RNAi-mediated Knockdown of Male Fertility Genes in the Queensland Fruit Fly *Bactrocera tryoni* (Diptera: Tephritidae)

by

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ABSTRACT

The Queensland Fruit Fly (QFF) *Bactrocera tryoni* is Australia's most important horticultural pest. The Sterile Insect Technique (SIT) has been used to control this species for decades, but current radiation sterilizing methods can reduce the insects' abilities to compete for mates. In this study, RNA interference (RNAi) techniques were examined for their potential to sterilize male QFFs without adversely affecting their mating competitiveness. QFF adults were injected or fed double-stranded RNAs (dsRNAs) targeting spermatogenesis genes (*tssk1*, *topi* and *trxt*); quantitative reverse-transcriptase PCR analyses confirmed that transcript levels were reduced 60-80% for all three genes following injections, while feeding produced a 69.1% gene knockdown for *tssk1* only. Flies fed with *tssk1* and *topi* dsRNA produced 70% fewer viable offspring than the negative controls. Accessory gland protein transcripts were either unaffected or were upregulated in RNAi-treated males. These findings suggest that RNAi technology could enhance SIT efficacy in these pest insects.

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To my dad and grandpa

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1. INTRODUCTION

One of the main threats to modern horticulture is the invasion of crops by pest organisms, especially insects. Increasing globalization over the last few decades, due largely to increased international trade and tourism, has significantly limited our ability to control the spread of invasive insects (Suckling *et al.*, 2014). For many years, insecticides have been widely used to control these organisms, but there are growing concerns about the off-target effects of these chemicals on other species (Gamble *et al.*, 2010). The development of new ecologically-friendly methods of pest insect control is a high priority issue for scientists and conservationists alike.

1.1. Sterile Insect Technique for pest insect management

The Sterile Insect Technique (SIT) is considered an ideal biological control method for pest insects, since it is species-specific and does not involve dissemination of any chemical substances (Juan-Blasco *et al.*, 2014; Suckling *et al.*, 2014; Leftwich *et al.*, 2014). Originally proposed in the mid-1950s (Knipling, 1955), it typically involves the release of large numbers of sterile males, previously exposed to radiation, into the wild populations, with the goal of females mating with them and thereby reducing the number of viable offspring (Dominiak *et al.*, 2011). This technique is most effective in controlling species where females only mate once in their life. In these cases, mating with a sterile male completely eliminates the reproductive output of the female. In species where females mate multiply, the SIT has a smaller effect on the reproductive output of females because they can mate with a fertile male after mating with a sterile male.

Since its postulation, SIT has been used in the eradication or reduction of invasive and endemic pest insects in many countries. In the Netherlands Antilles (an island of 440 km^2),

sterile New world screwworm flies (*Cochliomyia hominivorax*) were released at 400 flies/km^2 each week by airplane for a 9-week period. After just three weeks, about 70% of new egg cases found were sterile. After the next three weeks of releases, sterility was 84%. And by the end of the third 3-week period, very few egg cases were to be found, and all were sterile (Bowman, 2006). Following this early success, *Cochliomyia hominivorax* populations were subsequently eliminated from North America to Panama using SIT (Suckling *et al.*, 2014).

In Africa, on the island of Zanzibar, the tsetse fly (*Glossina* sp.) was eliminated within four years by use of the SIT in a manner similar to that employed for screwworm. Almost eight million sterile male flies were released during the eradication campaign, with an average of 70,000 sterile males produced every week. At the end of 1997, an independent expert group confirmed that since September 1996, not a single wild fly had been captured in the once heavily infested areas of Zanzibar (Bowman, 2006).

In horticulture applications, SIT has been used especially for the control of fruit fly species. Since its implementation in 1987, the state of California has reduced the number of Mediterranean fruit flies (*Ceratitis capitata*) by 96%, and has similarly observed a 96% reduction in the total number of fly infestations across the state. Moreover, Chile's National Fruit Fly Program has successfully kept the country free of economic species of fruit flies, and remains one of the best fruit fly control programs in the world. In the 1970s, Japan successfully used SIT to eradicate the melon fly in island communities for the first time, with no harm to the health of the public or the environment. In Mexico, after four years of intensive eradication activities (1977–1982), the Mediterranean fruit fly was eradicated from an infested area of 640,000 hectares (6400 km^2) in the state of Chiapas. This was the first time that a tephritid fruit fly population was eradicated at a continental level, in a region of difficult topography, high ecological diversity, and using an environment-friendly technology (Enkerlin *et al.*, 2015).

Modern fruit fly management program officials suggest the use of genetic sexing strains (GSS) to improve SIT, by removing females from the production line before release of insects into the environment. Removal of the females might be achieved using sex linked differences in a phenotypic attribute, if available, such as pupal color, dieldrin resistance, malathion resistance, alcohol resistance or temperature sensitivity (Meats *et al.*, 2002). Elimination of females has important implications in terms of reduced rearing, transport, and release costs, and significantly increased effectiveness of SIT, as sterile males appear to disperse farther in the absence of sterile females, and do not waste their energy on attempting to mate with sterile females. In addition, by not releasing any females, damage of fruits caused by the "stings" of sterile females will be avoided, and a high sex-ratio of sterile males to wild females can be maintained (Haq *et al.*, 2013).

Recently, a new approach for genetic sexing was described by Leftwich *et al.* (2014), in which a strain of male insects carrying a female-specific lethal transgene was released. Matings of wild females with these genetically-modified males produced no viable female offspring, thereby decreasing the reproductive potential of the wild population; however, this method has only been tested in small population cages, and further research is needed to verify its efficacy at a larger scale. This technology, sometimes referred to as Release of Insects carrying a Dominant Lethal (RIDL), relies upon the integration of a lethality gene, usually regulated by a tetracycline-controlled transactivator (tTa) into the host's genome (Thomas *et al.*, 2000; Heinrich & Scott, 2000; Gong *et al.*, 2005). However, use of this method does not cause an immediate decrease in insect abundance, but the effects are observed in subsequent generations. In addition, since the resulting G_1 males will be heterozygous, the lethal gene will be lost eventually, assuming that it is still possible for new wild females to migrate into the population. Consequently, the RIDL method still requires continued production and release of insects until the population is adequately suppressed.

Hammond *et al.* (2016) recently developed a synthetic gene drive system designed to produce sterile females in the human malaria vector mosquito *Anopheles gambiae*. The process relied on homing endonuclease genes, which encode proteins that can recognize and cleave a 15- to 30-bp recognition sequence. When these homing endonucleases identify a chromosome containing a specific, uninterrupted recognition sequence, they induce a double-strand break (DSB), which is later repaired by homologous recombination using the homologous chromosome as a template. As a result, a heterozygote is transformed into a homozygote, allowing a mutant allele to increase its frequency and easily spread through the population. By targeting a female fertility gene with a homing endonuclease sequence, the research team developed a strain of mosquitoes that could promote the spread of a recessive sterility gene, engineered using CRISPR technology, into the population. If successful, this technology could potentially drive through the population to eliminate disease-bearing mosquitoes. Before this technology is used in the field, more effort will be required to identify the best genetic constructs to apply, to identify potential resistance mechanisms that might arise, and most importantly, extensive discussion is needed to consider whether such an extinction technology should be applied at all.

It is possible that a combination of SIT, RIDL and a gene drive system has the potential to control and eliminate a pest insect population effectively. The combined effect of these methods will possibly allow the release of only sterile males into a wild population and also maintain the female lethality gene in subsequent generations. Future studies should focus on the applicability of these technologies in the same pest management program.

1.2. *Bactrocera tryoni*: Biology of the species

The Queensland fruit fly (QFF) *Bactrocera tryoni* (Diptera: Tephritidae) is the most important pest in horticultural regions of eastern Australia, and is known to infest more than

100 native and introduced host plants (Dominiak *et al.*, 2015). Between 2003 and 2008, $128.7 M were spent on fruit fly control measures in Australia (Mo *et al.*, 2014). This species originates from the tropical rainforests of the north-eastern parts of the country, but with its successful spread to cultivated fruits and vegetables in more temperate regions of the country, it has become a costly pest (Reynolds & Orchard, 2015). The polyphagous nature of this pest, the climatic suitability of many regions within the country, and the expansion of the insect's cultivated host plants are considered the main factors contributing to this species' success.

Tephritid fruit flies typically emerge with immature reproductive organs and must forage for the nutrients required to complete their reproductive development and maturation in early adulthood (Fletcher, 1987; Taylor *et al.*, 2013). Fletcher (1987) determined that carbohydrates, amino acids, minerals, sterols, vitamins and water are the main components required in the adult fruit fly diet, but Taylor *et al.* (2013) has noted that while the importance of nutrition in the biology of tephritid fruit flies is clear, the question of how fruit flies, including QFF, obtain these nutrients in nature remains poorly understood. They have suggested that members of the Dacinae subfamily (in which QFF is included) most likely feed on extrafloral exudates, plant leachates and bacteria of the family Enterobacteriaceae. Weldon & Taylor (2011) found that wild and domesticated QFF can forage on bat guano, bird feces and pollen in the laboratory; however, the effect of these elements on reproductive development or longevity diminish when they are provided as a supplement to sugar.

Yeast hydrolysate (YH) seems to have a beneficial impact on several parameters of QFF biology, such as reproductive development, sexual activity, copula duration, sperm storage, remating inhibition and longevity (Taylor *et al.*, 2013). For instance, YH-fed QFF females show a rapid increase in growth of ovaries and accessory glands and accumulation of fat around the spermathecae, while females provided only sugar and water show negligible development of these reproductive tissues. Although males and females both benefit from the inclusion of

YH in their diet, females appear to have a greater need of this dietary supplement (Taylor *et al.*, 2013). The development of an artificial diet for QFF that provides all the nutritional requirements to the insects will be essential for any SIT program, where millions of vigorous insects must be produced on a regular basis.

QFFs are sexually active only in the evening, initiating copulations within approximately 30 min of dusk (Tychsen, 1977). Males call and court females with a set of acoustic, pheromonal and visual signals (Bellas & Fletcher 1979; Mankin *et al.*, 2008). Copula duration has not been linked to any measures of post-copulatory performance, such as sperm transfer, fertility or female sexual inhibition, but Taylor *et al.* (2013) consider that mating duration may impact male fitness, as long copulations may serve as mate guarding to prevent females from remating on the same evening, and thereby ensure a male's paternity. Pérez-Staples *et al.* (2010) suggest that copula duration in QFF appears to be largely under female control, and hence, weak or sterile males could potentially be discriminated against by female controlling this aspect of mating behavior.

Female QFFs have two sperm storage organs, a pair of spermathecae and a ventral receptacle (*bursa copulatrix*). Spermathecae are used for long-term storage and contain the majority of sperm, while the ventral receptacle appears to serve as a short-term storage organ, containing comparatively few sperm (Dallai *et al.*, 1993; Pérez-Staples *et al.*, 2007). Two studies (Harmer *et al.*, 2006; Radhakrishnan *et al.*, 2009) noted that, not surprisingly, female QFFs that mated with radiation-treated male QFFs stored far fewer sperm than the mates of fertile male QFF. Taylor *et al.* (2013) speculate that it is highly likely that low sperm numbers transferred by sterile males could induce females to later remate with other males, until the female receives a normal complement of sperm.

Radhakrishnan & Taylor (2007) provide strong evidence supporting the involvement of accessory gland fluids transferred with the ejaculate as mediators of sexual inhibition. Proteins

and other biochemical components from the male ejaculate pass through the female reproductive tract and disperse through her body while the pair mates (Radhakrishnan *et al.*, 2008). Virgin females injected with extracts from male accessory glands exhibit sexual inhibition similar to that of mated females. After mating, male accessory glands are greatly diminished in size and recover their original size gradually over the following day (Radhakrishnan & Taylor, 2008). These changes in accessory gland dimensions presumably reflect volume of contents, as fluids are transferred to females during copulation and then replenished in time for the next mating opportunity (Radhakrishnan *et al.*, 2008). Wei *et al.* (2015a) identified a list of accessory gland proteins in *B. dorsalis* that includes a juvenile hormone-binding protein, which is reported to promote sexual maturation in adult insects (Kolodziejczyk *et al.*, 2003).

SIT has been used for the management of QFF in Australia since the 1960s, but since the early 1990s, there has been a significant increase in interest in the use of this technique for QFF control, supported by the establishment of the Fruit Fly Exclusion Zone (FFEZ) in south-eastern Australia (Fanson *et al.*, 2014). Since its founding in 1996, the Elizabeth Macarthur Agricultural Institute (EMAI) (Camden, NSW, Australia) has produced more than 3.8 billion fruit flies to be used in SIT.

Despite the renewed interest in SIT for QFFs, the radiation sterilizing method used in SIT for QFF management has not proven to be ideal, since it shows some side effects that diminish the efficacy of the technique (Collins *et al.*, 2008). Radiation is often used to sterilize male insects released in SIT programs, but according to Collins *et al.* (2008) and Dominiak *et al.* (2014), this treatment can affect the ability of these insects to compete with wild males for mates. Hybrid crosses between closely related species can also produce sterility (Klassen & Curtis, 2005). However, this method of producing sterile males has not been pursued since it

was first proposed in the 1940s, as it is not clear whether hybrids would compete effectively with non-hybrids for the appropriate species' mates.

1.3. RNA Interference: silencing mechanism and applications

Raphael *et al.* (2004) suggested that the use of RNA interference (RNAi) knockdown technologies could be an effective approach with potential applications in pest insect control. RNAi is a sequence-specific post-transcriptional gene silencing process elicited by double-stranded RNA (dsRNA) that occurs widely among plants and animals (Kim *et al.*, 2015). Since its discovery in 1998, RNAi has revolutionized functional genomics due to its relatively easy use and its power as a reverse genetic tool. RNAi-high throughput screening is being used in fully genome-sequenced organisms to elucidate gene function related to numerous medically and agriculturally relevant topics. In addition, RNAi can be applied to organisms lacking genetic tools as it does not require genetic transformation methods, and it can be performed *in vivo*, allowing for the study of tissue-specific and life-stage specific phenotypes associated with gene expression, as well as complex phenotypes that cannot be modeled in cell-based assays.

