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Tese

Fruit fly management research, transcriptome analysis and first evidence of RNAi in *Anastrepha fraterculus* (Diptera: Tephritidae)

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Abstract

DIAS, Naymã Pinto. **Fruit fly management research, transcriptome analysis and first evidence of RNAi in *Anastrepha fraterculus* (Diptera: Tephritidae).** 2019. 144f. Tese (Doutorado) - Programa de Pós-Graduação em Fitossanidade. Universidade Federal de Pelotas, Pelotas.

Fruit fly species from Tephritidae family are key pests of many horticultural crops and affect a range of countries. The puncture for oviposition and the larval development cause direct damage to fruits. In South America, the South American fruit fly, *Anastrepha fraterculus* (Wiedemann, 1830) (Diptera: Tephritidae) is one of the most economically important species. The fruit flies' management has been carried out in different ways in the world. Though chemical control is the more frequent tactic used for fruit flies, the research information is very dispersed. The RNA interference (RNAi) technique is being exploited to pest control through of the silencing of genes which have vital functions in insects, but the efficiency depends on the sensitivity of the target insect to RNAi and of the presence of some essential genes. Thus, the aims this thesis were: a) systematically review the research about fruit fly's management, including monitoring and control tactics and b) obtain transcriptome to development stages of *A. fraterculus* to screening of RNAi machinery genes and target-genes and design an affordable method for RNAi assays in larval stages of *A. fraterculus*. In the first study, were used Web of Science Core Collection, Science Direct, PubMed, and Scopus to generate a database of publications that assess fruit fly management. For each publication, were collected the full reference and extracted information on the monitoring and control tactics, fruit fly species studied, methodological approaches used and the country where the study was performed. In the second study, was obtained the transcriptome of development stages of *A. fraterculus* and was screened for RNAi machinery genes, as well as the duplication or loss of genes and novel target genes to dsRNA delivery bioassays. The soaking assay in larvae was performed to evaluate the gene-silencing of *V-ATPase* and the *Dicer-2* and *Argonaute-2* expression after dsRNA delivery, and the stability of dsRNA with an in vitro incubation. Through of the systematic review were selected 533 research studies of fruit fly management, which were conducted in 41 countries for 43 fruit fly species. Forty six percent of the studies were from countries of North America and the biological control was the most commonly studied control tactic (29%), followed by chemical control (20%). In the RNAi-study, were identified 55 genes related to the RNAi machinery with duplication and loss for some genes and selected 143 different target-genes related to biological processes involved in post-embryonic growth/development and reproduction of *A. fraterculus*. Larvae

soaked in dsRNA solution showed a strong knockdown of V-ATPase after 48 h and the expression of *Dicer-2* and *Argonaute-2* responded with an increase to exposure of dsRNA. The data demonstrated the existence of a functional RNAi machinery and an easy robust physiological bioassay with the larval stages that can be used for screening of target-genes for RNAi-based control of fruit fly pests. This is the first study that provides evidence of a functional RNAi machinery in *A. fraterculus*.

Keywords: Systematic review, RNA-Seq, RNA interference, RNAi-functional, South American fruit fly

Resumo

DIAS, Naymã Pinto. **Pesquisa de manejo de moscas-das-frutas, análise do transcriptoma e primeira evidência de RNAi em *Anastrepha fraterculus* (Diptera: Tephritidae).** 2019. 144f. Tese (Doutorado) - Programa de Pós-Graduação em Fitossanidade. Universidade Federal de Pelotas, Pelotas.

As espécies de moscas-das-frutas da família Tephritidae são pragas-chave de muitas culturas hortícolas e afetam uma série de países. A punctura para oviposição causam e o desenvolvimento larval danos diretos aos frutos. Na América do Sul, a mosca-das-frutas sul-americana, *Anastrepha fraterculus* (Wiedemann, 1830) (Diptera: Tephritidae) é uma das espécies de maior importância econômica. O manejo de moscas-das-frutas tem sido realizado de diferentes maneiras no mundo. Embora o controle químico seja a tática mais frequente usada para moscas-das-frutas, as informações da pesquisa são muito dispersas. A técnica de RNA de interferência (RNAi) está sendo explorada para o controle de pragas através do silenciamento de genes que possuem funções vitais em insetos, mas a sua eficiência depende da sensibilidade do inseto-alvo ao RNAi e da presença de alguns genes essenciais. Assim, os objetivos desta tese foram: a) revisar sistematicamente a pesquisa sobre o manejo de moscas-das-frutas, incluindo monitoramento e táticas de controle and b) obter o transcriptoma dos estágios de desenvolvimento de *A. fraterculus* para o rastreamento de genes de maquinaria de RNAi e genes-alvo e projetar um método acessível para ensaios de RNAi em estágios larvais de *A. fraterculus*. No primeiro estudo, utilizou-se o Web of Science Core Collection, Science Direct, PubMed e Scopus para gerar um banco de dados de publicações que avaliaram o manejo de moscas-das-frutas. Para cada publicação foram coletadas as referências completas e extraídas as informações sobre monitoramento e táticas de controle, as espécies de moscas-das-frutas estudadas, as abordagens metodológicas utilizadas e o país onde o estudo foi realizado. No segundo estudo, foi obtido o transcriptoma dos estágios de desenvolvimento de *A. fraterculus* e foi rastreado para genes de maquinaria de RNAi, bem como a duplicação ou perda de genes e novos genes alvo para bioensaios de entrega de dsRNA. O ensaio de imersão em larvas foi realizado para avaliar o silenciamento gênico da V-ATPase e a expressão de Dicer-2 e Argonaute-2 após a entrega do dsRNA, e a estabilidade do dsRNA com uma incubação *in vitro*. Através da revisão sistemática foram selecionados 533 estudos de pesquisa de manejo de moscas-das-frutas, que foram realizados em 41 países para 43 espécies de moscas-das-frutas. Quarenta e seis por cento dos estudos eram de países da América do Norte e o controle biológico foi a tática de controle mais comumente estudada (29%), seguida pelo controle químico (20%). No estudo de

RNAi, foram identificados 55 genes relacionados à maquinaria de RNAi com duplicação e perda para alguns genes e foram selecionados 143 genes alvos diferentes relacionados a processos biológicos envolvidos no crescimento / desenvolvimento pós-embrionário e reprodução de *A. fraterculus*. Larvas embebidas em solução de dsRNA mostraram um forte knockdown de V-ATPase após 48 h e a expressão de Dicer-2 e Argonaute-2 respondeu com um aumento na exposição de dsRNA. Os dados demonstraram a existência de uma maquinaria funcional de RNAi e um bioensaio fisiológico robusto e fácil com os estágios larvais, que pode ser usado para o rastreamento de genes-alvo para o controle da mosca-das-frutas sul-americana baseado em RNAi. Este é o primeiro estudo que fornece evidências de uma maquinaria funcional de RNAi em *A. fraterculus*.

Palavras-chave: Revisão sistemática, RNA-Seq, RNA de interferência, RNAi-funcional, mosca-das-frutas Sul-americana

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Summary

General Introduction

Fruit fly species from Tephritidae family are key pests of horticultural crops affecting a range of countries, through massive costs from crop losses, loss of market access, regulatory compliance costs and pesticide usage (SUCKLING et al., 2016). The adaptation to various regions, high polyphagia, and rapid reproduction are key characteristics of these pests (SARWAR, 2015). The puncture for oviposition and the larval development cause direct damage to fruits, leading to production losses of 40% up to 80%, depending on locality, variety and season (ALUJA, 1994; KIBIRA et al., 2010).

The Tephritidae family has around 40 fruit fly species considered as pests, highlighting *Ceratitis capitata* (Wiedemann, 1824), *Bactrocera dorsalis* (Hendel, 1912), *Bactrocera oleae* (Rossi, 1790), *Bactrocera tryoni* (Froggatt, 1897), *Anastrepha ludens* (Loew, 1873) and *Anastrepha fraterculus* (Wiedemann, 1830). In South America, *A. fraterculus,* commonly known as South American fruit fly (SA fruit fly) is one of the most economically important species, causing losses around USD 2 billion per year (MALAVASI; ZUCCHI; SUGAYAMA, 2000; MACEDO et al., 2017).

The fruit flies' control has been carried out in different ways in the world. The main tactics include the male annihilation technique (MAT); whereby is deployed a large number of devices with para-pheromone male lures combined with a killing agent; the sterile insect technique (SIT); whereby a large number of sterile males are released to mate with conspecific females, biological control tactics, fruit destruction, and more frequently insecticide sprays or bait sprays, in which a food attractant is used to lure flies to an insecticide (SUCKLING et al., 2016). However, the chemical control of fruit flies is becoming increasingly difficult, as formerly effective but broad-spectrum neurotoxic and systemic-acting

insecticides have been banned from the market (BÖCKMANN et al., 2014). In addition, due to progressively more stringent restrictions on the use of insecticides and the increasing demand for healthy food around the world, new environmentally friendly techniques for fruit fly control are arising (NAVARRO-LLOPIS et al., 2011).

Crop protection scientists have allocated a great deal of intellectual energy into seeking of more refined strategies to reduce crop losses such as transgenic crops expressing *Bacillus thuringiensis* (Bt) toxins and more recently gene silencing through RNA interference (RNAi) (GATEHOUSE et al., 2011; CAGLIARI et al., 2018). The application of the RNAi technology did not go unnoticed in agriculture. Since the discovery of RNAi in the nematode *Caenorhabditis elegans* (Maupas, 1900) and its regulatory potentials, it has become evident that RNAi has immense potential in opening a new vista for crop protection (FIRE et al., 1998; BASNET; KAMBLE, 2018; CAGLIARI et al., 2018). Nevertheless, one of the biggest challenges for the RNAi technology is to make possible that target organisms' uptake intact and active molecules that will trigger an RNAi pathway (CAGLIARI et al., 2018).

RNAi is a natural process present in eukaryotic cells for gene regulation and antiviral defense. The RNAi mechanism targeting technology to pest control involves initially the introduction of double-stranded RNA (dsRNA) in the cell. These molecules are then recognized in the cytoplasm and are processed by the enzyme Dicer-2 (Dcr-2) into small interfering RNAs (siRNAs) of 18–24 pb (TIJSTERMAN; PLASTERK, 2004). The siRNAs are loaded by Dicer-2 and R2D2 into the RNA-induced silencing complex (RISC) containing the catalytic component Argonaute-2 (Ago-2). So, one strand of the siRNA is released and the remaining strand (the guide strand) binds to its complementary mRNA (mRNA) leading to either cleavage of the mRNA or inhibition of its translation (HAMMOND et al., 2000; ZOTTI et al., 2018). Conserved proteins Dicers and Argonautes are involved in various RNAi pathways, as well as several auxiliary proteins that also participate in these processes to stabilize RNAi-related multiprotein complexes and bring specificity to the reactions (BERNSTEIN et al., 2001).

The RNAi mechanism is being exploited to silence genes which have vital functions in insects by delivery of dsRNA molecules, leading to lethal phenotypes or reduction in growth or development (WHYARD et al., 2009; HUVENNE;

SMAGGHE 2010). The dsRNA delivery to insects can be performed through various methods, including injection, feeding, soaking or transgenic plants, and can include nanoparticles and transfection agents, as virus and bacteria (CHRISTIAENS et al., 2018). Despite, the technique efficiency depends on the sensitivity of the target insect to RNAi (HUVENNE; SMAGGHE, 2010; SCOTT et al., 2013; WYNANT et al., 2014). The RNAi systemic response (intercellular spreading of RNAi) varies among insects of different orders. For example, *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae) has a robust systemic RNAi, but a similar system has so far not been identified in *Drosophila melanogaster* (Meigen, 1930) (TOMOYASU et al., 2008). This last species has been used as a model for RNAi studies in Diptera, but because it is low sensibility to dsRNA uptake by cells, it is necessary to use transfection agents for delivery of dsRNA molecules (TANING et al., 2016; CHRISTIAENS et al., 2018). For *C. elegans*, SID-1 and SID-2 genes are involved in the uptake and spread of the RNAi across cells. Homologs of SID-1 are present in insects of different orders, such as Orthoptera, Hemiptera, Coleoptera, Lepidoptera, and Hymenoptera, but not are found in Diptera species (DOWLING et al., 2016).

Although *A. fraterculus* is one of the main pests of fruit crops in the South American continent, the lack of genetic information is still a barrier to understanding this species. Over the past few decades, a great deal of research has been conducted on the basic ecological and biological characteristics of SA fruit fly (CLADERA et al., 2014), but the genetic information of this species is still limited. Thus, the availability of transcriptomes of insects little studied allows the evaluation and identification of genes that can be potentially used for pest control using different biotechnological approaches (GARCIA et al., 2017). Recently, the head transcriptome of *A. fraterculus* was performed to identify fixed single nucleotide polymorphisms (SNPs) for two closely related species of the *fraterculus* group (REZENDE et al., 2016). Several studies in the context to develop RNAi to control of fruit flies species were conducted so far, but only for *Anastrepha suspensa* (Loew, 1862) (SCHETELIG et al., 2012), *B. dorsalis* (CHEN et al., 2008, 2011, LI et al., 2011, 2017; LIU et al., 2015; PENG et al., 2015; SHEN et al., 2013; SUGANYA et al., 2010, 2011; XIE et al., 2017; ZHENG et al., 2012, 2015), *Bactrocera minax* (Enderlein, 1920) (XIONG et al., 2016) and *C. capitata (*GABRIELI et al., 2016; MECCARIELLO et al., 2019).

Thus, considering that the information about the fruit fly control tactics is very dispersed and the adaptability of the approaches to control pests must be taken into consideration prior to the deployment of new technologies, the aims this thesis include: a) systematically review the research about fruit flies' management, including monitoring and control tactics and b) obtain transcriptome to development stages of *A. fraterculus* to screening of RNAi machinery genes and target-genes and design an affordable method for RNAi assays in larval stages of *A. fraterculus*.

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Fruit fly management research: A systematic review of monitoring and control tactics in the world

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Abstract

Several fruit fly species are invasive pests that damage quality fruits in horticultural crops and cause significant value losses. The management of fruit flies is challenging due to their biology, adaptation to various regions and wide range of hosts. We assessed the historical and current approaches of fruit fly management research worldwide, and we established the current knowledge of fruit flies by systematically reviewing research on monitoring and control tactics, according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. We performed a systematic review of research outputs from 1952 to 2017, by developing an a priori defined set of criteria for subsequent replication of the review process. This review showed 4,900 publications, of which 533 publications matched the criteria. The selected research studies were conducted in 41 countries for 43 fruit fly species of economic importance. Although 46% of the studies were from countries of North America, analysis of the control tactics and studied species showed a wide geographical distribution. Biological control was the most commonly studied control tactic (29%), followed by chemical control (20%), behavioral control, including SIT (18%), and quarantine treatments (17%). Studies on fruit flies continue to be published and provide useful knowledge in the areas of monitoring and control tactics. The limitations and prospects for fruit fly management were analyzed, and we highlight recommendations that will improve future studies.

Keywords: control methods; horticultural crops; integrated pest management; quarantine pests; Tephritidae

1. Introduction

Horticultural crops constitute a significant segment of the global agricultural production. The importance of horticulture can be substantiated by its high export value, high yield and returns per unit area (Ravichandra, 2014). Several species of fruit flies (Diptera: Tephritidae) are invasive pests of horticultural crops worldwide, due to their adaptation to various regions, high polyphagia and rapid reproduction (Sarwar, 2015).

Fruit flies cause direct damage to fruits and vegetables by the puncture for oviposition by the female and the larval development inside the fruit (Aluja, 1994). These pests cause direct damage to important export crops leading to losses of 40% up to 80%, depending on locality, variety and season (Kibira et al., 2010). The presence of these pest species limits access to international markets due to quarantine restrictions imposed by importing countries (Lanzavecchia et al., 2014).

Few insects have greater impact on the international marketing of horticultural produce than tephritid fruit flies (Hendrichs, 1996). Countries that harbor these important pests spend millions of dollars each year on control and have trade sanctions imposed by rigorous treatments of products prior to export. Such treatments are effective, but the volume of imported horticultural produce into countries free of these pests raises biosecurity concerns (Dhami et al., 2016). To remain free of fruit flies, New Zealand, for example, spends approximately NZ $1.4 million each year in post-border surveillance alone (Dhami et al., 2016). However, in fruit fly-free countries, such as Chile, this status contributes to the export of up to 50% of fruit production (Retamales and Sepúlveda, 2011).

The management of fruit flies is challenging because third-instar larvae leave decaying fruits and drop to the ground to pupate in the soil; consequently, both larvae and pupae in fruits and soils are protected from surface-applied insecticides (Heve et al., 2016). The control of fruit flies is becoming increasingly difficult in many countries, as formerly effective broad-spectrum and systemic-acting insecticides are removed from the market (Böckmann et al., 2014).

Due to progressively more stringent restrictions on the use of insecticides and the increasing demand for healthy food around the world, new environmentally friendly techniques for fruit fly control are arising (Navarro-Llopis et al., 2011). In addition, given the dependence of fruit fly distribution and abundance on climate variables, there are also concerns about the intensification of the climate changes that will facilitate the occurrence of more frequent outbreaks in horticultural regions (Sultana et al., 2017).

In fruit fly management, more than one tactic is frequently required. Each of these tactics has different advantages and disadvantages, and its adoption may or not be available for every case (Suckling et al., 2016). For example, the Male Annihilation Technique (MAT) is applied for some *Bactrocera* species but not for other species, owing to the lack of suitable lures. Additionally, the Sterile Insect Technique (SIT) requires the mass rearing of the target pest and geographic isolation of the release zone (Suckling et al., 2016).

Therefore, it is important to examine the current and historical approaches to fruit fly management research worldwide to enable researchers to evaluate the effectiveness of current research approaches and, if needed, develop more appropriate research protocols. The objective of the present study was to establish the current knowledge on fruit fly management by systematically

reviewing research on monitoring and control tactics used for local and regional management of these pests. There is one overarching research question in the present systematic review that can be divided into a series of more focused questions: How has monitoring and control tactics research been conducted worldwide?

- What fruit fly control tactics have been/were studied?
- What methodological approaches were examined?
- What fruit fly species were targeted?
- What localities were studied?
- What are the challenges for fruit fly management?
- What are the prospects for fruit fly management?
- What are the potential knowledge gaps in fruit fly research?

2. Material and methods

2.1 Database sources

We used Web of Science Core Collection, Science Direct, PubMed and Scopus to generate a database of publications that assess fruit fly monitoring and control tactics efforts in a pest management context. The search was limited to these four databases because they contained research articles that were available in full text and had undergone peer-review by scientists. The search was limited to publications written in English, Spanish and Portuguese published in journals from 1952-2017.

2.2 Search term

We divided fruit fly monitoring and control tactics into nine categories: 1) monitoring and detection; 2) control with natural product insecticides; 3) bioinsecticides; 4) chemical control; 5) biological control; 6) behavioral control; 7) mechanical control; 8) quarantine; and 9) genetic control. The description of each category is shown in Supplementary information (Supplementary Material 1). We used the following search terms: ("fruit fly" AND "monitoring"), ("fruit fly" AND "natural products"), ("fruit fly" AND "bait"), ("fruit fly" AND "insecticide control"), ("fruit fly" AND "biological control"), ("fruit fly" AND "sterile insect technique"), ("fruit fly" AND "male annihilation technique"), ("fruit fly" AND "mass-trapping"), ("fruit fly" AND "quarantine control"), ("fruit fly" AND "irradiation") and ("fruit fly" AND "RNAi").

2.3 Article screening

The search generated 4,900 records (last access date: 13 December 2017), and the results were imported into a library of Mendeley Reference Manager. We removed duplicates, reviews, conference proceedings, editorial material and book chapters. The remaining records were retrieved in full text and inspected in detail. For study inclusion, three criteria were determined: 1) studies with Tephritidae fruit fly species; 2) fruit fly monitoring studies (excluding faunal analysis studies), and 3) studies that used one or more tactics for fruit fly control and assessed effects on biology, physiology and/or behavior (excluding studies of rearing techniques).

We followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (Moher et al., 2009) (PRISMA statement and Checklist)

guidelines in including or excluding publications during screening stages. A checklist of the systematic review is shown in Supplementary Material 2.

2.4 Data extraction

For each publication, we collected the full reference and extracted information on the monitoring and control tactics used, the fruit fly species studied, the methodological approach used and the country where the study was performed. Studies that included the species *Bactrocera invadens* (Drew, Tsuruta and White), *Bactrocera papayae* (Drew and Hancock) and *Bactrocera philippinensis* (Drew and Hancock) were added to studies of *Bactrocera dorsalis* (Hendel), the current synonymized species (Hendrichs et al., 2015; Schutze et al., 2015). The methodological approaches used in each study were categorized into laboratory, semifield, field or combined approaches. The combined approach used more than one methodology (e.g., field and laboratory). For studies lacking information on where the research was performed, we used the location of the first author's institution.

2.5 Data analysis

The extracted data were subjected to descriptive analysis (proc UNIVARIATE) and principal component analysis (PCA) (proc PRINCOMP). The PCA was performed to examine any intrinsic variation in the fruit fly studies and whether any clustering was presented. The PCA was performed on the countries (41 variables), species (43 variables), methodological approaches (4 variables) and monitoring and control methods (9 variables) extracted from the studies dataset (Supplementary Material 3). The data for each category were

148 transformed by standardized Euclidean distance analysis prior to PCA, to
149 stabilize the variance of the measured variables and thus give the variables
150 approximately equal weight in the PCA. The statistical analysis was performed
151 using SAS (version 9.0, SAS Institute Inc., Cary, NC, USA) and the results were
152 fitted using Sigma Plot®.
153
154 **3. Results**
155 A total of 533 publications matched the criteria and were included in the
156 analysis. Full references for all publications and extracted data are presented in
157 Supplementary Material 3. Figure 1 shows the flow diagram for the systematic
158 review.
159
160 *3.1 Publication years*
161 A significant increase in the number of published studies has been
162 observed since the 1990s (Fig. 2). However, more than half of the studies were
163 published within the last seven years (n= 290 studies), demonstrating a rapid
164 expansion of fruit fly research since 2010.
165
166 *3.2 Geographical distribution of studies*
167 Research studies were conducted in 41 countries (Fig. 3). However, 46%
168 of the studies were from countries of North America (n = 248), mainly United
169 States of America (U.S.A.) (n = 173) and Mexico (n = 61). In Europe (n = 93),
170 most of the studies were from Spain (n = 39). Thirteen percent of the studies were
171 from Asia (n = 71), mainly in China (n = 31). Nine percent of the research studies
172 were from South America (n = 47), while seven percent of the studies were from

Oceania (n = 40), and six percent of the studies were from Africa (n = 35). In South America, 64% of the studies were from Brazil (n = 31), and in Oceania, 39 studies were from Australia, and one study was from French Polynesia. In Africa, the studies were distributed in eight countries, but most studies were from Kenya and Egypt (n = 9). Publications from the U.S.A. and Spain included monitoring studies and all control tactics searched (Supplementary Material 3). Publications from Central American countries did not meet the present study criteria. The principal control tactics and fruit fly species researched in countries with more than 10 studies found in the present review are shown in Table 1.

3.3 Fruit fly species

A total of 43 fruit fly species were found in the studies (Table 2). The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) was the fruit fly species most studied, with 180 studies, followed by *Anastrepha ludens* (Loew) with 73 studies and *B. dorsalis* with 72 studies. Considering only the fruit fly genus, 37% of the species studied belong to the genus *Ceratitis* or *Bactrocera,* followed by *Anastrepha* (32%), *Rhagoletis* (10%), *Zeugodacus* (8%), *Dacus* (1.1%) and *Toxotrypana* (0.2%).

3.4 Methodological approaches

A total of 343 studies used laboratory approaches, 12 studies used semifield approaches and 241 used field approaches. Fifty-seven studies used combined approaches.

197 *3.5 Monitoring and control tactics*

198 Biological control was the most commonly studied control tactic (29%, n =
199 154 studies), followed by chemical control (20%, n = 108), behavioral control,
200 including SIT (18%, n = 95), quarantine treatments (17%, n = 89), bioinsecticides
201 (13%, n = 71), control with natural product insecticides (7%, n = 36), mechanical
202 control (6%, n = 31) and genetic control (3%, n = 17). Monitoring was found in
203 14% (n= 75) of studies (Table 3).

204

205 *3.6 Statistical analysis*

206 The PCA separated the methodological approaches into three groups. The
207 first two principal components explained 97.40% (PCI= 82.16% and PCII=
208 15.24%) of the total variance (Fig. 4). For monitoring and control methods, the
209 first two principal components explained 81.54% (PCI= 69.73% and PCII=
210 11.84%) of the total variance, and the PCA showed four groups for this category
211 (Fig. 5). The association tendency for these findings is shown in the Discussion.
212 For countries and species, the PCA did not showed a separation among the
213 categories.

214

215 **4. Discussion**

216 *4.1 Publication years*

217 The first fruit fly study found in the present systematic review was
218 published in 1952 (Steiner, 1952) and refers to the use of bait spray for control of
219 *B. dorsalis* in Hawaii. Subsequently, the number of publications remained low
220 until the late 1980s. The construction of mass rearing of sterile insects and
221 parasitoids seems to have stimulated fruit fly research in the 1990s. The first fruit

fly production and sterilization facility (MOSCAMED) was installed in Mexico (Metapa de Domínguez, Chiapas) in 1979, shortly after the introduction of *C. capitata* in Guatemala and Mexico in 1976 and 1977, respectively (Enkerlin et al., 2017). In 1992, Mexico initiated a national fruit fly control program against native *Anastrepha* species, based on the application of selective toxic baits, the use of the SIT and the augmentative releases of parasitoids to develop fruit fly-free areas (Enkerlin et al., 2017; Montoya et al., 2007). For this purpose, the MOSCAFRUT mass rearing center was built in Metapa de Domínguez to produce sterile flies of two *Anastrepha* species [*A. ludens* and *Anastrepha obliqua* (Macquart)] and the endoparasitoid *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae) (Enkerlin et al., 2017). Additionally, other countries, such as Guatemala (Enkerlin et al., 2017), Argentina (Longo et al., 2000) and Chile (Enkerlin et al., 2003) also established fruit fly centers.

Numbers of publications started to increase substantially in the 1990s, which also coincides with the first eradication attempts of invasive fruit fly species. Because of the control programs established in the 1980s and 1990s, the eradication of important species, such as *C. capitata* in southern Mexico (1982) (Hendrichs et al., 1993) and northern Chile (1995) (Olalquiaga and Lobos, 1993) and *Zeugodacus* (Zeugodacus) *cucurbitae* (Coquillett) (formerly *Bactrocera* (Zeugodacus) *cucurbitae*) in southern Japan (1993) (Kuba et al., 1996), was achieved through SIT and bait spray (Suckling et al., 2016).

4.2 Geographical distribution of studies

Studies performed in Argentina, Brazil, and Kenya were mainly related to biological control with parasitoids. In South America, most studies were

conducted in Brazil using the parasitoid *D. longicaudata*. This parasitoid was introduced in Brazil in 1994, and the studies found in the present review are related to parasitism capacity (Alvarenga et al., 2005; Meirelles et al., 2016), dispersion patterns (Paranhos et al., 2007), competition with native parasitoids (Paranhos et al., 2013) and interaction with other control tactics (Alvarenga et al., 2012).

Fruit fly research with bait spray was performed in the U.S.A, Israel, and Mexico, the latter having conducted the same number of studies with bait spray as with biological control tactics. Italy, Spain, and Egypt also used biological tactics (except parasitoids) in research. Research with natural product insecticides was performed in India, and the mass-trapping tactic was performed in Greece. Australia had the most publications related to male annihilation technique (MAT).

Recent technological advances in fruit fly control research were reported in China (Ali et al., 2017; Chen et al., 2008, 2011; Shen et al., 2013; Peng et al., 2015; Suganya et al., 2010, 2011; Zheng et al., 2012; Xiong et al., 2016). These studies examined the use of RNA interference in species native to the Asian continent, such as *B. dorsalis*.

4.3 Fruit fly species

Most studies of fruit fly control included the Mediterranean fruit fly *C. capitata.* Its high polyphagia and ability to adapt to wide-ranging climate conditions better than most other species of tropical fruit flies contribute its rank of first among economically important fruit fly species (Liquido et al., 1990). The Mediterranean fruit fly infests over 300 species of cultivated and wild fruits,

vegetables and nuts, the widest known host range of any pest fruit fly (Leftwich et al., 2014). Although endemic to Africa, this species is currently present on all continents (Szyniszewska and Tatem, 2014). This species was included in the main control tactics found in the present review (Table 3).

The species *B. dorsalis* and *A. ludens* were among the species with the highest number of publications. Native to Asia, *B. dorsalis* was included in studies performed in 14 countries, and research focused on various tactics; only mechanical control was not found in this review. *B. dorsalis* was the main species researched in MAT and RNAi studies (Table 3). Studies of *A. ludens* were concentrated in Mexico and U.S.A. *Anastrepha ludens*, together with *C. capitata*, were the main species included in studies of quarantine treatments using irradiation.

The melon fruit fly, *Z. cucurbitae*, was highlighted among the most studied species of the Tephritidae family. This species was included in 67% of the control tactics analyzed. *Zeogodacus cucurbitae* is a widely distributed and harmful pest, mainly affecting cucurbitaceous crops (Shishir et al., 2015). The damage caused by the larvae feeding on the fruit can reach 90% of the crop yield (Ryckewaert et al., 2010).

4.4 Methodological approaches

Laboratory studies were more common, followed by field studies, performed in 33 and 36 countries, respectively. Studies that included semifield assays were performed in six countries. Additionally, 10% of the studies used more than one approach. In the PCA, laboratory and field approaches showed separation of the semifield and combined approaches (Fig. 4).

297 The fruit fly management studies found in the present review that were
298 conducted in the laboratory were important to determine the essential aspects of
299 control tactics, and included studies on doses and efficacy of phytosanitary
300 treatments (Sharp and Polavarapu, 1999; Hallman and Thomas, 2010), effects
301 on the biological parameters (Juan-Blasco et al., 2013; Rempoulakis et al., 2015),
302 selection of attractants for traps (Katsoyannos et al., 2000), performance and
303 potential of biological control agents (Bokonon-Ganta et al., 2005). However, field
304 studies were critical to evaluate the response of fruit flies to control tactics under
305 uncontrolled conditions (Aluja et al., 2009; Ali et al., 2016).
306
307 *4.5 Fruit fly monitoring*
308 Prevention is one of the most effective strategies for fruit fly management
309 (Aluja, 1999). The monitoring of fruit flies is crucial to determine the population
310 dynamics, compare infestation levels between different sites and evaluate the
311 effectiveness of a control tactic (Eliopoulos, 2007; Enkerlin et al., 1996). However,
312 only 14% of the studies presented results for monitoring fruit flies (14%). Most
313 monitoring studies were performed in Mexico and could be assigned to a single
314 category, monitoring with traps (Lasa et al., 2014; Malo et al., 2012). These
315 studies were mainly conducted in *C. capitata* (Table 3).
316 The present review also found studies using polymerase chain reaction
317 (PCR) for detecting the DNA of fruit flies and biological control agents (Dhami et
318 al., 2016; Mathé-Hubert et al., 2013; Rejili et al., 2016), and this tool has been
319 widely used for various pest groups. PCR-based assays provide a highly
320 sensitive, rapid and accurate technique to detect pests in various biosecurity and

ecological applications (Dhami et al., 2016). This tool was used for five fruit fly species.

The correct identification of insects is a basic premise for pest management. However, the identification of fruit flies is manually performed by few specialists through morphological analysis. Brazilian researchers implemented a classifier multimodal fusion approach, using two types of images (wings and aculei), generating promising results for the identification of *Anastrepha* species. The results showed more than 98% classification accuracy, which is remarkable, despite the technical problems (Faria et al., 2014).

The risk of not detecting early or not responding immediately to the detections of exotic fruit flies can be illustrated by cases where eradication failed, such as *B. carambolae* in Suriname. This example illustrates the lag phase from initial detection in infested fruits in 1975 to species identification in 1986 and confirmation that the specimen had come from South-east Asia four years later (Suckling et al., 2016). Forecasting models of pests, such as CLIMEX (Sridhar et al., 2017), and VARMAX (Chuang et al., 2014), can enable the monitoring of fruit flies to make preemptive and effective pest management decisions prior to the occurrence of real problems (Chuang et al., 2014).

Fruit fly monitoring with traps is currently performed with manual weekly counting. However, this method is costly and time-consuming, resulting in a suboptimal spraying frequency (overdue or unnecessary spraying) (Goldshtein et al., 2017). Recently, an online method was proposed for the detection of infested fruits in orchards. An algorithm has been developed to identify spots generated in hyperspectral images of mangoes infested with fruit fly larvae. The algorithm incorporates background removal, application of a Gaussian blur, thresholding,

and particle count analysis to identify the locations of infestations. This study demonstrates the feasibility of hyperspectral imaging for fruit fly detection while highlighting the need for technology with improved resolution and signal to noise ratio to enable the detection of single larvae (Haff et al., 2013).

In this context, efforts to develop automatic insect traps have been intensified and accelerated. A recent study showed the first automatic trap for *C. capitata* monitoring, with optical sensors for detecting and counting dead or stunted flies (Goldshtein et al., 2017). The automatic and conventional traps had similar trapping efficiencies under field conditions. The accuracy of the automatic trap counts ranged between 88% and 100% and the overestimate rate was three flies, mostly due to ants and rain. However, the authors emphasized that any change in trap shape and components may have adverse effects on pheromone release or the attractiveness of traps to the insect, which in turn alters the efficiency of the traps (Epsky et al., 1999; Kehat et al., 1994). Moreover, unlike imaging systems, in automatic traps, the insects are not identified; therefore, the lure must be specific to the target pest to avoid erroneous counts caused by non-target species.

4.6 Fruit fly control tactics

Although various control tactics are available for fruit fly management, the present results demonstrate that most of the published studies focused on biological control, followed by chemical, behavioral control (including SIT) and quarantine treatments.

370 4.6.1 Biological control

371 Studies of biological control were performed for 29 fruit fly species in 26
372 countries, highlighting the use of parasitoids (Supplementary Material 3).
373 Parasitoids of the Braconidae family were the main natural enemies of fruit flies
374 studied and included *D. longicaudata* and *Psyttalia* spp. [*Psyttalia concolor,*
375 *Psyttalia fletcheri, Psyttalia lounsburyi, Psyttalia ponerophaga* and *Psyttalia*
376 *humilis* (Silvestri)] (Bon et al., 2016; Miranda et al., 2008; Mohamed et al., 2008;
377 Montoya et al., 2016; Ovruski et al., 2007; Ovruski and Schliserman, 2012;
378 Spinner et al., 2011). The egg parasitoid, *Fopius arisanus* (Sonan)
379 (Hymenoptera: Braconidae), and the pupal parasitoids *Coptera haywardi*
380 Loiácono (Hymenoptera: Diapriidae) and *Aganaspis daci* (Weld) (Hymenoptera:
381 Figitidae) are considered as alternative species to fruit fly biological control with
382 larval parasitoids (Ali et al., 2014, 2016; Appiah et al., 2014; Cancino et al., 2014;
383 Guillén et al., 2002; Zamek et al., 2012).

384 Research in Latin America has included biological control with native
385 parasitoids of the Neotropical region. These studies mainly include assays of
386 interspecific competition, such as the species *Doryctobracon areolatus*
387 (Szepligeti), *D. crawfordi* (Viereck) and *Utetes anastrephae* (Viereck) (Aluja et al.,
388 2013; Miranda et al., 2015; Paranhos et al., 2013). Some studies included the
389 evaluation of the efficacy of augmentative releases of parasitoids using *D.*
390 *longicaudata* and *D. tryony* (Cameron).

391 The control with entomopathogenic fungi has shown interesting results.
392 For *Rhagoletis cerasi* (L.), the control with *Beauveria bassiana* (Balsamo)
393 Vuillemin, *Isaria fumosorosea* (Wize) and *Metarhizium anisopliae* Sorokin caused
394 90-100% mortality and had the strongest influence on fecundity in laboratory

(Daniel and Wyss, 2009). In field tests, the infestation of this species in cherry trees was reduced by 65% using foliar applications of *Beauveria bassiana* (Daniel and Wyss, 2010). Promising results were obtained for the control of *C. capitata* (Castillo et al., 2000; Toledo et al., 2017; Yousef et al., 2014), *Bactrocera oleae* (Gmelin) (Yousef et al., 2013) and *Z. cucurbitae* (Sookar et al., 2014) using entomophatogenic fungi species.

Recently, the pathogenicity of three formulations of *B. bassiana* and their applications in autoinoculation devices and by means of sterile males as vectors, was tested for the control of *C. capitata* in coffee-producing areas of Guatemala (Toledo et al., 2017). The release of sterile male vectors was more effective than the autoinoculation devices in terms of transmitting the conidia to the wild population, but the total population reduction was over 90% for both treatments. The median survival time between the sterile male vectors and the autoinoculation devices was similar, which is considered suitable for strategies, as this enables the vector to live for enough time to disseminate the inoculum among wild individuals (Toledo et al., 2007; Flores et al., 2013). Higher virulence would reduce the chances for horizontal transmission for the control of pest populations in specific patches or hot spots where additional control tactic is required. However, the inoculation of sterile males is still controversial because of its possible effects on quality control parameters and higher cost of this approach, giving rise to a new proposal of integrating the SIT with the use of autoinoculation devices, where a synergistic effect may occur (Montoya, Personal communication).

Entomopathogenic nematodes, such as *Heterorhabditis* spp. (Rhabditida: Heterorhabditidae) and *Steinernema* spp. (Rhabditida: Steinernematidae), were

used for control of larvae and pupae of various fruit fly species. The present review found studies with *A. fraterculus* (Barbosa-Negrisoli et al., 2009; Foelkel et al., 2017)*, A. ludens* (Lezama-Gutiérrez et al., 2006)*, A. suspensa* (Heve et al., 2016)*, B. oleae* (Torrini et al., 2017)*, B. tryoni* (Langford et al., 2014), *C. capitata* (Malan and Manrakhan, 2009), *Ceratitis rosa* Karsh (Malan and Manrakhan, 2009), *Dacus ciliatus* Loew (Kamali et al., 2013) and *R. cerasi* (Kepenecki et al., 2015). The results were variable for each fruit fly species, with mortalities between 14-96%. Some studies suggest that soil type is a critical factor that should be considered when selecting the nematode species and planning fruit fly biological control strategies (Lezama-Gutiérrez et al., 2006).

4.6.2 Chemical control

Chemical control studies included the use of baits (spray or station) and insecticide pulverization. The bait spray consists of an attractant mixed with an insecticide (Roessler, 1989). Bait stations are defined as discrete containers of attractants and toxins that attract the pest to the insecticide (Heath et al., 2009). In this case, the toxin can kill, sterilize or infect the target insect (Navarro-LIopis et al., 2010). The application of bait sprays with insecticide should be considered a lure-and-kill method but using higher amounts of insecticide (Navarro-Llopis et al., 2012).

Chemical control was used against 21 fruit fly species in 20 countries. The bait spray and station were the main tactics included in all chemical control studies, except in Spain, that included mainly the insecticide pulverization tactic (Supplementary Material 3). The efficacy of insecticides (such as imidacloprid, chlorpyrifos, thiacloprid, malathion, zeta-cypermethrin and fipronil) was also

studied with *A. fraterculus*, *A. ludens*, *A. suspensa*, *Z. cucurbitae*, *B. dorsalis*, *C. capitata* and *Rhagoletis indifferens* Curran (Conway and Forrester, 2011; Harter et al., 2015; Juan-Blasco et al., 2013; Liburd et al., 2004; Yee and Alston, 2006, 2012).

