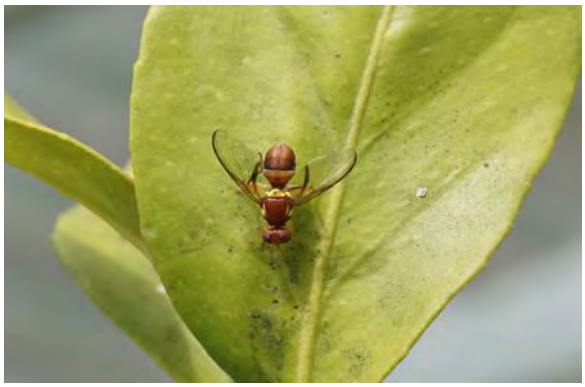
Dietary Methoprene and Caffeine as pre-release supplements for Queensland fruit fly *Bactrocera tryoni* (Froggatt) Sterile Insect Technique



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Master of Science in Entomology

Master of Research in Biological Sciences

A thesis submitted in fulfilment of the requirements for the degree of

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Summary

Sterile insect technique (SIT) is a sustainable pest management technique that involves releasing millions of sterile insects to suppress reproduction of pest populations. SIT has been employed to manage outbreaks of Queensland fruit fly (*Bactrocera tryoni* Froggatt, 'Q-fly'), the most difficult and costly challenge to market access for most Australian fruit growers. Due to a long adult maturation phase, immature Q-flies released in SIT can take a week or longer after release to attain sexual maturity. This delay from release until maturation can result in large proportion of sterile flies dying before becoming sexually active, and this can substantially constrain the efficacy of SIT. Treatments that promote early sexual maturity, as well as mating performance and longevity, provide valuable means of increasing abundance of sexually mature sterile males. Building on this understanding, my PhD investigated the viability of the juvenile hormone analogue methoprene and caffeine as pre-release treatments for Q-fly SIT. In laboratory trials, incorporation of a mosquito larvicide Nomoz (containings 40% s-methoprene) in a diet of sugar and yeast hydrolysate was found to substantially increase mating propensity of young male Q-flies; this effect was comparable to the effects obtained using analytical standard methoprene, providing an economic and practical method of methoprene application. The effect of methoprene on sexual activity of young male Q-flies is not only a behavioral response, but rather methoprene treatments resulted in accelerated maturation of reproductive organs. The effect of methoprene on mating propensity of young male Q-flies was confirmed in field cage studies that simulate field conditions; young treated sterile males (5-7 days old) sexually outcompeted untreated mature males. The effects of methoprene treatment on mating extended to post-copulatory success; the additional matings obtained by young treated males exhibited typical efficacy at inducing sexual inhibition in their mates. Contrasting potent effects on males, no effects of methoprene treatment on mating propensity or development were evident for females either in the laboratory or in field cages. The effects of methoprene treatment were not limited to sexual development physiologically but had a broad effect on physiology that present risks for operational use. Methoprene supplementation resulted in greatly increased activity levels in young flies, and reduced ability to tolerate challenges of nutritional and desiccation stress. In addition, potential of pre-release caffeine as a novel means to accelerate sexual maturation in Q-fly was demonstrated in one of my PhD projects. Dietary supplementation of newly emerged males with analytical caffeine significantly enhanced mating propensity and promoted

development of ejaculatory apodeme and testis in male Q-fly. Overall, my thesis demonstrates substantial potential of dietary methoprene and caffeine treatment to enhance the performance of Q-fly SIT through (1) increasing the proportion of released males that mature and contribute to the program and (2) by biasing operational sex ratio of released flies in favour of sterile males due to sex differences in response to methoprene application.

Keywords: Queensland fruit fly, sterile insect technique, methoprene, caffeine, pre-release treatment, sexual maturation.

Statement of Originality

I would like to declare that the work in this thesis entitled "**Dietary Methoprene and caffeine as pre-release supplements for Queensland fruit fly *Bactrocera tryoni* (Froggatt) Sterile Insect Technique**" has not previously been submitted for a higher research degree or diploma (except part of chapter two and chapter four) in any other universities; it is only being submitted to Macquarie University.

Part of chapter two and chapter four of my PhD thesis has already been examined as my MRes thesis (http://minerva.mq.edu.au:8080/vital/access/manager/Repository/mq:44536). Detailed declaration about the novelty and originality of chapter two and chapter four in my PhD thesis have been provided at the beginning of respective chapters.

To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Saleh Mohammad Adnan
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November 27, 2019

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Chapter 1 General introduction

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- PWT provided feedback on the text (10%).

Chapter 2 is entitled "Dietary methoprene supplement promotes early sexual maturation of male Queensland fruit fly *Bactrocera tryoni*" and has been published in *Journal of Pest Science* (September 2018, Volume 91, Issue 4, pp 1441–1454).

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- VM (10%), HA (5%), RM (5%), and IF (5%) assisted with conducting experiments.
- PWT jointly conceived and designed the work (10%), assisted with data analysis (20%) and provided substantial feedback on the manuscript (20%).

Chapter 3 is entitled "Accelerated sexual maturation in methoprene-treated sterile and fertile male Queensland fruit flies, and mosquito larvicide as an economical and effective source of methoprene" and has been accepted to be published in *Journal of Economic Entomology*.

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Chapter 4 is entitled "Dietary methoprene treatment promotes rapid development of reproductive organs in male Queensland fruit fly".

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- DPS assisted with data analysis (5%) and provided substantial feedback on the manuscript (5%).
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Chapter 5 is entitled "Dietary methoprene enhances sexual competitiveness of sterile male Queensland fruit flies in field cages" and has been accepted to be published in *Journal of Pest Science*.

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Chapter 6 is entitled "Methoprene-induced matings of young Queensland fruit fly males are effective at inducing sexual inhibition in females".

- SMA conceived and designed the work (90%), conducted the experiments (85%), analysed the data (85%) and wrote the manuscript (85%).
- PR (5%), and IF (10%) assisted with conducting experiments.
- PWT jointly conceived and designed the work (10%), assisted with data analysis (15%) and provided substantial feedback on the manuscript (15%).

Chapter 7 is entitled "Dietary yeast hydrolysate and methoprene supplements elevate locomotor activity of mass-reared Queensland fruit flies".

- SMA conceived and designed the work (90%), conducted the experiments (90%), analysed the data (75%) and wrote the manuscript (90%).
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Chapter 8 is entitled "Methoprene treatment increases development rate but also increases starvation and desiccation risk for Queensland fruit fly".

- SMA conceived and designed the work (90%), conducted the experiments (80%), analysed the data (90%) and wrote the manuscript (90%).
- PR (5%) and IF (15%) assisted with conducting experiments.
- PWT jointly conceived and designed the work (10%), assisted with data analysis (10%), and provided substantial feedback on the manuscript (10%).

Chapter 9 is entitled "Dietary caffeine supplements as a novel promotor of sexual development for fruit fly sterile insect technique".

- SMA conceived and designed the work (90%), conducted the experiments (60%), analysed the data (90%) and wrote the manuscript (90%).
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- PWT jointly conceived and designed the work (10%), assisted with data analysis (10%), and provided substantial feedback on the manuscript (10%).

Chapter 10 General Conclusions

- SMA conceived and outlined this chapter (100%), wrote this chapter (90%).
- PWT provided feedback on the text (10%).

Chapter One

Image captured by author only

General Introduction

1.1 Fruit flies

Tephritid fruit flies (Diptera: Tephritidae) present the greatest challenges to global fruit and vegetables production and trades by reducing the yield of marketable crops and triggering quarantines and restrictions on international trade in most tropical and subtropical regions and in some temperate regions of the world (Christenson and Foote, 1960; Hardy, 1969, 1983; Leblanc et al., 2013). The family Tephritidae comprises approximately 4,600 species, around 350 of which are reported as economically important horticultural pests (Christenson and Foote, 1960; White and Elson-Harris, 1992; Dhillon et al., 2005). Fruit flies belonging to genera *Anastrepha* (native to America), *Bactrocera* (native to Asia and Oceania), *Ceratitis* (native to Africa) and *Rhagoletis* (native to Eurasia and America) (Fletcher, 1987; Aluja, 1994; Drew, 2004; Drew et al., 2005) include the most economically important tephritid pests, whereas other genera, such as *Dacus*, *Zeugodacus*, and *Toxotrypana,* include relatively few fruit pests. In Australia, the average annual value of crops susceptible to fruit flies is around $4.8 billion (PHA, 2018), and the National Fruit Fly Strategy has identified 46 species as 'high priority pests' of concern (PHA, 2008). Most of these species are exotic to Australia, primarily found in South-East Asia and the South Pacific (PHA, 2008, 2018) and are yet to establish populations in Australia. Australia has ca. 80 native fruit fly species (Drew, 1989) with 11 economically significant pest species (Hancock, 2000; PHA, 2008, 2018).

1.2 Queensland fruit fly status and its management

Of the nine *Bactrocera* species that are currently present in Australia, Queensland fruit fly (Q-fly), *Bactrocera tryoni* (Froggatt), presents the greatest challenge to interstate and international market access for horticultural commodities produced in eastern Australia (PHA, 2008; Dominiak and Daniels, 2012; Dominiak and Mapson, 2017; Fig 1). Q-fly imposes significant costs in terms of yield loss and lost trade as well as monitoring, quarantine regulation, and control (Bateman, 1991; Sutherst et al., 2000). While tropical and subtropical coastal Queensland and northern New South Wales is considered the original range for Q-fly (Gilchrist et al., 2006), this species has significantly expanded its range in eastern Australia and is also present in Northern Territory (Clarke et al., 2011), New Caledonia (White and Elson-Harris, 1994), Tahiti (Leblanc et al., 2011), and South Pacific island nations (Drew et al., 1978; Meats, 1981; Gilchrist et al., 2006; Dominiak and Daniels, 2012). Q fly larvae was first reported to infest native fruits in 1819 in tracts of tropical

rainforests along the coastal areas of Queensland and northern NSW (May, 1962; Drew, 1989), but changes in rainforest distribution as well as commercial cultivation of introduced hosts such as peaches, apricots, nectarines, grapes, oranges and pears, have enabled Q-fly to substantially expand its host range and distribution (May, 1962; Bateman, 1968; Maelzer, 1990; Gilchrist and Meats, 2009). In recent times, incursions have been reported in Tasmania, (e.g., Flinders Island; https://www.dpipwe.ras.gov.au.) and in New Zealand (Biosecurity New Zealand, 2019; https://www.biosecurity.govt.nz.). Q-fly presents an increasing threat to the "fruit fly free" horticultural regions of South Australia, Tasmania (Sutherst, 2000; Sultana et al., 2017) and also to New Zealand.

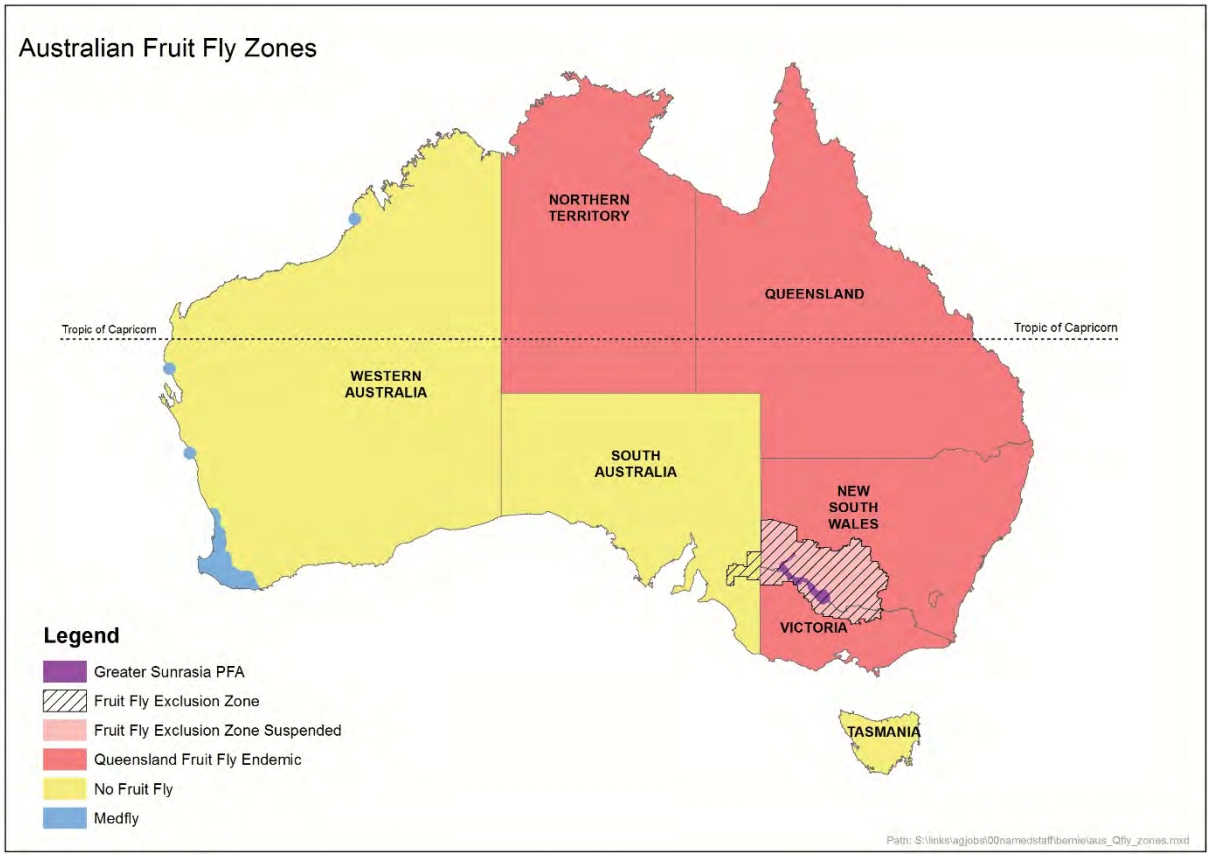

Fig 1. Distribution of Queensland fruit fly *Bactrocera tryoni* and Mediterranean fruit fly *Ceratitis capitata* in Australia (Dominiak and Mapson, 2017).

Q-flies undergo complete metamorphosis through four life stages: egg (Anderson 1962, 1963a, b), three larval instars (Anderson 1963b, 1964), pupa, and adult. Adult females lay eggs (sting) under the skin of fruits and vegetables. The newly hatched larvae start developing inside the fruit and late third instar larvae exit the fruit to pupate in the soil. Eight

to ten days after pupating, flies emerge as sexually immature adults (Bateman, 1972; Meats, 1981; Fletcher, 1987). During oviposition, female flies introduce bacteria into the fruits, and infested fruits start to decay and often drop prematurely. Infested fruits are readily recognised, since rots develop rapidly and the skin around the sting marks becomes discoloured. Q-fly infests more than 100 native and introduced hosts including citrus, pome, stone fruits, tomato, banana, coffee, berries and tropical fruits, and fruiting vegetables (May, 1953, 1957, 1960; Drew, 1989; Hancock et al., 2000). A total export value of $432 million has been estimated for 25 economically important Q-fly hosts (PHA, 2009). In Australia, the Queensland fruit fly, *Bactrocera tryoni* (Froggatt), or 'Q-fly', presents a costly challenge to Australia's $13.5 billion horticulture industry, affecting a vast diversity of commercial and non-commercial crops (Hancock et al., 2000, Clarke et al., 2011. Dominiak and Daniels, 2012). However, the above figures do not include costs to backyard growers, costs of restricted access to domestic or international markets, and flow-on costs to related industries, such as food retailers and processors, or the wine industry. Also, impact on ecosystem as well as human health due to repeated application of synthetic insecticides to control Q-fly populations remains invisible.