Wilson & Doudna (2013) comprehensively describe the RNAi phenomenon as follows. The three pathways of RNAi (miRNA, siRNA, piRNA) share a common mode of action: The main effector is a ribonucleoprotein complex comprising an Argonaute family protein bound to a single-stranded 20-30 nucleotide RNA that grants specificity via base-pairing interactions with the gene target. This is known as the RNA-induced silencing complex (RISC) and it drives silencing of a target mRNA via degradation and/or transcriptional repression, depending on the host species.

DsRNAs may be endogenous, but they typically arise via viral infection or other exogenous sources. Once in the cytoplasm, dsRNAs are trimmed down to a siRNA duplex of the appropriate size for loading onto an Argonaute protein (Wilson & Doudna, 2013); this is

typically performed by a Dicer enzyme. Dicers are large endoribonucleases containing a helicase domain and an internally dimerized pair of RNase III domains (Kim *et al.*, 2006). According to Wilson & Doudna (2013), the resulting siRNA is a duplex of 21 to 25 nucleotide strands, bearing a two nucleotides overhang at each 3’ terminus and a phosphate group at each recessed 5’ terminus. With this enzymatic step and the subsequent loading of the RNA duplex onto Argonaute, Dicer may be aided by a dsRNA-binding protein (dsRBP) such as TAR RNA-binding protein (TRBP). These three proteins (Dicer, Argonaute and a dsRBP) constitute the RISC-loading complex (RLC), which is responsible for generating diced dsRNA and loading it onto Argonaute (Redfern *et al.*, 2013). Once the dsRNA helix is presented to Argonaute, the 3’ terminus and 5’ phosphate of the guide strand are bound by the protein’s PAZ and MID domains, respectively. RISC loading is coincident with the strand selection step, wherein one strand of the duplex is bound to Argonaute to direct silencing and the other strand is discarded. These strands are known as the guide and passenger strands, respectively, and their selection is a key determinant of the silencing that follows. The transient complex consisting of Argonaute bound to the guide strand and a passenger strand that has yet to be cleaved and/or dissociate is known as the pre-RISC assembly.

RISC performs cellular surveillance, binding single-stranded RNA (ssRNA) such as mRNA with complementarity to the Argonaute-bound guide strand. Guide strand nucleotides 2–6 constitute the seed sequence and initialize binding to the target. In cases of perfect complementarity (siRNA duplexes feature perfect base-pairing), RISC binds to target sequences and silences them via the slicing activity of Argonaute (Wilson & Doudna, 2013). Hannon (2002) described the ability of RNAi to amplify throughout an organism even when triggered by very small quantities of dsRNA, a systemic phenomenon seen in the nematode worm *Caenorhabditis elegans* and plants. He also described the “transitive RNA”, in which dsRNAs targeting the 3’ portion of a transcript results in suppression of that mRNA and in the

production of siRNAs homologous to the targeted region. In addition, siRNAs complementary to regions of the transcript upstream from the area targeted directly by the silencing trigger also appear and accumulate, presumably due to the action of an RNA-dependent RNA polymerase (RdRP). If these siRNAs are complementary to other RNAs, those are also targeted. However, Hannon pointed out that this phenomenon is not present in flies, which was later supported by Roignant *et al.* (2003) who demonstrated the absence of transitive and systemic responses in *Drosophila*. RNAi mechanisms clearly differ across species, and until RNAi is examined in more species, it is not possible to determine the optimal efficacy of RNAi in any particular target species.

There are growing numbers of studies that demonstrate that ingested dsRNA can induce RNAi in insects (Pitino *et al.*, 2011; Ratzka *et al.*, 2013; Whyard *et al.*, 2009, 2015). Li *et al.* (2011), Chen *et al.* (2015) and Zheng *et al.* (2015) have shown significant gene knockdown in the Oriental fruit fly *B. dorsalis* (a species closely related to QFF) through dsRNA feeding; however, it is not yet clear how the dsRNA enters the insect's cells. Two pathways for dsRNA uptake in insects have been described (Huvenne & Smagghe, 2010) - the transmembrane channel-mediated uptake mechanism based on *Caenorhabditis elegans*' SID-1 protein and an alternative endocytosis-mediated uptake mechanism. It is not known which insect species use just one or both of these dsRNA uptake pathways. SID-like proteins have been identified in many different insects (reviewed in Huvenne & Smagghe; 2010), but their involvement in dsRNA uptake is still poorly understood. Jose (2015) pointed out that the presence of SID-1-like genes in an organism is not necessarily an indicator of dsRNA uptake through a SID-1-dependent mechanism. *Drosophila* and mosquitoes can take up dsRNA despite the loss of SID-1 homologs in the dipteran lineage, so endocytotic mechanisms are most likely the primary means of dsRNA entry into cells in these insects.

RNAi has been used in QFF only to modify the sex ratio by altering the expression of the sex-determining gene *doublesex* (*dsx*) (Raphael *et al.*, 2004), which encodes a key terminal transcription factor in the sex-determination pathway that controls male and female sexual differentiation in many insects (Mysore *et al.*, 2015). QFF embryos injected with dsRNA targeting the male-specific exon of *dsx* developed as females, while the embryos injected with female-specific *dsx* dsRNA almost entirely developed as phenotypic males. RNAi in QFFs may prove useful in a SIT program where sex-sorting of the sterilized insects to isolate males could be achieved without much effort.

1.4. Spermatogenesis in insects: genes involved in male fertility

Sterility in male insects could be achieved by affecting testis development or spermatogenesis at different stages. Spermatogenesis has not been widely studied in many insects, but in those that have been examined, the process appears similar in various species, although the structures of the tissues and the timing of events can be highly variable (Dumser, 1980). In *Drosophila*, spermatogenesis begins by early larval stages, although the germline stem cells niche is formed during the late stages of embryogenesis (Le Bras & Van Doren, 2006). Typically, both germline and somatic stem cells are located at the apical tip of the testis, and under homeostatic conditions, stem cells divide asymmetrically to produce two cells: one cell maintains stem cell characteristics, while the other daughter cell initiates differentiation (Demarco *et al.*, 2014). The differentiating daughter cell is called a gonialblast, and it undergoes mitotic amplification divisions to generate a cyst of spermatogonia, which will differentiate into spermatocytes and ultimately, spermatids. Elongation and maturation of the spermatids is called spermiogenesis and is the final stage of spermatogenesis (Demarco *et al.*, 2014), after which the sperm is transported to the seminal vesicle.

Like all developmental processes, spermatogenesis is regulated by genes, some of which are expressed only in gonadal tissue, while others are also expressed elsewhere in the body, depending on their specific function. Several studies have examined the genetic regulation of spermatogenesis in insects (Perezgasga *et al.*, 2004; Barreau *et al.*, 2008; White-Cooper, 2010; Moon *et al.*, 2011; Volpi *et al.*, 2013), identifying the variety of genes involved in this process. Microarray analysis comparing different *Drosophila melanogaster* adult tissues revealed that ~50% of the genes in the genome are expressed in testes and 8% of the transcripts detected in adults are testis-specific, while a further 5% are testis-enriched (White-Cooper, 2010). Among the genes reported in previous studies as important in germ cell formation in *D. melanogaster* are: *mst77F*, which is involved in chromatin condensation during late spermatogenesis (Barckmann *et al.*, 2013); *Testis-specific serine/threonine-protein kinase 1* (*tssk1*), which has been suggested to play an important role in post-meiotic chromatin remodeling and male fertility (Xu *et al.*, 2008); *βtub85D*, which encodes for the β-tubulin protein and functions in meiotic spindle and axoneme structure (White-Copper, 2010); and dynein, another protein that plays a key role in male fertility, since it provides the propulsive force necessary for sperm motility. Wei *et al.* (2015b) and Dong *et al.* (2016) presented lists of testis-specific genes in *B. dorsalis* involved in spermatogenesis, which include: *tssk1* (previously mentioned) and 2; *cyclin B* and *J*, required for meiosis during germ cell development; *topi*, a transcription factor for differentiation genes; *Nep4*, important for sperm-egg fusion and subsequent fertilization; *fer3*, involved in iron transport and storage in the germ line; and *Hsp70-2*, which presumably acts in the cellular defense mechanism of the testis. It is worth noting that genes involved in gametogenesis, especially in spermatogenesis, are typically evolving faster than other genes (Haerty *et al.*, 2007), and thus may contribute to species reproductive isolation. This feature may assist in the identification of dsRNAs that target only QFF, and not other species, which

could be an important factor to consider if this technology is to receive approval by government regulatory agencies.

1.5. Research objectives:

Given that the current radiation-based sterilization method in SIT can affect sterile male viability and mating competitiveness, and RNA interference has proven to be effective in silencing gene expression in other *Bactrocera* species (Li *et al.*, 2011; Chen *et al.*, 2015; Zheng *et al.*, 2015; Dong *et al.*, 2016), the present study aims to develop sterile males of *Bactrocera tryoni* suitable for SIT by silencing genes involved in spermatogenesis and male fertility using RNAi Knockdown technologies. To help achieve this ultimate goal, the following research objectives were proposed:

1. Identify testis-specific genes involved in spermatogenesis and male fertility in *B. tryoni*.

During this study, I set out to address the following questions: Do QFFs have genes that appear orthologous to spermatogenesis genes in other insects? Do some of these genes show testis-specific expression that suggests they are relevant to spermatogenesis? Which spermatogenesis genes would be most effective in producing sterile males?

For the selection of putative spermatogenesis genes in *B. tryoni,* I considered three main premises. First, I did not choose any genes identified by Whyard *et al.* (2015), as those genes were being investigated by Australian colleagues. Second, I selected genes that other studies had already validated as relevant to testis development/function in other insects. And lastly, I chose genes predicted to be involved in different stages of spermatogenesis.

2. Identify methods of dsRNA delivery to *B. tryoni* that can induce RNAi and assess whether spermatogenesis gene transcripts can be reduced.

To validate the function of the spermatogenesis genes identified in Objective 1, dsRNAs targeting different genes were delivered to QFFs using different methods to determine which one provided the most effective knockdown.

3. Assess whether feeding males with spermatogenesis-specific dsRNAs can induce sterility.

As feeding would be an ideal method to deliver dsRNA for the production of sterile QFFs, I assessed whether dsRNAs move from the digestive tract to the gonads and result in knockdown of the target genes. Additionally, I examined how long the RNAi effect lasts.

4. Assess possible disruptions of physiological parameters, such as accessory gland proteins levels, in male QFFs caused by RNAi-mediated knockdown.

To address this question, I examined whether males that were treated with spermatogenesis-specific dsRNAs showed any perturbation in male accessory gland protein gene expression.

The use of this new technology to sterilize males could have huge beneficial impacts on the economics and practice of SIT to control fruit flies, without the possible adverse environmental impact and low social acceptability of other approaches, such as genetic modification of flies. It could also provide a platform technology for managing a wide range of other agricultural pest insects.

2. MATERIALS AND METHODS

2.1. Insect culture

Adult flies were reared at 28°C, 75% relative humidity with a photoperiod of 14:10 h (light:dark) and were provided sugar cubes and water. A Torula yeast paste was also provided to promote egg development. Eggs were laid on Macintosh apple skins and transferred to yeast-agar artificial diet (protocol provided by the Commonwealth Scientific and Industrial Research Organization (CSIRO), see Appendix II for details). Wandering larvae were transferred to Petri dishes with autoclaved sand to allow larvae to pupate, and pupae were then transferred to the colony cages (30 cm x 30 cm x 30 cm) or placed in individual cotton-stoppered vials (25 ml) for treatment.

2.2. RNA extractions

Adult flies were dissected to collect specific organs as needed. The tissues were immediately immersed in 100 µL of Lysis Buffer with β-mercaptoethanol 2% and stored at −80 °C until required. Total RNA was extracted using QIAshredder (Qiagen) columns to homogenize tissues and a GeneJET RNA purification kit (see Thermo Fisher Scientific's protocol for details). RNA was quantified by measuring the absorbance at 260 nm using a Biochrom NanoVue UV-Vis spectrophotometer. The absorbance ratio of optical density (OD 260/280) was used to assess the purity of the RNA.

2.3. Preparation of cDNA

Genomic DNA was removed from RNA preparations using a DNase I, RNase-free kit. cDNA was synthesized with a Quanta BIOSCIENCES qScript cDNA Supermix, which provides all necessary components (except RNA template) for first-strand synthesis including:

buffer, dNTPs, $MgCl_2$, primers, RNase inhibitor protein, qScript reverse transcriptase and stabilizers. The purity of the cDNA was verified by PCR amplification using a Lucigen EconoTaq PLUS 2X Master Mix (following manufacturer's protocol) and specific primers, and subsequent 1.5% agarose gel electrophoresis.

2.4. Testis-specific gene transcript quantitative determination

Previous studies about genes involved in spermatogenesis in other insects were reviewed to create the list of genes to target in *B. tryoni*. Orthologs of genes that had been previously reported as testis-specific in other species were identified in the QFF using the BLAST tool of the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/), with comparisons to the genomes of *B. tryoni*, *B. dorsalis* and *D. melanogaster*.

QRT-PCR was used to determine the expression patterns of genes within *B. tryoni*. Total RNA from eggs, small larvae, medium larvae, large larvae, pupae, 1-day-old female heads and abdomens, 1-day-old male heads and abdomens, 7-day-old female heads and abdomens, 7-day-old male head and abdomens, ovaries, and testes of *B. tryoni* was isolated to determine the developmental profile of every gene under study. cDNA was synthesized from DNase I-treated total RNA using a qScript cDNA Supermix Kit. qRT-PCR was performed using primers **(Table 1)** designed from sequences acquired from the assembled *B. tryoni* genome database (http://www.ncbi.nlm.nih.gov/genome/15403; GenBank: JHQJ00000000.1). As an internal control, a fragment of the *actin-2* gene was also amplified using specific primers **(Table 1)**. This single reference gene was selected for these studies as it had been found by other researchers within the lab to have consistently high amplification efficiencies (>90%) in all tissues examined. QRT-PCR amplifications were performed using the BIORAD CFX Connect Real-Time PCR System in a 15 µL volume containing 2.0 µL cDNA template, 7.5 µL SsoFast

Evagreen Supermix, 1 μL of each primer (10 μM) and 3.5 μL of nuclease-free water. The reaction conditions were: one cycle at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 55 °C for 30 s. Melting curve analysis from 55 to 95 °C was applied to all reactions to ensure specificity and consistency of all generated products. Controls without RT were included to confirm that genomic DNA was thoroughly removed. All reactions were repeated in duplicate (experimental replicates) and three biological replicates were performed for each gene targeted with dsRNA, to examine more extensively their tissue specificity. Quantification of the transcript level was conducted according to the $2^{-\Delta\Delta \text{ cycle threshold value}}$ method (Livak & Schmittgen, 2001).