In a recent study, bait spray was used in a perimeter control approach in non-crop vegetation for the management of *Zeugodacus cucumis* (French) in Australia. Control in *Z. cucumis* in vegetable crops presents different challenges, since flies use these crops only for oviposition, spending most of their time in shelters outside the growing area (Senior et al., 2015). Thus, the application of bait spray to plants used as shelter is an important tool for the control of fruit flies (Senior et al., 2015). A similar study was performed for *B. tryoni* and *Z. cucumis* through the application of bait in eight plant species and applied at three heights. When protein bait was applied at different heights, *B. tryoni* primarily responded to bait placed in the upper part of the plants, whereas *Z. cucumis* preferred bait placed lower on the plants. These results have implications for the optimal placement of protein bait for control of fruit flies in vegetable crops and suggest that the two species exhibit different foraging behaviors (Senior et al., 2017).

Insecticide resistance studies with fruit flies have focused mainly on the following species: *C. capitata* (Arouri et al., 2015; Magaña et al., 2007), *B. oleae* (Kakani et al., 2010), *B. dorsalis* (Zhang et al., 2014) and *Z. cucurbitae* (Hsu et al., 2015). Knowledge of the underlying molecular mechanisms associated with insecticide resistance is relatively limited in Tephritidae species (Vontas et al., 2011). This limitation may be due to shortage of genome and transcriptome data, currently described for few species, as *B. dorsalis* (Shen et al., 2011), *B. oleae* (Pavlidi et al., 2013, 2017), *C. capitata* (Gomulski et al., 2012; Salvemini et al.,

2014), *Z. cucurbitae* (Sim et al., 2015) and *Bactrocera minax* (Enderlein) (Dong et al., 2014).

The rate of insecticide resistance development may vary among Tephritid fruit fly species for several reasons, including genetic/biological differences (number of generations, life cycle, fecundity, polygamy, migration and dispersal rates) and operational factors (selection pressure – type of applications: bait vs. cover sprays, role of refugia) in different ecological situations (Vontas et al., 2011). For example, spinosad sprays have led to resistance development in *B. oleae* after 10 years of use in California (Kakani et al., 2010), likely due to the limited selection pressure imposed by the bioinsecticide bait applications. However, resistance has now evolved and is becoming a problem to chemical products, such as the case of *C. capitata* in Spain where malathion and lambda-cyhalothrin resistance levels have led to field failures (Arouri et al., 2015; Magaña et al., 2007).

4.6.3 Behavioral control

The behavioral control studies included two main tactics, SIT and MAT. These studies included 20 fruit fly species in 24 countries. Studies of SIT included 12 fruit fly species, mainly *C. capitata, A. ludens* and *B. dorsalis* (Supplementary Material 3). The geographical distribution of these studies was mainly concentrated in Latin America, U.S.A. and Australia. For *Rhagoletis* species, only *R. mendax* was included in SIT studies. Many studies that included SIT evaluated basic factors of sterile insects, such as mating competitiveness, capacity of dispersion, survival, fertility, and basic parameters for application techniques

494 (irradiation doses and efficacy) (Barry et al., 2004; Dominiak et al., 2014; McInnis
495 and Wong, 1990; McInnis et al., 2002; Rempoulakis et al., 2015).

496 In its application, SIT still faces challenges, such as the determination of
497 sterile fly release densities required to achieve effective sterile to wild ratios for
498 the suppression or eradication of wild populations (Aluja, 1994). This aspect was
499 recently evaluated in *A. ludens* (Flores et al., 2014) and *A. obliqua* (Flores et al.,
500 2017) in mango orchards. The decline of sterility in fertile females was evaluated
501 using different ratios of sterile: fertile males under field cage conditions. The
502 trajectory of sterility slowed down after a sterile: wild ratio of 30:1 in *A. ludens*. A
503 10:1 sterile: wild ratio induced approximately 80% sterility in *A. obliqua* cohorts.
504 For *C. capitata*, a strong negative relationship between the proportion of sperm
505 and offspring was established by Juan-Blasco et al. (2014). In this study, the
506 proportion of V8 sperm in spermathecae increased with temperature and with the
507 number of V8 males released but leveled off between ratios of wild females to
508 wild males to V8 males of 1:1:10 and 1:1:20. In all seasons, except winter (no
509 offspring), viable offspring increased with temperature and was lowest for ratio
510 1:1:20.

511 Some studies have evaluated the performance of parasitoids reared in a
512 sterile fruit fly, such as *P. concolor* reared on larvae of *C. capitata* (Hepdurgun et
513 al., 2009), *P. humillis* reared in *B. oleae* (Yokoyama et al., 2012) and *D.*
514 *longicaudata* reared in *C. capitata* (Viscarret et al., 2012) and *A. fraterculus*
515 (Costa et al., 2016). Other studies included the evaluation of anti-predator
516 behavior of irradiated larvae of *A. ludens* (González-López et al., 2015; Rao et
517 al., 2014), the production of pheromones in irradiated males of *A. suspensa*
518 (Ponce et al., 1993), and the structure of the intestinal microbiota of *C. capitata*

519 (Ami et al., 2009). The inhibition of protein expression in irradiated pupae of *B.*
520 *dorsalis* was recently described (Chang et al., 2015).

521 Studies of MAT were performed in 17 countries for 16 fruit fly species. *B.*
522 *dorsalis* was the main species included in MAT studies (Table 3). These studies
523 evaluated the use of attractants and insecticides for male capture (Ndlela et al.,
524 2016; Reynolds et al., 2016; Vargas et al., 2012, 2015). The impact of methyl
525 eugenol and malathion, used for MAT was evaluated on non-target insects during
526 the eradication program for *Bactrocera carambolae* Drew and Hancock
527 (Vayssières et al., 2007). The results demonstrated that the use of blocks
528 impregnated with methyl eugenol and malathion had no more impact on non-
529 target insects than a non-impregnated block.

530 Studies aiming to integrate MAT with other techniques, such as SIT, bait
531 spray, parasitoids and the removal of infested fruits, were found in the present
532 review (Barclay et al., 2014; Shelly and Villalobos, 1995; Vargas et al., 2010).
533 This may be a function of scale, as MAT is sufficient for small populations, while
534 bait sprays, for example, are included to kill reproducing females in hot spots of
535 larger populations (Suckling et al., 2016). Additionally, the MAT involves minimal
536 cost and labor as it does not require frequent application (Lloyd et al., 2010).

537

538 4.6.4 Quarantine treatments

539 Studies that included quarantine treatments were performed for 23 species
540 in 14 countries (Supplementary Material 3). Irradiation was the tactic most used
541 for 20 species, mainly *C. capitata* and *A. ludens* (Table 3). Factors for fruit
542 irradiation control efficacy, such as radiation doses, were determined for various
543 fruit fly species, including *A. fraterculus* (Allinghi et al., 2007), *A. ludens* (Hallman

544 and Worley, 1999), *A. obliqua* (Hallman and Worley, 1999), *B. latifrons* (Follett et
545 al., 2011), *B. tryoni* (Collins et al., 2009), *B. zonata* (Draz et al., 2016), *C. capitata*
546 (Mansour and Franz, 1996), *D. ciliates* (Rempoulakis et al., 2015) and *R. mendax*
547 (Sharp and Polavarapu, 1999).

548 The temperature was the second quarantine treatment researched for 12
549 species, mainly *C. capitata* (Table 3). In *Anastrepha grandis* (Macquart),
550 temperature treatment was applied to determine the development stage more
551 tolerant to cold in zucchini squash [*Cucurbita pepo* L. (Cucurbitaceae)]. The
552 authors found that the 3rd instar was the most tolerant stage, and the time
553 required for a cold treatment in zucchini squash when treated at a minimum of
554 1.0 °C was estimated at ~23 d (Hallman et al., 2017). However, the estimated
555 time of 23 d needs to be confirmed by large-scale testing before it should be used
556 commercially.

557

558 4.6.5 Bioinsecticides

559 Studies that included bioinsecticides were performed in 17 countries for 18
560 fruit fly species, mainly *C. capitata*, *R. indifferens* and *A. ludens* (Supplementary
561 Material 3). These studies included formulated bio-based products, e.g spinosad-
562 based (GF-120™); a fermentation byproduct of the bacteria *Saccharopolyspora*
563 *spinosa* Mertz & Yao (Thompson et al., 2000) and plant-derived, e.g. neem
564 (Nimbicidine®).

565 The main studies related to control with bioinsecticides evaluated the use
566 of spinosad-based baits. These studies evaluated factors such as residual control
567 and lethal concentrations (Flores et al., 2011), attractiveness and efficacy of baits
568 (Mangan et al., 2006; Prokopy et al., 2003; Yee et al., 2007), toxicity to fruit flies

(Michaud, 2003) and effects on foraging and biological parameters of fruit fly species (Barry et al., 2003; González-Cobos et al., 2016*)*. The main biological parameters evaluated were emergence, mortality, and oviposition (Barry and Polavarapu, 2005; Yee and Chapman, 2005; Yee and Alston, 2006a; Yee, 2011).

Some studies have evaluated the toxicity of baits and insecticides to beneficial insects, such as parasitoids of tephritids *F. arisanus*, *P. fletcheri, Diachasmimorpha tryoni* (Cameron) and *D. longicaudata* (Liburd et al., 2004; Stark et al., 2004; Urbaneja et al., 2009; Wang et al., 2005;) and other natural enemies (Michaud, 2003). These studies confirmed that adult *F. arisanus*, the major parasitoid of *C. capitata* in Hawaii (as a model species), do not feed directly on GF-120™ in either the presence or the absence of honey and water resources in the laboratory (Wang et al., 2005). Other natural enemies also showed similar results (Michaud, 2003).

Studies with *Apis mellifera* L. (Hymenoptera, Apidae) demonstrated that the bait GF-120™ was toxic to honey bees at varying levels, depending on exposure and drying time (Edwards et al., 2003). In another study, Gómez-Escobar et al. (2014) showed that GF-120™ repels *Trigona fulviventris* (Guérin) and *Scaptotrigona mexicana* (Guérin-Meneville). This same study, the repellency was not as marked for *A. mellifera*, when GF-120™ was combined with highly nutritious substances, such as honey. These results suggest that area-wide application of GF-120™ should be carefully monitored, mainly in situations where the release or conservation of parasitoids and other beneficial insects are a prime concern (Wang et al., 2005).

593 4.6.6 Control with natural product insecticides

594 Natural product insecticides were used for control of 12 fruit fly species in
595 16 countries (Supplementary Material 3). These studies included mainly plant
596 and fungi extracts.

597 Plant-derived insecticides, such as azadirachtins, were included in these
598 studies (Singh, 2003; Silva et al., 2013). The interaction of neem used for *C.*
599 *capitata* control and the use of parasitoids *D. longicaudata* was also evaluated.
600 Both the botanical insecticide and the parasitism caused larval/pupal mortality
601 and reduced the emergence of *C. capitata* flies. However, the neem negatively
602 affected parasitoid emergence and the effect of parasitism coupled to neem did
603 not provide greater reduction in *C. capitata* emergence than when parasitism was
604 used alone (Alvarenga et al., 2012). The PCA showed that the control with natural
605 product insecticides and biological control were included in the same group (Fig.
606 5).

607

608 4.6.7 Mechanical control

609 The mechanical control studies included mass-trapping, fruit bagging, and
610 clipping of infested fruits. This method was researched in 11 countries for eight
611 species, mainly *C. capitata* and *B. oleae*. Mass trapping was the main tactic
612 included in these studies. This tactic has the potential to minimize or avoid the
613 use of insecticides and has attracted interest due to their efficacy, specificity and
614 low environmental impact (Navarro-Llopis et al., 2008; Martínez-Ferrer et al.
615 2010). Mass trapping consists of the use of traps and baits that release specific
616 volatile substances that attract insects to the trap, in which fruit flies are captured
617 and killed (El-Sayed et al., 2009; Martinez-Ferrer et al., 2012). However, for some

618 fruit fly species, the use of mass trapping as a control tool depends on the
619 availability of an effective and cheap attractant (Villalobos et al., 2017).
620 Additionally, this technique is most applicable where the cost of labor is low as it
621 is labor intensive. In the PCA, mechanical control showed separation from other
622 methods, likely because this technique was found for a few species in this review
623 (Fig. 5).
624
625 4.6.8 Genetic control
626 Genetic control involved the use of RNA interference (RNAi), which is a
627 mechanism of gene regulation and an antiviral defense system in cells, resulting
628 in the sequence-specific degradation of mRNAs (Huvenne and Smagghe, 2010;
629 Palli, 2012). The present review found studies of RNAi with *B. dorsalis* (Chen et
630 al., 2008), *B. minax* (Xiong et al., 2016), *A. suspensa* (Schetelig et al., 2012) and
631 *C. capitata* (Gabrieli et al., 2016). In these studies, the silencing and expression
632 of genes, such as *transformer* (tra), *trehalose-6-phosphate synthase* (TPS), *yolk*
633 *protein* (YP), *doublesex* (dsx), and *odorant receptor co-receptor* (Orco), among
634 others, were evaluated. The effects of genetic control on biological parameters,
635 sex determination and behavior were evaluated. These studies were performed
636 in four countries, with 82% of the studies performed in China in *B. dorsalis*
637 (Supplementary Material 3). As with mechanical control, the PCA showed
638 separation of genetic control from the other methods (Fig. 5).
639
640 *4.7 Limitations and prospects*
641 Fruit fly monitoring was included in some studies, with Mexico being the
642 country that performed most of such studies, mainly using traps. Studies of

643 monitoring with automatic traps showed potential to improve the effectiveness
644 and efficiency of monitoring (Goldshtein et al., 2017). These traps reduce human
645 involvement using cameras and communication technology and may reduce
646 costs in locations with high labor costs (Suckling et al., 2016), but this alternative
647 is still not commercially available. The mapping of population fluctuation, using
648 tools such as geographic information systems, was highly recommended for fruit
649 fly management (Nestel et al., 1997). However, these tools require adjustments
650 for specific field configurations and conditions and are dependent on the
651 development of specific attractants for fruit fly detection.

652 The present systematic review found many studies that included the use
653 of biological, chemical and behavioral control. Studies with entomopathogenic
654 fungi species showed promising results for biological control of fruit flies. The
655 entomopathogenic fungi, *M. anisopliae*, was used to investigate horizontal
656 transmission capacity among fruit fly adults during mating. The results showed
657 the capacity of transmission from treated flies to non-treated flies, resulting in high
658 mortality and the reduction of the number of eggs produced by fruit fly females
659 (Quesada-Moraga et al., 2008; Sookar et al., 2014). The results of pathogenicity
660 indicate that entomopathogenic fungi could be utilized with different modes of
661 application, such as cover or bait spray (Beris et al., 2013) or infection traps
662 (Navarro-Llopis et al., 2015).

663 Although many studies have included the use of attractants, such as bait
664 stations, mass trapping, and MAT, studies that include specific attractants remain
665 scarce. It is a problem particularly for the *Anastrepha* species, where there is not
666 a dry trap for monitoring these species. Inclusion in the surveillance networks of
667 food-based lures that capture both females and males is useful. However, food-

668 based lures often lack species specificity, although their deployment is essential
669 to detect species (Suckling et al., 2016).
670 Although many studies have included the use of attractants for application
671 in tactics, such as bait stations, mass trapping, and MAT, studies that include
672 specific attractants remain scarce. Male fruit flies are usually attracted by
673 parapheromones (IAEA, 2003). In contrast, lures for attracting female fruit flies
674 into traps are based primarily on food or host lures (Dominiak and Nicol, 2010).
675 Inclusion in monitoring networks of food-based lures that capture both females
676 and males is useful. However, although their deployment is essential to detect
677 species, food-based lures often lack specificity (Suckling et al., 2016). For *B.*
678 *tryoni*, wet-food-based McPhail traps collected more males than females despite
679 their reputation as being a specialist female lure (Dominiak and Nicol, 2010). It is
680 a problem particularly for the *Anastrepha* species, where a dry trap for these
681 species is not available.
682 Among recent technologies, RNAi is a promising tactic to control target
683 species (Andrade and Hunter, 2017). The RNAi effectiveness varies depending
684 on the species and target gene. Therefore, success in pest control mediated by
685 RNAi requires validation for each species and stage of development prior to its
686 use as a pest control tool (Taning et al., 2016). Similarly, it is essential to identify
687 an appropriate delivery method for the cropping system and pest. For most
688 horticultural crops, topically applied RNAi (e.g., Spray Induced Gene Silencing)
689 (Wang and Jin, 2017), could be an interesting alternative for use by growers
690 (Andrade and Hunter, 2017). To this end, the stability and uptake of the dsRNA
691 in the field must be improved (e.g., nanoparticles, such as nanosheets) (Mitter et
692 al., 2017), and the factors governing the systemic movement of dsRNA within the

693 plant need to be understood (Wang and Jin, 2017). The increase in the number
694 of the fruit fly transcriptome studies has contributed to the progress of RNAi-
695 based assays. Thus, progress in the identification of target gene studies for fruit
696 flies will stimulate the advancement in the generation of application technology
697 for the control of fruit flies.
698
699 **5. Conclusions**
700 Studies on fruit flies continue to increase and provide useful knowledge to
701 those working in the areas of monitoring and control tactics. From the 1950s to
702 the present day, there has been an emphasis on chemical control research,
703 especially the use of baits (Conway and Forrester, 2011; Díaz-Fleischer et al.,
704 2017; Steiner, 1952). However, the continued use of insecticides is increasingly
705 limited, making it necessary to evaluate other control strategies for inclusion in
706 fruit fly management.
707 Many advances in biological control tactics, SIT, quarantine treatments
708 and next-generation tools have been described (Ali et al., 2016, 2017; Aluja et
709 al., 2013; Bachmann et al., 2015; Cancino et al., 2014; Castanon-Rodriguez et
710 al., 2014; Landeta-Escamilla et al., 2016; Montoya et al., 2000;). The future of
711 fruit fly management research will require a continued emphasis on the principles
712 of Integrated Pest Management (IPM) and a broadening of the focus beyond pest
713 control. We highlight several recommendations that may improve future studies
714 on fruit fly management:
715 - We encourage researchers and technicians to disclose their unpublished
716 knowledge in peer-reviewed journals.

- We encourage researchers and funding organizations to establish and fund long-term studies. The present analysis shows that many tools for monitoring and control tactics showed promising results but need further research to confirm their effectiveness in the field (Chen et al., 2011; Chuang et al., 2014; Goldshtein et al., 2017; Haff et al., 2013).

- More monitoring studies are needed to provide useful knowledge on species detection and population density (Katsoyannos et al., 1999).

- We recommend that the studies include the risk evaluation of the control tactic on non-target species, such as beneficial insects (Cobo et al., 2015).

- We recommend a connection between researchers and commercial companies to meet the current needs of fruit fly management.

