomens of *Euthrix potatoria* (Lepidoptera: Lasiocampidae), and differences in $\delta^{15}N$ were observed in wing and thorax. These authors concluded that fat tissue can contribute to decreases in $\delta\ ^{13}C$ and changes in $\delta^{15}N$. Other possible explanations for the difference observed in isotopic composition within sexes of some insect's species could be the different behavior in nutrient use and in reproductive strategies, such as different ways for allocation and oxidation of dietary amino acids and fatty acids (LEVIN; MITRA; DAVIDOWITZ, 2016; LEVIN; MCCUE; DAVIDOWITZ, 2017a, 2017b). Although no difference was observed between the isotopic compositions between the sexes of *A. fraterculus* in this study, future analyses of other body structures instead of whole body could help to elucidate the existence of any differences of isotopic signatures between sexes.

Isotopic compositions of consumers can be predicted with certain accuracy if the isotopic signatures of the diet are well known (PETERSON; FRY, 1987; POST, 2002). However, the SIA is only useful for SIT in situations where two isotopically distinct dietary sources are available to discriminate the origin of the consumers (HOOD-NOWOTNY et al., 2009; 2016). More distinct, the better. Carbon isotopic ratio of consumers is usually similar to that of their diets and the isotopic ratio of nitrogen is commonly used to estimate trophic positions (DENIRO; EPSTEIN, 1978; 1981; MINAGAWA; WADA, 1984). Data interpretation of SIA depends also on trophic discrimination factor (TDF) and turnover rates. Ratios of stable isotopes can switch between diet and consumer due to metabolic processes and differential digestion or fractionation process during nutrients assimilation (TIESZEN et al., 1983; DUCATTI et al., 2002; HOLÁ et al., 2015). This process of isotope discrimination can be characterized by the TDF, consisting in an enrichment or depletion of the heavier isotope of the sample in chemical reactions (GALIMOV, 1985; EHLERING, 1991; MARTÍNEZ DEL RIO et al., 2009; HOLÁ et al., 2015). Variations in metabolic rate of different tissues of an animal can influence the TDF and turnover rate (TIESZEN et al., 1983; HOBSON; CLARK, 1992; VANDER ZANDEN et al., 2015), being also subject to diet quality, consumer's nutritional status, lipid extraction and other factors (MINAGAWA; WADA, 1984; VANDERKLIFT; PONSARD, 2003).

Either discrimination factor or the turnover rate of isotopes in insects tissues (whole-body in the case) are poorly know. When the *C* and *N* of the diet are incorporated into a particular consumer's tissue, a specific fractionation in the isotope ratio is expected and it needs to be evaluated for correct data interpretation (KENNED; KROUSE, 1990; CAUT; ANGULO; COURCHAMP, 2009). Until now, TDF and turnover rates of *A. fraterculus* flies had not been studied. According to the literature, the trophic fractionation of $\delta^{13}C$ is ~ 0‰ - 1‰ per trophic level (ROUNICK; WINTERBOURN, 1996; POST, 2002). In a study involving 22 terrestrial herbivorous arthropods feeding on 18 different host plants, Spence and Rosenheim (2005) observed a range from -3.5‰ to 1.9‰ for $\delta^{13}C$ enrichments across plants to herbivores (mean of -0.5 ± 0.3‰) and -0.2‰ to 6.6‰ for $\delta^{15}N$ (mean of 1.9 ± 0.8‰). In this study, the observed TDFs ranged from -2.7‰ to 2.5‰ for $\delta^{13}C$ and from 0.9‰ to 2.1‰ for $\delta^{15}N$.