Table 1. PCR primers used for RT-PCR and qRT-PCR analyses:

Gene	Primer	Primer Sequence
Putative testis-specific genes		
Testis-specific serine/threonine kinase 1 (tssk1)	QFFtssk RNAi F	TTTCGACAACCTCCAATCGCCAAC
	QFFtssk RNAi R	CGCATAGGAACCCTCGCCAATTT
	QFFtssk qRT F	CGCCGCCTCCTCAACTAAATGAT
	QFFtssk qRT R	CCTTGGTCCACGACCAGACAAAT
Matotopetli (topi)	QFFtopi RNAi F	ACGCTTCTTAACTGGCTCCGTTT
	QFFtopi RNAi R	GGCATTATTGGCATGCTGCTGTT
	QFFtopi qRT F	CATTTGGATGCGAACGCTCGTTTAATG
	QFFtopi qRT R	GGCATTATTGGCATGCTGCTGTT
Thioredoxin T (trxt)	QFFtrxt RNAi F	GATCTGGATAAGAAGCTGGAGGAAGC
	QFFtrxt RNAi R	TCGGCTTCCACAATTTCTGGCATATT
	QFFtrxt qRT F	GCCGAGAAAGCGATTGTGTTGAAAG
	QFFtrxt qRT R	TGAGTTGCCGCCAACGAATACA
Light chain dynein 1 (LCD1)	QFFLCD qRT F	GGCATTACAGCGCCAAAGAAGT
	QFFLCD qRT R	AAATTGCACGAAAGCCCTTGCT
Male-specific RNA 87F (mst87F)	QFFmst qRT F	GGCTCTTGCTGTAGTCCCTGTT
	QFFmst qRT R	ACGTCTAGCAACGGCCACA
Testis-specific gene 10 protein-like	QFF10p qRT F	TCAGGAACTGCGTCACAATCTCAC
	QFF10p qRT R	CTGCTTACAATGTCTGCGAGGGATAG
Cyclin B	QFFcycB qRT F	TGAGGAAAGCACCACCGACTAAC
	QFFcycB qRT R	AAGGCGCTTACTCAGCAGCAAA
Ferritin 3 (Fer3)	QFFfer3 qRT F	CTCTACGCCTGGCAATCACATACA
	QFFfer3 qRT R	GTGCCCATTCATTCCCGCATAAAG
CG8349	QFF8349 qRT F	GCGGCGTGTGTCGACAATTTAT
	QFF8349 qRT R	CAGCACCAGATCTTCGTCAGACTT
House-keeping reference gene		
Actin	QFFact qRT F	CCATGCCATTCTCCGTTTGGATTTG
	QFFact qRT R	AGCTGTGGTGGTGAACGAGTAG
Accessory gland protein genes		
Heat shock protein cognate 3 (isoform A)	QFFhsc3 qRT F	AGGAAACCGCTGAGGCTTATCTTG
	QFFhsc3 qRT R	ATCTTTGGTGGCCTGACGTTGA
Protein disulfide isomerase	QFFdiso qRT F	AGGGCGAACACACTGTTGAGAA

	QFFdiso qRT R	GATCTTGGAAGCTGATTCGTGGTTGA
Antigen 5 precursor	QFFan5p qRT F	GAACACAGTGGCGAGTGGTAAGA
	QFFan5p qRT R	CGTCGTGCTTCATTTGGCATTGTT
Odorant binding protein 2	QFFobp2 qRT F	ATCATGCCGGTCACTCCGATTATG
	QFFobp2 qRT R	TCTGGAAAGCTCCACTGCTTGT
CG5867	QFF5867 qRT F	GGTCATAGCAAGTCGTAAGGTGGAAA
	QFF5867 qRT R	TGCAACTTCACGTGATCGTCATACA

2.5. Preparation of dsRNA

For each gene selected for RNAi targeting, cDNA was synthesized from the RNA preparations and was then used as template for PCR amplification of fragments of ~350 bp in length, using gene-specific primers (**Table 1**, referred to as RNAi primers). The PCR products were ligated into the cloning vector pJET/blunt, and later excised from pJET using XbaI and XhoI restriction enzymes, then ligated into a similarly-digested plasmid pL4440, a vector possessing convergent T7 promoters. DNA templates for *in vitro* transcription of each of the gene fragments in pL4440 were PCR-amplified using the following pL4440-specific primers: pL4440F (ACCTGGCTTATCGAA) and pL4440R (TAAAACGACGGCCAGT). PCR products were purified using a GeneJET Gel Extraction Kit. The MEGAscript RNAi kit (Ambion) was then used for *in vitro* transcription and purification of dsRNAs. DsRNA targeting a non-QFF gene, *green fluorescent protein* (*gfp;* kindly provided by Alison Taylor), was used as negative control in all trials. The dsRNA sequences for all targeted genes are provided in Appendix I.

2.6. Delivering dsRNA to QFF adult males

DsRNA solutions were diluted with molecular grade water to a concentration of 1.0 µg/µL. Ten freshly eclosed male adults (less than 12 h) were injected with 2.0 µL of dsRNA solution and then placed into individual *Drosophila* vials (25.0 mL). To assess for RNAi, they were allowed to develop for three days after injection on a 10% sugar water solution diet and RNA was then extracted from dissected testes and subjected to qRT-PCR analyses as described

above, where the transcription levels of the genes selected were compared to control flies that were injected with *gfp*-dsRNA.

A different set of ten male adults was also placed in individual vials and fed daily with a dose of 2.0 µL of dsRNA dissolved in 10.0 µL of 10% sugar water for ten days (the 12.0 µL droplet of dsRNA-sugar water solution was placed at the bottom of the vial daily), after which RNA from dissected testes was extracted for subsequent quantitative analysis.

2.7. QFF fertility assays

Ten males previously treated with dsRNA for a period of ten days were given two virgin mates and kept in 750 mL plastic cups for three weeks (one male with two females in each cup). Flies were provided 10% sugar water *ad libitum*, and Torula yeast paste to promote egg development. A piece of Macintosh apple skin was also placed in the cup to allow females to lay their eggs and thereby assess fertility and fecundity, and it was changed every two days. Flies were observed daily to record the date of the first mating attempt. Apples with eggs resulting from mating were transferred to the yeast-agar medium and incubated at 28°C for five days. Viable larvae were then counted and information about the male in question and the date the apple was given was recorded. If no eggs were produced, or all eggs failed to hatch, the dsRNA-treated insect was considered sterile. Total values of number of viable offspring produced by dsRNA-treated flies were compared to control individuals, to detect a possible reduction of fecundity.

Cages (15 cm x 15 cm x 15 cm) were used for small population mating competitions with five males and five females. In these competition mating assays, varying proportions of dsRNA-treated males: control males (5:0, 3:2, 2:3 and 0:5) were mixed with the five females. Flies were given a piece of apple on day 7 and on day 14, post eclosion. Apples with eggs

resulting from mating were transferred to the yeast- agar medium and incubated at 28°C for five days, and viable larvae were then counted. The number of offspring was determined.

2.8. Accessory gland protein gene expression assessment

Ten males were fed with dsRNA for ten consecutive days (see procedure above). Due to the diffuse nature of the accessory glands in the QFF and their difficult visualization during dissections, total RNA was extracted from the entire reproductive system and the final portions of the gut tract. The cDNA produced from these RNA preparations served as template for qRT-PCR.

From the list of proteins found in the accessory gland secretions of the Oriental Fruit Fly *B. dorsalis* presented by Wei *et al.* (2015a), the five most abundant were selected and their nucleotide sequences were blasted against the genome of the QFF. Specific primers were then designed for the orthologous sequences found **(Table 1)**, and were later used during the quantification of the gene expression (qRT-PCR protocol previously presented). The transcription level of the selected accessory gland proteins in dsRNA-treated flies was compared to control flies, to detect a possible disruption of normal values after sterilization with RNAi.

2.9. Statistical analysis

Significant differences between treatments and controls during the spermatogenesis genes knockdown trials (through both injections and oral delivery) were evaluated using a t-test for two independent sample groups. A Tukey test was performed to analyze the average of total number of larvae per dsRNA-treated male during the mating assays, and detect specific differences among treatment groups. Normality and Homogeneity of variances were tested using Kolmogorov-Smirnov test and Levene's test, respectively. In cases where variables did

not meet the Normality and Homogeneity premises, they were $\log_{10}$ transformed. If variables still would not meet the premises after transformation, non-parametric Mann-Whitney U and Kruskal-Wallis tests were used. All statistical analyzes were performed in the STATISTICA 7.0 software with a significance level of 0.05.

3. RESULTS

3.1. Testis-specific genes potentially involved in *B. tryoni* spermatogenesis:

After reviewing literature about genes involved in spermatogenesis in other insects and using BLAST to search for similar sequences within the two *Bactrocera* genomes (*B. tryoni* and *B. dorsalis*), a total of nine genes were selected for further analysis to examine their potential role in QFF spermatogenesis: *Testis-specific serine/threonine-protein kinase 1* (*tssk1*) (Wei *et al.*, 2015b); *Matotopetli* (*topi*) (Perezgasga *et al.*, 2004); *Thioredoxin T* (*trxt*) (Svensson *et al.*, 2003); *Dynein light chain-1* (*dlc1*) (Ghosh-Roy *et al.*, 2005); *Male-specific RNA 87F* (*mst87f*) (Barckmann *et al.*, 2013); *Testis-specific gene 10 protein-like* (*10p*) (Wei *et al.*, 2015b); *Cyclin B* (Wei *et al.*, 2015b); *Ferritin 3* (*fer3*) (Wei *et al.*, 2015b); and *CG8349* (Perezgasga *et al.*, 2004). The putative *B. tryoni* genes were more than 93% identical to genes of *B. dorsalis* and ranged between 67 and 78% identical to the putative orthologues in *D. melanogaster* genome **(Table 2)**.

Table 2. Percentages of nucleotide similarity between the genes of interest in *Bactrocera tryoni* and the genomes of *B. dorsalis* and *Drosophila melanogaster*. Sequence identity analyses were performed using the BLAST program of the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Gene	% of nucleotide identity with *B. dorsalis*	% of nucleotide identity with *D. melanogaster*
Tssk1	97.5	75.0
Topi	97.1	78.0
Trxt	96.4	70.0
Dlc1	94.7	77.0
Mst87f	99.0	75.0
10p	97.8	69.0
Cyclin B	93.3	69.0
Fer3	97.8	75.0
CG8349	94.7	67.0

The expression pattern of the target genes in different tissues of *B. tryoni* was determined using RT-qPCR, and confirmed that all genes, except for *fer3* and *CG8349*, are testis-specific, showing higher transcript levels in the testis and slightly higher transcript levels in the male abdomen **(Fig. 1)**.

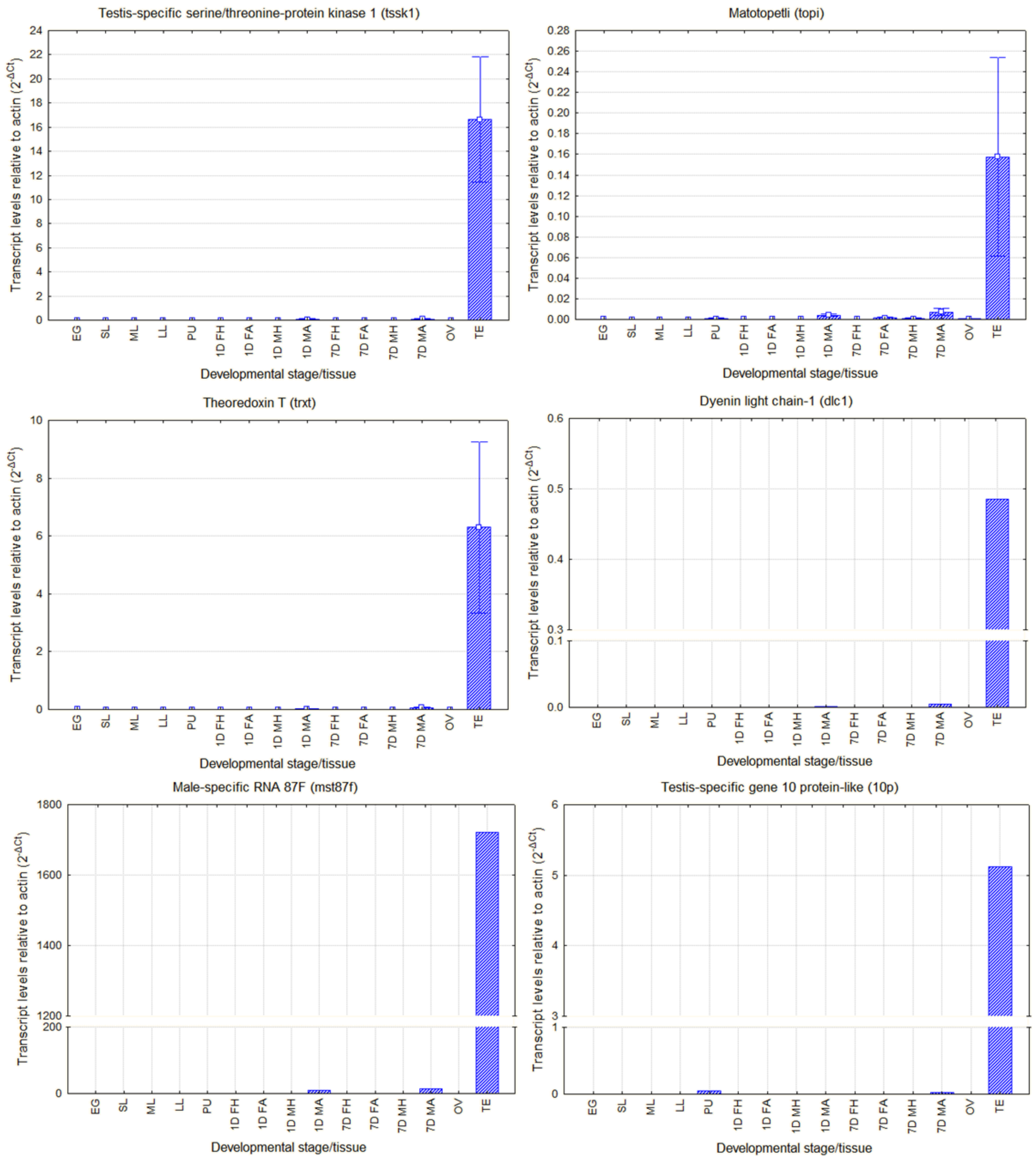

Figure 1. Transcript levels of putative spermatogenesis genes, relative to actin, in different tissues and stages of development of *Bactrocera tryoni* (EG: eggs; SL: small larvae; ML: medium larvae; LL: large larvae; PU: pupae; 1D FM: 1-day-old female heads; 1D FA: 1-day-old female abdomens; 1D MH: 1-day-old male heads; 1D MA: 1-day-old male abdomens; 7D FM: 7-day-old female heads; 7D FA: 7-day-old female abdomens; 7D MH: 7-day-old male heads; 7D MA: 7-day-old male abdomens; OV: ovaries; TE: testis). The values represent the mean and standard error of three biological replicates for *tssk1*, *topi* and *trxt*. For the remaining genes examined, N = 1.