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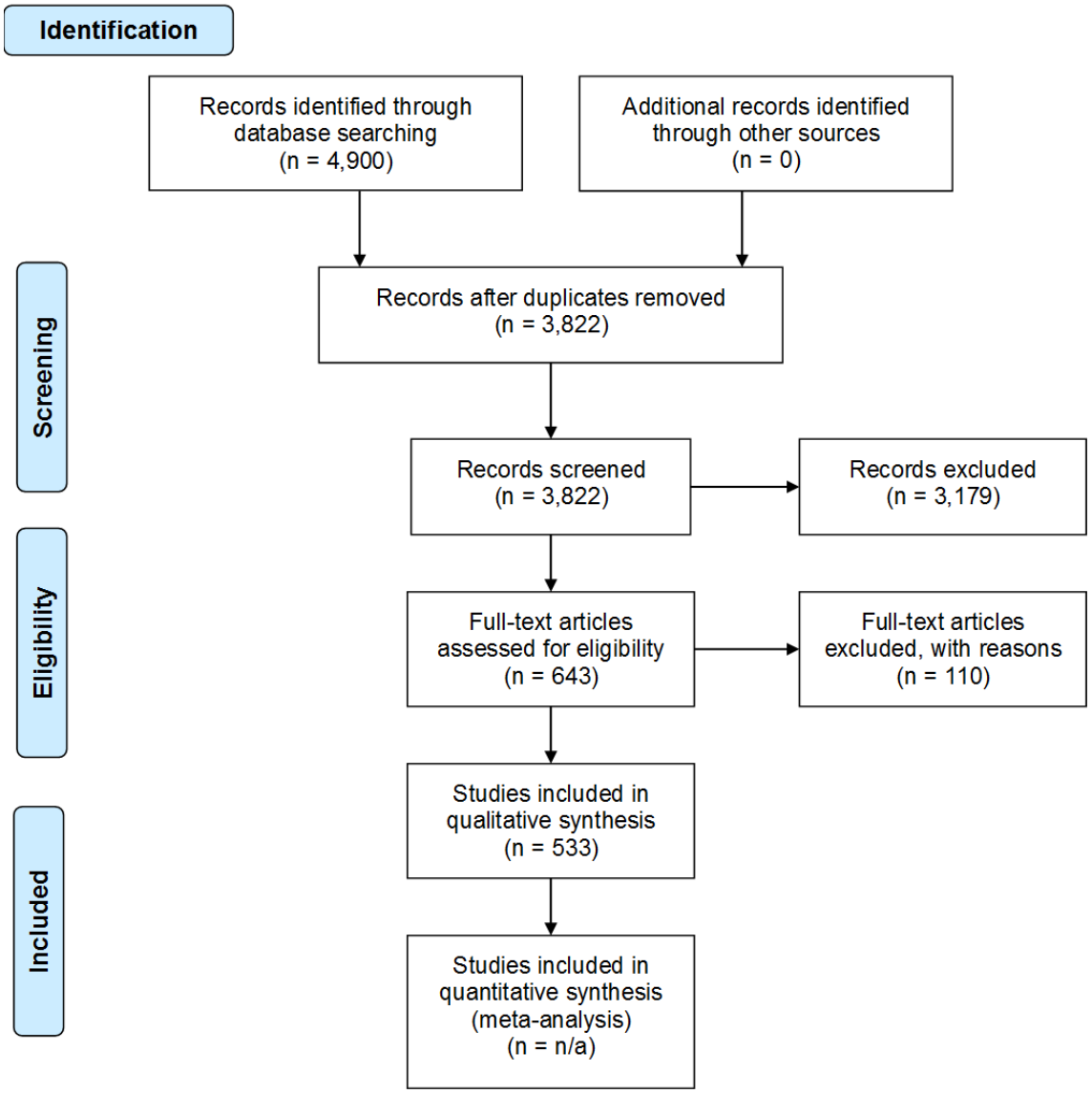


Fig. 1 PRISMA flow diagram. Flow diagram illustrating search strategy.

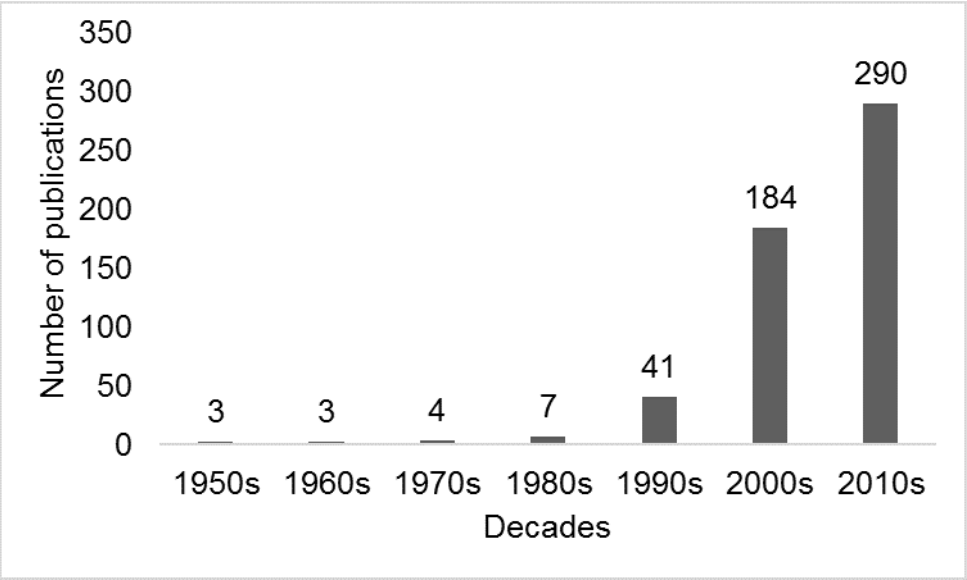


Fig. 2 Temporal trend of fruit fly management research. Studies of monitoring and control tactics of fruit flies from 1952 to 2017 by decade. Last access date 13 December 2017.

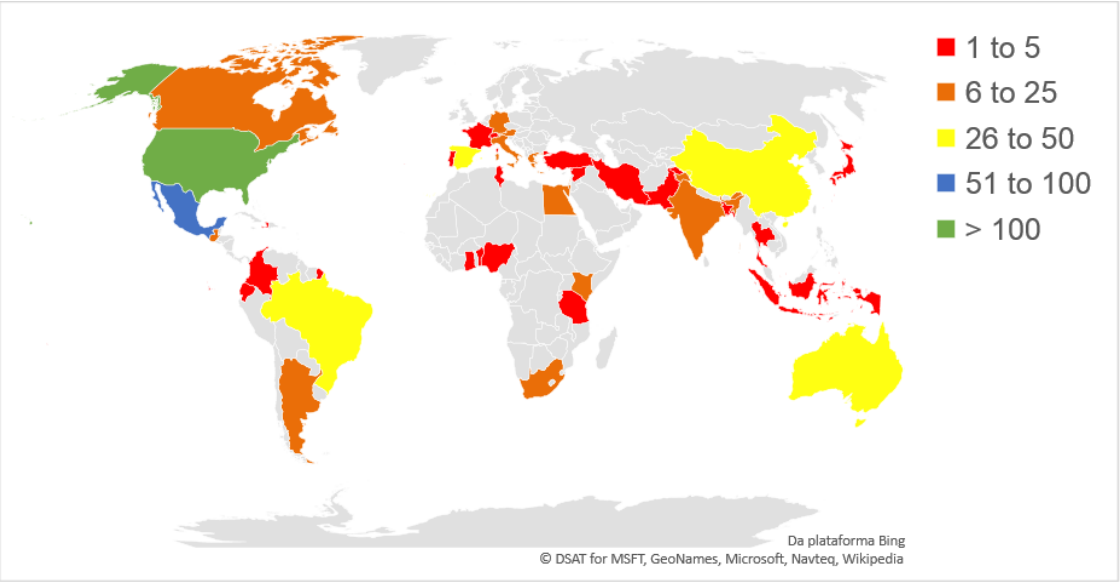
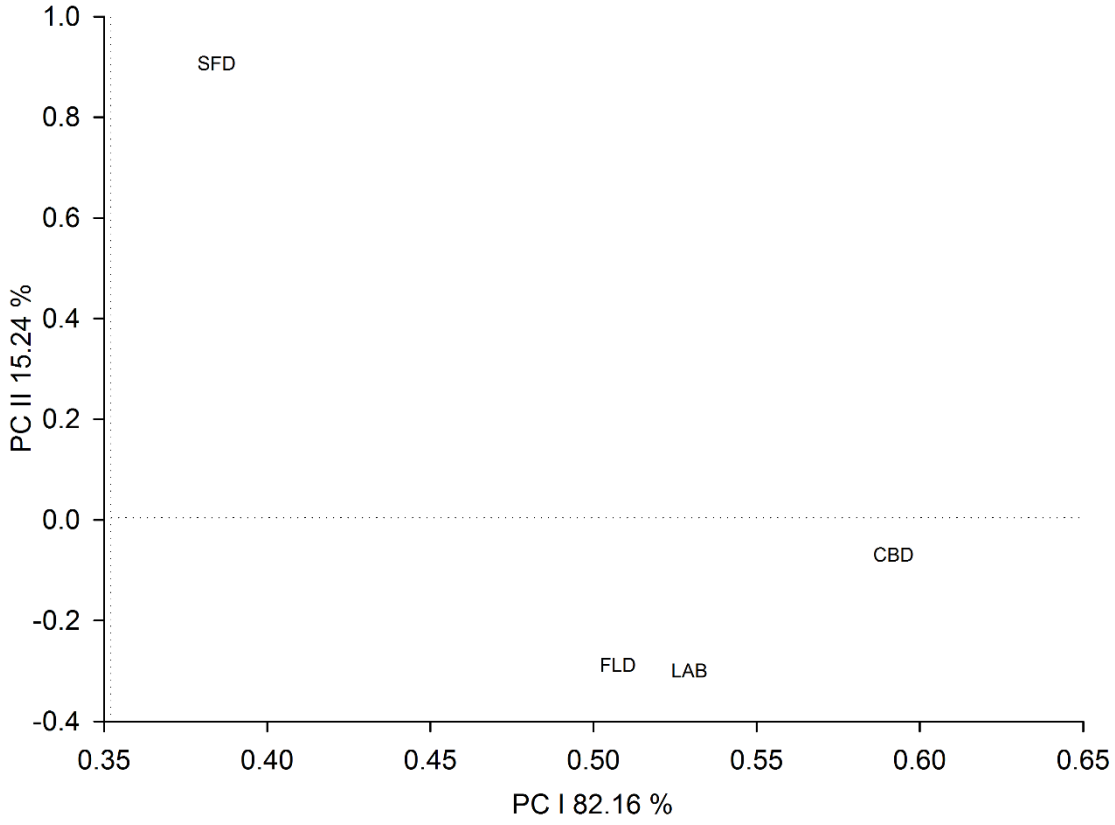


Fig. 3 Geographical distribution of fruit fly management research. Studies of monitoring and control tactics of fruit flies. The number of studies from each country is indicated by category.



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1512 **Fig. 4 Principal component analysis of methodological approaches used in**

1513 **fruit fly studies.** CBD: combined approaches; FLD: field; LAB: laboratory and

1514 SFD: semifield.

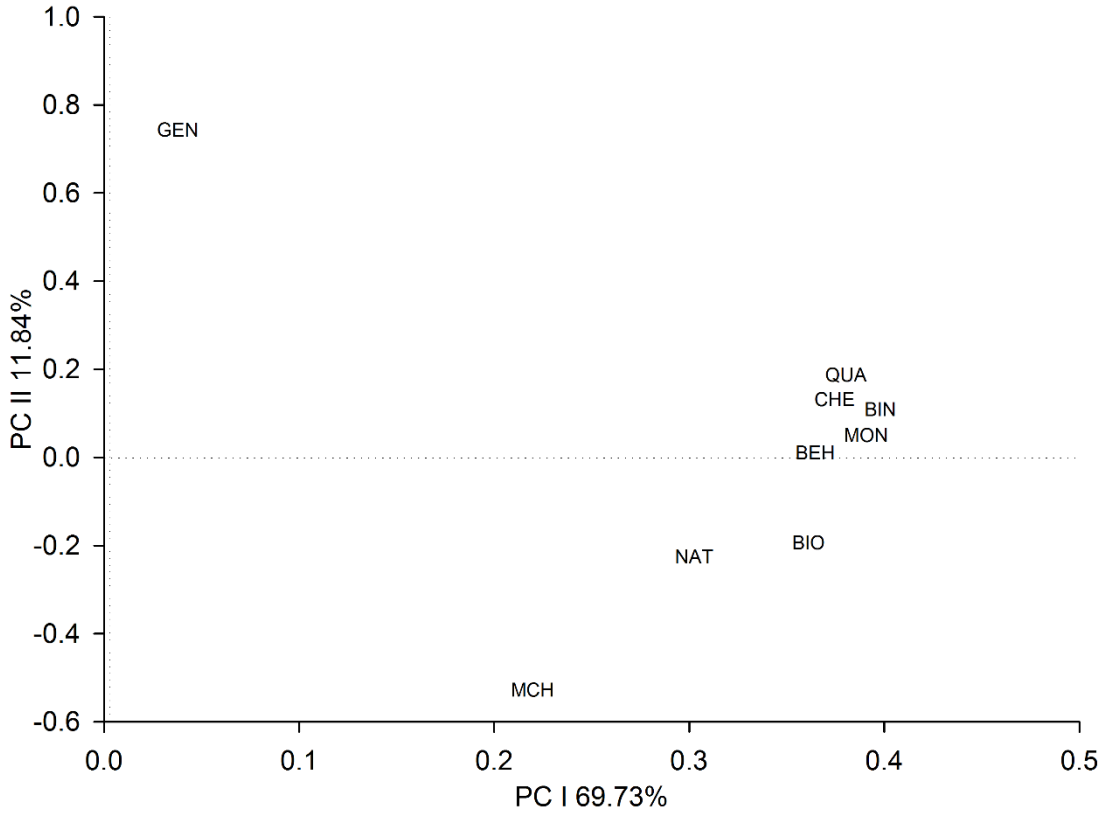

Fig. 5 Principal component analysis for control methods used in fruit fly studies. BEH: behavioral control; BIO: biological control; BIN: bioinsecticides; CHE: chemical control; GEN: genetic control; MCH: mechanical control; MON: monitoring and detection; NAT: control with natural product insecticides and QUA: quarantine treatments.

Table 1 Principal control tactics and fruit fly species researched in countries with more than 10 studies found in the review.

Country[a]	Principal control tactic	Fruit fly species
USA	Parasitoids and baits [b]	*Ceratitis capitata*
MEX	Biological tactics	*Anastrepha ludens*
AUS	Male Annihilation Technique	*Bactrocera tryoni*
ESP	Other biological agents [c]	*Ceratitis capitata*
BRA	Parasitoids	*Anastrepha fraterculus*
CHN	RNA interference	*Bactrocera dorsalis*
GRC	Mass-trapping	*Bactrocera oleae*
ARG	Parasitoids	*Anastrepha fraterculus*
ITY	Other biological agents [c]	*Ceratitis capitata*
ISR	Several tactics [d]	*Ceratitis capitata*

[a] USA: United States of America; MEX: Mexico; AUS: Australia; ESP: Spain; BRA: Brazil; CHN: China; GRC: Greece; ARG: Argentina; ITY: Italy; ISR: Israel.
[b] Bait spray and station of bioinsecticides and chemical products
[c] Predators, bacteria, viruses, fungi and nematodes
[d] Bait spray and station of bioinsecticides and chemical products, pulverization of chemical products, SIT and temperature

Table 2 Number of studies examining the monitoring and control tactics of fruit fly species.

Fruit fly species	n studies
Ceratitis capitata	180
Anastrepha ludens	73
Bactrocera dorsalis	72
Bactrocera oleae	49
Zeugodacus cucurbitae	40
Bactrocera tryoni	29
Anastrepha fraterculus	28
Anastrepha obliqua	25
Anastrepha suspensa	18
Ragholetis indifferens	18
Ragholetis pomonella	14
Bactrocera zonata	11
Ragholetis cerasi	10
Ragholetis mendax	10
Bactrocera invadens	9
Ceratitis rosa	8
Anastrepha serpentina	7
Ceratitis cosyra	7
Dacus ciliatus	6
Anastrepha spp.[a]	6
Bactrocera carambolae	5
Bactrocera minax	4
Bactrocera papayae	3
Bactrocera spp.[a]	3
Bactrocera tau	3
Zeugodacus cucumis	3
Anastrepha sorurcula	2
Anastrepha leptozona	2
Bactrocera correcta	2
Bactrocera latifrons	2
Anastrepha grandis	1
Anastrepha punensis	1
Anastrepha spatulata	1
Anastrepha distincta	1
Anastrepha chiclayae	1
Anastrepha striata	1
Anastrepha schultzi	1
Anastrepha zenildae	1
Bactrocera jarvisi	1
Bactrocera neohumeralis	1
Bactrocera philippinensis	1
Ceratitis anonae	1
Ceratitis fasciventris	1
Ragholetis cingulata	1
Toxotrypana curvicauda	1

[a] species not specified in the studies.

Table 3 Studies on monitoring and control tactics of fruit flies and principal fruit fly species researched in each tactic.

	Monitoring and control tactics	n studies	Fruit fly species
Monitoring and detection	Fruits	2	*Anastrepha* and *Rhagoletis* species [a]
	Traps	59	*Ceratitis capitata*
	PCR	7	*Bactorcera dorsalis* and *Bactrocera oleae*
	Automatic	7	*Bactrocera dorsalis*
Natural products	Bait spray and bait station	8	*Ceratitis capitata*
	Pulverization	21	*Ceratitis capitata*
	Biofilm, feeding and injection	7	*Zeugodacus cucurbitae*
Bioinsecticides	Bait spray and bait station	50	*Ceratitis capitata*
	Pulverization	20	*Ceratitis capitata*
	Feeding	1	*Bactrocera dorsalis* and *Zeugodacus cucurbitae*
Chemical	Bait spray and bait station	68	*Ceratitis capitata*
	Pulverization	40	*Ceratitis capitata*
Biological	Parasitoids	84	*Ceratitis capitata*
	Predators, bacteria, viruses, fungi and nematodes	70	*Ceratitis capitata*
Behavior	Sterile Insect Technique	52	*Ceratitis capitata*
	Male Annihilation Technique	43	*Bactrocera dorsalis*
Mechanical	Mass-trapping	26	*Bactrocera oleae* and *Ceratitis capitata*
	Fruit bagging and clipping infested fruits	5	*Anastrepha fraterculus*, *Ceratitis capitata* and *Zeugodacus cucurbitae*
Quarantine	Modified atmosphere	8	*Anastrepha ludens*
	Temperature	30	*Ceratitis capitata*
	Irradiation	48	*Anastrepha ludens* and *Ceratitis capitata*
	Metabolic stress	1	*Bactrocera dorsalis, Ceratitis capitata* and *Zeugodacus cucurbitae*
	Microwave	1	*Anastrepha ludens*
	Pulsed electric field	1	*Anastrepha ludens*
Genetic	RNA interference	17	*Bactrocera dorsalis*

Supplementary Material 1

Category description used in the systematic review (.xls)

Supplementary Material 2

Systematic Review Checklist by PRISMA (.docx)

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4-5
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	N/A
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5-7, S1 Table
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	5
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	5

Section/topic	#	Checklist item	Reported on page #
METHODS			
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	5
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	5-7
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	N/A
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	N/A
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	5
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	N/A
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	7-8
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	8, S3
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	8-10, S3
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	N/A
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	N/A
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	N/A
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	N/A

Section/topic	#	Checklist item	Reported on page #
RESULTS			
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	10, Fig.4-5
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	10-27
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	27-30
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	30-31
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	Funding statement

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Supplementary Material 3

Studies dataset – Information about monitoring and control methods, species, methodological approaches and countries extracted from 533 studies (.xls)

Article 2 – Frontiers in Physiology (Submitted)

The South American fruit fly: A new pest model with RNAi-sensitive larval stages

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Abstract

The RNA interference (RNAi) technology has been widely used in the development of approaches for pest control. The presence of some essential genes, the so-called core genes, in the RNAi machinery is crucial for its efficiency and robust response in gene silencing. Thus, our study was designed to verify whether the RNAi machinery is functional in the South-American (SA) fruit fly *Anastrepha fraterculus* (Diptera: Tephritidae) and whether the sensitivity to uptake dsRNA could induce an RNAi response in this fruit fly species. To prepare a transcriptome database of the SA fruit fly, total RNA was extracted from all the different developmental stages as eggs, larvae, pupae and

female and male adults for later cDNA synthesis and Illumina sequencing. After the d*e novo* assembly and gene annotation, the transcriptome was screened for RNAi pathway genes, as well as the duplication or loss of genes and novel target genes to dsRNA delivery bioassays. The soaking assay in larvae was performed to evaluate the gene-silencing of *V-ATPase* and the *Dicer-2* and *Argonaute-2* expression after dsRNA delivery, and the stability of dsRNA with an in vitro incubation. We identified 55 genes related to the RNAi machinery with duplication and loss for some genes and selected 143 different target genes related to biological processes involved in post-embryonic growth/development and reproduction of *A. fraterculus*. Larvae soaked in dsRNA solution showed a strong knockdown of V-ATPase after 48 h and the expression of *Dicer-2* and *Argonaute-2* responded with an increase upon the exposure to dsRNA. Our data demonstrated the existence of a functional RNAi machinery and an easy robust physiological bioassay with the larval stages that can further be used for screening of target genes at *in vivo* organisms' level for RNAi-based control of fruit fly pests. This is the first study that provides evidence of a functional siRNA machinery in the SA fruit fly.