Virtually, flies retain the isotopic signature that they obtain during the larval period (HOOD-NOWOTNY et al., 2009). Our results only deviate from the results of the study of Spence and Rosenheim (2005) for the difference between $\delta^{13}C$ values of the flies and apple ($\Delta^{13}C$= 2.5‰). In its turn, $\delta^{15}N$ signals presented the expected increase (MIN et al., 2006; SATO; AZUMA, 2016). Dietary protein values may influence the trophic enrichness by consumers, in which a positive relation between the dietary protein content and $\delta^{15}N$ values can be observed (KELLY; MARTÍNEZ DEL RIO, 2010). However, there is no certainty that food quality should be inversely related to consumer $\delta^{15}N$ enrichment (SPENCE; ROSENHEIM, 2005) and the results obtained here were lower than that described in other studies, in which the $\delta^{15}N$ values tended to increase with trophic levels by 3‰ to 5‰ (MINAGAWA; WADA, 1984).

In holometabolous insects, nutrient acquisition may derive either from larval or adult feeding and the ratio of stable isotopes can change during metamorphosis (MIN et al., 2006; SATO; AZUMA, 2016). In the present study, these factors were not evaluated in immature stages to understand the whole process of isotopic discrimination in all flies' biological cycle. According to Doi et al. (2007), the physiological condition of active growth phase or pre- or post-molting is important and may influence on stable isotope ratios in animals. These authors showed that *N* isotope enrichment of the arthropod larval stages was significantly lower than that of adults, supporting the importance of the metamorphosis effects in SIA (DOI et al., 2007). In another recent experiment with *Euthrix potatoria* (Lepidoptera: Lasiocampidae), Sato and Azuma (2016) also showed that $\delta^{15}N$ was higher in adults than in

larvae due to the excretion of *copious meconium* (abundant ^{14}N) and the $\delta^{13}C$ value decrease with insect metamorphosis.

Other concern for SIA was investigated in this study: the effects of attractive and preservative substances in the stable isotopes ratios of the samples (PONSARD; AMLOU, 1999; BARROW; BJORNDAL; REICH, 2008). Beside the discrimination of the laboratory-flies from wild ones by SIA, it is imperative to know if the attractive substances and preservation methodologies used to capture and preserve wild flies could affect their isotopic composition, since aggregation of isotopes from other sources could result in improperly interpretation of isotopic signatures (PONSARD; AMLOU, 1999). Preservation methods such as freezing and ethanol, and the storage duration significantly affected stable isotope values in cricket samples in the study of Jesus et al. (2015). These authors showed that freezing depleted and chemical preservatives caused enrichment of $\delta^{13}C$ and $\delta^{15}N$. Others chemical substances such as formalin and ethanol also affected the isotopic composition of preserved specimens in the study of Sarakinos et al. (2002). Similar results were demonstrated by Hogsden and McHugh (2017) with preservation methods (ethanol and freezing) that significantly affected $\delta^{13}C$ and $\delta^{15}N$ values of 10 common New Zealand stream invertebrates, showing enrichment of 1-2‰ in $\delta^{13}C$ from ethanol-preserved and lipid-extracted samples. According to Hogsden and McHugh (2017), samples subjected to organic solvents may have altered isotopic signals due to loss of dissolved lipids.

Our results corroborate previous studies, demonstrating that depending on attractive and preservative substance and the time that the captured flies remained immersed in the trap, the isotopic signatures ($\delta^{13}C$ and $\delta^{15}N$) of the laboratory-flies could be affected. The grape juice caused the most of $\delta^{13}C$ depletion, even after only one day of immersion. In its turn, the flies immersed for 1 day in CeraTrap™ had their $\delta^{13}C$ values not significantly altered, but CeraTrap™ was the substance that most impacted the $\delta^{15}N$ values of the immersed flies. For $\delta^{15}N$ values, all the substances tested caused enrichment, and the flies immersed in CeraTrap™ followed by absolute ethanol for 7 days presented depletion for both $\delta^{13}C$ and $\delta^{15}N$ values. Absolute ethanol had less impact in the isotopic composition of the laboratory-flies, causing both enrichment and depletion in $\delta^{13}C$ values, but only enrichment in the $\delta^{15}N$ values.