For decades growers have relied on 'traditional' methods to protect crops from Q-fly infestation (Bateman, 1966a, b; Jessup et al., 2007; Dominiak and Ekman, 2013; Suckling et al., 2016; Stringer et al., 2017). These include cover sprays of the organophosphate insecticides, especially with dimethoate and fenthion (APVMA, 2011, 2012; Reynolds et al., 2017) along with moderate use of other approaches, such as lure and kill technique primarily with protein bait sprays (Lloyd et al., 2003; Balagawi et al., 2014), male annihilation technique (MAT) using cue-lure as a potent male attractant (Monro and Richardson, 1969; Dominiak et al., 2003), natural enemies as biological control agents (Snowball et al., 1962a,b; Snowball, 1966; Ero et al., 2011), and sterile insect technique (SIT) (Monro, 1961; Monro and Osborn, 1967; Bateman, 1991; Meats et al., 2003). However, owing to concerns about environmental safety as well as human health, the Australian Pesticides and Veterinary Medicines Authority (APVMA) has significantly restricted the use of dimethoate (APVMA, 2015a) and fenthion (APVMA, 2015b). These restrictions together with increasing threat to major southern fruit-producing regions and the remaining fruit fly-free regions (Clarke et al., 2011; Stringer et al. 2017) have promoted the importance of sustainable approaches, such as SIT, to defend the remaining fruit fly free regions and to integrate SIT as part of an overall management strategy for endemic regions (Stringer et al., 2017).

1.3 SIT and pre-release treatments

Amongst the available alternative and effective control measures for Q-fly, SIT is growing rapidly in priority. In SIT programs, millions of insects are mass-reared, sterilized (usually by irradiation), and released in the field to induce reproductive failure in pest populations (Knipling, 1955; Benelli et al., 2014; Stringer et al., 2017). Over generations, the wild population is reduced (Knipling, 1955). SIT has been used to eradicate screwworm in north and central America (Kafsur et al., 1987; Vargas-Terán et al., 2005) and has proven to be an efficient and sustainable management approach to manage some of the most damaging fruit fly pests, including Mediterranean fruit fly, or 'medfly' *Ceratitis capitata* (Enkerlin et al., 2015), Mexican fruit fly *Anastrepha ludens* (Orozco- Dávila et al., 2007, 2015), Oriental fruit fly *Bactrocera dorsalis* (Vargas et al., 2010) and Queensland fruit fly *Bactrocera tryoni* (Monro, 1961; Monro and Osborn, 1967; Sproul et al., 1992, Dominiak and Ekman, 2013, Stringer et al., 2017). SIT for Q-fly was first investigated in the 1960s (Monro, 1961, Monro and Osborn, 1967) and has been used to suppress outbreaks for more than two decades, including a successful eradication in Western Australia in 1989 (Sproul et al., 1992; Dominiak et al., 2000; Meats et al., 2003; Reynolds and van der Rijt, 2011; Reynolds et al., 2012; Fanson et al., 2014). A substantial renewal and improvement of Q-fly SIT is currently underway.

Effectiveness of SIT programs is mostly associated with the ability of released sterile males to participate in mating activity. Therefore, abundance of sexually mature sterile males and sexual performance of these males are key factors in the success of SIT programs (Hendrichs et al., 2002). In SIT programs, sterile Q-flies are usually released at 2 or 3 days of age, when still sexually immature, and attain maturation in a week or longer after release. This long adult maturation phases in Q-fly (Pérez-Staples et al., 2008, 2009, 2011), similar to other *Bactrocera* and *Anastrepha* species (Liedo et al., 2013), together with high mortality caused by predators, pathogens, and abiotic stressors (Meats, 1998; Weldon and Meats, 2010), can sharply diminish the abundance of sexually mature sterile Q-flies. A very large proportion of released males die before maturing and so fail to participate as control agents in the field (Meats, 1998). To increase sexual development before release, flies could be held for additional days, but this would bring about additional operational costs (food, space, and

staff) and may increase physical damage and stress to the flies (Teal and Gómez-Simuta, 2002). To minimise pre-release holding periods, there is significant interest in pre-release treatments that promote mating performance of young released males and increase post release survival in the field, so that a greater proportion of released flies participate in SIT. (Yuval et al., 2007; Pereira et al., 2013b; Akter et al., 2017).

Adult tephritid fruit flies typically emerge with immature reproductive organs and need to forage for the nutrients to complete reproductive development (Fletcher, 1987; Wheeler, 1996; Drew and Yuval, 2000). Like many other fruit flies, Q-flies are anautogenous and require a source of protein to complete reproductive development (Vijaysegaran et al., 2002; Pérez-Staples et al., 2008, 2009, 2011). In nature, fruit flies obtain nutrition from bacteria, yeasts, plant leachates, fruit juices, pollen, and faeces (Hendrichs et al., 1993; Drew and Yuval, 2000). Most mass-rearing programs provide adult tephritid flies yeast hydrolysate (YH) as a rich source of amino acids, carbohydrates, sterols, vitamins, and minerals (Barry et al., 2007; Lee et al., 2008; Chang, 2009; Fanson and Taylor, 2012). Positive effects of post teneral YH feeding on reproductive development and mating propensity have been reported in numerous species, including *A. ludens* (Pereira et al., 2013a), *A. obliqua* (Liedo et al., 2013), *A. suspensa* (Pereira et al., 2009), *A. serpentina* (Aluja et al., 2001), *Z. cucurbitae* (Haq et al., 2010a, b), *B. dorsalis* (previously *philippinensis*) (Obra and Resilva, 2013), *C. capitata* (Yuval et al. 2002), and *Rhagoletis pomonella* (Webster and Stoffolano, 1978). In Q-fly provision of YH along with sucrose promotes development (Vijaysegaran et al., 2002; Meats and Leighton, 2004, Pérez-Staples et al., 2011; Weldon and Taylor, 2011), enhances mating probability (Pérez-Staples et al., 2007), increases ability of males inhibit sexual receptivity in mates (Meats et al., 2004; Pérez-Staples et al., 2008), and increases longevity (Pérez-Staples et al., 2007; Prabhu et al., 2008; Fanson et al., 2009; Taylor et al., 2013).

While significant benefits accrue from providing the released flies nutritional resources to support development, additional treatments that can further decrease the delay between release and maturity remain of particular interest. To date, several pre-release treatments, particularly plant semiochemicals, have been explored for their effects on sexual performance of already mature fruit flies, such as methyl eugenol to which males of some species are attracted and from which they gain substantial sexual benefits (Shelly and Edu, 2008; Shelly et al., 2005, 2008, 2010a, b; Ji et al., 2013; Orankanok et al., 2013; Haq et al.,

2018). In addition, exposure to fruit compounds, orange oil, and ginger root oil has been found to increase mating performance in *A. ludens* and *A. obliqua* (Liedo et al., 2013), in *A. fraterculus* (Vera et al., 2013), and *C. capitata* (Shelly et al., 2004, 2007, 2010b; Papadopoulos et al., 2006; Bricenõ et al., 2007; Shelly, 2008; Juan-Blasco et al., 2013; Paranhos et al., 2013; Steiner et al., 2013; Kouloussis et al., 2013). Moreover**,** dietary access to cuelure, an analogue of raspberry ketone, has been found to increase expression of energetic pathways and to increase sexual performance in mature male Q-flies for 2 - 3 days (Kumaran et al., 2014). Akter et al. (2017) found that raspberry ketone mixed in the diet of immature male Q-flies induces acceleration of sexual maturation that is a close match to the effects of methoprene (Collins et al., 2014).

1.4 Methoprene as pre-release supplement in SIT

Juvenile hormone (JH) functions in the endocrine system of insects and regulates numerous physiological processes, including larval development, metamorphosis, diapause, and reproduction (Vogel et al., 1979; Riddiford and Ashburner, 1991; Wyatt and Davey, 1996; Gilbert et al., 2000; Dubrovsky, 2005). JH not only regulates sexual receptivity, oocyte maturation, egg deposition, and ovarian vitellogenin in females (Shemshedini and Wilson, 1993; Ringo, 2002; Dubrovsky, 2005), but also it promotes the development and maturation of reproductive accessory glands in males, enhancing their growth and the production of glandular secretions (Yin et al., 1999, Wilson et al., 2003), and the synthesis of sex pheromones (Rantala et al., 2003). Because of physiological role played by JH in insect reproductive development, an analogue of JH, methoprene, has been explored as a treatment to accelerate development of adult fruit flies for use in SIT programs (Teal et al., 2000, 2013). While provision of methoprene in addition to YH rich adult diet significantly accelerated sexual maturation in some fruit flies, including *Z. cucurbitae* (Haq et al., 2010a, b), *A. fraterculus* (Segura et al., 2009, 2013; Abraham et al., 2013; Liendo et al., 2013), *A. ludens* (Gómez et al., 2013; Pereira et al., 2013a; Gómez-Simuta et al., 2017), and *A. suspensa* (Pereira et al., 2009, 2010), as well as Q-fly (Collins et al., 2014), such effect of methoprene on sexual maturation of *B. dorsalis* and *C. capitata* was not evident (Shelly et al., 2009). However, development of effective and practical delivery systems to apply methoprene treatment has not been straightforward (Teal et al., 2013). In early studies, methoprene dissolved in acetone was applied topically to adult flies (e.g. Teal et al., 2000) or to pupae by dipping them in methoprene-acetone solutions (Pereira et al., 2013a), and

these treatments were very effective in Q-fly (Collins et al., 2014). However, several drawbacks are associated with acetone as a carrier for methoprene application. Firstly, acetone solution is not a practical delivery method for operational SIT owing to its toxicity and flammability as well as disposal issues (Pereira et al., 2013a; Segura et al., 2013). Secondly, in the context of the SIT, the topical application of millions of adults with methoprene is not feasible. In addition, Q-fly dipping of pupae in acetone solution resulted in reduced adult emergence, increased wing deformity, and reduced flight ability (Collins et al., 2014). Seeking an alternative approach, dietary treatment with methoprene was explored by Teal et al. (2007), providing immature adults of *A. suspensa* and *C. capitata* access to an agar-based diet containing sugar, YH, and methoprene. While this method was found effective at improving sexual competitiveness, it was considered impractical owing to handling and legal issues (Teal et al., 2013). Mainly two limitations were associated with this method for operational use at fly emergence and release facilities: adding protein hydrolysate to the wet agar-sugar diet makes the diet sticky for sterile flies, causing significant mortality. Secondly, such a diet would waste a considerable amount of methoprene, because the amount of agar provided to flies in production facilities, used as a carrier for the water, far exceeds the amount consumed by the flies. Meanwhile, additional investigations of dietary approaches were carried out by Aluja et al. (2009), Gómez et al. (2013) and Gómez-Simuta et al. (2017), with promising effects for *A. ludens* and marginally in *A. serpentina*. Given these promising studies of other species, dietary administration of methoprene is a promising avenue to explore for deployment of methoprene as a pre-release treatment for Q-fly SIT programs.

1.5 Caffeine

So far, several compounds such methoprene, Rk, methyl eugenol, have been reported as metabolic enhancer in multiple fruit fly species, while potential of caffeine as metabolic enhancer has not been explored much. Caffeine is a worldwide-recognised psychoactive drug known for its stimulatory properties (Alhaider et al. 2010; Penetar et al. 1993). Being a metabolic enhancer, caffeine renders diversified impacts on wider range of taxa including insects (Alhaider et al., 2010; Penetar et al., 1993; Andretic et al., 2008; Nall et al., 2016; Roehrs and Roth, 2008; Nishi et al., 2010). In vertebrates, caffeine mostly acts on the central nervous system, whereas it influences energy reserves and metabolic activity in invertebrates (Cruz et al., 2016; Nehlig et al., 1993). In addition to the familiar effects on humans,

caffeine showed potential effect on sexual maturation in tephritid fruit flies by elevating mating propensity. In a previous study with *C. capitata*, Arita and Kaneshiro (1988) reported that males *C. capitata* emerged from Arabian coffee, (*Coffea arabica* L.) were sexually more succesful in securing matings with females than the males emerged from Jerusalem cherries (*Solanum pseudocapsicum* L.). In addition, Aquino et al. (2016) incorporated guarana powder, which contains caffiene, into adult diet of laboratory *C. capitata*, and compared mating competitiveness against untreated laboratory and wild flies. Males that were given access to guarana supplements outperformed other males. Therefore, both of the scenarios raised a possibility that stimulatory effect of caffeine could translate into elevated sexual effort that would match the effect of metabolic enhancers like methoprene or RK in Q-fly (Adnan et al., 2018; Akter et al., 2017).

1.6 Thesis objectives

Fruit flies usually have long adult maturation phases and high mortality rates in the field, such that a quite small proportion of the released flies might survive to mature and contribute to SIT (Liedo et al., 2002); Q-flies are no exception (Pérez-Staples et al., 2008; Weldon et al., 2008; Reynolds et al., 2012). To minimize the delay between release and maturity and to ensure participation of a large portion of released flies in mating activity, methoprene has been investigated as a potential enhancer of sexual development of mass reared sterile flies including Q-fly (Pereira et al., 2009; Segura et al., 2009; Gómez et al., 2013; Collins et al., 2014). However, methods of delivering methoprene in large-scale operational settings are not well developed and require validation and refinement. While most studies have focused on accelerating sexual maturation, potential broader impacts of methoprene treatment on other physiological traits such as stress tolerance and activity levels have not been considered at all. It is important to develop a richer understanding of the effects of methoprene treatment on the physiology and performance of fruit flies, including Q-fly. In addition, to gain insight into the role of caffeine in sexual development, it is imperative to investigate whether sexual maturation of male Q-flies is accelerated by caffeine supplements. Therefore, my PhD projects aim to:

1. Investigate the effects of dietary incorporation of Nomoz (a cheap source methoprene) mixed with YH-rich adult diet on pre-copulatory and post copulatory sexual performance of Q-flies in laboratory and outdoor settings.

2. Investigate the effects of methoprene treatment on eco-physiological fitness of Q-flies.
3. Investigate the effects of caffeine treatment on sexual development of male Q-flies.

1.7 Thesis Structure

This thesis is formatted following a 'thesis by publication' style and it comprises nine chapters. The structure of the thesis is briefly outlined below:

Chapter 1 This chapter reviews the economic significance of Q-fly in Australian horticulture, ongoing management tactics, and the growing role of SIT as a sustainable control option, and the potential of methoprene and caffeine as pre-release treatment that can improve the efficacy of Q-fly SIT.

Chapter 2 is entitled "Dietary methoprene supplement promotes early sexual maturation of male Queensland fruit fly *Bactrocera tryoni*" and has been published in *Journal of Pest Science* (September 2018, Volume 91, Issue 4, pp 1441–1454).

Chapter 3 is entitled "Accelerated sexual maturation in methoprene-treated sterile and fertile male Queensland fruit flies, and mosquito larvicide as an economical and effective source of methoprene" and has been accepted to be published in *Journal of Economic Entomology*.