1 Introduction

The South American fruit fly (SA fruit fly), *Anastrepha fraterculus*, is one of the main polyphagous pests of fruit crops. This species is distributed from southern United States (Texas) and Mexico to Argentina and is associated with 116 plant species only in Brazil (Zucchi, 2008). Oviposition and larval feeding of *A. fraterculus* cause the damage, that leads to accelerated ripening and premature fruit dropping (Aluja, 1994). Importantly, its presence limits access to international markets due to quarantine restrictions imposed by fruit-fly-free countries (Lanzavecchia et al., 2014). The losses caused by fruit flies can exceed USD 2 billion, and in Brazil, it is estimated that the economic losses are between $120 and 200 million USD per year (Macedo et al., 2017).

Currently, the only control tactic available for *A. fraterculus* is the use of bait sprays (Cladera et al., 2014). However, the chemical control of SA fruit fly is becoming increasingly difficult, as formerly effective but broad-spectrum neurotoxic and systemic-acting insecticides have been banned from the market (Böckmann et al., 2014). Also, the fruit growers are seeking new economic fruit fly control options, especially environmentally sustainable tactics (Sarles et al., 2015). Thus, the RNA interference (RNAi) is a promising alternative strategy for controlling crop pests that shows the advantage of using the insect's systemic gene-silencing machinery to suppress essential gene expression (Andrade and Hunter, 2017; Katoch et al., 2013). Double-stranded RNA (dsRNA) is the RNAi trigger molecule that primes the post-transcriptional down regulation of a target gene (Elbashir et al., 2001). Characteristics such as highly specific targeting and lack of environmental persistence make RNAi approaches desirable for crop protection against fruit fly pests (Huvenne and Smagghe, 2010; Zotti et al., 2018).

Efficient RNAi-induced gene silencing in insects requires some essential factors, such as dsRNA processing by RNAi enzymes, cellular uptake of dsRNA and expression of the core RNAi machinery (Huvenne and Smagghe, 2010; Wang et al., 2016). *Drosophila* species have been used as a model for RNAi studies in Diptera. However, this species shows low sensibility to dsRNA uptake by cells, it is necessary to use transfection agents for delivery of dsRNA molecules (Taning et al., 2016; Christiaens et al., 2018). Soaking of *Drosophila melanogaster* larvae for a period of 1 h with naked dsRNA resulted in only 5-8% of knockdown for *b-glucuronidase* (*gus*) gene (Whyard et al., 2009). In *Drosophila suzukii* larvae, the RNAi efficiency varied between 20-40% in a study using dsRNA

formulated with transfection reagent (Taning et al. 2016). For *Bactrocera dorsalis*, Shi et al. (2017) found knockdown around 50% in larval stages. This fact raises the question about variability in uptake routes and uptake mechanisms between different species within of Diptera (Whyard et al., 2009).

Thus, an increased understanding of the RNAi pathway in target insect can provide information to use this technology effectively (Vélez et al., 2016). Therefore, in order to evaluate the potential of RNAi as a tool in the control of the SA fruit fly, there is both the need for adequate genetic information concerning RNAi core genes and more insight into the silencing process by RNAi.

This paper is the first reporting on RNAi bioassays in the SA fruit fly together with a transcriptome analysis over the different developmental stages of eggs, larvae, pupae, and female and male adults. Our aim was to provide a genetic database to better understand this important pest insect and to screen for the genes related to the RNAi machinery, as well as the duplication or loss of genes and novel target genes to dsRNA delivery bioassays. Hence, we had a specific interest in genes related to insect-specific biological processes involved in post-embryonic growth/development and reproduction as potential future insecticidal target genes. In addition, we wanted to develop a miniaturized setup by soaking the SA fruit fly larvae. In case successful it is an easy robust physiological bioassay with the larval stages that can further be used to screen for interesting target genes at *in vivo* organisms' level for RNAi-based control of fruit fly pests. In the steps to validate the RNAi response, we first investigated the *Dicer-2* and *Argonaute-2* expression after dsRNA delivery, and then tested the gene-silencing of *V-ATPase* and if this effect correlated with insect mortality. Finally, we measured the stability of dsRNA with an *in vitro* incubation in insect juice to better understand the impact of metabolic degradation of dsRNA in the *in vivo* RNAi efficacy with fruit flies. This study will so be the first one providing evidence of a functional siRNA machinery in the SA fruit fly.

2 Material and Methods

2.1 SA fruit fly colony and maintenance

A colony of *A. fraterculus* was originally field-collected in 2015 from an orchard of strawberry guava (*Psidium cattleianum*) in Pelotas, Rio Grande do Sul, Brazil (31º40'47" S e 52º26'24" W) and was reared for thirteen generations before use for the experiments. SA fruit fly stages were maintained under standard conditions (temperature: 25±1°C; RH: 70±10% and 14L:10D photoperiod). The rearing methods were the same as those described by Gonçalves et al. (2013).

2.2 RNA extraction, cDNA library, and RNA-Seq

Total RNA was extracted from eggs, larvae (first-, second- and third-instar), pupae and adults (female and male) of SA fruit fly using the RNAzol (GeneCopoeia, Rockville, MD) and treated with DNase I (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. The RNA samples were pooled to cDNA synthesis. The RNA quality and concentration were examined on the Agilent 2100 Bioanalyzer and cDNA library was constructed using the TruSeq RNA Sample Prep kit (Illumina, San Diego, CA) protocol. The library was sequenced (RNA-Seq) using the Illumina HiSeq2500 platform using V4 by paired-end reads in one lane with read lengths of 2x125bp. Raw sequence data were

submitted to the Short Read Archive (SRA) of the NCBI database (accession number SRP157027).

2.3 Quality control and de novo assembly

All reads were trimmed for quality and length using the software Trimmomatic and the quality was checked using the software FastQC. High-quality reads had a Phred score over 30 across more than 70% of the bases. The high-quality reads were *de novo* assembled using Trinity software since there is no reference genome sequence for *A. fraterculus*. This software uses a Bruijn graph algorithm and was executed using default settings, a k-mer length of 25.

2.4 Transcriptome analysis and target genes database

The contigs generated by Trinity were aligned to the UniProt database using Diamond algorithm (Buchfink et al., 2015) and only those with hits on insects (E-value threshold of 1e-10) were selected for further analysis. For functional categorization by Gene Ontology (GO), a second similarity search was performed to annotate the contigs generated by searching the UniProt database with the Diamond. The gene generated identifiers were used as input in QuickGo from EBI and to calculate GO terms. A database was generated for novel target genes related to post-embryonic growth and development of the SA fruit fly larvae and the reproduction events in adults. The ID genes were searched in QuickGo using the GO terms related to biological processes: larval development (GO:0002164), imaginal disc morphogenesis (GO:0007560), post-embryonic development (GO:0009791), female sex differentiation (GO:0046660), sexual reproduction (GO:0019953), genital disc anterior/posterior pattern formation (GO:0035224) and oviposition (GO:0018991). The *D. melanogaster* sequences corresponding to the ID genes found were recovered in UniProt database and were used as a query to search the transcriptome from *A. fraterculus* using the tblastn tool with a threshold bit score ≥150 and E-value ≤1e-5 (Supplementary Material 1).

2.5 Identification of RNAi machinery genes

A list of RNAi-related genes, as employed by Swevers et al. (2013), Prentice et al. (2015) and Yoon et al. (2016), was selected, covering the RNAi core machinery, auxiliary factors (RISC), dsRNA uptake, nucleases, antiviral RNAi, intracellular transport, and lipid metabolism. Homologous sequences from *D. melanogaster* corresponding to RNAi-related genes were obtained in UniProt database and were used as a query to search the transcriptome from SA fruit fly (Supplementary Material 2). Alternatively, sequences of *Drosophila* and Tephritidae species were used in the absence of sequences of *D. melanogaster* (Supplementary Material 2). The program ORF Finder from NCBI was used to detect open reading frames. The protein domains were predicted by NCBI Conserved Domains using the Conserved Domain Database (CDD) (Supplementary Material 2). A similarity search was performed using the BLASTp against the NCBI database to confirm the identity of the RNAi-related genes (Supplementary Material 4).

2.6 Potential loss and duplication of RNAi-related genes

We screened the SA fruit fly transcriptome for the copy number of the ten RNAi pathway genes found using tblastn tool. The number of copies was based in the number of genes obtained by Trinity assembly. The distribution of these genes was compared to insects

related, following the results showed by Dowling et al. (2016). We also searched for genes for a systemic RNAi response, as *SID-1* found in cells of *Caenorhabditis elegans* (Winston et al., 2002).

2.7 Phylogenetic analysis

A phylogenetic analysis was constructed to provide an additional confirmation of the main siRNA machinery genes (*Dicer-2* and *Argonaute-2*) and the candidate gene silencing (*Vacuolar-proton-ATPase*) from the *A. fraterculus* transcriptome. Phylogenetic trees were constructed using the Neighbor-Joining method with the MEGA X software. Bootstrapping was used to estimate the reliability of phylogenetic reconstructions (1000 replicates). The selected species and accession numbers of the sequences used for phylogenetic analysis are showed in Supplementary Table S4.

2.8 dsRNA synthesis

The *A. fraterculus* transcriptome was searched for the *Vacuolar-proton-ATPase V0-domain* (*V-ATPase V0*) sequence using the homologous sequence from *D. melanogaster* as a query. Primers were designed from the *A. fraterculus* transcriptome sequences using Primer3 (http://primer3.ut.ee/). The *V-ATPase V0* fragment (483 pb) was amplified by PCR using cDNA second-instar larvae of *A. fraterculus* as a template, prepared with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). For dsRNA synthesis of *Green Fluorescent Protein* (*GFP*), a 560 bp *GFP* fragment was amplified by PCR using plasmid pIG1783f. The GFP amplicon was confirmed by Sanger sequencing. The primers used for the PCR are listed in Supplementary Table S1.

The dsRNA templates were produced by PCR using primers with a T7 promoter region at the 5' end of each primer (Supplementary Table S1). The PCR products were used for in vitro transcription and purification using MEGAscript kit (Ambion, Austin, TX) according to the manufacturer's instructions. Synthesized dsRNA products were quantitated by a Nanovue spectrophotometer (GE Healthcare, Little Chalfont, UK) at 260 nm and the integrity was confirmed by electrophoresis on 1% agarose gel.

2.9 RNAi by soaking of larval stages

The soaking treatment was performed using second-instar larvae of *A. fraterculus*. The dsRNA of *V-ATPase V0* (ds*VTP*) was diluted with RNase-free water to yield a concentration of 500 ng/µl, considering the data reported by Whyard et al. (2009). The ds*GFP* in the same concentration was used as control for the soaking assays. The insects were starved for 1 h and each larva was soaked in a 200 µl-tube with 25 µl of dsRNA solution for a period of 30 min. After soaking, the treated larvae were transferred to artificial diet (Nunes et al., 2013). The mortality of the insects was monitored over a 7-day period.

Larvae of *A. fraterculus* were stored at −80°C at 24, 48 and 72 h after soaking with dsRNA for the RNAi silencing efficiency assay. The RNA was extracted of three biological replicates to each time, using RNAzol (GeneCopoeia, Rockville, MD) following the manufacturer's instructions. After, the RNA samples were incubated with 10 U DNase I (Invitrogen, Carlsbad, CA) at 37 °C for 30 min. The RNA was quantified using a Nanovue spectrophotometer (GE Healthcare, Little Chalfont, UK) and verified by 2% agarose gel

electrophoresis. First strand cDNA was produced from 2 µg RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA).

2.10 Measurement of RNAi efficacy

Real-time Quantitative PCR analysis (qPCR) was performed to evaluated RNAi efficacy using a LightCycler 480 (Roche Life Science, Switzerland). The primers used in the analysis (Supplementary Table S1) were validated with a standard curve based on a serial dilution (1:1, 1:5, 1:25 and 1:125) of cDNA to determine the primer annealing efficiency and a melting curve analysis. The reactions included 5 µl of EvaGreen 2X qPCR MasterMix (ABM, Canada), 0.3 µl (10 µM) of forward primer, 0.3 µl (10 µM) of reverse primer, 3.4 µl of nuclease-free water and 1 µl of cDNA, in a total volume of 10 µl. The amplification conditions were 10 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 45 s at 59 °C and 30 s at 77 °C, interrupted by the dissociation curve with denaturation at 95 °C (5 s), cooling at 70 °C (1 min) and gradually heating at 0.11 °C steps up to 95 °C and cooling at 40 °C (30 s). The reactions were set-up in 96-wells microliter plates (Roche Life Science, Indianapolis, IN), using the cDNA dilution of 1:25, with three technical replicates and no-template controls. Relative mRNA expression of the V-ATPase gene was normalized to the endogenous reference genes α-tubulin and actin by the equation ratio 2-ΔΔCt (Livak and Schmittgen, 2001). The data were analyzed using analysis of variance (one-way ANOVA) and t-Test ($p \leq 0.05$).

2.11 Expression of siRNA genes *Dcr-2* and *Ago-2* upon exposure to dsRNA

To investigate the regulation of expression of siRNA pathway genes during the SA fruit fly RNAi bioassay, the expression of *Dicer-2* (*Dcr-2*) and *Argonaute-2* (*Ago-2*) in response to soaking with ds*GFP* was determined. The *Dcr-2* and *Ago-2* sequences found in the *A. fraterculus* transcriptome were used for primers design using the Primer3. The primers used for the qPCR are listed in Supplementary Table S1. The qPCR analysis was performed as described above and the expression responses were measured at 24, 48 and 72 h after larvae soaking with ds*GPF*.

2.12 dsRNA degradation assay

Body fluid (lumen contents and hemolymph) was collected from 5 second-instar larvae in 1.5 ml-tubes. The supernatant was removed by centrifugation at 13,000 rpm for 10 min at 4 °C. For the degradation assay, 20 µl of ds*GFP* solution (500 ng/µl dsRNA) was mixed with 2 µl of body fluid and incubated at 25 °C. Aliquots of 5 µl were collected at 0, 1, 2 and 4 h after incubation and a same volume of EDTA (10 mM) was added to stop the enzymatic reaction. The samples were stocked at -80 °C until the analysis. The results were verified by 1.5% agarose gel electrophoresis and the bands were analyzed using the Gel Analyzer software.

3 Results

3.1 SA fruit fly transcriptome analysis

The RNA sequencing generated a total of 103,808,135 reads of 125 bp long. The assembled transcriptome consisted of 163,359 transcripts, which accounted for 84,105 contigs (Supplementary Table S2). Of all contigs, 72,388 are from Eukaryote. The length

distribution of Eukaryote contigs in *A. fraterculus* transcriptome is shown in Supplementary Figure S1.

The Diamond analysis produced 73,193 hits, representing 45% of the total contigs (Supplementary Figure S2). For those sequences with a significant match, 72% of the contigs were most similar to sequences from fruit fly species: 17% to the *Ceratitis capitata*, 16% to the *Zeugodacus cucurbitae*, 15% to the *B. dorsalis* and *Bactrocera latifrons*, 9% to the *Bactrocera tryoni*, and 28% to other organisms. The species distribution of top 30 hits is shown in Supplementary Table S3. For those sequences with a significant match, of the contigs were most similar to sequences from Diptera, with featured for 55% to *Bactrocera*, 16% to *Ceratitis*, 3% to *Drosophila*, 1% to *Tabanus*, 0.9% to *Glossina*, 0.8% to *Lucilia* and 20% to other insect genera.

The Diamond similarity searches were performed against the UniProt database in order to classify the generated contigs. The resulting identifiers from this search were used to calculate GO terms, which were grouped into three main categories: molecular function (48%), biological process (31%) and cellular component (20%). A total of 167,729 predicted GO terms were obtained. On the most dominant GO terms within the molecular function, it was nucleic acid binding (11,734; 7%), for the biological processes it was RNA-dependent DNA biosynthetic process (4,070; 2%), and for the cellular component, it was the membrane (10,584; 6%) (**Figure 1**).

3.2 Target genes related to post-embryonic growth/development and reproduction events

We selected 143 different target genes related to biological processes involved in post-embryonic growth/development and reproduction of *A. fraterculus*. Preferably sequences were selected with annotations reviewed by Swiss-Prot and with experimental evidences. The target genes selected are involved in 5 biological processes: larval development (54 genes), imaginal disc morphogenesis (22 genes), post embryonic development (12 genes), sexual reproduction (44 genes), female sex differentiation (2), genital disc anterior/posterior pattern formation (2) and oviposition (7). The results are shown in Supplementary Material 1.

3.3 RNAi machinery genes are present in SA fruit fly

We identified 55 genes related to the RNAi machinery in *A. fraterculus* transcriptome of this study (**Table 1**). The components of the miRNA, siRNA and piRNA pathways, auxiliary factors (RISC), dsRNA uptake, intracellular transport, antiviral RNAi, nucleases, and lipid metabolism showed most conserved protein domains (Supplementary Material 2). The number of the copies at which these genes were found in *A. fraterculus*, is shown in **Figure 2.**

A BLASTp similarity search was performed against the NCBI database and the sequences of *Rhagoletis zephyria*, *B. dorsalis*, and *C. capitata* showed the closest similarity to *A. fraterculus* (Supplementary Material 4). The phylogenetic analysis showed that the siRNA pathway gene sequences (*Dcr-2* and *Ago-2*) from *A. fraterculus* transcriptome were classified in the same clade of *D. melanogaster* (**Figure 3**) and the *V-ATPase* sequence in the same of *B. dorsalis* clade (**Figure 4**). The *V-ATPase* sequence was grouped only with insect sequences, indicating the dsRNA sequence specificity.

3.4 Gene silencing and mortality in larval stages induced by dsRNA soaking

Larvae of *A. fraterculus* soaked in a concentration of 500 ng/µl of dsVTP, showed a robust gene silencing as early as 24 h after exposure to dsRNA. The dsVTP soaking resulted in an 85% knockdown relative to ds*GFP* control and increased to 100% after 48 h (**Figure 5**). The silencing effect persisted up to 72 h ($p \leq 0.05$). The mortality of *A. fraterculus* was evaluated for a period of 7 days, when larvae reached the pupal stage. Larval mortality started one day post-soaking (dps), with 5% mortality in larvae soaked with dsVTP. The mortality induced by dsVTP became evident at 2 days (19%) and rose further to 40% at 7 dps (**Figure 6**). While the mortality in larvae soaked with ds*GFP* (control) was 14% at 7 dps.

3.5 Expression of siRNA pathway genes *Dcr-2* and *Ago-2* in response to dsRNA

The expression of the siRNA genes after the dsRNA soaking in the SA fruit fly larvae confirmed the robust response of the *V-ATPase* gene. The *Dcr-2* mRNA levels were upregulated on the first 24 h after the dsRNA soaking and increased after 48 h; at that moment the *V-ATPase* mRNA levels were completely downregulated (**Figure 7A**). The *Ago-2* mRNA levels needed a long time to show an upregulation: The *Ago-2* upregulation was significant at 72 h after soaking (**Figure 7B**).

3.6 dsRNA degradation in *A. fraterculus* larvae

We analyzed the degradation of ds*GFP* by the dsRNases present in the body fluids (lumen contents and hemolymph) from *A. fraterculus* larvae. After 1 and 2 h of incubation period, no significant degradation of dsRNA was observed (**Figure 8**). However, after a longer incubation of 4 hours, approximately 40% of the body fluid band intensity was reduced when compared with the start of the incubation (0 h).