Despite the effects of some attractive and preservative substances in immersed flies' isotopic signals, it was still possible to distinguish flies reared on artificial diets from the wild ones (Tables 7 and 8). Therefore, the influence level of attractive and preservative substances

in the isotopic composition of laboratory-flies did not significantly compromised our isotope marking methodology for *A. fraterculus* with C_4-based larval diets.

Laboratory diet switching experiments were also carried out in order to investigate the turnover rate and to explain the contributions of the C_3 and C_4 sources in the flies' diet over time, being important to understand spatio-temporal relationships between host and consumers in the field (YOHANNES; ROTHHAUPT, 2017). Most of the turnover studies using diets with natural $\delta^{13}C$ variations focus on the exchange rate of tissue's carbon from the intake diets with different isotopic ratios, being important to determine when the insects switched between different diets and environments (MARTINEZ DEL RIO; CARLETON, 2012; MADEIRA et al., 2013).

Isotopic turnover occurs as a mainly result of tissue growth and catabolic turnover (FRY; ARNOLD, 1982), and the period over which consumer's isotopic composition will reflect the isotopic composition of its diet can depend on the isotopic turnover rate in the specific tissue analyzed and the time required to shift the isotope ratio from one source to another (TIESZEN et al., 1983; HOBSON; CLARK, 1992). Our equation of carbon proportion derived from papaya showed that about 50% of the carbon in the *A. fraterculus* is structural and 50% is metabolically active, agreeing with previously proportions reported for male mosquito *Anopheles arabiensis* (HOOD-NOWOTNY et al., 2006) and *Ceratitis capitata* (HOOD-NOWOTNY et al., 2009).

According to Phillips and Eldridge (2006), animals experience a change in their isotopic composition at some point in their life cycle because of movement to new environments and dietary shift with a different isotopic background. In the case of the factory-reared flies, when released in the field, the predominant diet switch to host fruits (C_3-based diets like fruit's juices and pulp), and also honeydews, extrafloral glandular secretions, bird faeces and leaf leachates (ALUJA; BIRKE, 1993; CELEDONIO; ALUJA; LIEDO, 1995; JÁCOME; ALUJA; LIEDO, 1999). In this context, evaluation of the turnover rate of *A. fraterculus* flies and the persistence of the carbon isotope tracer during adult life is crucial for monitoring purposes.

In this study, *A. fraterculus* were reared initially on a C_4-based diet and then switched to a C_3-based diet and otherwise (C_3 to C_4-based diet). The diet switching moment was chosen to reflect the usual release time and the long pre-copulatory period of the males (SEGURA et al., 2013), simulating the transition from an artificial diet to fruits (C_3-based diet). After diet switching, the isotopic composition of their tissues began to change to reflect that of their recent diet, as a result of metabolic turnover observed in $\delta^{13}C$ signals

(HOBSON; CLARK, 1992). The faster the metabolism, the faster the isotopic turnover (HOBSON; CLARK, 1992; OSTROM; COLUNGA-GARCIA; GAGE, 1997; PRASIFKA; HEINZ, 2004; PHILLIPS; ELDRIDGE, 2006). Our data indicated that, after diet switching, most of the treatments showed significant difference in $\delta^{13}C$ values over 15 days of evaluation with well fitted isotopical curves by the applied model ($r^2 > 60\%$). The exception occurred in the treatments in which adults were initially fed on the Gainesville diet (honey and agar). This could be explained because the honey source was from citrus (C_3-plant), presenting less variation in $\delta^{13}C$ signals between the diets switched. Therefore, adult diets with a C_3-honey (or other C_3-based diet) must be avoided for monitoring purposes, once $\delta^{13}C$ values are closer to the signals found in wild flies.