Chapter 4 is entitled "Dietary methoprene treatment promotes rapid development of reproductive organs in male Queensland fruit fly" and has been formatted in accordance with style of *Journal of Insect Physiology*.

Chapter 5 is entitled "Dietary methoprene enhances sexual competitiveness of sterile male Queensland fruit flies in field cages" and has been accepted to be published in *Journal of Pest Science*.

Chapter 6 is entitled "Methoprene-induced matings of young Queensland fruit fly males are effective at inducing sexual inhibition in females" and has been formatted in accordance with style of *Journal of Applied Entomology*.

Chapter 7 is entitled "Dietary yeast hydrolysate and methoprene supplements elevate locomotor activity of mass-reared Queensland fruit flies" and has been formatted in accordance with style of *Journal of Insect Physiology*.

Chapter 8 is entitled "Methoprene treatment increases development rate but also increases starvation and desiccation risk for Queensland fruit fly" and has been formatted in accordance with style of *Journal of Insect Physiology*.

Chapter 9 is entitled "Dietary caffeine supplements as a novel promotor of sexual development for fruit fly sterile insect technique" and has been formatted in accordance with style of *Journal of Pest Science*.

Chapter 10 draws general conclusion based on results found in previous chapters and suggests possible relevant future research scopes and orientations.

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Chapter Two

Image captured by author only

This chapter has been published in *Journal of Pest Science*

Declaration

Chapter two of my PhD includes and builds upon findings reported in my MRes thesis (http://minerva.mq.edu.au:8080/vital/access/manager/Repository/mq:44536). Even though similarity remains between chapter two of my PhD thesis and my MRes thesis, several additional experiments were conducted during PhD to investigate the effect of Methoprene and Yeast hydrolysate on sexual maturation and longevity of Q-fly and substantial changes were made to the presentation. The collection of additional data for Chapters two of my PhD comprised the three months of data collection in my PhD, followed by several months of re-analysis and writing. Novelty and development of research reported in Chapter two of my PhD thesis is been outlined below:

1. Experiments and data collection: I conducted two additional replicates of experiments to assess the effect of Methoprene and Yeast hydrolysate on Q-fly longevity during my PhD candidature while data on mating trials were obtained during my MRes.
2. Data analysis and visualisation: Data obtained during my MRes and from experiments conducted in the first three months of my PhD have been subjected to revised statistical analysis (For changes see Tables 1, 2, 3, 4 in Chapter two and also tables 4, 5, 6, 7 in my MRes thesis). In addition, I created new graphical presentations for Chapter two for better visualisation of analysed data and these are completely different from those reported in my MRes thesis (For changes See Figures 1, 2, 3, 4, 5, 6 in Chapter Two and also Figures 5, 6, 7, 8 in my MRes thesis).
3. Writing and conceptualisation: While there is substantial similarity in the issue addressed in Chapter two of my PhD and part of my MRes thesis, I very substantially modified the presentation and re-wrote most of the content (See abstract, introduction, results, discussion, conclusion, references section in Chapter two and also check with respective sections in MRes thesis). The additional work conducted in the PhD was required to bring the work to a suitable standard for publication.

Overall, while there is of course similarity because Chapter two builds upon work of my MRes thesis, Chapter two of my PhD thesis also includes substantial distinctiveness in terms of data collection, analysis, visualisation, and writing that goes well beyond the material examined in my MRes thesis.

Overview

Juvenile hormone analogue methoprene, has been investigated as a potential enhancer of sexual development of adult fruit flies for use in SIT programs. Provision of methoprene can substantially reduce the duration of the adult maturation phase in fruit flies so that a greater proportion of released flies might survive to mate, or pre-release holding periods can be reduced. So far, methoprene has most often been administered topically in acetone solution, which is toxic, flammable, and impractical for operational settings. As a practical alternative, in this chapter, I incorporated methoprene (0, 0.05, 0.1, and 0.5%) into Q-fly adult diet of sugar only or sugar mixed with yeast hydrolysate for 2 days, and then provided sugar only for the rest of the trial period and investigate whether dietary methoprene promotes sexual maturation in young Q-fly. In addition, longevity trails have been undertaken to assess effects of diet and methoprene supplements on Q-fly survival.

Dietary methoprene supplement promotes early sexual maturation of male Queensland fruit fly *Bactrocera tryoni*

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Short title: Dietary methoprene promotes fruit fly maturation

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Key Message

- Queensland fruit flies have long adult maturation phases and together with high mortality rates this can substantially reduce number of released flies that survive to mature and contribute to sterile insect technique (SIT) programs.
- Incorporation of methoprene, a juvenile hormone analogue, in adult diet for two days, simulating a pre-release holding period, significantly accelerated male but not female sexual development.
- Pre-release methoprene dietary supplements show potential to enhance Queensland fruit fly SIT by decreasing the delay between release and maturity, by increasing the proportion of males that attain sexual maturity in the field, and by biasing operational sex ratio of released flies.

Abstract

Sterile insect technique (SIT) is an environmentally benign pest management technique that relies on released sterile male insects mating with, and curtailing reproduction of, wild females. However, for species with high mortality rates and long adult maturation phases, a large proportion of the released insects can die before maturing and so fail to contribute to SIT. To counter this problem, inclusion of yeast hydrolysate in pre-release diets and treatment of pupae or adults with methoprene, a juvenile hormone analogue, have been investigated as means of accelerating development of some fruit flies, including Queensland fruit fly, *Bactrocera tryoni* (Froggatt) ('Q-fly'). Methoprene has most often been administered topically in acetone solution, which is toxic, flammable, and impractical for operational settings. As a practical alternative, we incorporated methoprene (0, 0.05, 0.1, and 0.5%) into Q-fly adult diet of sugar only or sugar mixed with yeast hydrolysate for two days, and then provided sugar only for the rest of the trial period. Mating performance of males and females was tested from 4 to 30 days of age. Flies provided sugar mixed with yeast hydrolysate had increased mating propensity in comparison to flies that were provided sugar only. At all ages and for both diets, all methoprene doses increased male mating probability. Methoprene treatment did not affect copula latency of males that received yeast hydrolysate, but males that received only sugar mated earlier if they had received 0.05% methoprene. Methoprene treatment of males was also associated with longer copulations, which may affect fertility of females that later remate. Females differed from males in that methoprene treatment did not significantly affect mating probability or latency, but resembled males in that methoprene treatment resulted in longer copulations. Sex differences in response to methoprene may lead to male-biased operational sex ratio when bisex Q-fly strains are used in SIT. Yeast hydrolysate increased longevity of both males and females, but methoprene treatment did not affect longevity. Overall, findings of the present study indicate that Q-fly sexual maturation can be accelerated, and SIT might hence be enhanced, by incorporation of methoprene and yeast hydrolysate in pre-release diet.

Keywords: Q-fly, Tephritidae, yeast hydrolysate, sterile insect technique, development

Introduction

Tephritid fruit flies are amongst the world's most damaging insect pests, constraining both production and trade in most regions. The Sterile Insect Technique (SIT) has been developed as an effective regional means of managing some of the most economically significant fruit fly species including *Ceratitis capitata* (Wiedemann) (Mediterranean fruit fly, or medfly, Reyes et al. 2007), *Zeugodacus* (previously *Bactrocera*) *cucurbitae* (Coquillett) (melon fly, Kakinohana 1994; Itô et al. 2003), *Bactrocera dorsalis* (Hendel) (Oriental fruit fly, Orankanok et al. 2007) and *Anastrepha ludens* (Loew) (Mexican fruit fly, Orozco-Dávila et al. 2015). SIT involves releasing millions of sterile male fruit flies that mate with wild females, inducing reproductive failure (Knipling 1955; Krafsur 1998; Benelli et al. 2014a, b). As a result, the reproductive potential of pest populations is reduced and, over generations, pest abundance declines.

The Queensland fruit fly, *Bactrocera tryoni* (Froggatt) or 'Q-fly' presents the most difficult and costly challenge to market access for fruit producers in eastern Australia, affecting a vast diversity of commercial and non-commercial crops (Hancock et al. 2000; Clarke et al. 2011; Dominiak and Daniels 2012). Following restrictions on the use of organophosphate insecticides owing to concerns about environmental and human health (Dominiak and Ekman 2013), there has been substantial interest and investment in development of SIT as a sustainable and environmentally benign solution to protect 'fruit fly free' regions from Q-fly outbreaks, as well as to suppress pest abundance in endemic areas. SIT has been used intermittently to manage Q-fly outbreaks for more than 20 years (Dominiak et al. 2000; Meats et al. 2003; Reynolds and van der Rijt 2011; Reynolds et al. 2012; Fanson et al. 2014). With current plans for more routine and widespread use of SIT, there is a need for research aimed at improving efficacy and efficiency.

SIT programs rely on the participation of released sterile males in mating activity. Sterile Q-flies are released as sexually immature adults and may take a week or longer after release to mature and be attracted to cue lure traps (Weldon et al. 2008; Reynolds et al. 2012). Abundance of released Q-flies can be quickly diminished by predators, pathogens, and abiotic stressors (Meats 1998; Weldon and Meats 2010). To counter slow development and high field mortality, there has been particular interest in practises that promote early sexual maturity, as well as mating performance and longevity, so that a greater proportion of

released flies participate in mating activity. The period during which sterile fruit flies are held as adults before release provides opportunity for provision of resources and application of treatments that can improve the performance of released sterile flies (Pereira et al. 2013b; Akter et al. 2017).

Like most fruit flies (Hendrichs and Prokopy 1994; Drew and Yuval 2000; Liedo et al. 2013), Q-flies are anautogenous, relying largely on nutritional resources acquired as adults, especially protein, in order to complete sexual development (Vijaysegaran et al. 2002; Weldon and Taylor 2011; Fanson and Taylor 2012). Yeast hydrolysate (YH) provides essential nutritional resources for somatic maintenance and development in Q-fly rearing programs (Monro and Osborn 1967; Dominiak et al. 2008; Fanson et al. 2014). As a pre-release nutritional supplement in SIT programs, YH may ensure that reproductive development is already well under way and supported before flies are released. In Q-fly, provision of YH together with sucrose has been found to sustain development (Meats and Leighton 2004; Pérez-Staples et al. 2011; Vijaysegaran et al. 2002; Weldon and Taylor 2011), enhance mating propensity and competitiveness (Pérez-Staples et al. 2007, 2009), increase longevity (Fanson et al. 2009; Pérez-Staples et al. 2007; Prabhu et al. 2008), and increase abundance of sexually mature (cuelure responsive) males in field releases (Reynolds et al. 2014) (for a review, see Taylor et al. 2013).

In addition to benefits from the nutritional resources of YH to sustain development, some fruit flies exhibit substantially accelerated sexual maturation when treated with methoprene, a juvenile hormone analogue, including *Z. cucurbitae* (Haq et al. 2010a,b), *Anastrepha fraterculus* (Wiedemann) (South American fruit fly, Segura et al. 2009; 2013; Liendo et al. 2013, Abraham et al. 2013), *A. ludens* (Gomez et al. 2013; Pereira et al. 2013a; Gomez-Simuta et al. 2017), and *Anastrepha suspensa* (Loew) (Caribbean fruit fly, Pereira et al. 2009, 2010), as well as Q-fly (Collins et al. 2014). A variety of methods have been used to apply methoprene (for a review, see Teal et al. 2013). Early studies dissolved methoprene in acetone that was applied directly to adult flies (e.g., Teal et al. 2000). Later studies found that similar effects could be obtained by dipping pupae into methoprene-acetone solutions (e.g., Pereira et al. 2013a). Both of these methods are effective for accelerating sexual maturation of Q-fly (Collins et al. 2014). While dipping of pupae is a more practical approach compared with topical treatment of adults, the use of acetone in a production setting still poses significant health hazards, owing to toxicity, volatility and flammability, as well as disposal

issues (Pereira et al. 2013a; Segura et al. 2013). Seeking more readily implemented approaches, Teal et al. (2007) provided immature adults of *A. suspensa* and *C. capitata* an agar-based diet containing sugar, YH and methoprene, and found this to be effective at improving sexual competitiveness. However, Teal et al. (2013) considered that this approach produced large amounts of waste, became sticky, and was not cost effective. Aluja et al. (2009) formulated slurries of methoprene in acetone mixed with diet, then dried off the acetone, and fed the methoprene-containing food to tested flies. This treatment produced clear positive effects for *A. ludens* (depending on larval host fruit), some positive effects for *Anastrepha serpentina* (Wiedemann) (sapodilla fruit fly), and no significant effects for *Anastrepha obliqua* (Macquart) (West Indian fruit fly) or *Anastrepha striata* (Schiner) (guava fruit fly). Gomez et al. (2013) and Gomez-Simuta et al. (2017) also used methoprene combined with food, but in this case as a water-soluble formulation mixed with sugar and YH, and this was very effective for *A. ludens* in the laboratory, in field cages, and in the field. Collins et al. (2014) demonstrated accelerated development in Q-fly treated with methoprene in an acetone solution application as immature adults or pupae, with substantially increased mating propensity in young males. However, treatment of Q-fly pupae with methoprene/acetone solution resulted in reduced adult emergence, increased prevalence of wing deformity, and reduced flight ability. A more practical and effective approach is needed before methoprene can be considered as a pre-release treatment for Q-fly SIT programs.

Building on the previous demonstration that methoprene treatment in acetone solution accelerates sexual development of Q-fly (Collins et al. 2014), and studies in which methoprene has been administered through the adult diet of *A. ludens* (Aluja et al. 2009; Gomez et al. 2013; Gomez-Simuta et al. 2017) and *A. suspensa* (Teal et al. 2007), in the present study we investigate whether sexual maturation of male and female Q-flies is accelerated by methoprene incorporated into the pre-release adult diet. Because single sex strains are not yet available for Q-fly, it is important to test effects of pre-release treatment on females as well as males. Some Q-fly SIT programs have provided the flies just sugar as food prior to release whereas others provided both sugar and YH (Reynolds et al. 2014), and so we compare effects of dietary methoprene supplements for flies that receive these two diets during a simulated pre-release treatment period that is consistent with common pre-release holding periods for Q-fly SIT (Reynolds and van der Rijt 2011). Further, to investigate potential toxic effects of methoprene treatment, we assess effects of diet and methoprene supplements on longevity.

Materials and methods

Insects

Fertile mass-reared Q-flies were obtained as pupae from the Fruit Fly Production Facility located at the Elizabeth Macarthur Agricultural Institute, New South Wales, Australia. Historically, Q-flies released in SIT programs have been sterilised using gamma radiation (Collins et al. 2009; Dominiak et al. 2014), but alternative approaches are under development and will likely replace gamma radiation. A new Q-fly SIT program currently under development proposes to sterilise flies using X-rays instead, but protocols remain under development. Further, existing and emerging technologies such as the use of female specific lethal transgenes (fsRIDL) (Leftwich et al. 2014) or RNAi disruption of spermatogenesis (Dong et al. 2016) may supersede the use of irradiation for sterility induction in some SIT programs. Fertile flies of a bisex strain provide a baseline from which general patterns can be determined.

At Macquarie University, all pupae and flies, and experiments, were maintained in controlled environment rooms of 25±0.5ºC and 65±5% RH. A L13:D11 h photoperiod, with flies experiencing a simulated dawn and dusk as the lights ramped up and down through an additional 0.5 h at the beginning and end of the light phase.