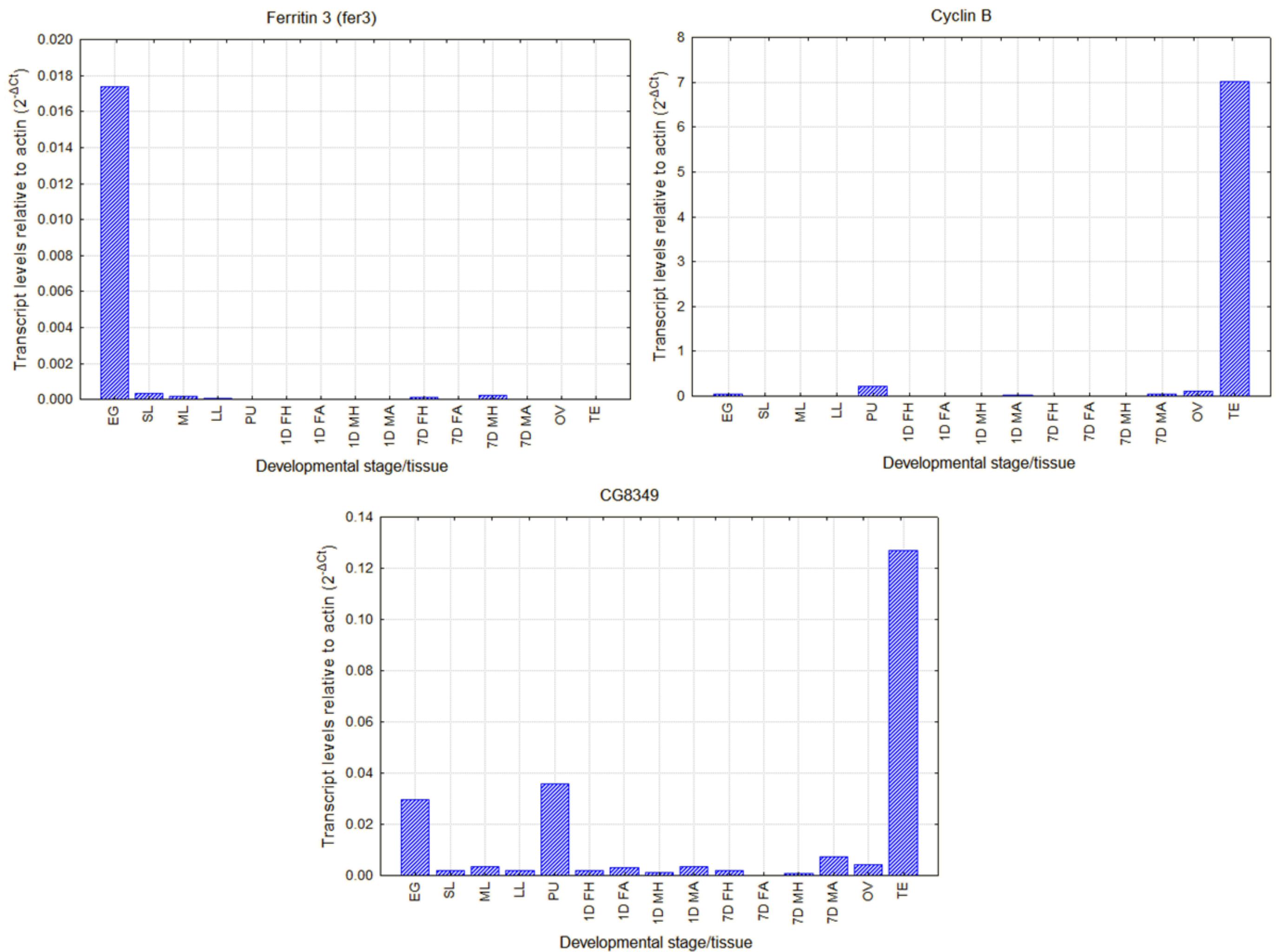

Figure 1 (cont.). Transcript levels of putative spermatogenesis genes, relative to actin, in different tissues and stages of development of *Bactrocera tryoni* (EG: eggs; SL: small larvae; ML: medium larvae; LL: large larvae; PU: pupae; 1D FM: 1-day-old female heads; 1D FA: 1-day-old female abdomens; 1D MH: 1-day-old male heads; 1D MA: 1-day-old male abdomens; 7D FM: 7-day-old female heads; 7D FA: 7-day-old female abdomens; 7D MH: 7-day-old male heads; 7D MA: 7-day-old male abdomens; OV: ovaries; TE: testis). The values represent the mean and standard error of three biological replicates for *tssk1*, *topi* and *trxt*. For the remaining genes examined, N = 1.

Based on their male and testis-specific expression profiles, and previously reported sterility in mutant males, *tssk1* (Xu *et al.*, 2008) and *topi* (Perezgasga *et al.*, 2004) were chosen as good target genes for RNAi-mediated knockdown through injections and oral delivery of dsRNA. *Trxt* was also chosen based on its role in protection against oxidative stress in the testis of *Drosophila*, although mutations of this gene have failed to produce sterile males in this species previously (Svensson *et al.*, 2003).

3.2. RNAi-mediated knockdown by injections of dsRNA to *B. tryoni*:

Injections of dsRNA targeting *tssk1*, *topi* and *trxt* in *B. tryoni* showed significant knockdown of all genes (t-test, $p < 0.05$) three days post-injection **(Fig. 2)**; *tssk1* showed a 60.1% knockdown ($t = -4.44$); *topi* showed a 58.8% knockdown ($t = -2.82$); and *trxt* had the biggest reduction of transcript levels, with 78.4% compared to *gfp* controls ($t = -3.80$).

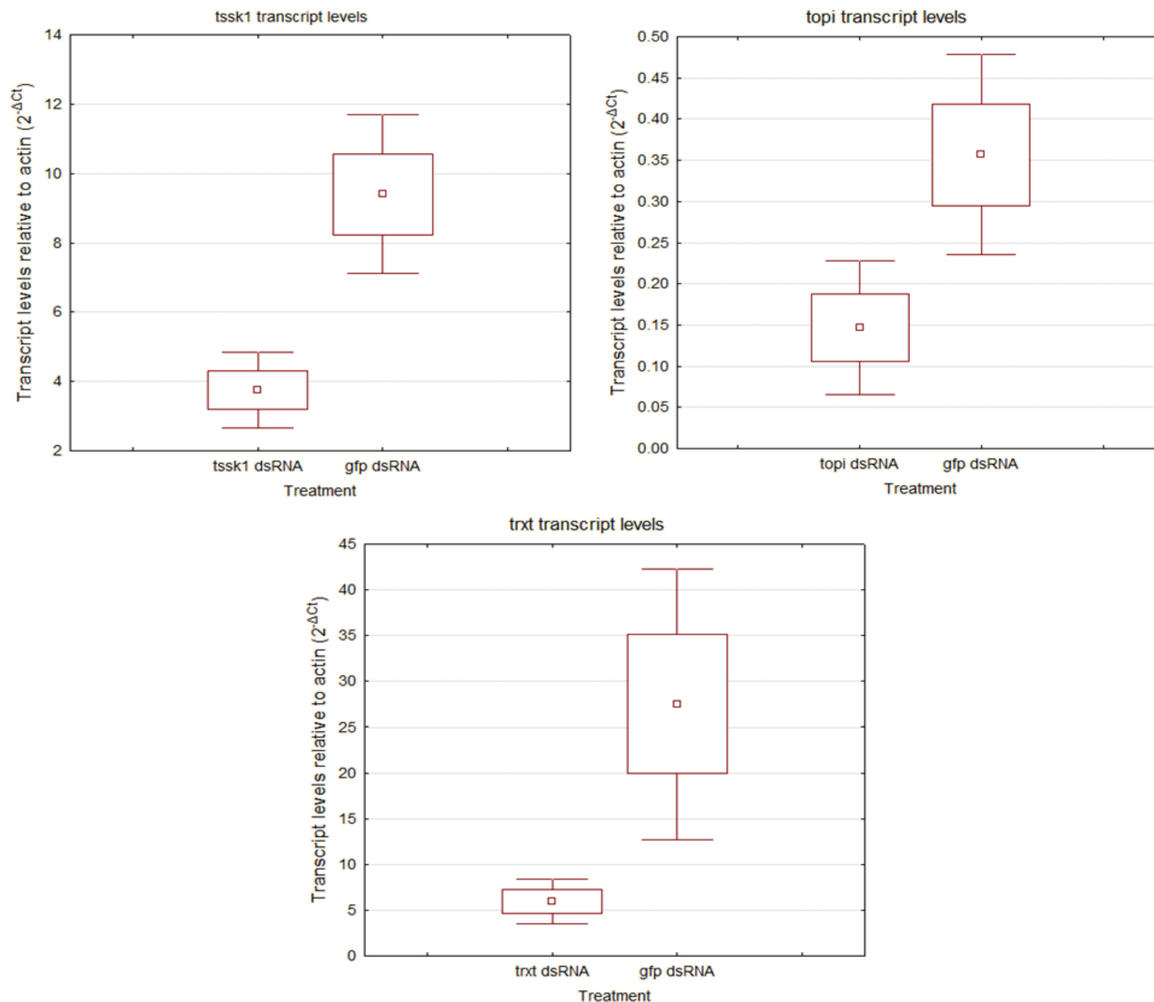

Figure 2. Transcript levels of *tssk1*, *topi* and *trxt*, relative to actin, in *Bactrocera tryoni* testis three days after dsRNA injections. Values represent the mean (square), standard error (rectangle), and the 96% confidence intervals (bars) of ten biological replicates.

3.3. RNAi-mediated knockdown by oral delivery of dsRNA to *B. tryoni*:

While oral delivery of dsRNA was successful to achieve a significant reduction of the transcript levels of *tssk1* (t-test, $p < 0.05$), no knockdown was detected for *topi* and *trxt* **(Fig. 3)**. *Tssk1* showed a 69.1% knockdown ($t = -5.77$). Flies treated with dsRNA targeting *topi* and

trxt showed an increase in the mRNA levels, compared to *gfp* control flies. Although this overexpression was not statistically significant in *topi*-treated flies (t-test t = 1.91, p = 0.07), it was in *trxt* treatments (t-test t = 3.75, p < 0.05).