Studies have demonstrated isotopic composition changes after a diet shift following an exponential model (HOBSON; CLARK 1992; TIESZEN et al., 1983; PHILLIPS; ELDRIDGE, 2006), however, Boltzmann sigmoidal regression model was the model that better explained the behavior of the isotopic results, allowing us to detect the gradual replacement of food sources with distinct carbon composition (BALESDENT; MARIOTTI, 1996). In the simulated field scenario (diet switched from C_4- to C_3-based diet), mainly the $\delta^{13}C$ values of the flies tended toward to the signal found in wild flies over time, after diet switching, indicating significant dietary changes (Figures from 20 to 26). Considering artificial diets, greater variation in $\delta^{13}C$ values was observed in flies from larval Diet I and initially fed on an mixture of sugar and hydrolyzed yeast in adult stage and switched to apple (depletion of 5.6‰), and less variation was observed in adult fed on the Gainesville diet switched to apple (depletion of 0.5‰). Hood-Nowotny et al. (2009) observed an average depletion of 4‰ in $\delta^{13}C$ signal of *C. capitata* in a C_4-C_3-diet switching experiment, dropping from -19‰ to -23‰ in 12 days. Therefore, when laboratory-flies are released, they slowly lose their characteristic $\delta^{13}C$ signal. Dropping in isotopic signal reflects the excretion of C_4-carbon and the uptake of C_3-carbon (HOOD-NOWOTNY et al., 2009). The opposite isotopic dynamics (excretion of C_3-carbon and the uptake of C_4-carbon) occur in the 'switched scenario' from C_3- to C_4-based diet (switched from papaya to sugar), although not likely to be observed in the field, the C_3-reared flies were not as distinctly marked as C_4-reared flies.

Knowledge of the turnover rates and transition phase from laboratory to field provides a basis for rational monitoring plan in a SIT program. Turnover results were expressed in half-life (HL), defined as the time ($t_{1/2}$) required to the fly reach the balance of 50% with its diet (TIESZEN et al., 1983; VANDER ZANDEN et al., 2015).

The new equilibrium is a mixed isotopic signature consisting of carbon from a diet source which after some time is partially substituted with carbon from another diet. In this study, it was observed that the diet shift was rapidly detectable ($t_{1/2}$< 10 days in most treatments) (GRATTON; FORBES, 2006; MCINTYRE; FLECKER, 2006; MARTÍNEZ DEL RIO; ANDERSON-SPRECHER, 2008; MARTINEZ DEL RIO; CARLETON, 2012). Considering only treatments where an artificial diet was switched for fruits (C_4- to C_3-based diet) (field scenario), half-lives of carbon were relevant for the study objectives and ranged from 6.2 days to 8.5 days in flies, considering the Boltzmann Sigmoidal Model (r^2 > 70%). The faster turnover rates obtained were observed at the treatments where adult flies were switched from sugar to apple. On the other scenario (C_3- to C_4-based diet), the treatment from papaya to sugar was fitted with low accuracy with the model applied, obtaining a fast turnover rate (*i.e.*, 7.8 days). According to Jácome, Aluja and Liedo (1999), the fast turnover rate obtained in this scenario could be explained by the 'junk food' theory, which stated that the insect prefers a high-energy food source such as sucrose ('junk food') instead of a high quality but probably less readily digestible food (open fruit). The high energy of the poor quality 'junk food' could reach a faster satiation ('junk food syndrome') and the nutritional compensation could affect some biological parameters such as fecundity, as verified by these authors.

In relation to isotopic signals of the fly's sexes, the δ^{13}C and δ^{15}N values from the males did not differ from the values of the females in the diet switching treatments (Tables 9 and 10). Comparing the HLs between sexes, females showed lower rates of metabolic turnover in most treatments in comparison to males (*i.e.*, the treatments with papaya and flies reared on Diet I). Turnover of stable isotopes in animals are still poorly understood and are related to many factors such as metabolic activity and physiological process of specific tissue, body size and temperature (TIESZEN et al., 1983; SWEETING et al., 2007; BUCHHEISTER; LATOUR, 2010). Gratton and Forbes (2006) observed that females had faster changes in δ^{13}C signals in fat and reproductive tissues. Recently, similar results were observed by Madeira et al. (2013) studying the predator *Orius majusculus* (Hemiptera: Anthocoridae), detecting different turnover rates between sexes after diet switching up to 20 days.