Diet and methoprene treatments

Adult Q-fly males and females were treated with the following combinations of diet and methoprene:

(1) Methoprene (0.05%) + sugar and YH (3:1)
(2) Methoprene (0.1%) + sugar and YH (3:1)
(3) Methoprene (0.5%) + sugar and YH (3:1)
(4) Methoprene (0.05%) + sugar
(5) Methoprene (0.1%) + sugar
(6) Methoprene (0.5%) + sugar
(7) No Methoprene, sugar and YH (3:1)
(8) No Methoprene, sugar

For the incorporation of methoprene into the adult diet, we selected NOMOZ® pellets (a trademark of Pacific Biologists, Brisbane, Queensland, Australia). Nomoz contains the active ingredient PROLINK®, which contains 40 g/kg (S)-methoprene. This product is locally available, being marketed as a mosquito larvicide. NOMOZ pellets were finely powdered using mortar and pestle. Then the powdered methoprene (as required on dry weight basis) was mixed into sugar and YH diet (3:1), or only sugar, using a blender. The combined diet and methoprene treatments were provided to flies as a blended dry mixture.

Experiment 1: Effect of methoprene and YH on mating performance

Approximately 6,000 pupae from a single day of pupation were placed in a mesh cage for adult emergence (Megaview Bugdorm 44545, 47.5 x 47.5 x 47.5 cm). Usually few flies emerge on the first day of emergence, and these were discarded. After the following 24 hours of emergence, unemerged pupae were removed from the cage and food containing one of three doses of methoprene (0.05, 0.1, and 0.5%) or a control (0%) was provided to the flies in each cage for next two days *ad libitum*. After two days, the treated food was replaced with sugar only in a 90 mm Petri dish. The flies were then sorted according to sex within 3 days after emerging by collecting and transferring individual flies in glass tubes. As a source of water, cages of flies were provided water soaked cotton wool in a 70-ml sample container.

To obtain mature flies (12-17 days old) to pair with treated flies, ca. 800 pupae from numerous pupation dates were placed in separate mesh cages for adult emergence (Megaview Bugdorm 44545, 47.5 x 47.5 x 47.5 cm). Cages were supplied with water soaked cotton wool in a 70-ml sample container and dry granular sucrose along with YH (3:1) as food on a 90 mm Petri dish *ad libitum*; this diet is effective at supporting Q-fly development (Vijaysegaran et al. 2002; Pérez-Staples et al. 2007, 2011). Adult flies were sorted according to sex within 3 days after emerging by collecting and transferring individual flies in glass tubes to transparent plastic 12-L cages that had a mesh-covered ca. 80cm^2 window for ventilation. Approximately 200 flies were sorted into each 12-L cage, with this relatively low density to avoid effects of crowding on longevity and mating performance. No calling, courting, or mating was observed in cages prior to separating the sexes.

Mating trials were conducted at 4, 6, 8, 10, 12, 15, 20, 25 and 30 days post emergence. On each mating day, at least four hours before the onset of dusk, ten males and ten females

from each treatment group were placed individually in transparent plastic 1.125 L containers with a mesh-covered window (ca. 28 cm^2) for ventilation. Each fly was paired with a sexually mature (12-17 days old) fly of the opposite sex as virgin flies of this age fed diet of sugar and YH show a high level of sexual receptivity (Pérez-Staples et al. 2007; Prabhu et al. 2008). Periodic observations were carried out from when pairs were set up, and continuous observations began 90 minutes prior to the onset of dusk. To assess copula latency, defined as time from the start of dusk till the onset of mating, the time of onset of copulation was recorded for each mating pair. To assess copula duration for each mating pair, observation continued until the last pair had separated. Thus 160 treated male and female flies were tested on each day, providing a total of 1,440 test pairs. The experiment was repeated twice using batches of pupae obtained two months apart.

Experiment 2: Effect of methoprene and YH on longevity

Application of treatments for longevity trials was similar to that of mating trials. Male and female flies were sorted within 3 days after emerging by transferring individual flies in glass tubes to group and individual longevity cages. No calling, courting, or mating were observed in cages prior to placing in the longevity trial cages.

In group cages, for each treatment five flies of either sex were placed in each of three 1.125 L cages having a mesh-covered window (ca. 28 cm^2) for ventilation (i.e., 15 male and 15 female flies for each treatment). Each cage was provided with water-soaked cotton and sugar in separate 35 mm Petri dishes. Group longevity trials were repeated four times, using pupae from different production batches.

In individual cages, for each of treatments 10 male and 10 female flies were placed singly in 70 ml containers that had 8-10 2-mm diameter holes for ventilation. Flies were provided with water-soaked cotton and a sugar solution dried onto 1-cm squares of porous paper as food. Flies were checked daily until all had died. Dead flies were removed from the cages daily. Individual longevity trials were repeated four times, using pupae from different production batches.

Statistical analysis

Mating probability (binary outcome) was assessed using nominal logistic regression with significance tested using likelihood ratio tests (*G*). Main effects included in the model were age of the flies (continuous), diet (binary) and methoprene dose (ordinal). Model parameter estimates were inspected to identify effects. Odds ratios were used to test differences between diets and methoprene treatments. Copula latency and copula duration (continuous outcomes) were analysed for each treatment using least squares regression including age (continuous), diet (binary) and methoprene dose (ordinal). For copula latency, values were rank transformed.

Longevity of flies was assessed for each treatment using least squares regression including diet (binary) and methoprene treatment (ordinal) as factors.

All analyses were performed using JMP Statistical Software Version 10.0.0 (SAS Institute, Cary, NC, USA). Because the mating experiments were repeated using flies from two batches of pupae, and the longevity experiments were repeated using flies from four batches of pupae, batch identity was included as a random effect to account for variation amongst batches. For the group longevity trials in which five flies were maintained in each cage, cage identity nested within replicate was also included as a random effect to account for cage level effects. In all analyses, initial models considered all interaction terms amongst fixed effects. Non-significant interaction terms were removed from each final model.

Results

Mating probability

For males, mating probability varied with age (polynomial), diet, and methoprene treatment, including a significant age × age × diet interaction (Table 1). For male flies on both diets, mating probability increased to a peak at ca. 20 days and then declined (Fig. 1), although flies provided YH exhibited a steeper increase in mating probability, and greater mating probability overall, compared with flies provided only sugar during the two days following emergence. All methoprene doses increased mating propensity, and there was no evidence of variation in this effect amongst the doses, between the diets, or with age (Table 1, Fig. 1).

For females, mating probability varied with age (polynomial) and diet, including a significant diet × age interaction but, importantly, did not vary significantly with methoprene treatment (Table 1, Fig. 2). As for male flies, for female flies on both diets mating probability increased to a peak at ca. 25 days and then declined (Fig. 2), although flies provided YH exhibited a steeper increase in mating probability, and greater mating probability overall, compared with flies provided only sugar during the two days following emergence.

Copula latency

In Q-fly matings are mostly initiated after the onset of dusk, but in laboratory studies it is common to have some matings start earlier. For males, 202 (27%) of 740 observed matings started before the onset of dusk. For females, 92 (20%) of 468 observed matings started before the onset of dusk.

In male flies, copula latency (rank transformed) varied significantly with age (polynomial) and diet × methoprene dose interaction (Table 2, Fig. 3). Copula latency decreased with fly age till 20 days and then increased. The diet × methoprene dose interaction was explored further using slice analyses. For flies that had received YH, methoprene treatment did not significantly affect mating latency ($F_{3, 729.1}$ = 1.809, P = 0.144) whereas for flies that received only sugar, there were significant effects of methoprene treatment ($F_{3, 729.1}$ = 5.462, P = 0.001); male flies that received 0.05% methoprene had significantly shorter copula latency than controls (t = -2.58, P = 0.010) but this was not the case for male flies that received 0.1% methoprene (t = 0.93, P = 0.355) or 0.5% methoprene (t = -1.18, P = 0.238). Diet during the first two days following emergence did not affect copula latency of male flies that received no methoprene ($F_{1,729}$=2.014, P=0.156) or received 0.05% methoprene ($F_{1,729}$=0.026, P=0.873), but did affect copula latency of male flies that received 0.1% methoprene ($F_{1,729.3}$=14.806, P<0.001) or 0.5% methoprene ($F_{1,729.1}$=5.068, P=0.025). For flies receiving 0.1% or 0.5% methoprene, copula latency was longer for flies that had received sugar only than for those that also received YH.

In female flies, copula latency (rank transformed) did not vary significantly with methoprene treatment but did vary with age in a diet-specific manner (i.e., significant diet × age interaction) (Table 2). Copula latency decreased with age (N=143, b=-3.98±1.6, $F_{1, 141}$ =

6.662, P=0.010) for females provided access to sugar only, but increased with age (N=325, b=2.13±0.94, $F_{1, 323}$ = 5.262, P=0.022) for females provided access to YH.

Copula duration

For males, copula duration varied significantly with age (polynomial), diet, and methoprene treatment (Table 3, Fig. 4). Copula duration increased as male flies aged, but then declined toward the end of the tested period and male flies that received access to YH during the first two days following emergence had significantly longer copulations than those with access to only sugar. Males treated with 0.05% or 0.5% methoprene had copulations that were significantly longer than controls and not different from each other. Males treated with 0.1% methoprene had copulations that were intermediate in duration, being not significantly different from either control or other methoprene doses.

Female copula duration varied with diet and methoprene treatment, not with age (Table 3, Fig. 5). Females that received access to YH during the first two days following emergence had significantly longer copulations than females with access to only sugar. Females treated with all doses of methoprene had copulations that were significantly longer than controls and not different from each other.

Experiment 2: Effect of methoprene and YH on longevity

In both group and individual cages, there was no evidence that methoprene at any dose influenced longevity (Table 4). In group cages, access to YH resulted in longer lifespan for both males (least squares mean ± se: S = 26.83 ± 2.28, S+YH = 41.87 ± 2.28) and females (least square mean ± se: S = 29.84 ± 2.16, S+YH = 44.16 ± 2.16). In individual cages, access to YH resulted in longer lifespan for males (least square mean ± se: S = 20.18 ± 1.15, S+YH = 24.63 ± 1.15) but not females (least square mean ± se: S = 21.94 ± 1.18, S+YH = 24.14 ± 1.18).

Discussion

Mating propensity

The incorporation of methoprene into Q-fly adult diet for two days following emergence increased mating propensity of males but had no significant effect on mating propensity of females. These findings contrast with Collins et al. (2014), who found that methoprene treatment promoted sexual development in both male and female Q-fly, with similar effects when adults were treated by topical application of methoprene /acetone solution and when pupae were treated by dipping in methoprene /acetone solution. As an analogue of juvenile hormone, methoprene affects a vast diversity of insect tissues, especially those related to development and reproduction in both males and females (Kelly et al. 1987; Happ 1992; Ringo 2002; Flatt et al. 2005; Riddiford 2012). Differences between studies in effects of methoprene treatment on female flies may reflect differences in the dose at affected tissues. Higher effective doses may be required to induce detectable effects in female Q-fly and may be more readily accomplished across the cuticle by topical treatment in solvent than by ingestion with food.

The benefit of methoprene treatment to male Q-fly in the present study closely matches the benefits reported by Collins et al. (2014) for topical treatment in acetone. It is possible that the level of effect observed in these studies is close to the maximum maturation and mating propensity benefit that can be achieved by pre-release methoprene treatment and so there may be little value in higher doses, especially if higher doses induce increased mating propensity in females. However, there may be more interest in exploring lower doses. All doses of methoprene used were effective at increasing male Q-fly mating propensity. This suggests that the minimum dose required to induce the full effect on mating propensity is below the lowest dose used in the present study (0.05%).

The sex differences in response to methoprene treatment in the present study are very similar to those reported for *A. fraterculus* by Segura et al. (2009) and Liendo et al. (2013), who also found strong effects for males and no significant effect for females. For *A. ludens*, Pereira et al (2013a) found positive effects of methoprene treatment on development of both males and females, but that the effects were much stronger for males. Similarly, Pérez-Staples et al. (2011) reported sex differences in the effects of access to YH for two days following emergence, as males gained a far greater boost in development than females. Segura et al. (2009), Liendo et al. (2013) and Pérez-Staples et al. (2011) all suggested a practical application of such sex differences in response to pre-release treatments as a physiological sexing system that minimizes mating between sterile males and sterile females

in releases of bisexual strains. That is, pre-release methoprene and YH treatment might bias operational sex ratio toward relatively greater numbers of sexually active males especially in the first week following release. Male-biased operational sex ratio can be beneficial for efficacy of SIT (McInnis et al. 1994; Rendón et al. 2004). In the absence of genetic sexing strains of Q-fly, pre-release treatments that promote male-biased operational sex ratio have potential to improve efficacy of Q-fly SIT.

As has been consistently reported in previous studies (e.g., Pérez-Staples et al. 2007, 2008, 2011), access to YH had a profound effect on Q-fly mating propensity in the present study. YH-fed males and females became sexually mature earlier, exhibited a steeper increase in mating propensity with age than flies that had been provided only sugar, and attained higher mating propensity overall. The effects of YH and methoprene on male mating propensity were additive, as YH increased mating levels for all methoprene treatments, and methoprene increased mating levels for flies on both diets. Similarly, Haq et al. (2010a, b), Pereira et al. (2009), and Pereira et al. (2013a) have reported positive effects of YH and additional positive effects of methoprene in YH-fed male *Z. cucurbitae*, *A. suspensa,* and *A. ludens,* respectively.

For Q-fly of both sexes on both diets, mating propensity followed a polynomial trend with age, peaking at 20-25 days and then declining. Very similar polynomial effects of age on mating propensity have been reported previously for Q-fly by Pérez-Staples et al. (2008) who, as in the present study, provided the flies dietary YH for just a short post-emergence period and then maintained the flies with only sugar. This pattern likely reflects age-related changes in the availability of nutritional resources. Flies had ample access to sugar as a source of energy throughout life but only had access to other nutrients either as residual carried over from the pupal stage (Nestel et al. 2004) or acquired over a two-day post-emergence period (Fanson and Taylor 2012). Alternatively, polynomial effects of age on mating propensity could reflect increased mating propensity with maturation and then a reduction in vigour as physical state of flies declines with senescence (Papadopoulos et al. 2002; Yap et al. 2015). In the Q-fly rearing program from which pupae were obtained, the flies are usually discarded after three to four weeks (Fruit Fly Production Facility Manager Dr Solomon Balagawi, pers. comm.). Reduced performance at older ages might be expected given strong selection for early reproduction and lack of selection for somatic maintenance beyond the imposed maximum adult lifespan of four weeks.

Copula latency

In the present study no effects of methoprene on copula latency were detected for female flies of either diet group or for male flies fed YH. This is consistent with Collins et al. (2014), who also did not detect any effects of methoprene on copula latency of male or female Q-flies that had received topical application of methoprene in acetone solution as pupae or young adults and had been fed YH. In contrast, Haq et al. (2010b) reported a decrease in copula latency of male *Z. cucurbitae* that had received topical application of methoprene and were fed YH. Haq et al. (2010b) provided the flies continuous access to YH throughout adult life, and it may be that the short period of access to YH in the present study and in Collins et al (2014) was not sufficient to support methoprene-related effects. In the present study it is interesting to note that an effect of methoprene treatment was observed, however, for male Q-flies that received only sugar as food; for these flies copula latency was significantly reduced when 0.05% methoprene was included in the diet but not when higher doses were used. It is not clear why methoprene affected copula latency only for males receiving this treatment combination.