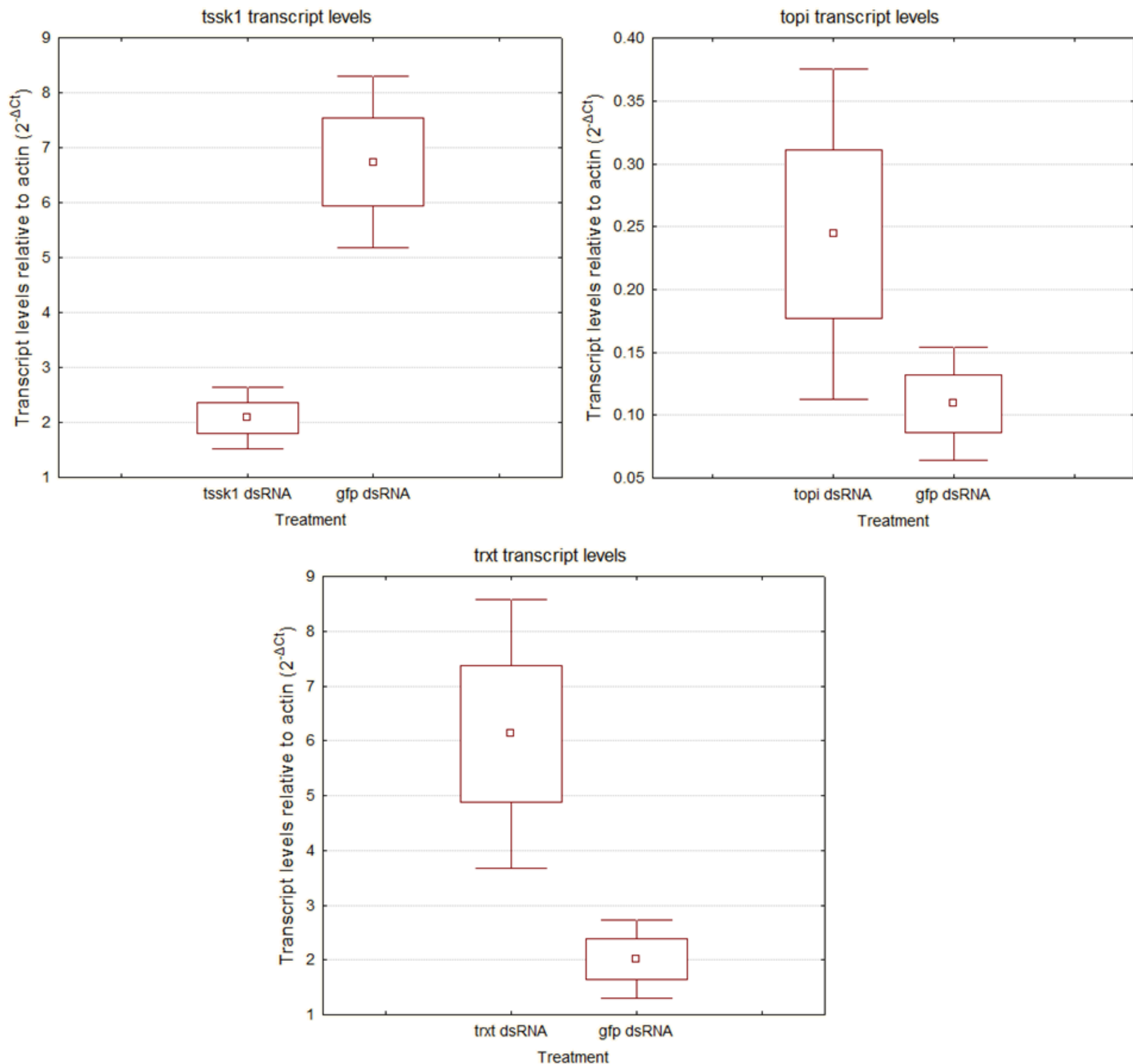

Figure 3. Transcript levels of *tssk1*, *topi* and *trxt*, relative to actin, in *Bactrocera tryoni* after ten days of continuous oral delivery of dsRNA. Values represent the mean (square), standard error (rectangle), and the 96% confidence intervals (bars) of ten biological replicates.

3.4. Mating assays:

Only eight males survived the feeding procedure and were used for the analysis. Different aspects of the male reproductive biology of *B. tryoni* were affected by oral delivery of dsRNA. After treatment, *gfp* control males were observed to attempt mating at approximately 14 days

of age **(Fig. 4)**, while the average age at which flies attempted mating from all three treatment groups was three days older (17 days). While the precise age at which flies mated was not determined, the date at which viable larvae were produced from the mating assays suggested that control flies had mated earlier, followed by the *trxt*-dsRNA treated group two days later, and finally, *tssk1*- and *topi*-dsRNA treated flies started laying eggs four days after that **(Fig. 4)**.

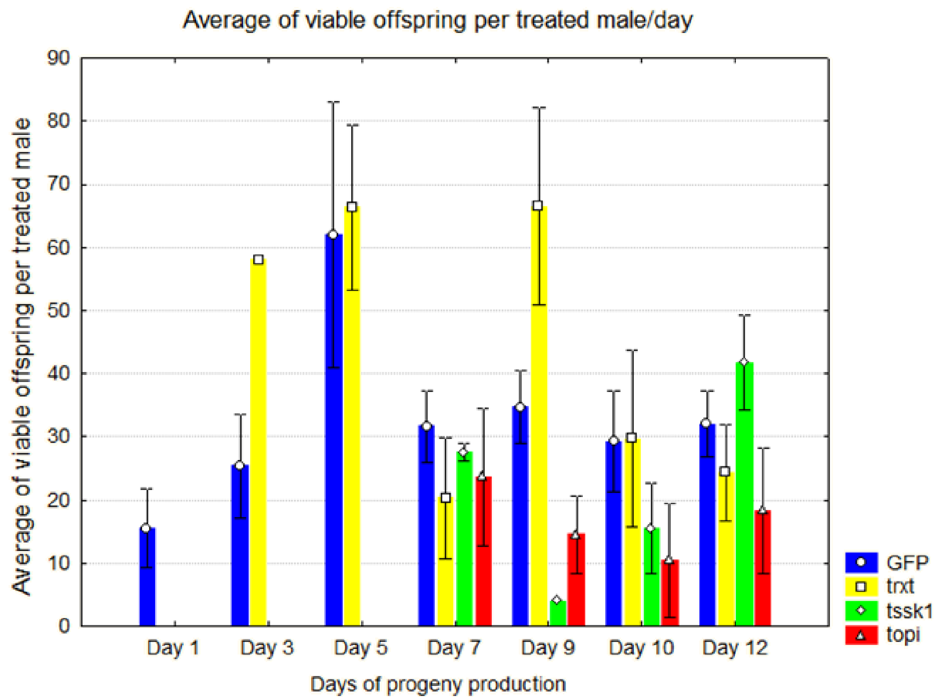

Figure 4. Average of viable offspring per dsRNA-treated male of *Bactrocera tryoni* per day during a two-week period (N = 8). Bars indicate the standard error.

During the last three dates of egg collection, it was observed that the number of larvae produced by males treated with *tssk1*-dsRNA had begun to increase **(Fig. 4)**, however, a Tukey test showed no significant differences in the average of viable offspring among the last three days of progeny production (MS = 216.31, $p > 0.05$).

The average number of progeny produced per male over this two-week period, depicted in Figure 4, does not fully describe the full impacts of the dsRNA treatments on the males. While all *gfp* control males were fertile and able to produce viable offspring, three *tssk1*-

dsRNA treated males, four *topi*-treated males, and two *trxt*-treated males were sterile and failed to produce viable offspring **(Fig. 5)**.

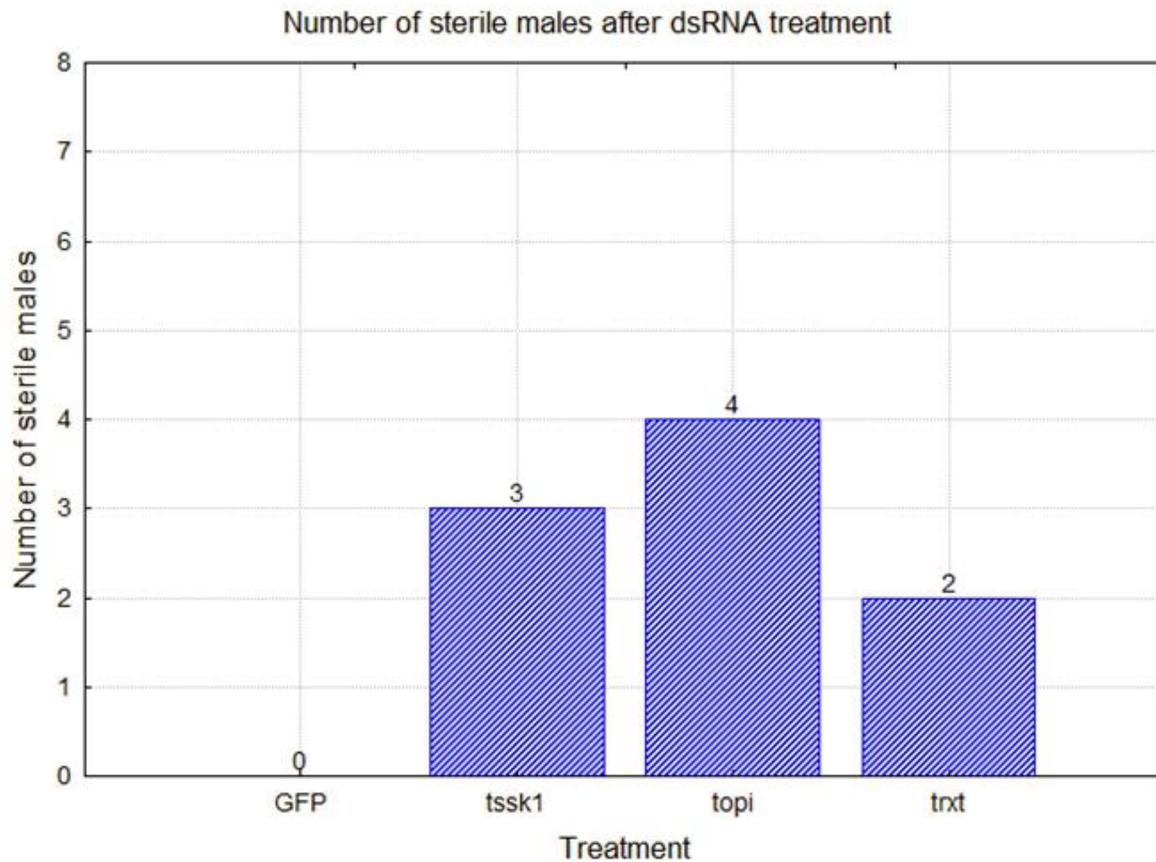

Figure 5. Sterility of males of *Bactrocera tryoni* after ten days of continuous oral delivery of *tssk1*-, *topi*- and *trxt*-dsRNA (N = 8).

A non-parametric Kruskal-Wallis test confirmed that there were significant differences in the average number of larvae produced by dsRNA-treated males ($H (3, N = 32) = 14.9$, $p < 0.05$). Fertile *tssk1*- and *topi*-treated flies had a 74.5% and 78.2% reduction of viable offspring, respectively; and *trxt*-treated males had a 40.8% reduction in progeny **(Fig. 6)**. *Tssk1*- and *topi*-treated flies differed from controls statistically (Kruskal-Wallis, $p < 0.05$); however, *trxt*-treated males did not differ from any of the other groups (Kruskal-Wallis, $p > 0.05$).

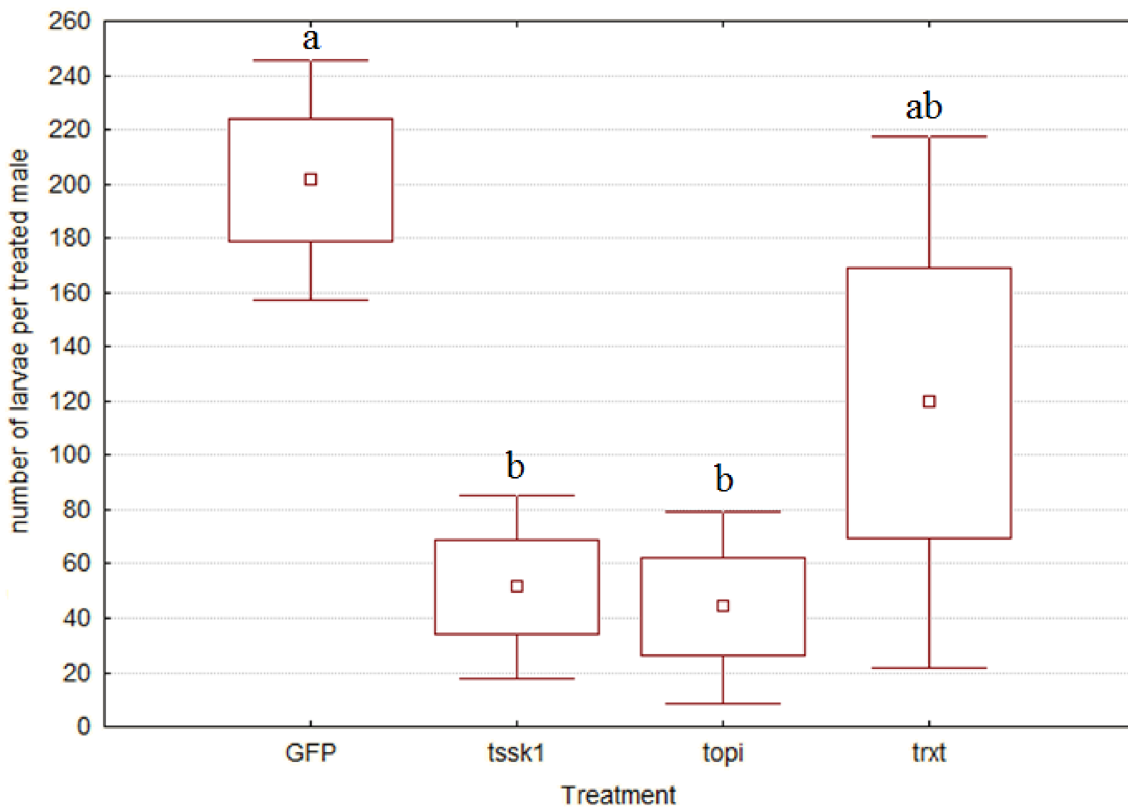

Figure 6. Average number of larvae per fertile male of *Bactrocera tryoni* treated with *tssk1*-, *topi*- and *trxt*-dsRNA (*gfp* N = 8; *tssk1* N = 5; *topi* N = 4; *trxt* N = 6). Values represent the mean (square), standard error (rectangle), and the 96% confidence intervals (bars), letters indicate the results of the Kruskal-Wallis test (statistical differences among groups).

When the total number of larvae produced by dsRNA-treated males was examined, a similar pattern of daily progeny production between *gfp* controls and *trxt*-dsRNA treated flies was observed. They both showed a peak of larval production when they were approximately 20 days old (soon after they started laying eggs), after which larval production decreased and stabilized **(Fig. 7A)**. This pattern was not observed in the other two treatment groups.

Cumulative values of total larvae per day showed a great reduction in the progeny of dsRNA-treated flies during the entire study. From the 1612 larvae produced by *gfp* control flies, progeny was reduced in *tssk1*- and *topi*-dsRNA treated flies by 84 and 89%, respectively, and in *trxt*-dsRNA treated males, progeny production was reduced 56% **(Fig. 7B)**.