4 Discussion

Although *A. fraterculus* is one of the main pests of fruit crops in the American continent, the lack of genetic information is still a barrier to understanding this species. Over the past few decades, a great deal of research has been conducted on the basic ecological and biological characteristics of SA fruit fly (Cladera et al., 2014), but the genetic information of this species is still limited. The availability of insect transcriptomes allows the evaluation and identification of genes that can be potentially used for pest control using different biotechnological approaches (Garcia et al., 2017; Sagri et al., 2014). Recently, the head transcriptome of *A. fraterculus* was characterized and this study aimed to identify fixed single nucleotide polymorphisms (SNPs) for two closely related species of the *fraterculus* group (Rezende et al., 2016). Several studies in the context to develop RNAi in the control of fruit flies species were conducted so far, but only for *Anastrepha suspensa* (Schetelig et al., 2012), *B. dorsalis* (Chen et al., 2008, 2011, Li et al., 2011, 2016; Liu et al., 2015; Peng et al., 2015; Shen et al., 2013; Suganya et al., 2010, 2011; Xie et al., 2017; Zheng et al., 2012, 2015), *Bactrocera minax* (Xiong et al., 2016) and *C. capitata* (Gabrieli et al., 2016).With this project, more than 84,000 new queries related to *A. fraterculus* have been made available. We also provide here a database of 143 novel target genes.

The Diamond search analysis showed the greatest number of non-significant hits, which indicates that the *A. fraterculus* transcriptome contains unknown sequences that are not

described in the protein sequences databases. Thus, the *A. fraterculus* transcriptome was screened for the presence of the most important genes related to the RNAi machinery and for further exploration of essential genes to be silenced through RNAi technology. Similarity searches were performed using as reference preferably the *D. melanogaster* sequences because it is the species more phylogenetically related to *A. fraterculus* with the complete genome sequenced and fully annotated (Adams et al., 2000). This is first study that provides evidence of a functional RNAi machinery in the SA fruit fly.

4.1 Novel target genes found in *A. fraterculus* transcriptome

The target genes selected are involved in post-embryonic growth/development (90 genes) and sexual reproduction (53 genes). Fruit fly pests cause direct damage to fruits and vegetables by the puncture for oviposition by the female and the larval development inside the fruit (Aluja, 1994). Thus, the use of RNAi techniques in insect post-embryonic development is crucial for crops protection. In insect evolution increasing functional separation has occurred between the larval phase which is associated with the growth and accumulation of reserves, and the adult stage whose functions are reproduction and dispersal (Gillott, 1980). In the holometabolous insects, like the fruit flies, considerable differentiation of adult tissues occurs during metamorphosis, often from imaginal discs that are a group of cells that remain embryonic through the larval life (Gillott, 1980). Therefore, genes involved in the formation of posterior organs during the larval stage, as for instance the ovipositor, are very interesting for RNAi studies. Examples of genes involved in the formation of the posterior organs found in the SA fruit fly transcriptome are: *hedgehog* (*hh*), *homeobox protein abdominal-A* (*abd-A*) and *homeobox protein abdominal-B* (*abd-B*), that are part of a developmental regulatory system that provides cells with specific positional identities on the anterior-posterior axis (Celniker et al., 1990).

Genes involved in reproductive events such as oviposition regulation can be also screened in the *A. fraterculus* database. The *sex peptide receptor* (*spr*), for example, is a gene involved in the suppression of mating receptivity and induces the egg laying (Yapici et al., 2008). These genes in association can be studied for dsRNA delivery sequentially or dsRNA-concatemerized, between other possibilities.

4.2 Three pathways of the RNAi in SA fruit fly

RNAi pathways are found throughout eukaryotic organisms and are thought to be present in the last common ancestor of extant eukaryotes (Ketting, 2011). RNAi may have originated as a means of anti-viral defense and other functions, such as gene regulation, are thought to have evolved later (Shabalina and Koonin, 2008). In insects, three RNAi pathways can be distinguished: miRNA, siRNA and piRNA, based on the types of *Dicers* (*Dcr*) or *Argonaute*s (*Ago*) and the small RNAs related. Thus, the miRNA pathway consists of nuclear *Dicer* (*Drosha*/*Pasha*), cytoplasmic *Dicer* (*Dcr-1*/*Loquacious*), and *Ago-1* as core proteins. The siRNA pathway is activated by exogenous dsRNA and involves *Dcr-2*/*R2D2* and *Ago-2*. The piRNA pathway is also involved in defense against transposable elements and is characterized by *Ago* proteins of the Piwi class (*Aubergine*/*Ago-3*) and its independence of *Dcr* (Taning et al., 2016). The different RNAi pathways have distinct components that are intimately integrated with other essential cellular processes such as translation, RNA processing, cytoskeleton function, transcriptional regulation, protein turnover, protein trafficking, splicing, nuclear import and export, DNA repair, and other mRNA degradation pathways (Yamanaka et al., 2013).

Once the dsRNA has found its way into the target tissues and cells, one of the first requirements for RNAi is the presence and availability of the RNAi machinery components (Christiaens and Smagghe, 2014). Sequences representing all core RNAi genes were identified in the *A. fraterculus* transcriptome with a bitscore ≥150 and E-value ≤1e-5. The main domains of the *Drosha* and *Dcr* proteins were found to be conserved in *A. fraterculus* (Supplementary Material 2). The *Dcr* domains found were amino-terminal DExH-box helicase domains, PAZ domain, two RNaseIII domains, and carboxy-terminal dsRNA-binding domain (dsRBD) (Carmell and Hannon, 2004). Some members of the *Dcr* family differ from this general arrangement; for instance, some lack a functional helicase domain or a PAZ domain, or the number of dsRBD can range from zero to two (Macrae et al., 2006), such the sequence of *Dcr-2* in *A. fraterculus*, that does not show an dsRBD domain.

Unlike *Dcr*, *Drosha* has no PAZ and amino-terminal DExH-box helicase domain. Two cofactors with the conserved domains DSRM, Pasha and Loquacious, were also identified in *A. fraterculus*. These proteins are required to interact with the RNaseIII genes *Drosha* and *Dcr-1*, respectively (Carmell and Hannon, 2004). For *R2D2*, we found sequences inside the threshold defined, but without conserved domains. *R2D2* can form the *Dcr-2*/*R2D2* complex with *Dcr-2* and bind to siRNA to enhance sequence-specific messenger RNA degradation mediated by the RNA-initiated silencing complex (RISC). In *Drosophila*, *R2D2* acted as a bridge between the initiation and effector steps of the RNAi pathway by facilitating siRNA passage from *Dcr* to RISC (Liu, 2003).

The *Ago* superfamily is segregated into two clades, the *Ago* and the Piwi. In *Drosophila*, there are two *Ago* members (*Ago-1* and *Ago-2*) and three Piwi members (Piwi, Aubergine, and Ago-3) (Cerutti et al., 2000; Cox et al., 2000). These insects, *Ago-2* mainly mediates siRNA-directed mRNA cleavage, and *Ago-1* is mostly involved in miRNA-directed translational inhibition. *Argonaute* proteins can silence their targets, certain *Argonautes* cleave the target mRNA while others affect their targets using alternative mechanisms (Ketting, 2011). The biogenesis of smRNA duplexes in flies is uncoupled from their loading into *Ago-1* or *Ago-2* but is governed by the structure of the duplex. Duplexes that contain bulks and mismatches are sorted into *Ago-1*, while duplexes with a greater double-stranded structure will be sorted into *Ago-2*. However, since increasing the *Dcr-2*/*R2D2* complex concentrations reduces the number of siRNAs loaded into *Ago-1*, it was demonstrated that sorting could create competition for the substrate (Förstemann et al., 2007). *Ago* proteins are characterized by the presence of a PAZ domain and a C-terminal Piwi domain (Cerutti et al., 2000). In the *A. fraterculus* transcriptome of this study, we have identified the five members of the *Ago* protein superfamily, with the PAZ and Piwi conserved domains.

The third pathway of RNAi, the piRNA, involves the proteins *Aubergine*, *Ago-3*, *Piwi* and *Zucchini* (Hartig et al., 2007). Zucchini is an endoribonuclease that has a role in piRNA maturation. When absent, transposons are no longer repressed and no piRNAs are detectable (Pane et al., 2007). In *A. fraterculus* we found sequences of *Zucchini* protein with the presence of conserved domains superfamily PLD (Phospholipase D).

4.3 Duplication and loss of the RNAi-related genes in *A. fraterculus*

While the basic structures of the RNAi pathways and associated proteins are similar throughout eukaryotes, substantial gene duplication and gene loss have occurred in various insects. Duplications may lead to sub-functionalization or neofunctionalization in

RNAi pathways and could explain observed differences in the efficacy of RNAi across different insect groups. Loss of core RNAi-related genes may also explain observed decreases in RNAi efficacy (Dowling et al., 2016).

Our transcriptome analysis indicated gene duplication and gene loss events in *A. fraterculus*. Possible duplicates of *Drosha*, *Ago-2* and *R2D2* were found in the SA fruit fly transcriptome compared to *D. melanogaster*. Dowling et al (2016) also found possible duplicates of *Ago-2* in transcriptomes of other order insects, as *Peruphasma schultei* (Phasmatodea), *Prorhinotermes simplex* (Isoptera) and *Pseudomallada prasinus* (Neuroptera). These authors suggested that *Ago-2* was present in two copies in the last common ancestor of insects. Is it possible that SA fruit fly has three copies to *Dcr-2*, while *D. melanogaster* has only one copy. It is known that insects inherited a complete RNAi system from their common ancestor and, over time, diversified and expanded this original system (Dowling et al., 2016). One example of this is the *Piwi*/*Aub* gene. In insects, the piRNA pathway acts as a defense against transposons in the germ line. *Ago-3* and *Aubergine* operate in a loop (termed the ping-pong amplification loop) which alternately are cleaving sense and antisense transcripts. Piwi binds to the resulting piRNAs generated by the loop (Siomi et al., 2011). In the *A. fraterculus* transcriptome of this study, this gene is present with two copies, while Hemiptera species as *Acyrthosiphon pisum* has eight copies for this piRNA gene. Possibly, homologs of both *Piwi/Aub* and *Ago-3* were present in the last common ancestor of insects in multiple copies (Dowling et al., 2016). Although we have used a mix of all developmental stages of SA fruit fly with eggs, larvae, pupae and adult males and females to generate a comprehensive transcriptome, it must be remarked that the firm conclusion that a gene is lost from a species cannot be made since the gene in question may not have been expressed or very lowly expressed, at the time the samples were collected (Dowling et al., 2016).

4.4 SA fruit fly has auxiliary factors (RISC)

We found 19 intracellular factors that are associated or regulate the activity of the RISC complex. In the RISC assembly pathway for exogenous RNAi in the *D. melanogaster*, the siRNA duplex is transferred from complex B to the RISC-loading complex (RLC), consisting of *Dcr-2* and *R2D2*, previously shown. Next, *C3PO* (*translin* and *TRAX*) are joined with the RLC and the RISC complex [consisting of the *Dcr-1*, *Tudor-Staphylococcal nuclease* (*Tudor-SN*), *vasa intronic gene* (*VIG*), *FMR*, and *Ago-2* subunits] to generate the holoRISC by a *Drc2–Ago-2* interaction (Jaendling and McFarlane, 2010). These sequences were found in our *A. fraterculus* transcriptome all with conserved main domains and with the identity between 49-82% compared to *D. melanogaster* (Supplementary Material 2).

The nucleases involved in piRNA biogenesis, *Armitage* and *Homeless* (*spindle-E*) showed long sequences (> 4,000 nc) in *A. fraterculus*, while *Maelstrom* was represented by rather small fragments. Genes that encode *Gawky*, an RNAi effector, *Staufen*, an RNA-binding protein, *Elp-1*, a component of the core elongator complex involved in the RNAi, and *Clp-1*, a kinase that can phosphorylate siRNAs, as well the *RNA helicases Rm62* and *Belle* also showed long sequences (Findley, 2003; Vagin et al., 2006). The DEAD-box RNA helicase *Belle* has a function in the endo-siRNA pathway, interacting with *Ago-2* and endo-siRNA-generating loci and is localized in condensing chromosomes in a *Dcr-2*- and *Ago-2*- dependent manner (Cauchi et al., 2008). Another, the DEAD-box RNA helicase *PRP16* has an important role in the pre-mRNA splicing and was found in *A.*

fraterculus transcriptome with an identity of 93% as compared to *Drosophila* sequences (Ansari and Schwer, 1995).

4.5 dsRNA uptake genes

Except for *SID-1*, all dsRNA uptake components were found in the *A. fraterculus* transcriptome. This confirms the idea that this gene is absent in Diptera. However, it is known that the mechanism of uptake for dsRNA in *Drosophila* is unique compared with a typical model organism of *C. elegans*, which uses *SID-1* to transport dsRNA into the cells. Although no *SID-1* orthologues were found in Diptera (Huvenne and Smagghe, 2010), instead two scavenger receptors, namely *SR-CI* and *Eater*, were proven to undertake the transport function in *Drosophila* (Ulvila et al., 2006). Scavenger receptors are known to act as receptors for large molecules and/or microbes and play a role in phagocytosis (Prentice et al., 2015). In *A. fraterculus*, genes belonging to *SID-1* were found only for Eater and *SR-CI* sequences, this last one with conserved domains (Supplementary Material 2). Other genes coding for proteins involved in endocytosis were found in *A. fraterculus*, including *HPS4* (*Hermansky-Pudlak Syndrome 4* protein), a factor involved in the regulation of the association of late endosomes with RNA-processing GW bodies, *FBX011* (F-box motif, Beta-helix motif), a regulator of endosome trafficking and the *clathrin heavy chain* (*chc*), which is required for clathrin-mediated endocytosis (Swevers et al., 2013).

4.6 Nucleases in SA fruit fly development transcriptome

Nucleases sequences were identified only for *Snipper*, a histone involved in mRNA metabolism, siRNA degradation, and apoptosis, and for the *Nibbler*, a nuclease involved in the processing of 3′ends of miRNAs in *Drosophila* (Swevers et al., 2013). We identified the conserved domains ERI-1 3' exoribonuclease for *Snipper* sequences in *A. fraterculus* transcriptome (Supplementary Material 2).

4.7 Presence of genes involved in RNAi efficacy

We found five intracellular transport components classified by Yoon et al. (2016). The components *Vha16* (*Vacuolar H+ ATPase 16kD subunit 1*) and *VhaSFD* (*Vacuolar [+] ATPase SFD subunit*) involved in proton transport, *Rab7* (*Small Rab GTPases*) involved in endocytosis process, *Light* involved in lysosomal transport and *Idlcp* involved in exocytosis process.

Four antiviral RNAi was found in our *A. fraterculus* transcriptome, *Ars2*, a regulator involved in innate immunity via the siRNAs processing machinery by restricting the viral RNA production, *CG4572*, a protease implicated in systemic silencing and antiviral RNAi, *Egghead* (e*gh*), a seven-transmembrane-domain glycosyltransferase with innate immunity against RNA virus and *ninaC*, a protein involved in vesicle transport. All antiviral RNAi components were identified with conserved main domains (Supplementary Material 2).

Involved in lipid metabolism, *Saposin* receptor was identified with *Saposin A* and *Saposin B* conserved domains in *A. fraterculus* (Supplementary Material 2). *Saposin* is a small lysosomal protein that serves as activator of various lysosomal lipid-degrading enzymes (Darmoise et al., 2010).

4.8 Evidence for the sensitivity of larval stages of *A. fraterculus* to RNAi

To demonstrate the functionality of the RNAi in *A. fraterculus*, dsRNA targeting *V-ATPase* was evaluated using the in-house developed soaking bioassay. *V-ATPases* are ubiquitous holoenzyme among eukaryotes (Finbow and Harrison, 1997). These enzymes are composed of two subcomplexes, the cytosolic V1-domain, where ATP binding and hydrolysis takes place, and a transmembranous V0-domain, through which protons are translocated (Vitavska et al., 2003). The *V-ATPase* sequence analyzed in *A. fraterculus* belongs to V0-domain (Supplementary Material 2). The *V-ATPases* utilize the energy derived from ATP hydrolysis to transport protons across intracellular and plasma membranes of eukaryotic cells (Nelson et al., 2000). Although the V0 complex plays a key role in translocating the proton, only few reports on targeting V0-domain were published in insect studies (Ahmed, 2016). We therefore synthesized a dsRNA targeting *V-ATPase V0*-domain gene and attempted to knockdown this gene by dsRNA fragment of 483 bp length.

The results presented here indicated that *A. fraterculus* is very sensitive to RNAi, as a small dose of dsRNA (500 ng) administered by soaking for 30 min could induce significant RNAi responses (target gene suppression and death). The uptake of dsRNA for some organisms is dependent of *SID-1* homolog (Saleh et al., 2006). However, in the *A. fraterculus* transcriptome, as well as in other dipterans, no *SID-1* homolog is present. Another mode of uptake of dsRNA known in insects is endocytosis. In *D. melanogaster* dsRNA uptake by receptor-mediated endocytosis has been demonstrated (Ulvila et al., 2006). Studies showed that insect cells can take up siRNA from the environment, and the siRNA could move systemically through the insect body (Wuriyanghan et al., 2011). Our results suggest that uptake of dsRNA through endocytosis might also occur in *A. fraterculus* instead of by a *SID-1*-based mechanism. Besides that, larvae of *A. fraterculus* showed to be more sensitive to dsRNA uptake than *Drosophila* larvae. Alternative explanations for successful RNAi using soaking as the delivery method could be the fact that the dsRNA is also absorbed through the tracheal system, through the intersegmental membranes of the thorax or taken up orally from the soaking solution (Gu and Knipple, 2013).

The effective response of gene silencing as showed by *A. fraterculus* at 48 h after dsRNA soaking, resulted in mortality of these larvae. The *V-ATPase* sequence from the *A. fraterculus* transcriptome contains the *VMA21*, a short domain that has two transmembrane helices (Supplementary Material 2). The product of the *VMA21* gene is an 8.5 kDa integral membrane with a C-terminal di-lysine motif that is required for retention in the endoplasmic reticulum, and disruption of the gene causes failure to assemble a stable Vo, rapid turnover of *Vph1p* subunit (that contains charged residues that are essential for proton translocation) and consequent loss of *V-ATPase* function (Hill and Stevens, 1994). In other dipterans species, the *V-ATPases* knockdown responses were variable. In *B. dorsalis*, the ingestion of 2000 ng *V-ATPase D* (V1-domain) dsRNA through diet caused only 35% of gene silencing after four days, (Li et al., 2011). The neonate larvae of *D. melanogaster* when soaked in 500 ng of *V-ATPase E* (V1-domain) dsRNA caused a decrease of 49% in gene expression and feeding larvae caused 56% knockdown with 70% mortality (Whyard et al., 2009). These studies suggest indeed that the silencing of *V-ATPase* subunits genes shows variable results according to targeted subunit and insect species.

4.9 *Dcr-2* and *Ago-2* respond to dsRNA exposure

To investigate the regulation of siRNA genes during an RNAi experiment, the expression of the two siRNA pathway genes following dsRNA soaking was determined. The upregulation of the *Dcr-2* at 24 h after the dsRNA soaking demonstrated that the RNAi response in *A. fraterculus* is active. The *Dcr-2* is a specialized ribonuclease that initiates RNAi by cleaving dsRNA substrates into small fragments of about 25 nucleotides in length (Macrae et al., 2006). In an intact *Dcr* enzyme, the distance between the PAZ and RNase III domains matches the length spanned by 25 base pairs of RNA. Thus, *Dicer* itself is a molecular ruler that recognizes dsRNA and cleaves a specified distance from the helical end (Macrae et al., 2006). The PAZ and RNase III domains from *Dcr-2* found in *A. fraterculus* transcriptome are shown in the Supplementary Material 2.

After *Dcr* processing, the siRNAs are then picked up by the RISC and are unwound to become a single strand that is referred to as the guide strand. The RISC complex along with the guide strand pairs with the homologous mRNA, which is then cleaved by *Ago-2*. PAZ and PIWI are the main domains of the *Ago-2* protein. The PAZ domain has been suggested to be involved in the RNA binding, whereas the PIWI domain is similar to RNase H in structure and function and causes the cleavage of the target mRNA. The *Ago-2* domains were found in the *A. fraterculus* transcriptome (Supplementary Material 2).