In addition to diet-dependent effects of methoprene treatment, male copula latency also varied significantly with age, decreasing in all treatments to ca. 20 days, and then increasing again. A similar trend has been reported previously by Pérez-Staples et al. (2007), who found that copula latency decreased with age in a group of flies tested up to 14 days of age and then increased with age in a second group of flies tested when 11-30 days of age. Similarly, Collins et al. (2014) tested flies up to 12 days of age and found that copula latency decreased over this period. That male Q-fly copula latency is lowest during the period of greatest mating propensity (compare Figures 1 and 3) supports interpretation of copula latency as generally related to sexual effort or attractiveness.

For female Q-fly, YH-fed and sugar-fed flies exhibited very different patterns in copula latency as they aged. Copula latency increased with age for YH-fed females but decreased with age for sugar-fed females. Despite generally similar methods, these patterns differ from those reported by Pérez-Staples et al. (2011), who found no significant effects of age and no differences between 48 hr YH-fed and sugar-fed in copula latency. Inconsistency across

studies in effects of diet on female copula latency may reflect differences in the fly colonies used in these studies or details of handling and experimental design.

Copula duration

Methoprene significantly increased copula duration of both male and female Q-fly, and this effect was detected for flies in both diet groups. This finding is partly consistent with results for *Z. cucurbitae*, in which the application of methoprene significantly increased copula duration of YH-fed males but not sugar-fed males (Haq et al. 2010b). In contrast to the effects of methoprene on Q-fly copula duration in the present study, Collins et al. (2014) did not detect any effects of methoprene treatment on copula duration of either sex. Similarly, Gomez-Simuta et al. (2017) found no effect of methoprene treatment on copula duration in *A. ludens*. In addition to effects of methoprene treatment, male and female Q-flies with access to YH had longer copulations than those provided only sugar, and these results are consistent with previous reports for Q-fly (Pérez-Staples et al., 2007, 2011) as well as for *Z. cucurbitae* (Haq et al. 2010b).

The significance of copula duration for fruit fly sexual selection and SIT is not straightforward. Pérez Staples et al. (2007) reported a tendency for short copulations to be more likely to result in no sperm storage, but such tendency was not found by Collins et al. (2012). Previous studies have consistently failed to detect evidence of association between copula duration and total number of sperm stored by female Q-flies (Harmer et al. 2006; Pérez-Staples et al. 2007; Radhakrishnan et al. 2009; Collins et al. 2012). Despite a lack of a relationship between copula duration and sperm storage, sterile males that have long copulations are more effective at reducing fertility of females that have mated previously with a fertile male (Collins et al. 2012). Accordingly, it is possible that methoprene-induced increases in copula duration would have positive implications for post-copulatory competitiveness of flies released in SIT programs.

Longevity

Access to YH increased longevity for both male and female Q-fly, and this corresponds with previous work on Q-fly (Pérez-Staples et al. 2008), and is a common tendency in numerous fruit fly species, including *Z. cucurbitae* (Haq and Hendrichs 2013), *Rhagoletis indifferens*

(Yee 2003), *A. serpentina* (Jacome et al. 1995), *A. ludens* and *A. obliqua* (Liedo et al. 2013). Importantly for the present study, there was no evidence that methoprene affects longevity of either male or female Q-fly, regardless of diet. Similarly, methoprene treatment alone had no adverse effects on longevity of *C. capitata* (Faria et al. 2008), *A. suspensa* (Pereira 2010), and *A. fraterculus* (Segura et al. 2013). Methoprene treatment has been reported to increase longevity of male *Z. cucurbitae* that were provided both sugar and YH for three days and then only sugar (Haq and Hendrichs 2013), an approach very similar to the present study. While we did not detect positive effects of methoprene on longevity, the absence of negative effects is promising.

Conclusions

Methoprene supplements added to diet for two days show promise as a means of accelerating sexual development of male Q-flies. This accelerated development has potential value in SIT programs, reducing the delay from release until sexual maturation and thereby increasing the proportion of released males participating in mating activity. Differential developmental responses of males and females to dietary methoprene supplements indicate some potential for male biased operational sexual ratio following methoprene treatment. The extent of such effects will likely depend on conditions following release, especially availability of nutrition.

Strain development for Q-fly SIT is currently in a state of transition and so the present study used conventional fertile flies from a bisex strain. Irradiation is used to sterilize Q-flies under current protocols, and so confirmation of our findings with irradiated flies is now a priority. As new approaches to sterility induction and unisex strains are developed for Q-fly, the final development of operational protocols will need to be developed from the platform established here using the specific types of fly to be used in SIT for this species.

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Author Contribution

Saleh Mohammad Adnan (SMA) conceived and designed the work, conducted experiments, analysed the data and wrote the manuscript. Vivian Mendez (VM) conducted experiments. Renata Morelli (RM) conducted experiments. Humayra Akter (HA) conducted experiments. Iffat Farhana (IF) conducted experiments. Phillip W. Taylor (PWT) analysed the data and wrote the manuscript. All authors read and approved this manuscript.

Compliance with Ethical Standards

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Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Table 1. Logistic regression analysis testing fixed effects of age (4, 6, 8, 10, 12, 15, 20, 25 and 30 days post emergence), diet (sugar or sugar+YH) and methoprene dose (0, 0.05, 0.1 and 0.5%) on male and female Q-fly mating probability.

	Source	*d.f.*	*G*	*P*
Males	Age	1	145.368	<0.001
N=1440	Age × Age	1	71.330	<0.001
	Dose	3	36.499	<0.001
	Diet	1	140.532	<0.001
	Age × Diet	1	0.026	0.873
	Age × Age × Diet	1	8.591	0.003
Females	Age	1	251.973	<0.001
N=1440	Age × Age	1	37.503	<0.001
	Dose	3	2.510	0.474
	Diet	1	136.955	<0.001
	Age × Diet	1	9.648	0.002

Table 2. Regression analysis testing fixed effects of age (4, 6, 8, 10, 12, 15, 20, 25 and 30 days post emergence), diet (sugar or sugar+YH) and methoprene dose (0, 0.05, 0.1 and 0.5%) on male and female Q-fly copula latency (rank transformed).

	Source	*d.f.*	*F*	*P*
Males	Age	1,729.1	6.841	0.009
N=740	Age × Age	1,729	15.953	<0.001
	Dose	3,729	5.789	<0.001
	Diet	1,729	2.014	0.156
	Diet × Dose	3,729.1	2.806	0.039
Females	Age	1,460.2	1.194	0.275
N=468	Dose	3,460.2	0.138	0.937
	Diet	1,436.3	0.123	0.726
	Age × Diet	1,435.3	11.602	<0.001

Table 3. Regression analysis testing fixed effects of age (4, 6, 8, 10, 12, 15, 20, 25 and 30 days post emergence), diet (sugar or sugar+YH) and methoprene dose (0, 0.05, 0.1 and 0.5%) on male and female Q-fly copula duration.

	Source	*d.f.*	*F*	*P*
Males	Age	1,732.2	28.875	<0.001
N=740	Age × Age	1,732.1	13.142	<0.001
	Dose	3,732	5.104	0.002
	Diet	1,732.4	43.302	<0.001
Females	Age	1,461.5	2.760	0.097
N=468	Dose	3,461.4	2.802	0.040
	Diet	1,462	9.578	0.002

Table 4. Analysis of variance testing effects of diet (sugar or sugar+YH) and methoprene dose (0, 0.05, 0.1 and 0.5%) on longevity of Q-fly housed in group cages or individual cages.

	Source	*d.f.*	*F*	*P*
Group cages				
Male	Dose	3,88	0.108	0.955
N=480	Diet	1,88	73.999	<0.001
Female	Dose	3,88	0.157	0.925
N=480	Diet	1,88	71.447	<0.001
Individual cages				
Male	Dose	3, 190.6	0.537	0.657
N=200	Diet	1, 190.6	18.242	<0.001
Female	Dose	3, 190.6	0.615	0.612
N=200	Diet	1,190.6	3.505	0.064

Figure

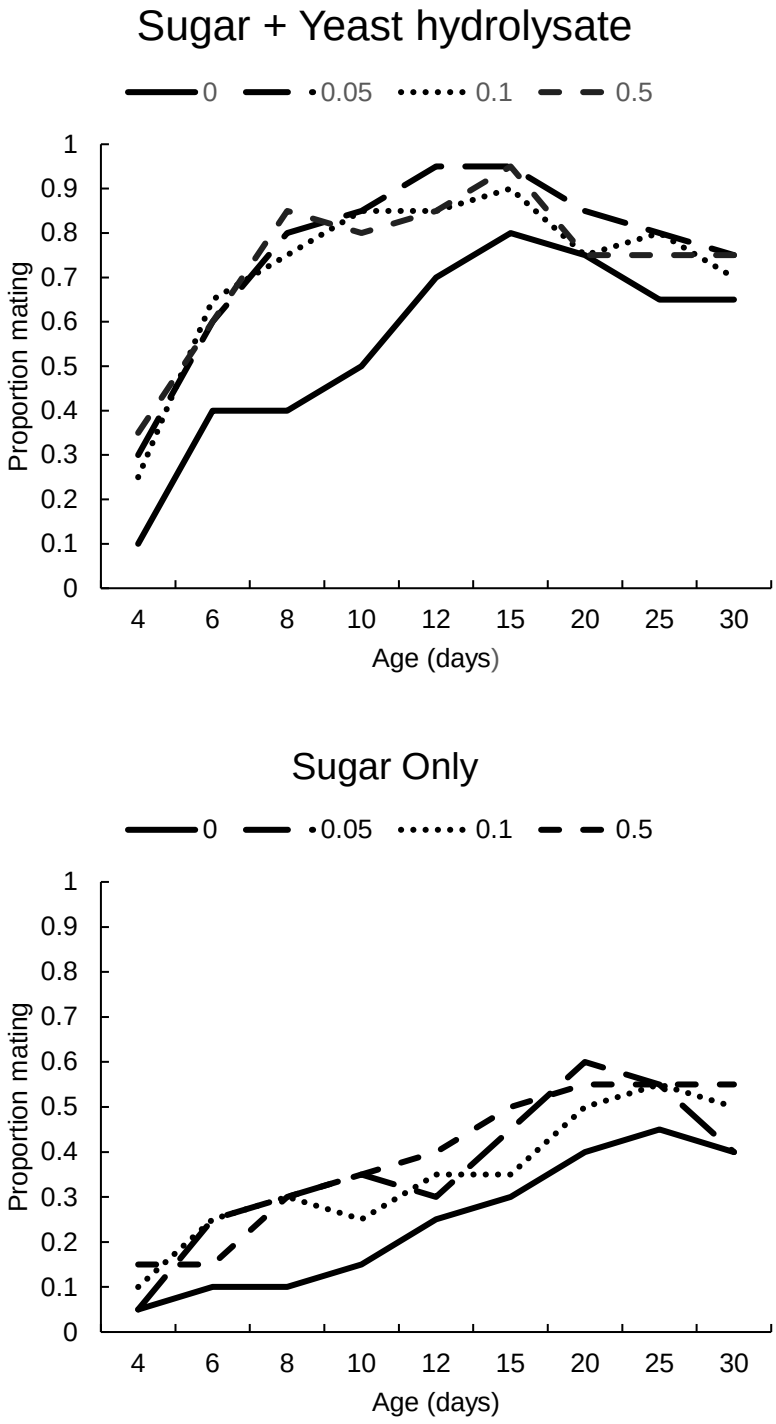
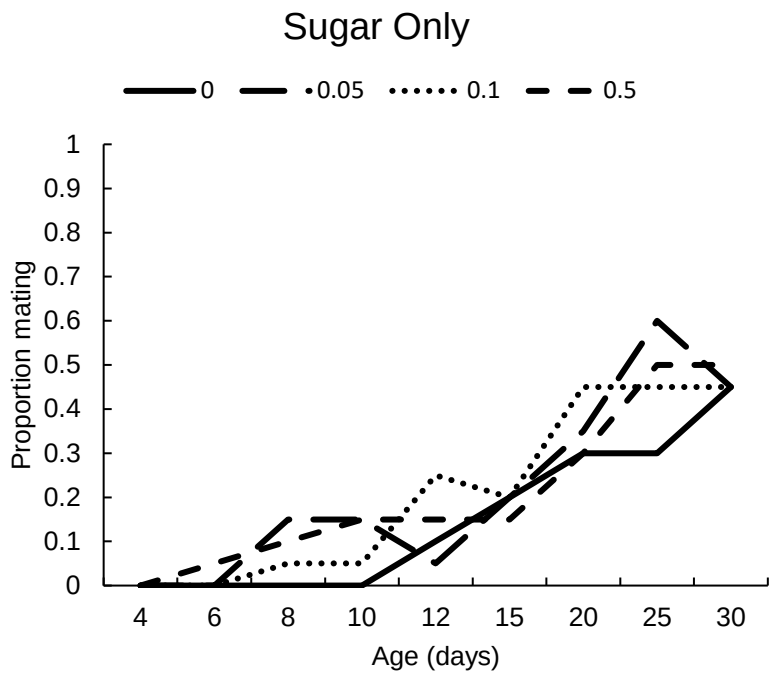


Figure 1. Relationship between age (4, 6, 8, 10, 12, 15, 20, 25 and 30 days post emergence) and proportion of male Q-flies mating (mean± SE) after being fed a diet of sugar only or sugar plus yeast hydrolysate combined with one of four levels of methoprene supplementation (0, 0.05, 0.1, 0.5 %) for two days after emergence and then sugar only.