Now, considering δ^{15}N values of the flies, signals showed slightly enrichment in most of the diet-switching treatments, with the highest enrichment observed in flies switched from papaya to sugar diet (Δ= 0.6‰) and the highest depletion was observed in flies from larval Diet II switched from the Gainesville diet to apple (Δ= -0.4‰). Turnover rates based on δ^{15}N

were not estimated in this study because the diet switching experiment was outlined in relation to the differences in carbon sources (C_3-C_4-based diets).

After 15 days of diet switching, $\delta^{13}C$ and $\delta^{15}N$ signals of flies from all treatments were still statistically different ($P< 0.01$) from the wild flies' signals. Despite of the differences observed in $\delta^{15}N$ signals of laboratory-flies from wild ones, stable isotopes of nitrogen were considered not suitable intrinsic markers as the stable isotopes of carbon, because of the similar $\delta^{15}N$ signals found between the origins of the flies. In contrast, C_4-based diets in the larval and adult stages were considered suitable tracers for *A. fraterculus*.

In this study, first experimentally derived TDF of stable carbon ($\Delta^{13}C$) and nitrogen isotopes ($\Delta^{15}N$), and turnover rates of $\delta^{13}C$ for *A. fraterculus* flies fed on different controlled diets were obtained, providing reliable dietary inferences based on isotopic data recorded over time. Despite of relative fast HL detected in diet switching experiment, all treatments continued to be statistically different from wild flies for $\delta^{13}C$ values, suggesting that it would be possible to distinguish with acceptable accuracy the laboratory-flies from wild ones for 15 days at least. The duration of the experiment was enough to reach the equilibrium condition in flies in most treatments.

The application of SIA in entomological investigations are still very scarce in Brazil and the results of the present study provides a powerful means to discriminate factory-flies reared on artificial diets against wild flies, becoming an alternative monitoring tool for a SIT program targeting *A. fraterculus*. Our study provided a foundation for future research using isotopic tracers to monitoring *A. fraterculus* in an integrated pest management program. Besides, future determinations of the isotopic signatures of hosts' fruits and *in loco* studies using SIA could help to elucidate the differences observed between wild flies from different sites and to improve our understanding of ecological issues such as the interaction between *A. fraterculus* and different hosts from within a region.

6 CONCLUSIONS

In view of the results of this study, it is possible to conclude that:

1) The *Anastrepha fraterculus* flies reared on artificial larval diets presented distinct isotopic signatures ($\delta^{13}C$ and $\delta^{15}N$) from flies whose larvae were reared on fruits and wild flies (C_3-based diets).
2) No significant differences in $\delta^{13}C$ and $\delta^{15}N$ signatures were found between males and females, neither from flies reared on different larval diets nor in the wild flies.
3) The estimated Trophic Discrimination Factors ranged from -2.7‰ to 2.5‰ for $\delta^{13}C$ and from 0.9‰ to 2.1‰ for $\delta^{15}N$.
4) The isotopic compositions ($\delta^{13}C$ and $\delta^{15}N$) of the flies whose larvae were reared on artificial diets can be affected by attractive and preservative substances and by the time that the captured flies remain immersed in the trap.
5) In general, for the most of the diets tested, switching the food source (C_4- to C_3-type and vice versa) led to a rapid change in $\delta^{13}C$ values of *Anastrepha fraterculus* flies ($t_{1/2}$< 10 days).
6) The laboratory-reared flies can unequivocally be distinguished from wild flies up to 15 days after the diet shift. Therefore, the Stable Isotope Analysis could be adopted as a complementary technique in the identification of laboratory-reared *Anastrepha fraterculus* flies that have been released in the field after 15 days at least.
7) The Stable Isotope Analysis showed potential to trace *A. fraterculus* flies reared under laboratory conditions, without restrictions imposed by traditional methods of capture and marking methods.

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