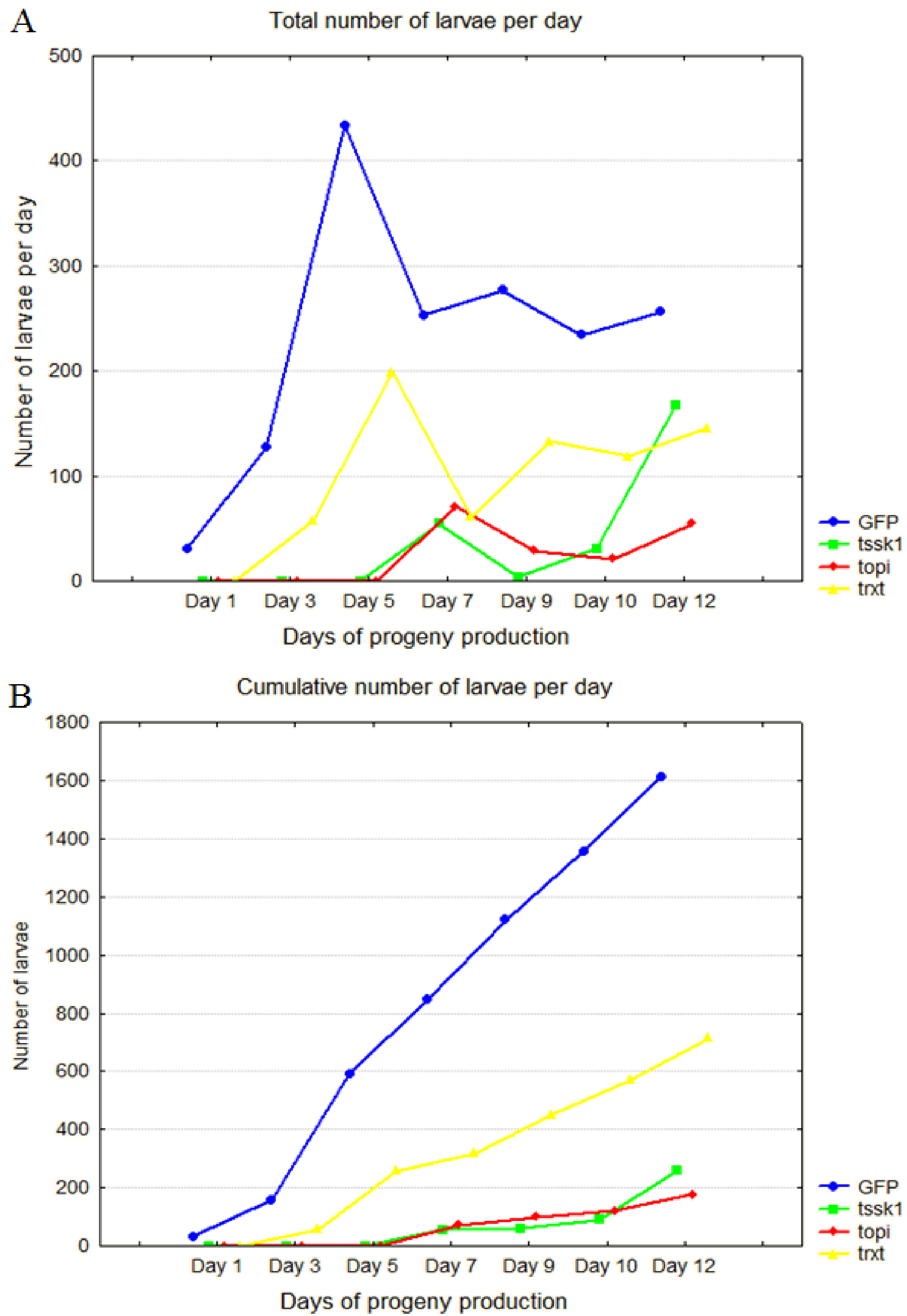

Figure 7. Number of larvae per *tssk1*-, *topi*- and *trxt*-dsRNA treated male of *Bactrocera tryoni* (*gfp* N = 8; *tssk1* N = 5; *topi* N = 4; *trxt* N = 6). **A:** Total values per day for all fertile males, **B:** Cumulative values per day for all fertile males.

Mating competition assays between dsRNA-treated and control males showed a reduction in the number of viable offspring produced in those cages seeded with dsRNA-

treated males. Cages with a higher ratio of *gfp*-treated flies produced more larvae in the progeny, while the opposite situation was observed in cages with higher ratios of *tssk1*- and *trxt*-treated flies **(Fig. 8)**; however, Tukey tests failed to detect significant differences in progeny production among the different cages (*tssk1* MS = 295.87, *trxt* MS = 379.88; $p > 0.05$). During trials with *topi*-dsRNA, some flies (both females and males) died for unknown reasons in the parental cages, which affected the assessment of the impact of dsRNA on the progeny.

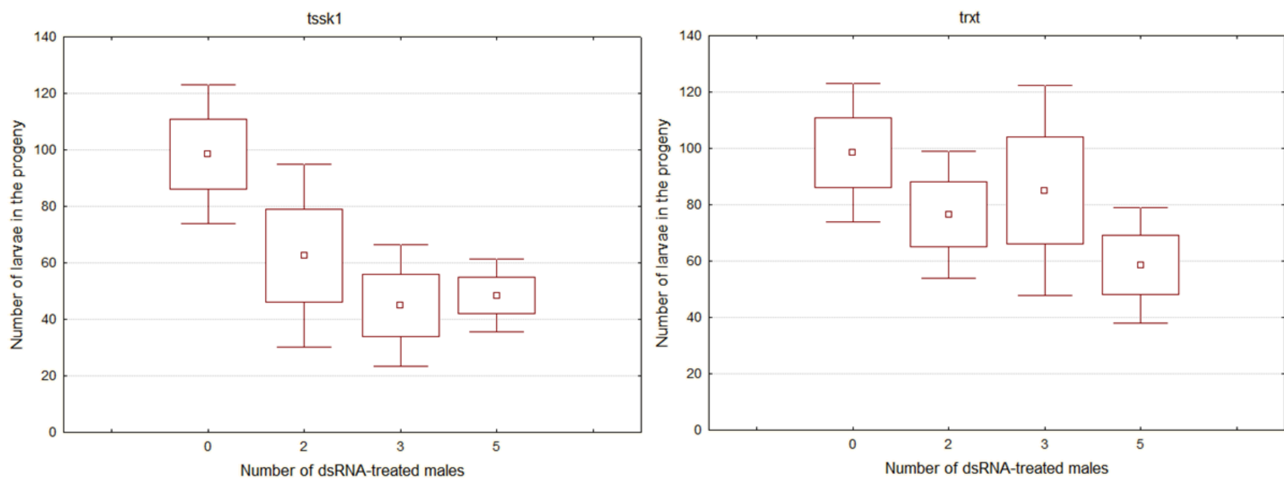

Figure 8. Number of viable offspring of *Bactrocera tryoni* produced in cages with different numbers of dsRNA-treated males. Values represent the mean (square), standard error (rectangle), and the 96% confidence intervals (bars) of the number of larvae collected from apples in two occasions.

3.5. Accessory gland protein gene expression assessment:

According to Wei *et al.* (2015a), CG5867, heat shock protein cognate 3 (Isoform A) (hsc3), protein disulfide isomerase (diso), antigen 5 precursor (an5p) and odorant binding protein 2 (obp2) are the five most abundant proteins in the male accessory glands of *B. dorsalis*. Orthologs of these proteins in the QFF were identified based on nucleotide identity **(Table 3)** and qRT-PCR primers were designed to measure the level of these genes' transcripts from male QFF reproductive tissues.

Table 3. Percentages of nucleotide similarity between the male accessory gland genes in *Bactrocera dorsalis* and the genome of *B. tryoni*. Sequence identity analyses were performed using the BLAST program of the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Gene	% of nucleotide identity with *B. dorsalis*
CG5867	87.2
hsc3	98.3
diso	97.7
an5p	74.5
obp2	84.5

Only eight males survived the feeding procedure and were used for the analysis. The QFF-specific qRT-PCR primers failed to amplify any product for *CG5867*, *hsc3* or *an5p* in *B. tryoni* male reproductive tissues. Perhaps the primers were targeting an intron sequence, since the genome of *B. tryoni* is not completely annotated. Further tests should use these primers on genomic DNA to confirm the presence of these genes in the QFF. On the other hand, *diso* and *obp2* were readily detected and in *tssk1*-dsRNA-treated males, transcript levels of these genes remained unaltered when compared to controls (t-test, $p > 0.05$; $t = -0.48$ and $t = 0.61$ respectively). Interestingly, in *topi*- and *trxt*-dsRNA-treated flies, both *diso* and *obp2* transcript levels were significantly increased (t-test, $p < 0.05$; Mann-Whitney U test ($p < 0.05$) for *obp2* in *topi*- and *gfp*-treated flies) **(Fig. 9)**.

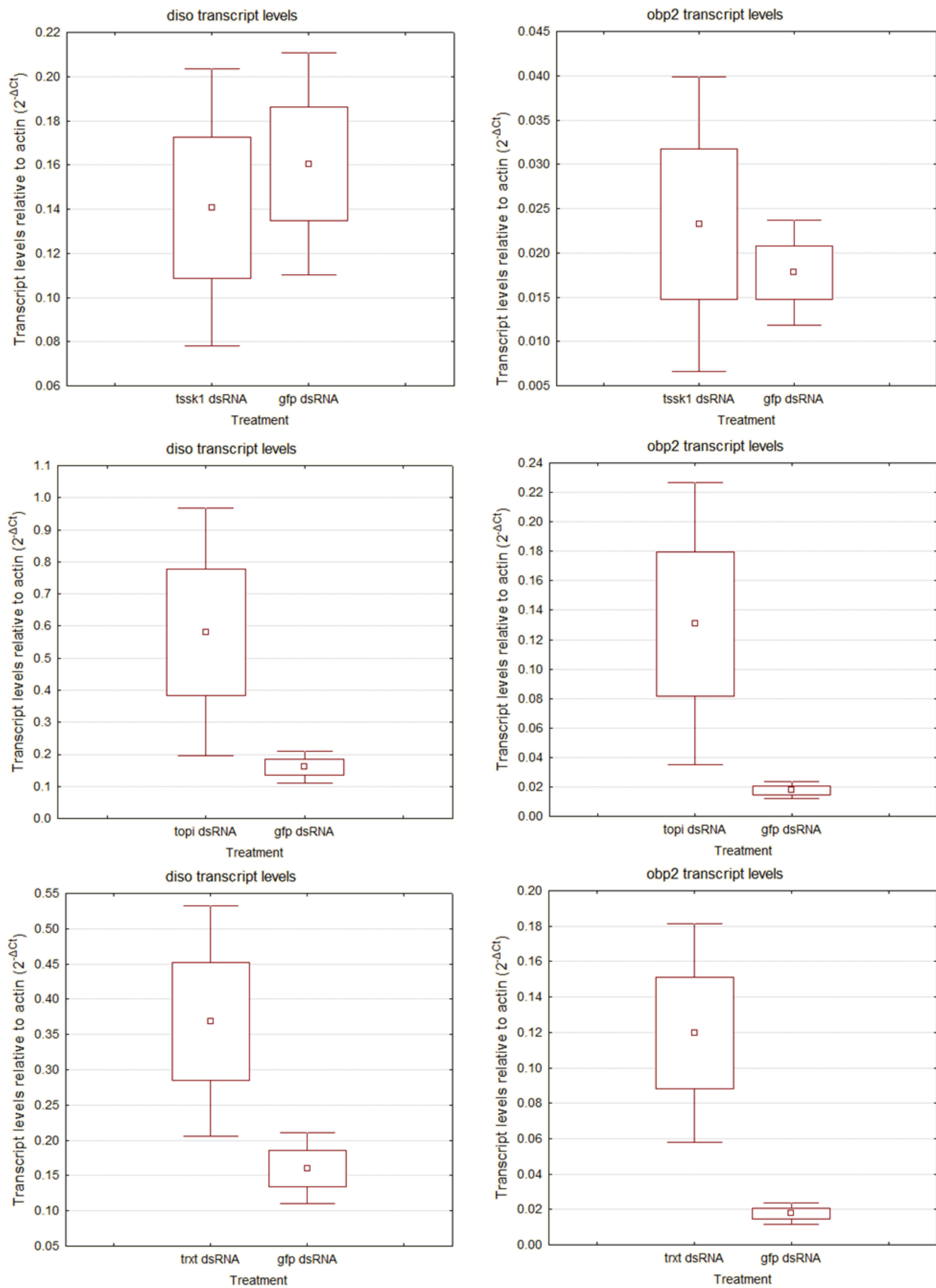

Figure 9. Transcript levels of *diso* and *obp2*, relative to actin, in *Bactrocera tryoni* after ten days of continuous oral delivery of dsRNA. Values represent the mean (square), standard error (rectangle), and the 96% confidence intervals (bars) of eight biological replicates.

4. DISCUSSION

SIT is a pesticide-free method used to control Australia's most serious horticultural pest, the Queensland fruit fly (QFF), but the use of radiation to sterilize males is considered less than ideal, as it can weaken the males and reduce their ability to actively compete with wild males in the field (Collins *et al.*, 2008; Dominiak *et al.*, 2014). One possible alternative to radiation sterilization is RNAi-mediated knockdown of male fertility genes, and in this study, I examined the efficacy of RNAi in QFF to produce sterile males that could compete with fertile males for mates.

Genes involved in spermatogenesis are obvious candidates to target for an RNAi-mediated male sterilization technology. There have been numerous studies that have examined genes associated with spermatogenesis in insects, with most focused on the model insect, *Drosophila melanogaster* (Moon *et al.*, 2011; Fabian & Brill, 2012; Volpi *et al.*, 2013; Rathke *et al.*, 2014). The availability of mutant stocks and the application of many molecular genetic tools in this model insect has enabled researchers to ascribe functions to many spermatogenesis genes, some of which have been confirmed by examination of testis or sperm development, or by examining impacts on fertility or fecundity (Perezgasga *et al.*, 2004; Brackmann *et al.*, 2013; Demarco *et al.*, 2014). Orthologues of spermatogenesis genes have been identified in close relatives of the QFF, the oriental fruit fly, *Bactrocera dorsalis* (Wei *et al.*, 2015b; Dong *et al.*, 2016), and the Mediterranean fruit fly, *Ceratitis capitata* (Sutton *et al.*, 2016; Economou *et al.*, 2017). In this study, I identified nine genes from the QFF genome that showed high sequence identity to *B. dorsalis* and *D. melanogaster* testis genes.