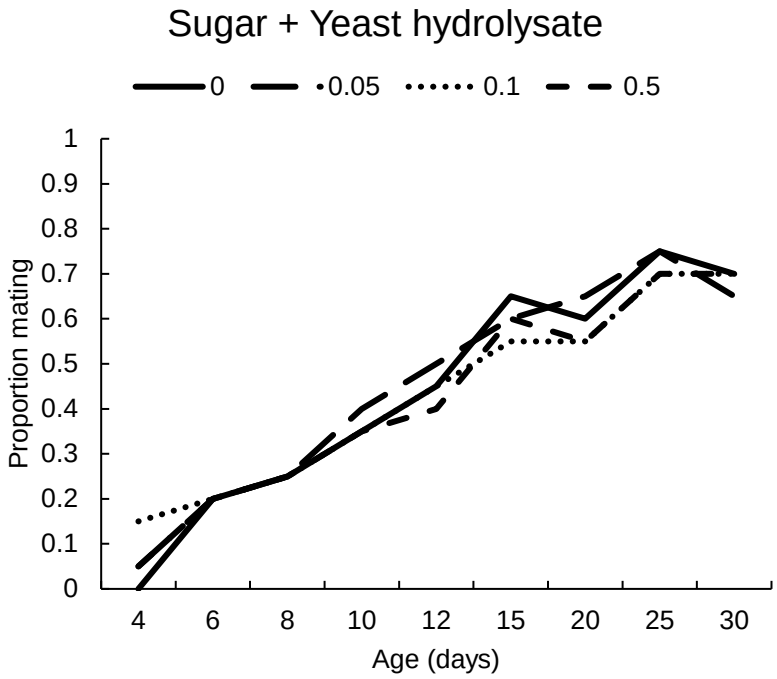
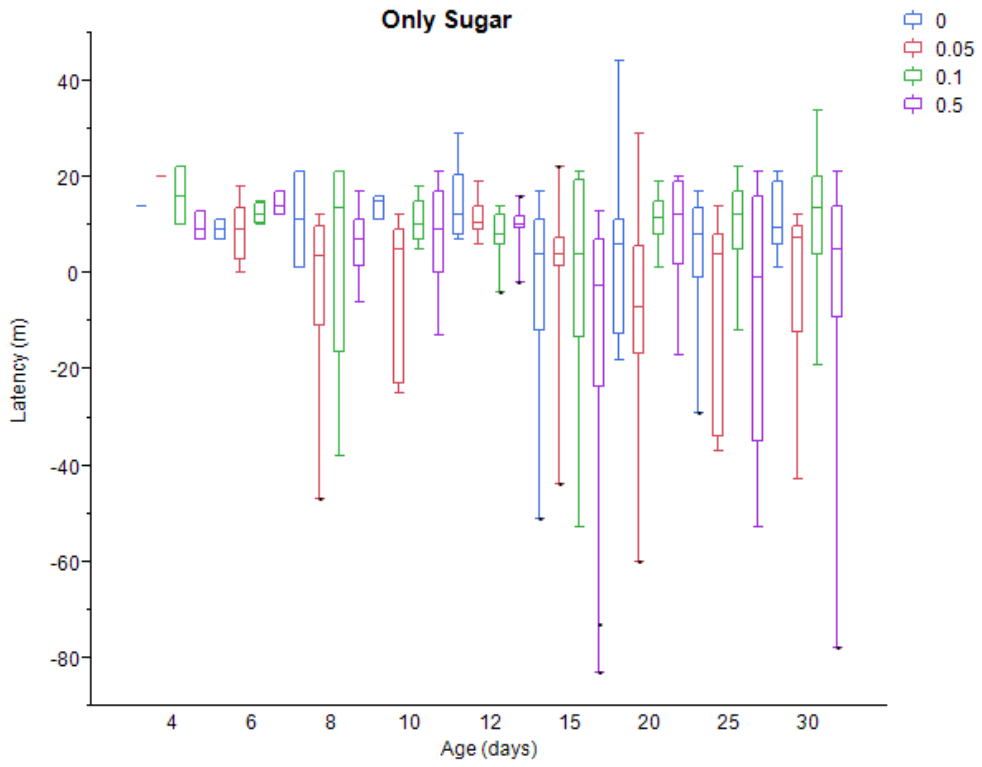


Figure 2. Relationship between age (4, 6, 8, 10, 12, 15, 20, 25 and 30 days post emergence) and proportion of female Q-flies mating (mean± SE) after being fed a diet of sugar only or sugar plus yeast hydrolysate combined with one of four levels of methoprene supplementation (0, 0.05, 0.1, 0.5 %) for two days after emergence and then sugar only



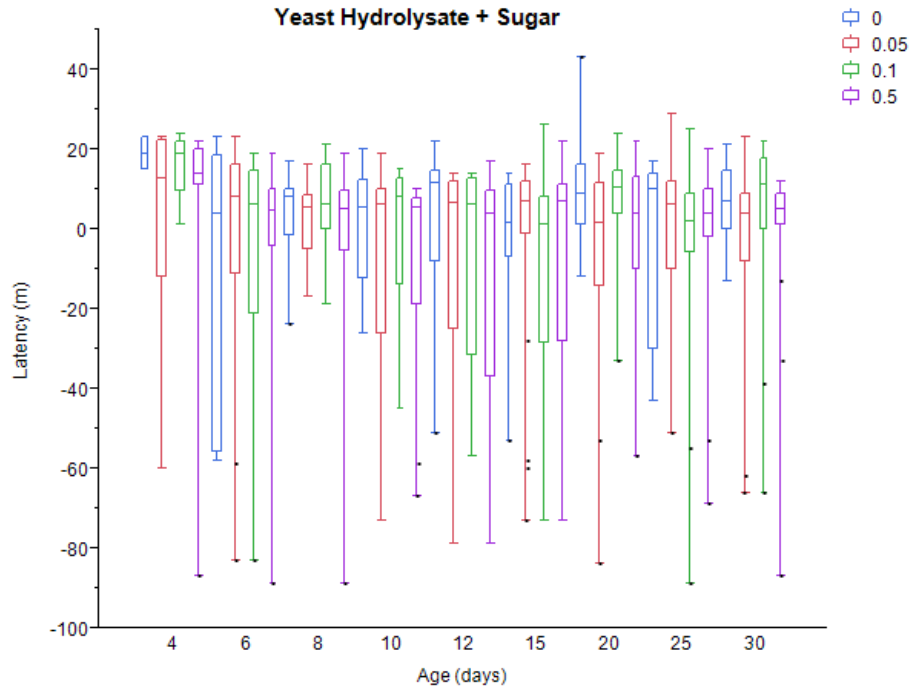


Figure 3. Relationship between age (4, 6, 8, 10, 12, 15, 20, 25 and 30 days post emergence) and copula latency (mean± SE) of male Q-flies after being fed a diet of sugar only or sugar plus yeast hydrolysate combined with one of four levels of methoprene supplementation (0, 0.05, 0.1, 0.5 %) for two days after emergence and then sugar only.

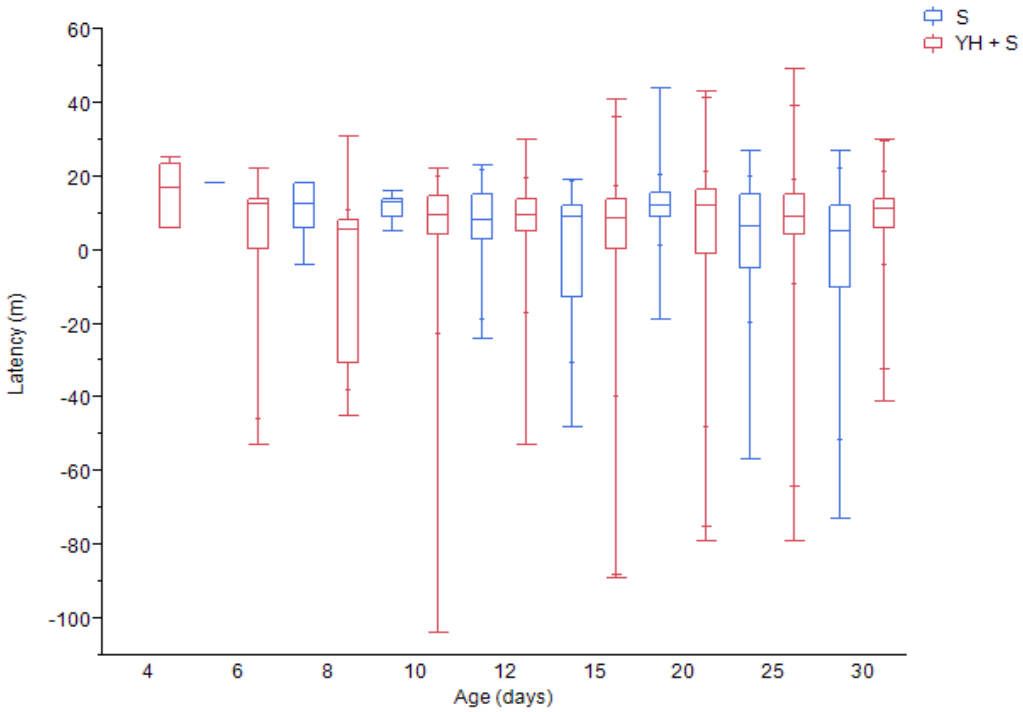


Figure 4. Relationship between age (4, 6, 8, 10, 12, 15, 20, 25 and 30 days post emergence) and copula latency (mean± SE) of female Q-flies after being fed a diet of sugar only or sugar plus yeast hydrolysate combined with one of four levels of methoprene supplementation (0, 0.05, 0.1, 0.5 %) for two days after emergence and then sugar only. Values estimated from standard least squares model in Table 2.

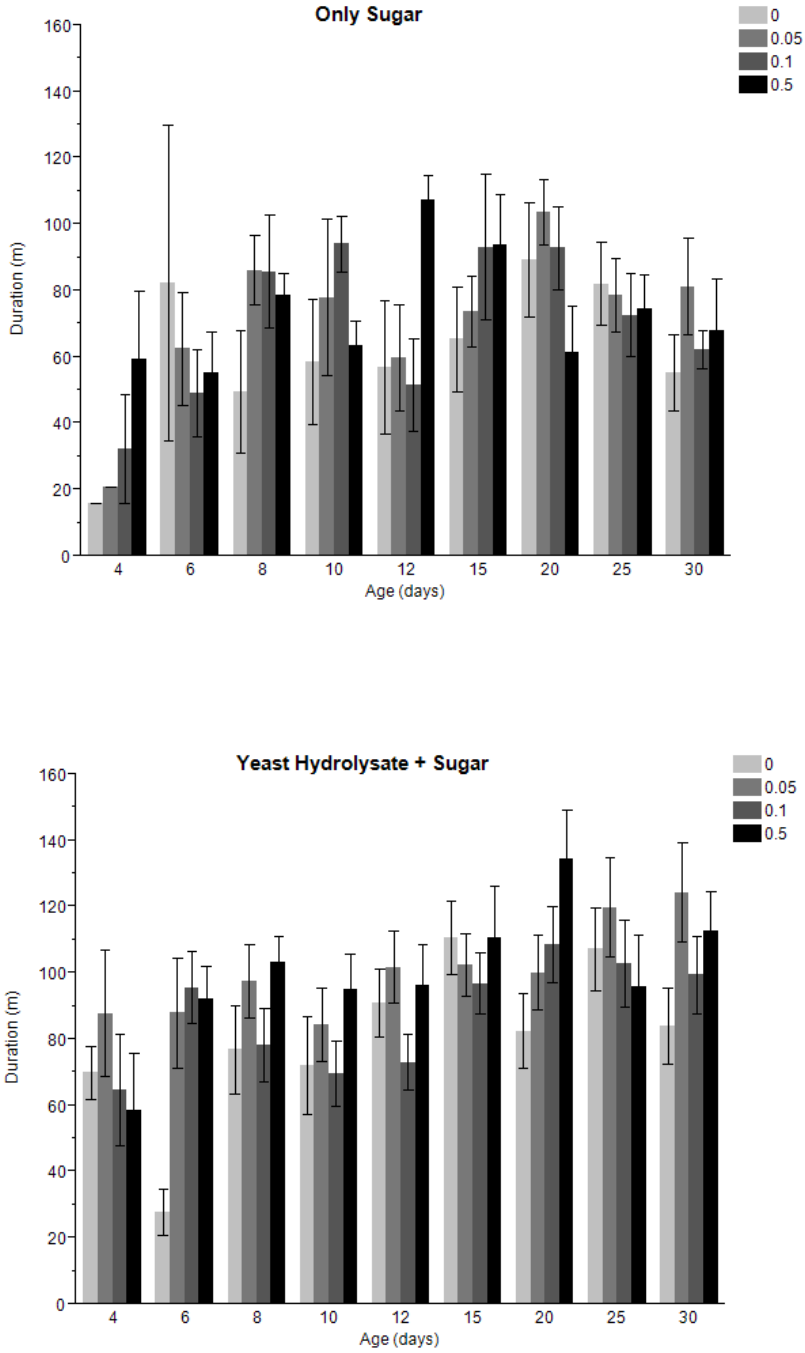


Figure 5. Relationship between age (4, 6, 8, 10, 12, 15, 20, 25 and 30 days post emergence) and copula duration (mean± SE) of male Q-flies after being fed a diet of sugar only or sugar plus yeast hydrolysate combined with one of four levels of methoprene supplementation (0, 0.05, 0.1, 0.5 %) for two days after emergence and then sugar only.

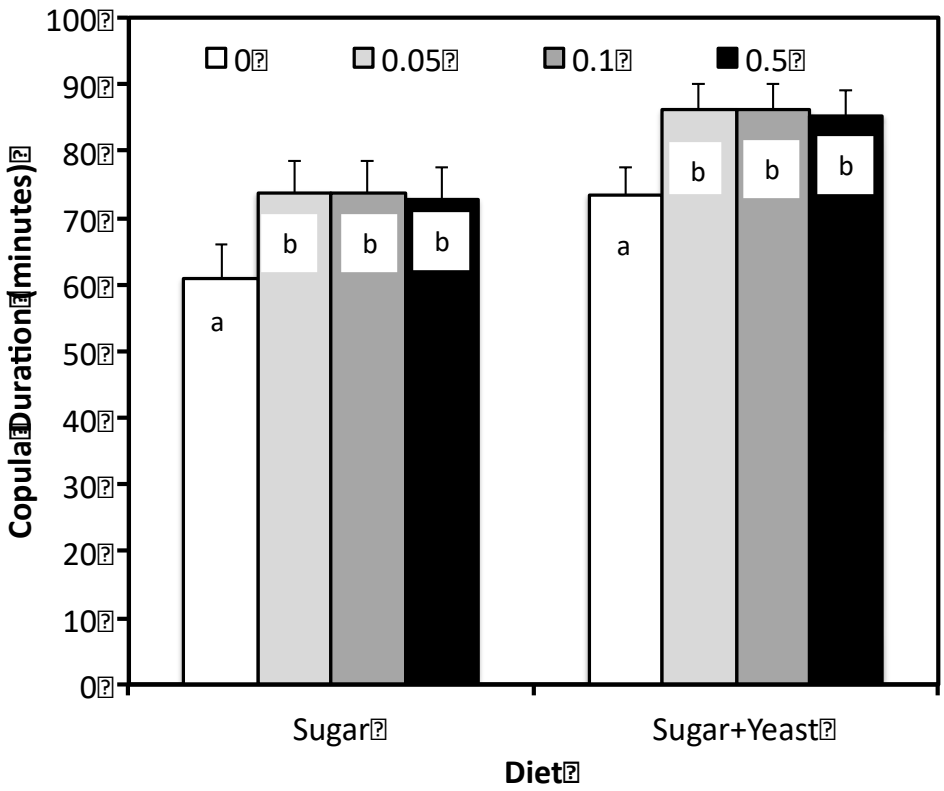


Figure 6. Copula duration (mean± SE) of female Q-flies after being fed a diet of sugar only or sugar plus yeast hydrolysate combined with one of four levels of methoprene supplementation (0, 0.05, 0.1, 0.5 %) for two days after emergence and then sugar only. Age was not a significant predictor of copula duration. Values estimated from standard least squares model in Table 3, with age set at 15 days. Letters denote significant differences amongst methoprene treatments within each diet treatment.