4.10 dsRNA is degraded in *A. fraterculus* body fluid

Only after 4 h of incubation, some degradation was observed of ds*GFP* (0.5 mg/ml) using body fluid from *A. fraterculus* larvae. Liu et al. (2012) verified ds*GFP* degradation only after 3 h of incubation using hemolymph of *Bomyx mori* larvae. On the other hand, the authors verified that ds*GFP* degradation in midgut juice occurred at less than 10 min. Christiaens et al. (2014) demonstrated a rapid and strong degradation of dsRNA after 1 h in aphid hemolymph (*A. pisum*).

Usually, a high concentration of body fluid from dipteran insects is required to degrade dsRNA. For *A. suspensa*, for example, Singh et al. (2017) showed that 4.44 mg/ml of body fluid was required to degrade 50% of dsRNA, while for *Spodoptera frugiperda* a very low concentration of hemolymph (0.11 mg/ml) was enough to degrade dsRNA within an hour. Singh et al. (2017) also suggested that the abundance or expression of genes coding for dsRNases can be lower in these insects when compared to that in insects from other orders. This was noted in the bioinformatics analyses, that showed only a nuclease (Snipper) involved in the siRNA degradation in the SA fruit fly life stage transcriptome, based on the lists previously reported (Prentice et al., 2015; Swevers et al., 2013; Yoon et al., 2016).

5 Conclusion

The present project made available more than 84,000 new queries related to the developmental of *A. fraterculus* and a database of 143 novel and different target genes to dsRNA delivery bioassays. This transcriptome database is a handy tool for research on the SA fruit fly, especially in studies with a focus on RNAi. The identification of the RNAi machinery genes combined with dsRNA soaking, siRNA genes expression and dsRNA degradation bioassays clearly demonstrated that an RNAi response is active in *A. fraterculus*. The presence of RNAi machinery and efficacy genes by transcriptome analysis confirm the RNAi functionality in *A. fraterculus* and the sensitivity of this species to take up dsRNA to induce an RNAi response. Interestingly, we demonstrated that soaking of the larval stages in ds*V-ATPase* lead to a strong gene-silencing and this

611 concurred with a strong mortality of 40%. This delivery of soaking demonstrates that
612 dsRNA delivery can also be efficient via dermal contact on the insect. Our data
613 demonstrated the existence of a functional RNAi machinery in *A. fraterculus* and an easy
614 robust physiological bioassay with the larval stages that can be used for *in vivo* screening
615 of target genes for RNAi-based control of fruit fly pests.

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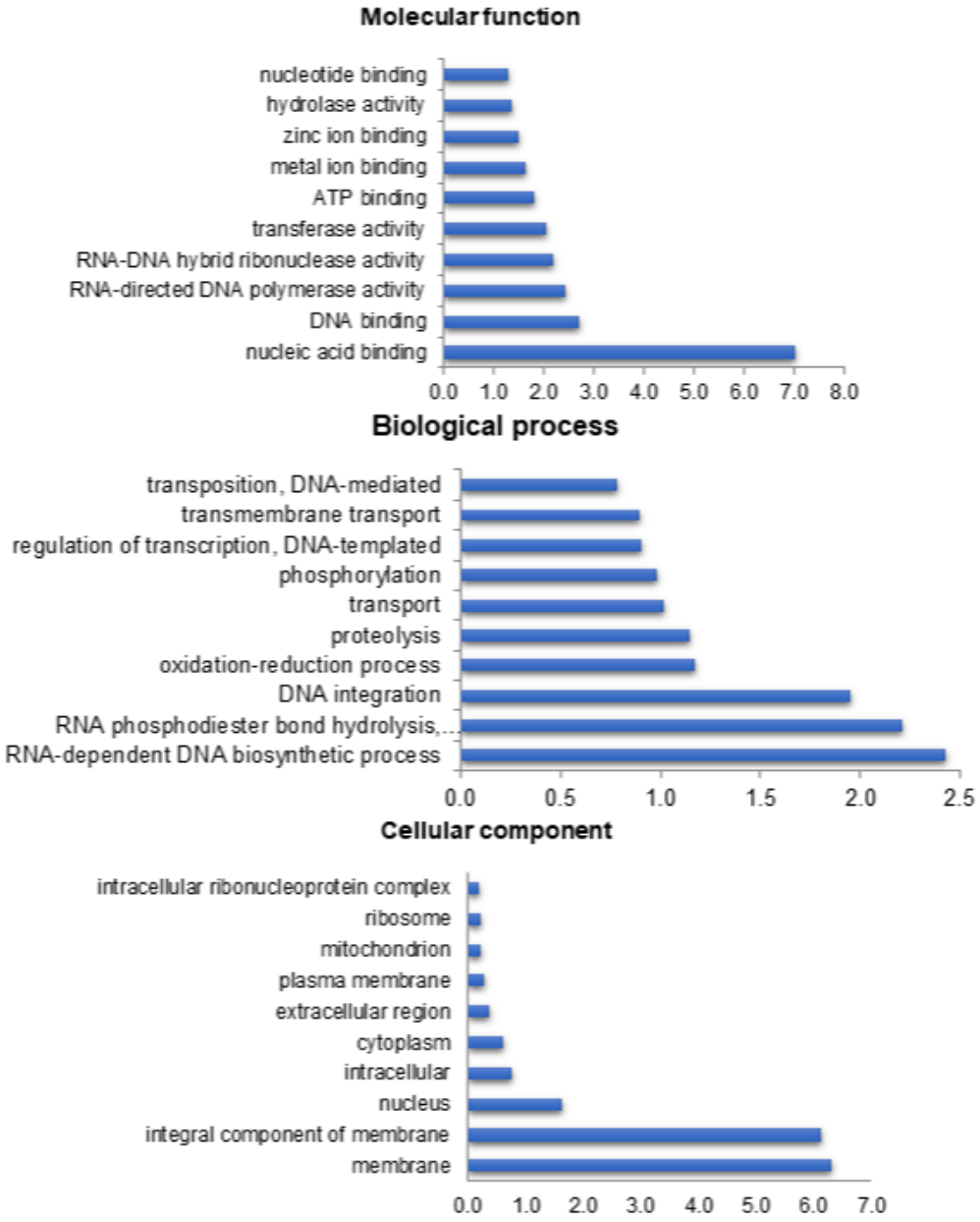
868

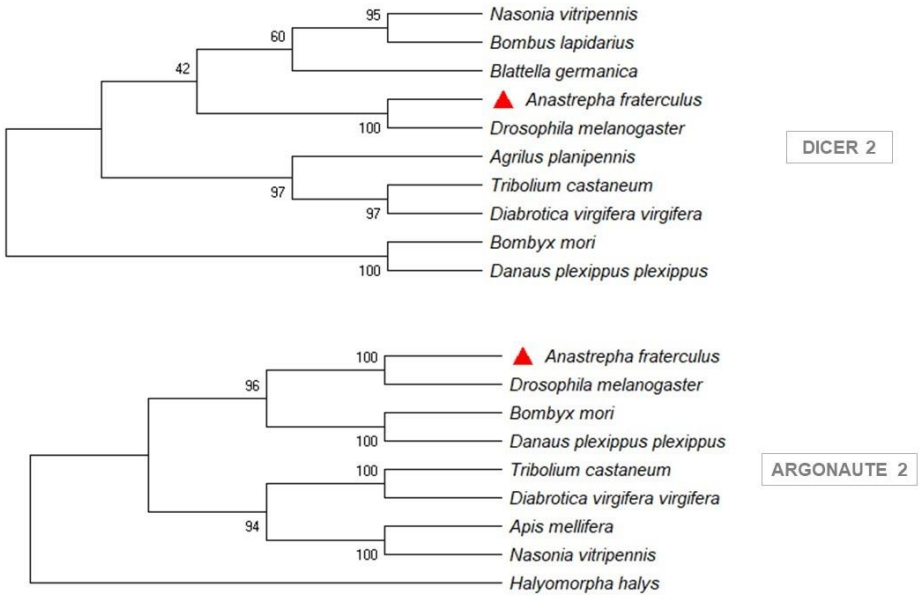


869

870 **Figure 1.** Percentage of *Anastrepha fraterculus* contigs assigned to a certain gene
871 ontology term as predicted by QuickGO from EBI. Top 10 terms are shown.

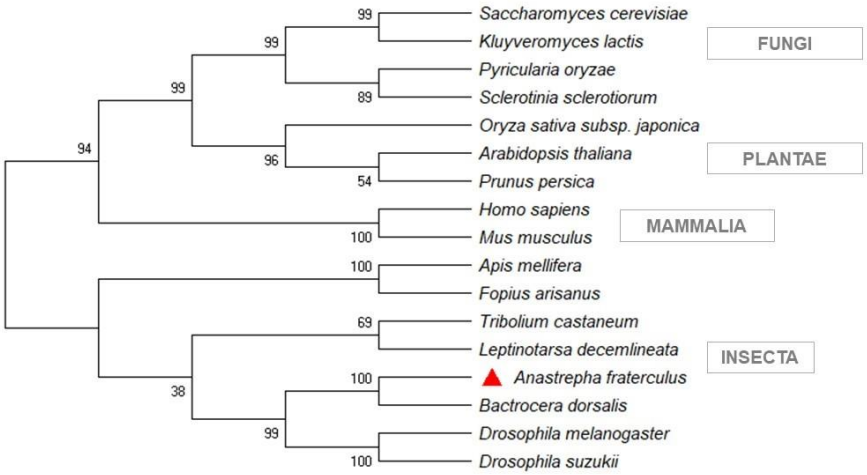
RNAi pathway	Gene	*A. fraterculus*	*Drosophila*	*Triboilum*	*Nasonia*	*Acyrthosiphon*
miRNA	Dicer-1	1 (=)	1	1	1	2
	Argonaute-1	1 (=)	1	1	1	2
	Loquacious	1 (=)	1	1	1	2
	Drosha	2 (+)	1	1	1	1
	Pasha	1 (=)	1	1	1	4
siRNA	Dicer-2	3 (+)	1	1	1	1
	Argonaute-2	2 (+)	1	2	2	1
	R2D2	2 (+)	1	2	1	1
piRNA	Aub/Piwi	2 (=)	2	1	2	8
	Argonaute-3	1 (=)	1	1	1	1
Sid	Sid-1	0 (=)	0	3	1	1

Figure 2. Copy number of the ten RNAi-related genes and *SID-1* found in *Anastrepha fraterculus* transcriptome by Trinity and in other insect species (showed by Dowling et al. 2016). The number of copies showed in *A. fraterculus* is compared to *Drosophila*. (=) same, (+) duplication (-) loss.



877

878 **Figure 3.** Phylogenetic trees of siRNA pathway genes, *Dicer 2* (*Dcr-2*) and *Argonaute 2*
879 (*Ago-2*). MEGA X was used to construct the phylogenetic trees with Neighbor-Joining
880 method. *Anastrepha fraterculus* sequences from transcriptome was marked with a red
881 triangle. All accession numbers are shown in Supplementary Table S4.



882

883 **Figure 4**. Phylogenetic tree of target gene of silencing, *V-ATPase*. MEGA X was used to
884 construct the phylogenetic tree with Neighbor-Joining method. *Anastrepha fraterculus*
885 sequence from transcriptome was marked with a red triangle. All accession numbers are
886 shown in Supplementary Table S4.

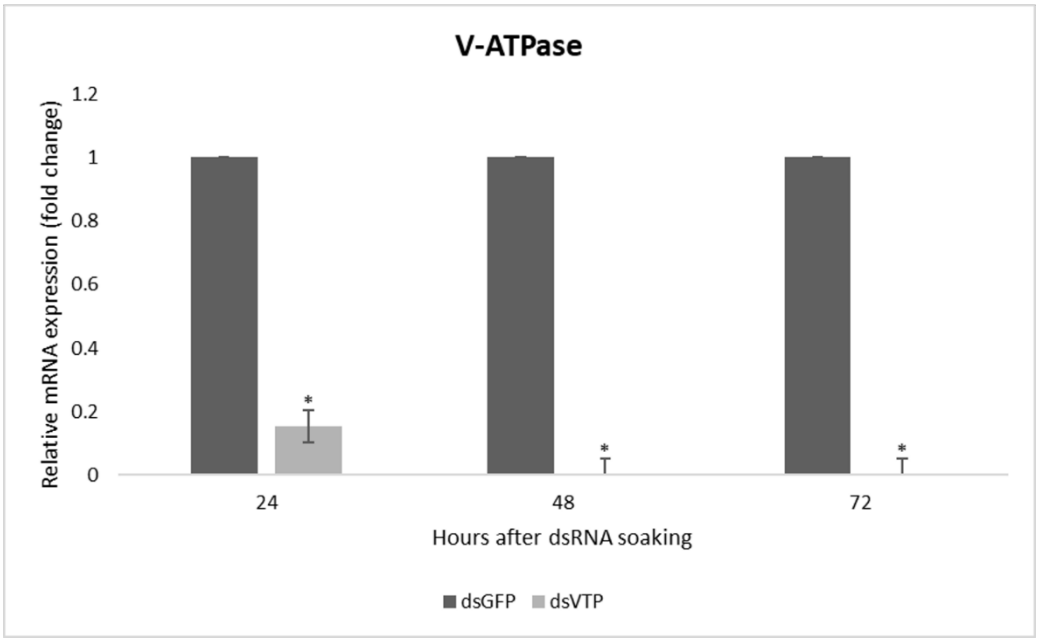


Figure 5. Relative mRNA expression of *V-ATPase* in *Anastrepha fraterculus* larvae after 24, 48 and 72 hours soaking in dsRNA (500 ng/µl). The mRNA levels were normalized using α-tubulin and actin as reference genes. The columns represent the mean ± SE (n = 3).

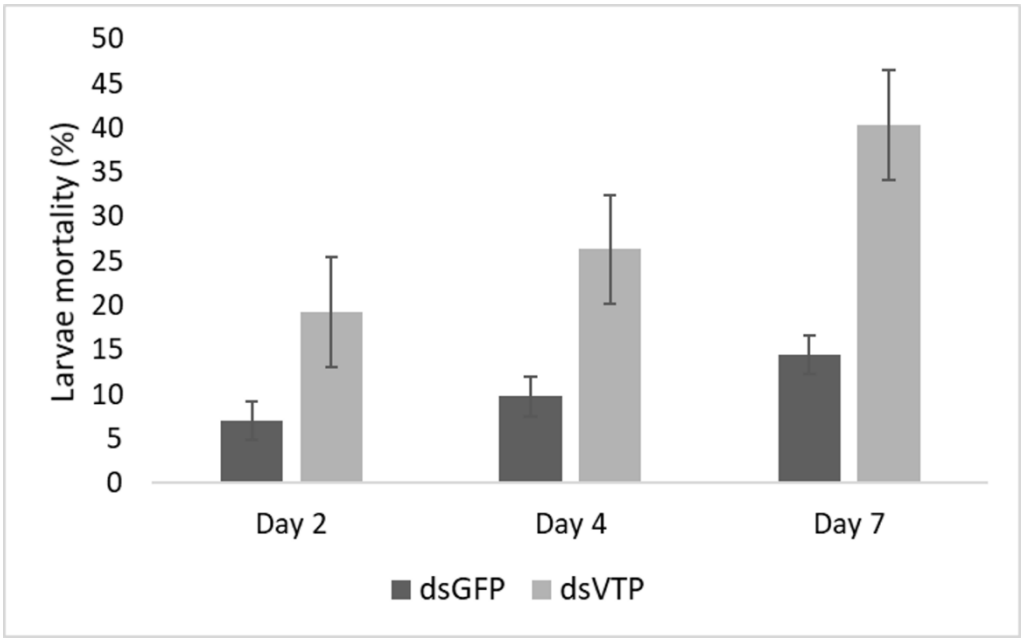


Figure 6. Mortality cumulative of *Anastrepha fraterculus* larvae (n = 57) after soaking in dsRNA solution (500 ng/µl) from *V-ATPase* (ds*VTP*) and *GFP* control (ds*GFP*) at 2, 4 and 7 days.

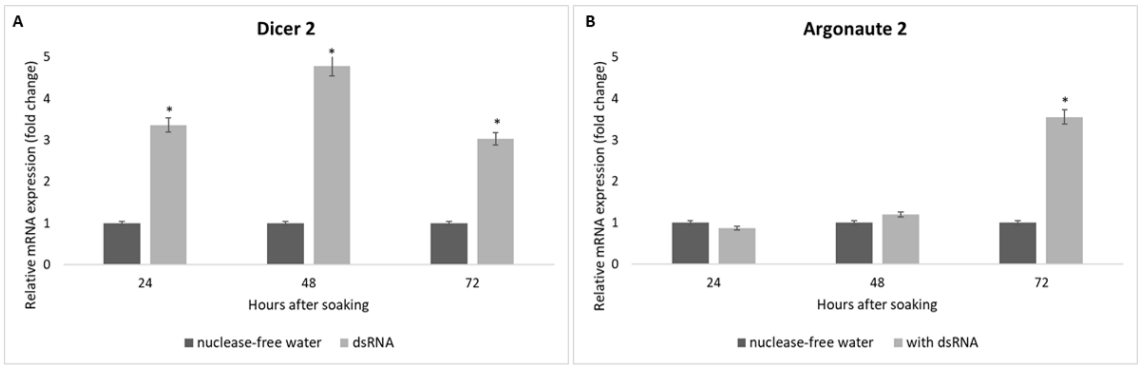


Figure 7. Relative mRNA expression of *Dicer-2* **(A)** and *Argonaute-2* **(B)** in *Anastrepha fraterculus* larvae in response to ds*GFP* soaking after 24, 48 and 72 hours (500 ng/µl). Nuclease-free water was used as control. The mRNA levels were normalized using α-*tubulin* and a*ctin* as reference genes. The columns represent the mean ± SE (n = 3). *p ≤ 0.05 (t-test).

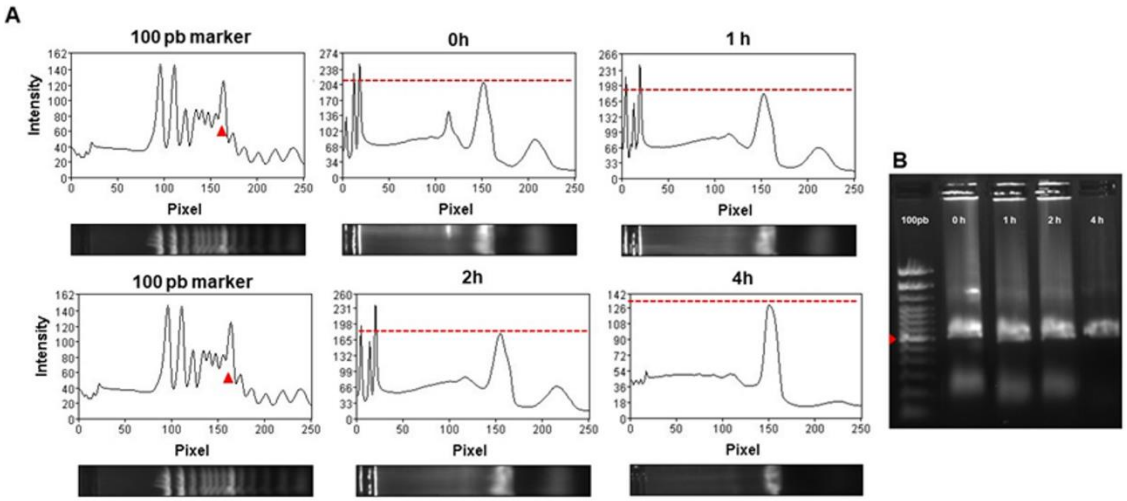


Figure 8. dsRNA degradation assay. The peak at 150 pixels (Δ) indicate the band intensity of the dsRNA when incubated **(A)**. Agarose gel image show the dsRNA (500 pb) degradation **(B)**. The triangle (Δ) indicate the fragment size of the ds*GFP*. Incubation of 20 µl (500 ng) ds*GFP* with 2 µl of body fluid from *Anastrepha fraterculus* larvae. Aliquots were removed at the times indicated. The samples were visualized by electrophoresis on a 1.5% agarose gel and analyzed using the Gel Analyzer software. Marker used was 100 pb.