The very high degree of nucleotide identity between the genes found in the QFF and *B. dorsalis* (over 90%), and the moderately similar sequence identity to corresponding genes in *D. melanogaster* (around 70%) made it possible to assign putative names to the QFF genes that

were examined in this study, which included: *Testis-specific serine/threonine-protein kinase 1* (*tssk1*); *Matotopetli* (*topi*), also referred to as *testis-specific zinc finger protein*; *Thioredoxin T* (*trxt*); *Dynein light chain-1* (*dlc1*); *Male-specific RNA 87F* (*mst87f*); *Testis-specific gene 10 protein-like*; *Cyclin B*; *Ferritin 3* (*fer3*); and *CG8349*. All of them are considered to be involved in sperm formation in *D. melanogaster* at different stages of gametogenesis. For instance: *tssk1* is expressed in the spermatids and it is critical in spermiogenesis (Wei *et al.*, 2015b); *dlc1* is involved in sperm individualization and sperm motility (Ghosh-Roy *et al.*, 2005); *mst87f* is expressed in young spermatocytes and acts in chromatin condensation at later stages (Barckmann *et al.*, 2013); *CG8349* is expressed in primary spermatocytes and the transcript persists into mid-late stages of spermatid elongation (Perezgasga *et al.*, 2004).

The high nucleotide identity of these genes with those of *B. dorsalis* suggests that these two species are closely related, as genes involved in spermatogenesis are reported to show faster mutation rates than non-reproduction genes (Haerty *et al.*, 2007). The degree of sequence similarity with genes in *D. melanogaster* was not nearly as high as that seen in *B. dorsalis*, but this degree of sequence identity has been noted for *topi* orthologues in other *Bactrocera* species, such as *B. oleae* (Dong *et al.*, 2016). This conservation is in accordance with the fact that these genes are involved in gametogenesis processes that are shared by many species, such as activation of the male germline differentiation and sperm maturation. Sutton *et al.* (2016) explored some genes in the male germline of *Aedes aegypti* and *C. capitata* and identified two testis-specific genes, *centrosomin* and a predicted zinc finger protein, which showed high nucleotide identity between the two species. In addition, these authors also reported that these two genes showed high homology to *D. melanogaster*, thus demonstrating their inter-species conservation.

Of the nine QFF genes that were examined, seven were found to be highly expressed in the testis, based on the qRT-PCR analyses of dissected tissues. This tissue-specificity of the

genes was a key factor in selecting the best targets for RNAi-mediated knockdown. To produce sterile males that would still be competitive in mating with females, it was considered ideal to target genes that are only expressed in the reproductive organs; that way, we would ensure that the disruption of gene expression only affected processes involved in fertility and/or fecundity and not impair the overall health of the insects. Using dsRNA to target a gene that is not specifically expressed in the gonads could affect various biological processes that could alter the fly's proper development, physiology, or behaviour, and thereby prevent the males from competing for mates. In addition, RNAi-targeting genes expressed in other tissues, in the worst-case scenario, could knock down genes essential for survival, which would serve no practical use for an SIT application. However, such a knockdown could be considered useful for other applications, such as the development of RNAi-based insecticides (Li *et al.*, 2011), provided effective methods of distributing the insecticide could be developed.

Fer3 and *CG8349* were initially identified as testis-specific genes in other insects (Perezgasga *et al.*, 2004; Metzendorf & Lind, 2010; Wei *et al.*, 2015b); however, they showed a considerable level of expression in eggs and pupae (*CG8349*) in *B. tryoni*. The gene expression patterns examined by these authors did not include early stages of development (i.e. embryos, larvae, pupae); total RNA was only obtained from adult tissues, which made the genes appear to be testis-specific. Since there is no information about when spermatogenesis begins in *B. tryoni*, I decided not to target these genes in my RNAi-mediated gene knockdown assays.

4.1. RNAi-mediated knockdown by injections of dsRNA to *B. tryoni*:

The reduction of *tssk1*, *topi* and *trxt* transcript levels in the testis of *B. tryoni* observed in this study after injections of dsRNA into the hemolymph provides evidence that RNAi occurs in this species, and can be induced by the introduction of foreign dsRNA into the system. Prior

to this study, the only other report of RNAi in QFF was that of Raphael *et al.* (2004), who provided preliminary evidence that injected dsRNA into embryos could affect the expression of *doublesex* (*dsx*). Direct injection of dsRNA into the hemocoel is a standard method of delivery of dsRNA to insects, as it ensures that each insect is provided a uniform dose. With this type of delivery system, it is possible to seriously injure the insect, and hence, it is a laborious method to ensure good survivorship, but nevertheless, a great many studies of RNAi in insects rely on this method to produce knockdown phenotypes (reviewed in Garbutt & Reynolds, 2012; Garbutt *et al.*, 2013; Whyard *et al.*, 2015; Van Ekert *et al.*, 2016).

Direct injection of dsRNA to induce RNAi has been described in one other tephritid fly, *B. dorsalis*, by several research groups. Injection of embryos of dsRNA demonstrated knockdown of the *transformer* (*tra*) gene (Peng *et al.*, 2015), and direct injection of larvae with *G6PI*- and *UAP*-dsRNA successfully knocked down its targeted genes (*Glucose-6-phosphate isomerase* and *UDP-N-acetylglucosamine pyrophosphorylase*, respectively) (Yang *et al.*, 2015), and direct injection of adults with *dsx* (Chen *et al.*, 2008) and *tra* (Peng *et al.*, 2015) dsRNAs resulted in knockdown of the targeted genes and perturbation of sex differentiation development. These latter two studies reduced gene expression by approximately 60%, which is comparable to the extent of knockdown that I observed for some of the genes targeted in QFF following dsRNA injections. Interestingly, Chen *et al.* (2008) observed that transcript knockdown could be detected as early as one hour after injections, and lasted for 6-10 days. Both of these latter studies share a similar methodology with mine, in that flies were injected on the first day of eclosion with a dose of 2.0 µg of dsRNA. In my study, I only examined transcript levels three days post-injection, although it seems reasonable to assume that the impacts of the dsRNA could persist considerably longer, based on these two other studies.

Injection of dsRNA has been observed to up-regulate the RNAi response in some insects. Garbutt & Reynolds (2012) reported increased expression of *dicer-2* and *argonaute-2* (genes

involved in the RNAi pathway) in response to injection of dsRNA in the larvae of the tobacco hornworm *Manduca sexta*. Both these genes increased their mRNA levels (*dicer-2* more significantly) in fat bodies, haemocytes and midgut following the introduction of dsRNA. Effects were first detected six hours after treatment and lasted about 18 hours. The responses of *dicer-2* and *argonaute-2* expression were also dose-dependent, with higher doses of dsRNA causing greater increases in these genes' transcripts. They also noted that *dicer-2* responded to a second injection six hours later, with a similar magnitude to the first injection, suggesting that this gene can respond to multiple doses of dsRNA. Other genes (such as *translin*, *VIG* and *TSN*) did not modify their expression after dsRNA treatment, indicating their roles in the RNAi pathway may be supplementary, while *dicer-2* and *argonaute-2* play the key roles.

Direct injections of dsRNA into the hemocoel do not always induce RNAi in insects, as the dsRNA can degrade before it reaches its cellular targets. Garbutt *et al.* (2013) studied the stability of dsRNA in the hemolymph of the German cockroach *Blattella germanica* and the tobacco hornworm, *M. sexta*, and observed that the dsRNA persisted for at least 24 hours in the cockroach (making it very susceptible to RNAi), while it was rapidly degraded in the tobacco hornworm, lasting for only about one hour (making this insect less sensitive to RNAi). The degradation of dsRNA in *M. sexta* was attributed to a hemolymph nuclease capable of degrading the dsRNA before it could induce RNAi. A small number of studies have identified dsRNases in the hemolymph of other insects, including locusts (Wang *et al.*, 2016), aphids (Christiaens *et al.*, 2014), and silk moths (Liu *et al.*, 2012), and in these insects, RNAi efficacy is low compared to other insects. The occurrence of RNAi in the QFF following dsRNA injections suggests that this species may lack or have very little dsRNase activity in the hemolymph.

4.2. RNAi-mediated knockdown by oral delivery of dsRNA to *B. tryoni*:

Oral delivery of dsRNA to QFF successfully reduced *tssk1* expression. Other researchers (Li *et al.*, 2011; Zheng *et al.*, 2015; Dong *et al.*, 2016) have observed similar results when feeding dsRNA to *B. dorsalis* targeting reproduction-related and other genes. However, in these studies, the flies were fed in communal cages, so the exact quantity of dsRNA ingested by each fly was unknown. In addition, they were continuously fed for different periods of time, ranging from 12 to 14 days. In the present study, the dose of dsRNA delivered to each *B. tryoni* male is known (2.0 µg/day for 10 days), which makes the determination of the effect of dsRNA on gene expression more accurate. Li *et al.* (2011) examined transcript levels 0, 1, 2, 4 and 7 days post-feeding, and observed that the extent of transcript knockdown at different points differed for each gene examined, even when dsRNA dose was the same for all. This finding suggests that any given gene's measured knockdown will be dependent on the gene's biological function and its level of transcriptional regulation, and may therefore show variable phenotypes, as some genes may not respond equally to the dose of dsRNA delivered.

The upregulation of *trxt* post-dsRNA feeding in *B. tryoni* is in accordance with findings reported by Li *et al.* (2011) and Dong *et al.* (2016). Li and colleagues observed that an overexpression of some of the dsRNA-targeted genes occurred after an initial knockdown was observed, when flies were continuously fed with dsRNA. In my study, QFFs fed with *topi* dsRNA showed no significant knockdown, and Dong *et al.* (2016) reported that *B. dorsalis* fed with *topi* dsRNA showed the same effect on transcript levels. Dong and colleagues suggested that an increase in transcript levels after dsRNA treatment may be due to a development of refractoriness to RNAi or a yet undefined immune response to exogenous dsRNA.

Li *et al.* (2015) have similarly suggested that exposure to dsRNA can induce refractoriness to subsequent dsRNA exposures in *B. dorsalis*. Refractoriness arising from continuous or frequent feeding on dsRNA might explain the apparent lack of knockdown of

topi and *trxt* in the QFF observed in my study. However, the same dsRNA feeding regimen successfully reduced *tssk1* transcripts, which would suggest that not all genes are equally affected by the same doses of dsRNA, and perhaps not all genes show refractoriness to dsRNA. Li and colleagues also noted that RNAi refractoriness diminishes with time, but it can nevertheless last up to 20 days. They proposed that the refractoriness duration could be related to the concentration of the first exposure dsRNA, and it's not sequence-specific. This suggestion is not supported by my observation of *tssk1* transcript knockdown in QFF, nor with the findings of Li *et al.* (2011), Zheng *et al.* (2015) and Dong *et al.* (2016), who also fed *B. dorsalis* a continuous supply of dsRNAs targeting different genes (all of them with the same dsRNA dose) and observed significant transcript knockdown. Perhaps the duration of RNAi refractoriness is influenced by the concentration of the first exposure dsRNA and if this is true, it might be different for every target gene, possibly in a sequence-specific manner.

Transcription upregulation after dsRNA treatment could also be due to a stress response or to tight regulation of certain genes' transcript levels. Following transcript knockdown by RNAi, cells could respond with an increase in the transcription rate of the gene in question, with the goal of overcoming the RNAi-mediated suppression. The mRNA levels could rise above average for a determined time, before gene expression stabilizes. It is possible that cells control the expression of some genes more tightly than others, depending on their biological functions; genes with key roles in a determined process could therefore recover from RNAi-mediated suppression faster than others with supplemental functions.

The disruption of gene expression caused by feeding *B. tryoni* with dsRNA presented in this study is evidence that dsRNA retains stability in the midgut of this species, at least long enough for it to be taken up by the gut cells and start the RNAi effect. The exact mechanism by which different insect epithelial cells in the midgut take dsRNA into their cytoplasm is still unknown, but Joga *et al.* (2016) suggest that this process must be very quick, due to the possible

presence of salivary enzymes in the gut tract that can degrade the dsRNA molecules. Wynant *et al.* (2014) identified some gut-specific dsRNases in the desert locust *Schistocerca gregaria*, and were able to increase the stability of dsRNA in the midgut juice of this species by previously injecting adults with dsRNA targeting these dsRNases. It is possible that *B. tryoni* possess some of these enzymes, so further studies are necessary to determine their presence and how they could affect RNAi efficiency in the QFF.

Much is still unknown about the movement of dsRNA in insects. Joga *et al.* (2016) presented a review in which they explain that the dissemination of dsRNA in nematodes requires the cooperation of SID-1 (a channel through which dsRNA passes to neighboring cells) and SID-2 proteins (which import dsRNA from the intestinal lumen through endocytosis). To date, *SID-1* seems to be present in most insects, while *SID-2* is not; furthermore, *Drosophila* and other dipterans lack *SID*-like genes. Ulvila *et al.* (2006) found some evidence of the involvement of scavenger receptors, such as *eater* and *SR-CI*, in dsRNA uptake in *Drosophila* S2 cells. Knockdown of these receptors significantly decreased the internalization of dsRNA by more than 90%. They also reported that *clathrin heavy chain* (*Chc*), a well-known component of the endocytic machinery, also plays a role in this process, since silencing of this gene also hindered RNAi in the cells.

Considering that *B. tryoni* is a dipteran, and according to the information presented above, probably lacks SID-like proteins, QFFs probably use endocytosis as their primary mechanism for dsRNA uptake and might also use an as yet, undiscovered transmembrane channel. Further studies are clearly required to uncover the dsRNA uptake mechanisms in this and other insects.

4.3. Mating assays:

An important finding of this study was that oral delivery of dsRNA targeting spermatogenesis genes successfully reduced the fertility and/or fecundity of *B. tryoni*. The production of some completely sterile males after feeding adults the dsRNA and the significant reduction in the number of progeny of *tssk1*- and *topi*-dsRNA-treated flies proved that the dsRNA can spread from the gut to the testis. The reduction in the progeny of *topi* dsRNA-treated males, even though no knockdown was detected, suggests that gene silencing occurred at an earlier point (prior to the day-10 qRT-PCR analysis) and was sufficient to affect sperm development. Dong *et al.* (2016) observed similar results in *B. dorsalis* after feeding adult flies with dsRNA targeting several genes involved in different phases of sperm formation, seeing a significant reduction in the number of eggs laid per day, egg hatching rates, number of sperm found in female spermathecae, and length of sperm. Further studies should try to elucidate if knockdown of *tssk1* in the QFF can also reduce these other aspects of male fertility.