Chapter Three

Image captured by author only

This chapter has been published in *Journal of Economic Entomology*

Overview

Effects of dietary treatment with NOMOZ® on sexual development of fertile Q-fly has been studied in chapter two, while effects on sterile flies those might be deployed in SIT programs are still needed to be explored. As irradiation used to induce sterility can have deleterious effects on physiology and behaviour of Q-fly, there is a risk that the promising effects of methoprene on fertile flies may not be evident in sterile flies. In chapter two, dietary NOMOZ® treatment accelerates sexual maturation of males but not of females, at the same time in previous studies topical application of analytical standard methoprene promoted sexual development in both males and females (Collins et al., 2014). Building on these key questions, I carried out mating trials to investigate whether methoprene from NOMOZ® and analytical standard performs differently in accelerating sexual maturation in both sexes of Q-fly; and are effects of methoprene identical for fertile and sterile flies. In addition, to investigate potential detrimental effects of methoprene treatment in fertile and sterile Q-flies, I carried out longevity assay to assess the separate and combined impact of irradiation and dietary methoprene supplements on longevity. Since all three concentrations of methoprene were significantly effective in accelerating mating performance, in this chapter only lowest dose of methoprene has been tested.

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Accelerated sexual maturation in methoprene-treated sterile and fertile male Queensland fruit flies, and mosquito larvicide as an economical and effective source of methoprene

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Abstract

Queensland fruit flies *Bactrocera tryoni* ('Q-fly') have long adult pre-reproductive development periods, which can present challenges for sterile insect technique (SIT) programs. Holding the sterile flies in release facilities is expensive for control programmes. Alternatively, releases of sexually immature males can lead to substantial mortality of sterile males before they mature. Recent studies have reported effectiveness of dietary supplementation with a mosquito larvicide (NOMOZ®) that contains S-methoprene, a juvenile hormone analogue, for accelerating sexual development of fertile Q-fly males. However, it is not known whether effects on sterile flies are comparable to effects on fertile flies, or whether effects of methoprene-containing larvicide are comparable to effects of analytical standard methoprene such has been used in most studies. Here we address both knowledge gaps, investigating the effects of analytical standard methoprene and NOMOZ® mixed with food and provided for 48 hours following emergence on sexual development and longevity of fertile and sterile Q-flies. Compared with controls, fertile and sterile male Q-flies that were provided diets supplemented with methoprene from either source exhibited substantially accelerated sexual development by 2 - 3 days and longer mating duration. Unlike males, females did not respond to methoprene treatment. While fertile and sterile flies were generally similar in sexual development and response to methoprene treatment, sterile flies of both sexes tended to have shorter copula duration than fertile flies. Neither methoprene supplements nor sterilisation affected longevity of flies. The present study confirms effectiveness of dietary methoprene supplements in accelerating sexual development of both fertile and sterile male (but not female) Q-flies, and also confirms that low-cost mosquito larvicides that contain methoprene can achieve effects similar to those for high-cost analytical grade methoprene as pre-release supplements for Q-fly SIT.

Key words: Juvenile hormone, mating, sterilisation, longevity, Tephritidae, *Bactrocera tryoni*.

1 INTRODUCTION

Tephritid fruit flies present some of the most serious challenges for production and trade of fruits and vegetables globally, affecting a vast diversity of commercial and non-commercial crops [e.g., Mediterranean fruit fly, 'medfly', *Ceratitis capitata* (Segura et al. 2006, Navarro-Campos et al. 2011, Szyniszewska and Tatem 2014); Mexican fruit fly, *Anastrepha ludens* and West Indian fruit fly, *A. obliqua* (Orozco et al. 2002, Orozco-Dávila et al. 2017); Oriental fruit fly, *Bactrocera dorsalis* (Vargas et al. 2010); Queensland fruit fly or 'Q-fly', *B. tryoni* (Suthurst et al. 2000, Clarke et al. 2011, Dominiak and Daniels 2012)]. Area-wide management programs are commonly deployed against tephritid fruit flies, and these sometimes include the use of sterile insect technique (SIT) as a sustainable management practise that has minimal non-target effects (Dyck et al.2005). SIT involves rearing and releasing millions of sterile fruit flies. Released males mate with females of pest populations, inducing reproductive failure (Knipling 1955, Krafsur 1998). As a result, reproduction of the pest populations is impeded, and over generations, pest abundance is consequently reduced. SIT has been used to combat Q-fly outbreaks in Australia for more than 20 years (Fisher 1996, Fanson et al. 2014).

SIT programs rely on released sterile males surviving to maturity and participating effectively in mating activity. Fruit flies commonly have an adult maturation phase lasting a week or longer (Webster and Stoffolano 1978, Raghu et al. 2003, Shelly et al. 2008), and this is the case for Q-fly. Sterile Q-flies are usually released as sexually immature adults at 2- 3 days of age (Reynolds and van der Rijt 2011) and may take up to an additional week to fully mature (Weldon et al. 2008, Pérez-Staples et al. 2011). Mortality rates of released sterile Q-flies are often high (Meats 1998, Reynolds et al. 2012), such that numbers may be greatly reduced by the time the flies mature and participate in mating competition against males of the wild, fertile, population. As with other fruit flies targeted for SIT, significant research effort has focused on interventions that improve the ability of sterile Q-flies to survive and mature, and to compete with wild fertile males (Taylor et al. 2013, Akter et al. 2017).

Reproductive development of insects is largely mediated by juvenile hormone (JH) (Vogel et al. 1979, Wyatt and Davey 1996, Gilbert et al. 2000, Wilson et al. 2003, Dubrovsky 2005). In a practical application of the physiological role played by JH in insects an analogue of JH, methoprene, has been investigated as a treatment to accelerate development of adult

fruit flies for use in SIT programs (Teal et al. 2000, 2013). JH acts on the endocrine system of insects, regulating numerous physiological processes, including larval development, metamorphosis, diapause, and reproduction (Vogel et al. 1979, Riddiford and Ashburner 1991, Wyatt and Davey 1996, Gilbert et al. 2000, Dubrovsky 2005). In most insects, JH mediates sexual maturation and development of reproductive tissues (Happ 1992, Wyatt and Davey 1996, Gilbert et al. 2000, Wilson et al. 2003). In females, JH regulates sexual receptivity, oocyte maturation, egg deposition, and ovarian vitellogenin (Shemshedini and Wilson 1993, Ringo 2002, Dubrovsky 2005). In males, JH promotes the development and maturation of reproductive accessory glands, enhancing their growth and the production of glandular secretions (Yin et al. 1999, Wilson et al. 2003), and the synthesis of sex pheromones (Rantala et al. 2003).

Treatment with methoprene can substantially reduce the duration of the adult maturation phase in fruit flies so that a greater proportion of released flies might survive to mate, or pre-release holding periods can be reduced. Methoprene has been applied in acetone solution to adults or pupae to accelerate maturation in *Zeugodacus cucurbitae* (Haq et al. 2010a, b), *A. fraterculus* (Segura et al. 2009, 2013; Abraham et al. 2013, Liendo et al. 2013), *A. ludens* (Gómez et al. 2013, Pereira et al. 2013), and *A. suspensa* (Teal et al. 2000, Pereira et al. 2010,). However, acetone solution is not a practical delivery method for operational SIT owing to its toxicity and flammability, and so dietary application has also been explored as an alternative approach for delivery of methoprene and has proven effective in *A. ludens* (Teal et al. 2007, 2013, Aluja et al. 2009, Gómez et al. 2013, Gómez-Simuta et al. 2017).

Drawing on approaches proven successful with other fruit fly species, Collins et al. (2014) treated sterile Q-fly pupae and recently emerged adults with topical applications of analytical grade methoprene (99.5% pure) dissolved in acetone. Application at both life stages was effective in accelerating sexual development of males and females, although treatment of pupae resulted in reduced adult emergence, increased prevalence of wing deformity, and reduced flight ability. As a practical alternative to topical treatment in acetone, Adnan et al. (2018) incorporated methoprene in the form of a commercially available mosquito larvicide 'NOMOZ®' (a trademark of Pacific Biologists, Brisbane, Queensland, Australia, containing 40% S-methoprene) into the diet provided to fertile Q-flies for 48 hours following emergence and found this to be effective in accelerating sexual development of males, but not of females. Such strong sex-specific effects of methoprene treatment, also

reported for *A. fraterculus* (Segura et al. 2009, Liendo et al. 2013), and to a lesser extent in *A. ludens* (Pereira et al. 2013), potentially bias operational sex ratio in favour of males in bisex SIT releases (Vreysen et al. 2006), which currently are the only available option for Q-flies. In addition to obviating the use of acetone, being less than 1% of the cost of analytical grade methoprene the use of a commercial insecticide product allows a very significant cost saving for methoprene treatment in SIT operations.

Several important questions remain unanswered, however. First, the study of Adnan et al. (2018) used fertile flies from laboratory strains, rather than sterile flies such as are deployed in SIT programs. Irradiation used to induce sterility can have significant effects on physiology and behaviour of fruit flies (Cayol 2001, Calkins and Parker 2005), including in Q-fly (Weldon 2005, Mankin et al. 2008, Collins et al. 2009, Weldon et al. 2010), and so there is a risk that the positive effects of methoprene on fertile flies may not be matched in sterile flies. Second, while Adnan et al. (2018) found positive effects of dietary treatment with NOMOZ® on development of males, it is not known whether the extent of these effects is as great as might be obtained with analytical grade methoprene. Further, while Collins et al. (2014) reported significant effects of methoprene treatment on sexual development of both males and females, Adnan et al. (2018) reported positive effects only in males. These differences may derive from differences in the source of methoprene used in these studies. Methoprene has been found not to affect longevity of some fruit flies including *A. suspensa* (Pereira et al. 2010), *A. fraterculus* (Segura et al. 2013), and Q-fly (Adnan et al. 2018), and to increase longevity of male *Zeugodacus cucurbitae* (Haq and Hendrichs 2013).

Building on the previous Q-fly studies of Collins et al. (2014) and Adnan et al. (2018), in the present study we investigate whether acceleration of sexual maturation in male and female Q-flies varies between analytical grade methoprene and NOMOZ® incorporated into the adult diet, and whether effects are similar for fertile and sterile flies. Further, to investigate potential detrimental effects of methoprene treatment in fertile and sterile Q-flies, we test the separate and combined impact of irradiation and dietary methoprene supplements on longevity.

2 MATERIALS AND METHODS

2.1 Insects

Mass-reared Q-flies were obtained as pupae from a colony maintained at Macquarie University, New South Wales, Australia (23 generations from wild). Experimental flies were reared on a gel based larval diet of Moadeli et al. (2017), that was derived from the liquid diet formulation of Chang et al. (2006). The colony and experiments were held in separate controlled environment rooms that were maintained at 25±0.5°C and 65±5% RH. The photoperiod was 13L:11D, with a simulated dawn and dusk as light level gradually increased and decreased for 0.5 h at the beginning and end of each light phase. To obtain sterile flies, 4000 pupae were sealed in zip lock plastic bags (100 x 150 mm) two days prior to emergence and maintained at 18° C for 18 hours to induce hypoxia. The pupae were then sterilised by exposure to 65 Gy of gamma radiation using a ^{60}Co irradiator at Macquarie University, New South Wales, Australia (Collins et al. 2009, Dominiak et al. 2014).

2.2 Methoprene treatment

Fertile and sterile adult male and female Q-flies were provided with the following treatments combined with a standard adult diet of yeast hydrolysate (MP Biomedicals) and cane sugar (1:3 by weight) *ad libitum* for 48hrs after emergence:

(1) Nomoz: Methoprene (0.05%) from NOMOZ® mosquito larvicide;
(2) Analytical methoprene: Methoprene (0.05%) from analytical standard dissolved in acetone;
(3) Control: No Methoprene or acetone;
(4) Acetone control: No Methoprene, only acetone.

The methoprene treatments were provided to flies as a blended dry mixture in the standard adult diet. For incorporation into the adult diet, NOMOZ® pellets were finely powdered using a mortar and pestle. The powdered NOMOZ® (as required on dry weight basis; 0.25 g of Nomoz was mixed with 19.75g of standard diet) using a blender (following Adnan et al., 2018). To incorporate analytical standard methoprene (Sigma Aldrich, CAS Number 40596-69-8), a methoprene solution (20 ml of acetone containing 10 mg of methoprene) was mixed with 20 g of standard adult diet and kept overnight in a fume cabinet

to allow the acetone to completely evaporate (following Aluja et al., 2009). The Control treatment comprised the standard adult diet without modification and the Acetone control treatment was prepared as for the Analytical methoprene treatment but using acetone only.

2.3 Experiment 1: Effect of methoprene treatment on mating behaviour

For adult emergence, approximately 1,000 fertile pupae were placed in each of four cages and 1,000 sterile pupae were placed in each of another four cages (Megaview Bugdorm 44545, 47.5 x 47.5 x 47.5 cm). As a source of water, each cage was provided water-soaked cotton wool in 70-ml sample containers. Few flies emerged on the first day of emergence, and these were discarded. After the following 24 hours of emergence, non-emerged pupae were discarded and standard diet containing one of the four different treatments was provided to each of the four cages of fertile and sterile flies (in a 90 mm diameter Petri dish). After two days of *ad libitum* feeding, the treated food was replaced with sugar only in a 90 mm Petri dish. The flies were sorted according to sex within 3 days after emerging by collecting and transferring individual flies in glass tubes and were housed in 12 L cages that had a mesh-covered ca. 80cm^2 window for ventilation. At two days of age, Q-flies are still immature although nutrition acquired before this time can promote development to maturation after this time, especially in males (Pérez-Staples et al. 2008, 2009, 2011). In each 12 L cage, 200 flies were provided with sugar only as food and water-soaked cotton wool in a 70-ml sample container.

To obtain mature flies to pair with treated flies, ca. 800 pupae from numerous pupation dates were placed in separate mesh cages for adult emergence (Megaview Bugdorm 44545). Cages were supplied with water-soaked cotton wool in a 70-ml sample container and the standard adult diet on a 90 mm Petri dish *ad libitum*; this diet is effective at supporting Q-fly development (Vijaysegaran et al. 2002, Pérez-Staples et al. 2007, 2011). Adult flies were sorted according to sex within 3 days after emerging by collecting and transferring individual flies in glass tubes to transparent plastic 12 L cages that had a mesh-covered ca. 80cm^2 window for ventilation. Approximately 200 flies were sorted into each 12 L cage, with this relatively low density to minimise effects of crowding on longevity and mating performance. No calling, courting, or mating was observed in cages prior to separating the sexes. These flies were paired with experimental flies when 12-17 days old, as virgin flies of this age that

have been provided the standard adult diet exhibit a high level of sexual receptivity (Pérez-Staples et al. 2007, Prabhu et al. 2008).

Mating trials were conducted when treated flies were 4, 6, 8, 10, 15, and 20 days old. On each mating day, at least four hours before the onset of dusk, 15 males and 15 females from each treatment group were placed individually in transparent plastic 1.125 L cages that had a mesh-covered window (ca. 28 cm^2) for ventilation. Each fly was paired with a sexually mature fly of the opposite sex. Periodic observations were carried out from when pairs were set up, and continuous observation began 90 minutes prior to the onset of dusk. To assess mating latency, the time of onset of mating was recorded for each mating pair. To assess mating duration for each mating pair, observation continued until the last pair had separated. Thus 240 treated male and female flies were tested on each day, providing a total of 1,440 test pairs in total across the six ages of testing. The experiment was repeated twice using batches of pupae obtained two months apart.

2.4 Experiment 2: Effect of methoprene on longevity

Application of treatments for longevity trials was the same as that of Experiment 1. For each treatment, ten flies were placed in each of three transparent plastic 1.125 L cages that had a mesh-covered window (ca. 28 cm^2) for ventilation (i.e., 30 fertile and sterile males and females for each treatment, 480 flies in total). Each cage was provided with water-soaked cotton wool and sugar in separate 35 mm Petri dishes. Longevity trials were repeated twice, using pupae from two different production batches. Mortality was checked daily until all flies had died.