Table 1. Overview of the presence of genes related to the RNAi pathways in the *Anastrepha fraterculus* transcriptome

	Contig	First hit tblastn	ID taxon homologue	Comparison to homologue	Identity (%)
miRNA					
Dicer-1	TRINITY_DN33861_c2_g1_i1	Endoribonuclease 9 [Drosophila melanogaster]	Q9VCU9	E= 0.0; bits= 2728	62
Argonaute-1	TRINITY_DN32900_c0_g1_i7	Argonaute-1, isoform A [Drosophila melanogaster]	Q32KD4	E= 0.0; bits= 1823	94
Loquacious	TRINITY_DN27977_c3_g1_i4	Loquacious [Drosophila melanogaster]	Q4TZM6	E= 6e-106; bits= 332	72
Drosha	TRINITY_DN30547_c4_g2_i1	Drosha [Drosophila melanogaster]	Q7KNF1	E= 0.0; bits= 1719	73
Pasha	TRINITY_DN28163_c0_g1_i6	Partner of drosha, isoform B [Drosophila melanogaster]	A0A0B4KI70	E= 0.0; bits= 809	70
Exportin-5	TRINITY_DN23399_c0_g1_i2	exportin-5 isoform X1 [Drosophila ficusphila]	A0A1W4VG06	E= 0.0; bits= 1634	67
siRNA					
Dicer-2	TRINITY_DN32516_c1_g2_i1	Dicer-2, isoform A [Drosophila melanogaster]	A1ZAW0	E= 0.0; bits= 1582	48
Argonaute-2	TRINITY_DN30039_c4_g1_i5	Protein argonaute-2 [Drosophila melanogaster]	Q9VUQ5	E= 0.0.; bits= 834	53
R2D2	TRINITY_DN28410_c0_g2_i4	R2D2 [Drosophila melanogaster]	Q2Q0K7	E= 9e-085; bits= 277	47
piRNA					
Argonaute-3	TRINITY_DN27717_c4_g1_i3	Protein argonaute-3 [Drosophila melanogaster]	Q7PLK0	E= 0.0; bits= 1056	57
Piwi	TRINITY_DN30302_c0_g2_i1	Protein piwi [Drosophila melanogaster]	Q9VKM1	E= 0.0; bits= 1046	63
Aubergine	TRINITY_DN30302_c0_g1_i1	Protein aubergine [Drosophila melanogaster]	O76922	E=0.0.; bits= 1081	64
Zucchini	TRINITY_DN31164_c0_g2_i2	Zucchini [Drosophila melanogaster]	L0CR90	E= 3e-053; bits= 183	42
Auxiliary factors (RISC)					
Tudor-SN	TRINITY_DN30816_c0_g1_i2	LD20211p [Drosophila melanogaster]	Q9W0S7	E= 0.0; bits= 1503	82
Vasa intronic (VIG)	TRINITY_DN23682_c0_g1_i2	LD07162 [Drosophila melanogaster]	Q9V426	E= 1e-066; bits= 233	49
FMR	TRINITY_DN33674_c0_g2_i3	Synaptic functional regulator FMR1 [Drosophila melanogaster]	Q9NFU0	E = 0.0; bits =750	74
Rm62	TRINITY_DN31247_c0_g1_i3	ATP-dependent RNA helicase p62 [Drosophila melanogaster]	P19109	E= 0.0; bits= 716	91
Translin	TRINITY_DN31480_c3_g3_i11	GM27569p [Drosophila melanogaster]	Q7JVK6	E= 2e-122; bits= 372	74
Translin associate fator X	TRINITY_DN24775_c0_g1_i2	translin-associated protein X [Drosophila ficusphila]	A0A1W4VFE4	E= 4e-124; bits= 367	61
Armitage	TRINITY_DN31912_c0_g1_i3	Probable RNA helicase armi [Drosophila melanogaster]	Q6J5K9	E= 0.0.; bits= 1164	50
Homeless (spindle-E)	TRINITY_DN31966_c0_g1_i1	ATP-dependent RNA helicase spindle-E [Drosophila melanogaster]	Q9VF26	E= 0.0; bits= 1281	48
Maelstrom	TRINITY_DN28061_c2_g2_i5	Protein maelstrom [Drosophila yakuba]	B4PIP5	E= 6e-085; bits= 279	38
HEN1	TRINITY_DN27986_c1_g1_i3	Small RNA 2'-O-methyltransferase [Drosophila melanogaster]	Q7K175	E= 3e-103; bits= 319	47
RNA helicase Belle	TRINITY_DN28586_c1_g3_i2	ATP-dependent RNA helicase bel [Drosophila melanogaster]	Q9VHP0	E= 0.0; bits= 892	86
PRP16	TRINITY_DN32795_c0_g2_i1	pre-mRNA-splicing factor ATP-dependent RNA [Drosophila ficusphila]	A0A1W4VUB2	E= 0.0; bits= 737	93
Gemin3	TRINITY_DN30190_c0_g1_i1	BcDNA.LD05563 [Drosophila melanogaster]	Q9V3C4	E= 3e-131 bits= 430	49
Gawky	TRINITY_DN27487_c0_g4_i19	Protein Gawky [Drosophila melanogaster]	Q8SY33	E= 0.0; bits= 803	55
Staufen	TRINITY_DN33993_c3_g1_i10	Maternal effect protein staufen [Drosophila melanogaster]	P25159	E= 2e-159; bits= 523	51
Clip 1	TRINITY_DN32205_c1_g4_i1	CLIP-associating protein [Drosophila melanogaster]	Q9NBD7	E= 0.0; bits= 1765	64
Elp-1	TRINITY_DN33357_c0_g1_i4	Putative elongator complex protein 1 [Drosophila melanogaster]	Q9VGK7	E= 0.0; bits= 1102	48
GLD-1	TRINITY_DN24535_c0_g1_i2	Protein held out wings [Drosophila melanogaster]	O01367	E= 0.0; bits= 527	86
ACO-1	TRINITY_DN30096_c0_g1_i6	1-aminocyclopropane-1-carboxylate oxidase [Bactrocera dorsalis]	A0A034VX75	E= 0.0; bits= 753	92
dsRNA uptake					
Scavenger receptor	TRINITY_DN31545_c2_g1_i7	Scavenger receptor isoform A [Drosophila melanogaster]	Q9VM10	E= 0.0; bits= 717	66
Eater	TRINITY_DN33643_c4_g2_i2	Eater [Drosophila melanogaster]	Q9VB78	E= 6e-107; bits= 370	41

Clathrin Heavy chain	TRINITY_DN29160_c0_g1_i4	Clathrin heavy chain [Drosophila melanogaster]	P29742	E= 0.0; bits= 3150	94
FBX011	TRINITY_DN32848_c4_g1_i12	GM01353p [Drosophila melanogaster]	Q6NQY0	E= 0.0; bits= 1540	86
HPS4 = CG4966	TRINITY_DN31238_c0_g1_i2	Hermansky-Pudlak syndrome 4 ortholog [Drosophila melanogaster]	A1ZAX6	E= 0.0; bits= 604	61
Adaptor protein 50 (Ap50)	TRINITY_DN29475_c0_g1_i1	AP-50 [Drosophila simulans]	B4R022	E= 0.0; bits= 899	99
TRF3	TRINITY_DN30474_c2_g1_i5	Similar to Drosophila transferrin (Fragment) [Drosophila yakuba]	Q6XHM9	E= 5e-098; bits= 294	77
Sortilin Like Receptor	TRINITY_DN26733_c0_g2_i34	Sortilin-related receptor (Fragment) [Bactrocera dorsalis]	A0A034V651	E= 0.0; bits= 856	79
Innexin2 (Gap Junction)	TRINITY_DN33133_c1_g1_i6	Innexin inx2 [Drosophila melanogaster]	Q9V427	E= 0.0; bits= 644	93
Low density lipoprotein	TRINITY_DN19392_c0_g3_i1	Low-density lipoprotein receptor-related [Drosophila melanogaster]	A1Z9D7	E= 0.0; bits= 1407	83
TRF2	TRINITY_DN32249_c1_g1_i3	LD22449p [Drosophila melanogaster]	Q9VTZ5	E= 0.0; bits= 1307	76
Intracellular transport					
Vha16	TRINITY_DN29956_c2_g1_i7	V-type proton ATPase 16 kDa subunit [Drosophila melanogaster]	P23380	E= 2e-088; bits= 284	95
VhaSFD	TRINITY_DN26174_c1_g1_i6	V-type proton ATPase subunit H [Drosophila melanogaster]	Q9V3J1	E= 0.0; bits= 675	90
Small Rab GTPases (Rab7)	TRINITY_DN30000_c1_g3_i9	CG5915 protein [Drosophila melanogaster]	O76742	E= 9e-125; bits= 371	87
Light	TRINITY_DN31345_c1_g2_i1	LD33620p [Drosophila melanogaster]	Q7PL76	E= 0.0; bits= 1113	67
Idlcp (Exocytocis)	TRINITY_DN46925_c0_g1_i1	Inner dynein arm light chain, axonemal [Drosophila melanogaster]	Q9VGG6	E= 1e-164; bits= 463	90
Antiviral RNAi					
SRRT = Ars2	TRINITY_DN31881_c2_g1_i5	Serrate RNA effector molecule homolog [Drosophila melanogaster]	Q9V9K7	E= 0.0.; bits= 1823	94
CG4572	TRINITY_DN33767_c1_g1_i2	Carboxypeptidase [Drosophila melanogaster]	Q9VDT5	E= 0.0; bits= 749	73
Egghead	TRINITY_DN32129_c1_g1_i5	Beta-1,4-mannosyltransferase egh [Drosophila melanogaster]	O01346	E= 0.0; bits= 863	94
ninaC	TRINITY_DN26176_c0_g1_i5	Neither inactivation nor afterpotential protein C [Drosophila melanogaster]	P10676	E= 0.0; bits= 1894	83
Nucleases					
Snipper	TRINITY_DN31391_c0_g1_i1	LD16074p [Drosophila melanogaster]	Q95RQ4	E= 7e-128; bits= 388	65
Nibbler	TRINITY_DN29782_c2_g2_i1	Exonuclease mut-7 homolog [Drosophila melanogaster]	Q9VIF1	E= 2e-152; bits= 475	44
Lipid metabolism					
Saposin receptor	TRINITY_DN32577_c3_g2_i1	Saposin-related, isoform B [Drosophila melanogaster]	Q8IMH4	E= 0.0; bits= 1021	58

Supplementary Material 1

Target-genes related to biological processes involved in post-embryonic growth/development and reproduction of *A. fraterculus (*.xls)

Supplementary Material 2

RNAi machinery genes - Sequences of *Anastrepha fraterculus*: Comparasion with *Drosophila* or Tephritidae species (132p) (.docx)

Supplementary Material 3

Table S1. Primers used in the South American fruit fly bioassays

Gene	Primer name	Primer sequence (5' to 3')	Product size (pb)
V-ATPase	dsvtp_F	TAATACGACTCACTATAGGGAGATGCATATTCGTTCAGGCACA	483
	dsvtp_R	TAATACGACTCACTATAGGGAGACAGCGCATTCAAAGTGGTCT	
	vtp_F	CCTTCCTCATGTTGTGCTCC	219
	vtp_R	CAGCGCATTCAAAGTGGTCT	
GFP	dsgfp_F	TAATACGACTCACTATAGGGAGATCGTGACCACCCTGACCTAC	560
	dsgfp_R	TAATACGACTCACTATAGGGAGATCGTCCATGCCGAGAGTGAT	
Actin	act_F	TACACTGGAACTAACGCGGT	212
	act_R	GTCGAACCACCACTCAACAC	
α-Tubulin	tub_F	CGAGGCCTCAAACATGATGG	155
	tub_R	GGCACCAGTCCACAAATTGT	
Dicer 2	dcr2_F	CCGTAGCACTTTCGTTAGA	122
	dcr2_R	GGCCGATATTCGTTGTTTG	
Argonaute 2	ago2_F	GCAGAGACAGACTCCTATTC	118
	ago2_R	GCTTCTTTGGGACGTAGAT	

The T7 RNA polymerase promoter is underlined.

Table S2. Overview of the Illumina sequencing and *de novo* assembly statistics of the life stages of *Anastrepha fraterculus*

Total of paired-end reads	103,808,135
Total of contigs	84,105
Total of transcripts	163,359
GC (%)	38,82
Contig N50	1,898
Average contig length (bp)	956.50
Median contig length (bp)	448.00
Total assembled bases	156,252,865

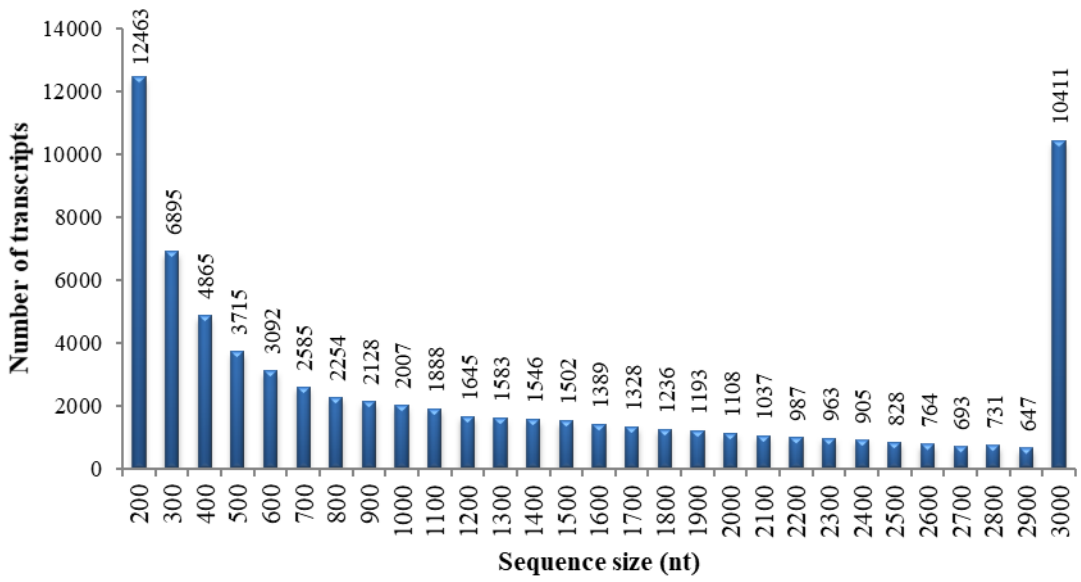


Figure S9. Length distribution of contigs in *Anastrepha fraterculus* transcriptome (only contigs of Eukaryote).

Figure S2. Distribution of Diamond similarity search. A) Distribution of the total hits against the UniProt-trEMBL database. B) Sequence comparison to insect species from the distribution of Diamond hits (E-value 1e-10).

Table S3. Species distribution of top 30 hits in Diamond searches (e-value 1e-10) of the data against the UniProt-trEMBL database.

Top30	Species	hits	(%)
1	*Ceratitis capitata*	12,050	16.46
2	*Zeugodacus cucurbitae*	11,463	15.66
3	*Bactrocera dorsalis*	11,226	15.34
4	*Bactrocera latifrons*	11,044	15.09
5	*Bactrocera tryoni*	6,883	9.40
6	*Tabanus bromius*	1,240	1.69
7	*Lasius niger*	1,141	1.56
8	*Acyrthosiphon pisum*	999	1.36
9	*Acromyrmex echinatior*	692	0.95
10	*Lucilia cuprina*	578	0.79
11	*Musca domestica*	503	0.69
12	*Lygus hesperus*	491	0.67
13	*Harpegnathos saltator*	487	0.67
14	*Drosophila ananassae*	450	0.61
15	*Corethrella appendiculata*	445	0.61
16	*Stomoxys calcitrans*	437	0.60
17	*Drosophila subobscura*	391	0.53
18	*Bombyx mori*	387	0.53
19	*Dufourea novaeangliae*	365	0.50
20	*Camponotus floridanus*	347	0.47
21	*Nasonia vitripennis*	346	0.47
22	*Drosophila melanogaster*	327	0.45
23	*Fopius arisanus*	324	0.44
24	*Cuerna arida*	277	0.38
25	*Lepeophtheirus salmonis*	262	0.36
26	*Rhodnius prolixus*	243	0.33
27	*Trachymyrmex zeteki*	241	0.33
28	*Trachymyrmex septentrionalis*	235	0.32
29	*Homalodisca liturata*	229	0.31
30	*Trachymyrmex cornetzi*	226	0.31

Table S4. Number accession of sequences used in phylogenetic analysis

Number accession	Species
Dicer-2	
TRINITY_DN32516_c1_g2_i1	*Anastrepha fraterculus*
ABB54747.1	*Drosophila melanogaster*
NP_001107840	*Tribolium castaneum*
AUM60046.1	*Diabrotica virgifera virgifera*
K7J5H5	*Nasonia vitripennis*
A0A172M4U9	*Bombus lapidarius*
NP_001180543.1	*Bombyx mori*
OWR42902.1	*Danaus plexippus plexippus*
CCF23094.1	*Blattella germanica*
AJF15703.1	*Agrilus planipennis*
Argonaute-2	
TRINITY_DN30039_c4_g1_i5	*Anastrepha fraterculus*
ADQ27048.1	*Drosophila melanogaster*
NP_001107828	*Tribolium castaneum*
AUM60042.1	*Diabrotica virgifera virgifera*
XP_395048.4	*Apis melífera*
XP_008214882.1	*Nasonia vitripennis*
NP_001036995	*Bombyx mori*
EHJ72821.1	*Danaus plexippus plexippus*
XP_024214272.1	*Halyomorpha halys*
V-ATPase	
TRINITY_DN27448_c0_g3_i1	*Anastrepha fraterculus*
XP_011205737.1	*Bactrocera dorsalis*
NP_788549.1	*Drosophila melanogaster*
XP_016934184.1	*Drosophila suzukii*
XP_015834455.1	*Tribolium castaneum*
XP_023015994.1	*Leptinotarsa decemlineata*
XP_001120244.1	*Apis mellifera*
XP_011304607.1	*Fopius arisanus*
NP_011619.3	*Saccharomyces cerevisiae*
XP_453740.2	*Kluyveromyces lactis*
NP_001017980.1	*Homo sapiens*
NP_001074825.1	*Mus musculus*
XP_003710030.1	*Pyricularia oryzae*
XP_001586304.1	*Sclerotinia sclerotiorum*
NP_565728.1	*Arabidopsis thaliana*
XP_015635612.1	*Oryza sativa* subsp. *japonica*
XP_007212280.1	*Prunus persica*

Supplementary Material 4

BLASTp for identify confirm of machinery genes - Sequences of *Anastrepha fraterculus* transcriptome (76p) (.docx)

Concluding Remarks

- The fruit fly management research had a significant increase in the last decade. Although most studies have been conducted in the U.S., the fruit fly research is being conducted in 41 countries.

- The three species more studied are *C. capitata*, *A. ludens* and *B. dorsalis*.

- The main methodological approach used in the fruit fly studies is laboratory approach.

- Fruit fly monitoring is included in few studies and the Biological control is the most commonly control tactic studied, highlighting the use of parasitoids.

- The RNAi technique is performed mainly in studies of *Bactrocera* species.

- The *A. fraterculus* transcriptome generated more than 84,000 new queries related to developmental stages.

- A database of 143 novel target-genes related to post-embryonic growth and development of *A. fraterculus* larval stages and the reproduction events in the male and female adults is available for RNAi-based research.

- The transcriptome analysis showed that *A. fraterculus* presents the three pathways of RNAi and 55 genes related to the RNAi machinery. This Dipteran has duplication to Drosha, Dicer-2, Argonaute-2, and R2D2 genes.

- The delivery by soaking of larval stages in dsRNA leads to a strong gene-silencing and this concurred with 40% of larval mortality.

- The RNAi efficacy is correlated with the increase Dicer-2 and Argonaute-2 expression, evidenced the activation of the siRNA pathway in *A. fraterculus*.

- The design an affordable and easy method for testing RNAi in larval stages of *A. fraterculus*.

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