While some completely sterile males were produced following knockdown of *tssk1*, there were still many males that showed only reduced fecundity, and for an SIT program, it will be highly desirable to decrease their fecundity more fully. Different dsRNA delivery approaches that increase the dsRNA stability in the digestive tract could increase the magnitude of gene suppression. Two possible methods to stabilize or protect the dsRNA in the gut are the use of polymeric nanoparticles and liposomes to deliver the dsRNA. Kumar *et al.* (2016) used chitosan/dsRNA nanoparticles to silence the wing development *gene vestigial* (*vg*) in *Aedes aegypti* mosquitoes; chitosan attaches to nucleic acids to form nanopolyplexes via ionic interactions, improving the extent of RNAi in the insects. Taning *et al.* (2016) use cationic liposomes to encapsulate dsRNAs targeting three different genes in the larvae of *Drosophila suzukii*. They observed efficient knockdown of the genes using these liposomes but feeding larvae with naked dsRNA was unsuccessful in suppressing gene expression.

Taning *et al.* (2016) also performed an experiment in which they fed *D. suzukii larvae* with two different dsRNAs simultaneously targeting two different vital genes. They observed a higher mortality rate in those flies fed with a combination of dsRNAs, relative to groups treated with only a single dsRNA. This finding suggests that targeting more than one spermatogenesis gene with different dsRNAs combined could provide a greater effect on the fertility and/or fecundity of *B. tryoni*.

The mating competition assays failed to show a significant reduction in the fecundity of the flies when *gfp*-treated individuals were mixed with dsRNA-treated flies from the treatment groups, although the slight reductions in fecundity that were observed suggest that with larger number of replicates and larger population cages, it might be possible to demonstrate that dsRNA-treated males can actively compete with untreated males. Mating competitiveness of sterile males is an important factor in the production of sterile insects suitable for SIT. In the mating competition assays, only five males were used in each cage (with varying proportions of dsRNA-treated insects), and at these densities, the impacts of the dsRNA were undetectable, unlike the previous experiment, where each dsRNA-treated male was assessed on his own, without competitors. It is important to note that the magnitude of gene knockdown was not the same in every treated individual. Hence, some flies may be more fecund than others; one fertile/high fecundity male fly can potentially mate with all the females in the cage, thereby masking the effect of the other sterile or low fecundity males.

It is also worth noting that the dsRNA-treated males appeared to become sexually active three days later than control males. This delay in mating activity could pose problems when using RNAi to produce male insects suitable for SIT, as the sterile males could prove less competitive than wild males in a natural mating system. However, we could prevent this from affecting the efficiency of the technique by simply ensuring that dsRNA-treated flies are released into wild populations once they have reached sexual maturity.

4.4. Accessory gland proteins analysis:

The analysis of the male accessory gland protein transcripts showed that after successful knockdown of the spermatogenesis gene *tssk1* in males of *B. tryoni*, *diso* and *obp2* transcript levels remained unaltered, when compared to control flies. This is an indicator that these males can transfer necessary proteins of the seminal fluid to females at the moment of copulation. Radhakrishnan & Taylor (2007) have argued the importance of these proteins for remating inhibition in females. Sterile male insects suitable for SIT should ideally be able to prevent females from remating with wild males, to ensure the efficiency of the technique, and transfer of male accessory gland proteins during mating may be essential for maximum efficacy of the sterilized males in reducing the population size.

In contrast, flies fed with *topi*- and *trxt*-dsRNA showed a significant overexpression of male accessory gland proteins. This observation suggests that attempted knockdown of some testis genes may have unexpected results on the male's overall reproductive capacity, which apart from disrupting their ability to produce viable offspring, could also affect the successful transfer of seminal fluid proteins during copula necessary for female remating inhibition. Interestingly, the *topi*-dsRNA-treated males did have reduced fecundity relative to the negative control males, and perhaps the increased male accessory gland protein expression in these males contributed to the overall reduction in egg production in the females. A closer examination of impacts of RNAi on other male accessory gland proteins and female fecundity would prove helpful in identifying the best RNAi targets to produce sterile QFF for SIT applications.

4.5. Final considerations:

My experimental results indicate that RNAi is operational in the Queensland fruit fly *B. tryoni*, as it does in most eukaryotic organisms (Hannon, 2002), and can be used to suppress

transcription of specific genes through delivery of exogenous dsRNA. Although many of the mechanisms by which these molecules act to prevent the translation of target mRNAs are still not entirely understood, it was proven that they are effective at knocking down spermatogenesis genes in the QFF and thereby reducing male fecundity. This result provides a strong incentive to continue to explore the potential applications of RNAi in pest management programs to control wild populations of *B. tryoni* in Australia.

Feeding assays showed that oral administration of dsRNA can successfully suppress the expression of a targeted gene; however, it might also result in an upregulation of some genes. Therefore, the selection of the best targets to produce sterile male flies suitable for SIT is vital for the efficiency of the technique. Due to the role of male accessory gland proteins in female remating inhibition, it is important to ensure that the accessory gland protein genes are not adversely affected by dsRNA treatment. Should they remain unaltered, dsRNA-treated male flies should be able to transfer those essential components of the seminal fluid during mating, and thereby compete more effectively with wild males for mates.

In order to promote the use of this RNAi technology in the global horticultural market, some challenges should be addressed. For example, a more significant reduction of the fertility and/or fecundity of the QFF could make this technology more appealing to investors. The use of nanoparticles and liposomes to deliver dsRNA to these insects could be a good approach for this purpose, since these RNA microcarriers have been suggested to increase the stability of dsRNA in the gut tract and facilitate their uptake by gut cells, thus increasing the efficiency of RNAi.

The elimination of females from the progeny during the QFF mass-rearing process becomes a necessity in modern SIT programs to increase their productivity. The results of the present study suggest that RNAi could also be used to target the female variant of genes involved in the sex determination pathway of *B. tryoni*, such as *doublesex* and *transformer*,

expecting to produce a shift in the sex ratio of the progeny towards a male predominance. Ideally, the elimination of females from the lab- or factory-bred populations should occur earlier in development, to reduce the costs of rearing both sexes. Feeding dsRNA to larvae has been effective in *B. dorsalis* (Li *et al.*, 2017), and hence, may prove effective in QFF to prevent female development.

In my study, I focused on delivering dsRNA to young adult insects, and observed a significant knockdown of the spermatogenesis genes. It would be interesting to assess whether larvae could be fed spermatogenesis gene-specific dsRNAs, to ensure that the dsRNA is within the tissues well before the spermatogenesis genes are transcribed. In my study, I targeted genes that appeared to be expressed only in adults, but it is quite possible that some gene expression occurred before the insects had ingested a sufficient dose of dsRNA to achieve full knockdown. By delivering dsRNA to the insects earlier, more complete sterility might be achieved. It will be interesting to determine whether the dsRNA persists throughout the lengthy pupation period of these insects, to determine whether larval feeding will improve the efficacy of the technique.

Future studies should also determine the number of dsRNA-treated males that should be released into the environment to reduce the QFF populations effectively. Shelly & McInnis (2016) reported that fruit flies with simple male courtships (such as *Bactrocera* spp.) require a lower sterile:wild male ratio than species with a complex courtship (like *C. capitata*). For *B. cucurbitae* populations, male ratios of 50:1 to 100:1 have produced up to 80% egg sterility (Shelly & McInnis, 2016); hence, this could be a good starting point in the study of dsRNA-treated:wild male ratio needed to control *B. tryoni* in Eastern Australia.

Overall, this study’s findings demonstrate that RNAi-mediated SIT for QFF control is worth considering, but there are still many parameters concerning target gene selection, dsRNA delivery and RNAi efficacy, and population responses to dsRNA-sterilized insects that must be explored before this technology is applied in the field.

5. CONCLUSIONS

1. Nine genes potentially involved in sperm formation at different phases of gametogenesis were found in *Bactrocera tryoni* based on their high degree of nucleotide identity with *B. dorsalis* and *Drosophila melanogaster*, and seven of them showed testis-specificity in the QFF.

2. The suppression of three spermatogenesis genes of *B. tryoni* after injections and one gene after oral delivery of dsRNA provides evidence that RNAi occurs in this species, and can be induced by the introduction of foreign dsRNA into the system; however, it can result in an upregulation of some accessory gland protein genes.

3. The production of some completely sterile males after feeding adults dsRNA and the significant reduction in the number of progeny of *tssk1*- and *topi*-treated flies proved that the dsRNA can spread from the gut to the testis and reduce the fertility and fecundity of *B. tryoni* males.

4. After successful knockdown of the spermatogenesis gene *tssk1* in males of *B. tryoni*, *diso* and *obp2* (accessory gland protein genes) transcript levels remained unaltered, but flies fed with *topi*- and *trxt*-dsRNA showed a significant overexpression of male accessory gland proteins. This observation suggests that attempted knockdown of some testis genes could result in a disruption of the male's overall reproductive capacity.

6. APPENDICES

Appendix I: dsRNA sequences

Green fluorescent protein

GAGAAGAACTCTTCACTGGAGTTGGTCCCAGTTCTTGTTGAATTAGATGGC
GATGTTAATGGGCAAAAATTCTCTGTCAGTGGAGAGGGTGAAGGTGATGCAACA
TACGGAAAACTTACCCTTAATTTTATTTGCACTACTGGGAAGCTACCTGTTCCAT
GGCCAACACTTGTCACTACTTTCTCTTATGGTGTTCAATGCTTCTCAAGATACCCA
GATCATATGAAACAGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTAC
AGGAAAGAACTATATTTTTCAAAGATGAC

Testis-specific serine/threonine kinase 1

CCGCGTAACGCATGAAGATGAAAATTTTCGGGCCACGCTGTAGTATACTGT
GTATCTGTATGATGTTTGGGTGATCGATTCTTGTTAGAATTTCCAATTCACGCGGA
AAGAATTTGTGTACGAAATCCGAGGGTGCCTTGGCCTTATCAATTATTTTGCAGG
CCAAATGTACACCATGACCCGCATCGTCGGCATAGCCAGCTGTGATGACGGTCG
CATAGGAACCCTCGCCAATTTTGTGTCCGATATTGTAGCCACGCTGAGCCAGGGC
ATCGATGTCGGAGCTGCGCGTATTCAGTTGGCGATTGGAGGTTGGGTACCCAATT
CGCCCTATAGTGAGTCGTATTACGCGCGCTC

Matotopetli

ACGCTTCTTAACTGGCTCCGTTTTCTATCAGCACCGTCTTATACATCGTGGC
GAGCGACGTTATGAATGTGAAGAGTGTGGTAAACGTTTTTATCGTGCAGATGCGC
TTAAGAATCATCAACGAATCCATACCGGTGAAAAACCATTTGGTTGTCTATTTTG

TACAAAGAATTTTCGTCAACGCGGCGATCGAGACAAGCATATAAGAGCTAGACA

TTCGCATTTGGATGCGAACGCTCGTTTAATGATGCAAATGCAAAAATTACAGTTG

GAAGCAGCAGCAGCTGCCGCTGCTGCTCAAGTAGTAGTTCAACAGCAGCATGCC

AATAATGCC

Thioredoxin T

GATCTGGATAAGAAGCTGGAGGAAGCCGTCGGCTCTGGTCAATTGGTGGTC

ATTGATTTTTTTGCAAACTGGTGTGGGCCATGTAAAATTATTAGTCCGAAACTCG

AAGAGTTGGCCACACAATATGCCGAGAAAGCGATTGTGTTGAAAGTCAATGTGG

ATGATTGTGAAGACATAGCCCTTGAGTACAACGTGACCAGTATGCCAACATTCGT

TTTTATCAAGGATAATCATGTTATTGATGTATTCGTTGGCGGCAACTCAGAGAAA

CTGGTCAAGAATATGGAGAAATATGTAGGCGAACCAATACCCGTAGAAAATATG

CCAGAAATTGTGGAAGCCGA

Appendix II: Fruit Fly Gel/Agar Diet

Ingredient	Amount	Percent Volume
Distilled H_2O	100ml	73.81%
Brewer's Yeast	20.40g	15.06%
White Sugar	12.18g	8.99%
Citric Acid *(product code: C2404)*	2.31g	1.70%
Nipagin	0.20g	0.15%
Sodium Benzoate *(product code: B3420)*	0.20g	0.15%
Wheat Germ Oil	0.20g	0.15%
Agar *(product code: A1296)*	1.00g	1.0%

<u>Notes</u>

- *Macquarie protocol:* above amount makes a total volume of **150ml** = 1 x rectangular takeaway container (17cm[l] x 12cm[w], 4cm[d]) for **250µl eggs**.
  250µl = **3500 eggs** = **2800 pupae** ie. 80% survival from egg to pupae

- *CSIRO protocol adapted for storage of diet in smaller containers:* **35ml** diet in 120ml 'castaway container' (depth of diet should be 1.2cm) for **58µl eggs.**
  58µl= 800 eggs = 640 pupae (assuming 80% survival from egg to pupae)
  Therefore 150ml of diet should make enough for 4 'castaway containers'

Method

1. Pour half of the water (50ml) into the blender
2. Add sugar, nipagin, sodium benzoate and citric acid to the blender and mix (all white powders), mix well
3. Add yeast to the blender and mix well again
4. Add 1g (1%) of agar to the remaining 50ml of water and dissolve carefully in the microwave
5. Add the wheat germ oil to the blender
6. Add agar to the blender and mix for a short time
7. Once agar has been added, dispense quickly into 'castaway containers'. Diet should be pH 3.5-4.
8. Allow diet to cool before securing lid onto container

Crack open lid at approximately 3 days after eggs added and remove lid completely when larvae are ready to pupate (ie. start exiting the media).

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