2.5 Statistical analysis

All analyses were conducted using JMP 14.1.0 (SAS Institute Inc 2018). Separate models were developed for males and females. For the mating data, we asked if the main variables affected the probability of mating, mating latency and mating duration. The same approach was taken with each model, except that the response variable and the error distribution differed. For mating probability, a general linear model was fitted with a binomial distribution. For mating latency and mating duration, a linear model was fitted following log-transformation. Mating latency was defined as the time from onset of mating until the end of

the 30-minute dusk phase as the log of this variable was normally distributed. For each model, the predictors were the same: sterility, age, and treatment, including interactions between all variables. A backward model selection process was then performed by removing non-significant interactions. Batch was also included in each model but was not included in interaction with any other variable. Post-hoc pairwise comparisons were then performed using estimated least-square means, correcting for multiple comparisons. Model assumptions were tested using graphical methods. For the GLM model of mating probability, we tested for overdispersion in the data, but none was found.

For the longevity data, a linear model was fitted following log-transformation. The predictors were sterilisation and treatment, and their interaction. If not significant, the interact term was removed. Batch was also included in each model as a fixed effect but was not included in interaction with any other variable. To account for cage level effects, cage identity was included as a random effect.

3 RESULTS

3.1 Mating Probability

For males, mating probability varied with an age x treatment interaction (Table 1, Fig 1). At 4, 10, 15 and 20 days of age there was no evidence of differences amongst the treatment groups. However, at 6 and 8 days of age males receiving either of the two methoprene treatments had significantly higher mating probability than both controls. At these ages, the two methoprene treatments were not different from each other and the two controls were not different from each other. Both methoprene treatments advanced sexual maturation by 2 - 3 days.

For females, mating probability varied with age, increasing as the flies matured, but was similar for all treatments as well as for fertile and sterile flies (Table 1, Fig 1).

3.2 Mating latency

For males, mating latency (median = 21 min, upper quartile = 29 min, lower quartile = 16 min, range = 1 - 107 min, log transformed) varied with age, as at 20 days of age, onset of mating was significantly later than at 8, 10, and 15 days of age (Table 2). There was a significant Sterility x Treatment interaction whereby fertile males receiving Nomoz tended to mate earlier than all other combinations except for fertile males receiving Analytical standard methoprene (Fig 2)

For females, mating latency (median = 17 min, upper quartile = 23 min, lower quartile = 11 min, range = 1 - 86 min, log transformed) varied with age with notably later matings at 6 and 8 days of age (Fig 3), but was similar for all methoprene treatments as well as for fertile and sterile flies (Table 2).

3.3 Mating duration

For males, mating duration (median = 63 min, upper quartile = 86 min, lower quartile = 43 min, range = 7 - 216 min, log transformed) varied with age, differed among the treatment groups and differed between fertile and sterile flies (Table 3). Mating duration tended to increase with age until plateauing at around 8 days of age (Fig 4). Fertile males tended to have longer mating duration than sterile males (Fig 4). Males from the two methoprene treatments did not differ in mating duration and had significantly longer mating duration than those receiving Acetone control. However, control flies mated for intermediate duration that were not significantly different from either two methoprene groups or acetone control (Fig 4).

For females, mating duration (median = 52 min, upper quartile = 67 min, lower quartile = 38 min, range = 10 - 161 min, log transformed) varied with age and differed for fertile and sterile flies, but did not vary amongst the methoprene treatments (Table 3). Mating duration was particularly short for those females that mated at 4 days of age (Fig 5). As was also the case for males, fertile females tended to have longer mating duration than sterile females (Fig 5).

3.4 Longevity

For males, longevity (median = 57 days, upper quartile = 77 days, lower quartile = 38 days, range = 10 - 133 days, log transformed) did not vary significantly with either methoprene

treatment ($F_{3,42}$ = 0.087, p = 0.967) or sterility ($F_{1,42}$ = 1.196, p = 0.280). Similarly, for females, longevity (median = 64 days, upper quartile = 86 days, lower quartile = 43 days, range = 12 - 135 days, log transformed) did not vary significantly with either methoprene treatment ($F_{3,42}$ = 0.027, p = 0.994) or sterility ($F_{1,42}$ < 0.001, p > 0.999).

4 DISCUSSION

The present study demonstrates the suitability of a mosquito larvicide that contains methoprene, NOMOZ®, as an effective alternative to analytical grade methoprene as a dietary supplement to accelerate the sexual development of male Q-flies. Mating propensity increased as male and female Q-flies matured, but males that were provided either of these methoprene supplements exhibited a much more rapid increase compared to flies from the control treatments. As was also reported by Adnan et al. (2018), methoprene supplements in adult diet did not affect the sexual development of Q-fly females. Further, both methoprene supplements were similarly effective in accelerating sexual development of fertile and sterile Q-flies.

Previous studies have reported positive effects of analytical grade methoprene on sexual maturation in males of *Bactrocera, Zeugodacus* and *Anastrepha* fruit flies (Pereira et al. 2009, 2013, Segura et al. 2009, 2013, Haq et al. 2010a, b, Abraham et al. 2013, Gómez et al. 2013, Liendo et al. 2013, Collins et al. 2014, Gómez-Simuta et al. 2017). In *A. fraterculus*, topical application of methoprene has been found to have strong positive effects on male development but to have no effect on female development (Segura et al. 2009, Liendo et al. 2013). Previous studies have yielded mixed responses for Q-flies. While topical application of analytical grade methoprene in acetone solution on adults or pupae was found to accelerate sexual maturation in both sterile males and females (Collins et al. 2014), dietary application of NOMOZ® was found to accelerate development of fertile males but not fertile females (Adnan et al. 2018). The present study confirms the findings of Adnan et al. (2018) for effects of dietary application of NOMOZ® on fertile males and females and also demonstrates (i) effects of NOMOZ® are similar for fertile and sterile flies as well as (ii) effects of dietary application of analytical grade methoprene are similar for fertile and sterile flies.

Having found close similarity in the present study for the two sources of methoprene used by Collins et al. (2014) and Adnan et al. (2018) we can exclude the simple explanation of methoprene source as cause for the differences in respose of females to treatment. Larval diet can affect the responses of fruit flies to methoprene (Aluja et al., 2009), but we can also exclude this as an explanation. The flies used by Collins et al. (2014) and Adnan et al. (2018) came from a mass-rearing facility where larvae are reared using a lucerne chaff diet (see Dominiak et al. 2008). However, similarity of results in studies of Adnan et al. (2018), using flies reared on lucerne chaff diet, and the present study, using flies reared on gel diet, indicates that larval diet does not explain the differences between studies in female response to methoprene. At least two further scenarios may explain why female maturation was unaffected by dietary methoprene supplementation in the present study and in Adnan et al. (2018) but was affected by topical methoprene application in the previous study of Collins et al. (2014). The facility from which flies were obtained by Collins et al. (2014) and Adnan et al. (2018) replaced the colony in the period between these studies. It may be that the colony used by Collins et al. (2014) differed substantially from those used by Adnan et al (2018) and in the present study, in ways that affected sensitivity of females to methoprene treatment. It may also be that the effects of methoprene may be stronger, or a higher effective dose delivered, in the case of topical treatment in acetone compared to ingestion with food, and therefore be sufficient to induce effects in females.

In addition to effect on mating propensity, methoprene supplementation showed some effects on mating latency. Males that were treated with NOMOZ® mated earlier than other treatment groups. This effect on mating latency contrasts with Collins et al. (2014) and Adnan et al. (2018), neither of which found significant effects of methoprene treatment on mating latency of Q-flies of either sex. However, earlier mating has been reported for male *Z. cucurbitae* that received topical application of methoprene (Haq et al. 2010). It is unclear why NOMOZ®-treated Q-flies exhibited a decrease in mating latency while others did not in the present study, especially given that the same treatment did not differ significantly from controls in the study of Adnan et al. (2018). Larval diet and level of domestication can affect the responses of fruit flies to methoprene, which can be an explanation. The flies used by Adnan et al. (2018) and in the present study were reared on different larval diet. In addition, the flies used in this study were obtained from a younger colony than that of flies tested in Adnan et al. (2018).

Methoprene supplementation resulted in increased mating duration of male Q-flies in the present study, consistent with the previous study of dietary supplementation in Q-fly (Adnan et al. 2018). Topical methoprene treatment has previously been found to increase mating duration in *Z. cucurbitae* males (Haq et al. 2010), while no effects of methoprene treatment of mating duration were reported following topical treatment of Q-fly by Collins et al. (2014) and *A. ludens* by Gomez-Simuta et al. (2017). Longer copulation can play a positive role in post-copulatory success for Q-fly SIT because longer copulations by sterile males yield higher levels of infertility in females that had previously mated with a fertile male or later remate with a fertile male (Collins et al. 2012).

Overall, both males and females exhibited reduced mating latency with increasing ages, which may indicate greater sexual vigour and courtship effort at the ages of greater mating propensity. Pérez-Staples et al. (2007) and Collins et al. (2014) have reported similar trends previously, whereby mating latency decreased with age in a group of flies tested up to 14 days and 12 days of age respectively. However, findings in the present study are partly consistent with Adnan et al. (2018) who found that mating latency in males decreased with age up to 20 days, then increased at 25 and 30 days, whereas mating latency in females increased with age. Furthermore, mating duration in both males and females increased as flies aged in the present study, which is consistent with Collins et al. (2014) but contradicts partially with Adnan et al. (2018), who reported that female mating duration was not age dependent while male mating duration increased with age.

Fertile and sterile flies exhibited very similar responses to methoprene supplements in the present study and there were few effects of sterility overall. Of particular note, sterile and fertile males were very similar in mating performance and were very similar in the positive effects of methoprene and diet supplements. The finding of similar mating propensity of fertile and sterile males is consistent with previous studies (Harmer et al. 2006, Pérez-Staples et al. 2007, Collins et al. 2008). However, while sterile male and female Q-flies had shorter copulations than their fertile counterparts in the present study, no differences were found by Harmer et al. (2006) or Pérez-Staples et al. (2007). The reasons for these differences amongst studies are not clear, but may relate to the origins, rearing or handling of the insects, or details of experimental design.

Irradiation and methoprene, regardless of the source, did not affect the longevity of Q-flies. Likewise, previous studies found that methoprene treatment had no adverse effects on longevity of Q-flies (Adnan et al. 2018), *C. capitata* (Faria et al. 2008), *A. suspensa* (Pereira et al. 2010), and *A. fraterculus* (Segura et al. 2013). In contrast, methoprene treatment has been reported to increase longevity of male *Z. cucurbitae* that were provided both sugar and YH for three days and then only sugar (Haq and Hendrichs 2013), an approach similar to the present study. Overall the absence of negative effects of methoprene treatment on longevity are promising for application of methoprene as a pre-release supplement for Q-fly SIT.

5 CONCLUSION

Analytical standard methoprene and a commercial mosquito larvicide containing methoprene both show significant potential to enhance Q-fly SIT by decreasing the delay between emergence and maturity. However, methoprene from larvicides, such as NOMOZ®, provide a more practical option for operational SIT. First, analytical grade methoprene is only soluble in organic solvents such as acetone which presents difficulties for operational settings (Pereira et al. 2013, Segura et al. 2013) but these issues are circumvented by use of water-soluble insecticidal formulations. Second, at less than 1% of the cost of Analytical standard methoprene, NOMOZ® (and similar products) offers significant economic advantages.

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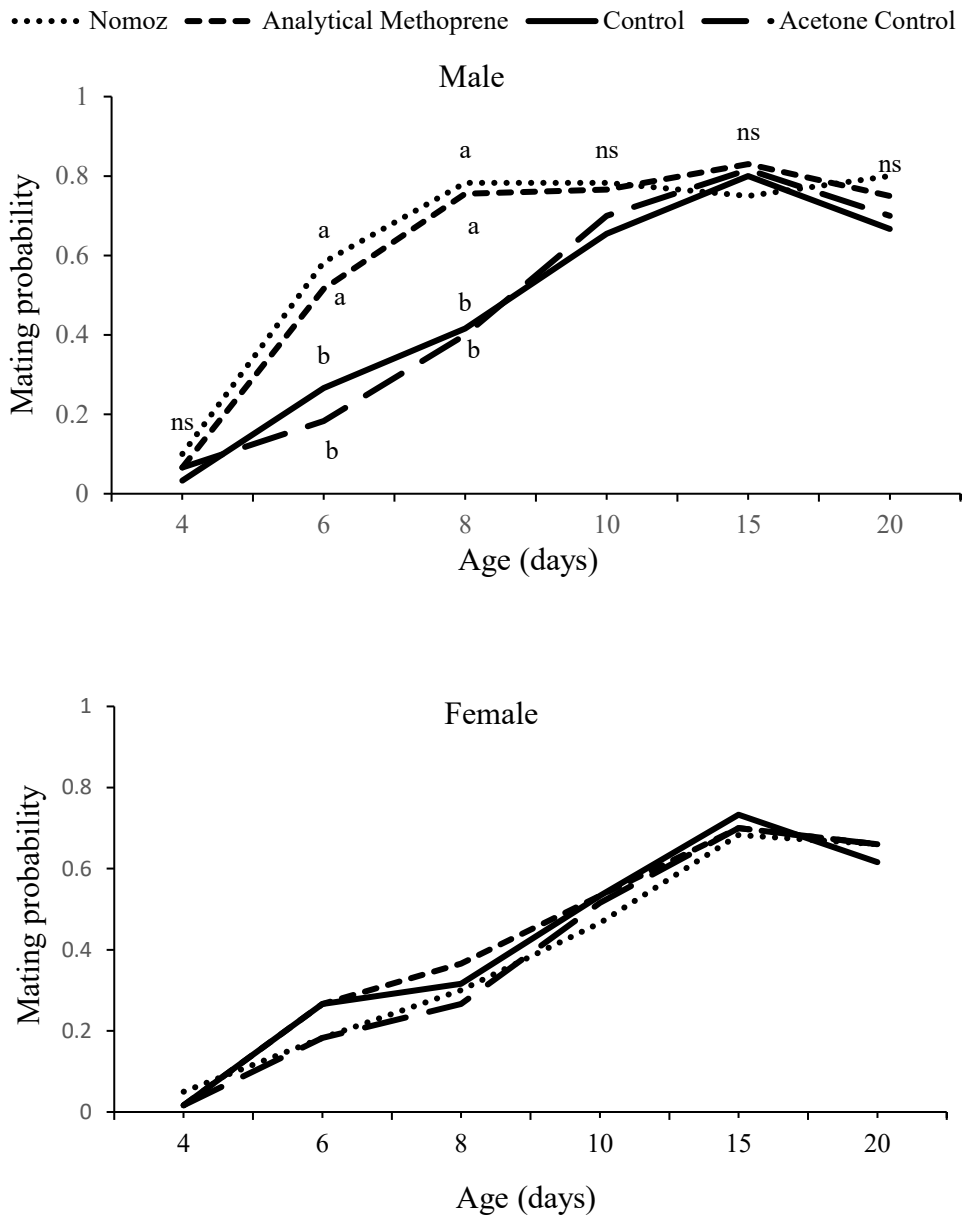
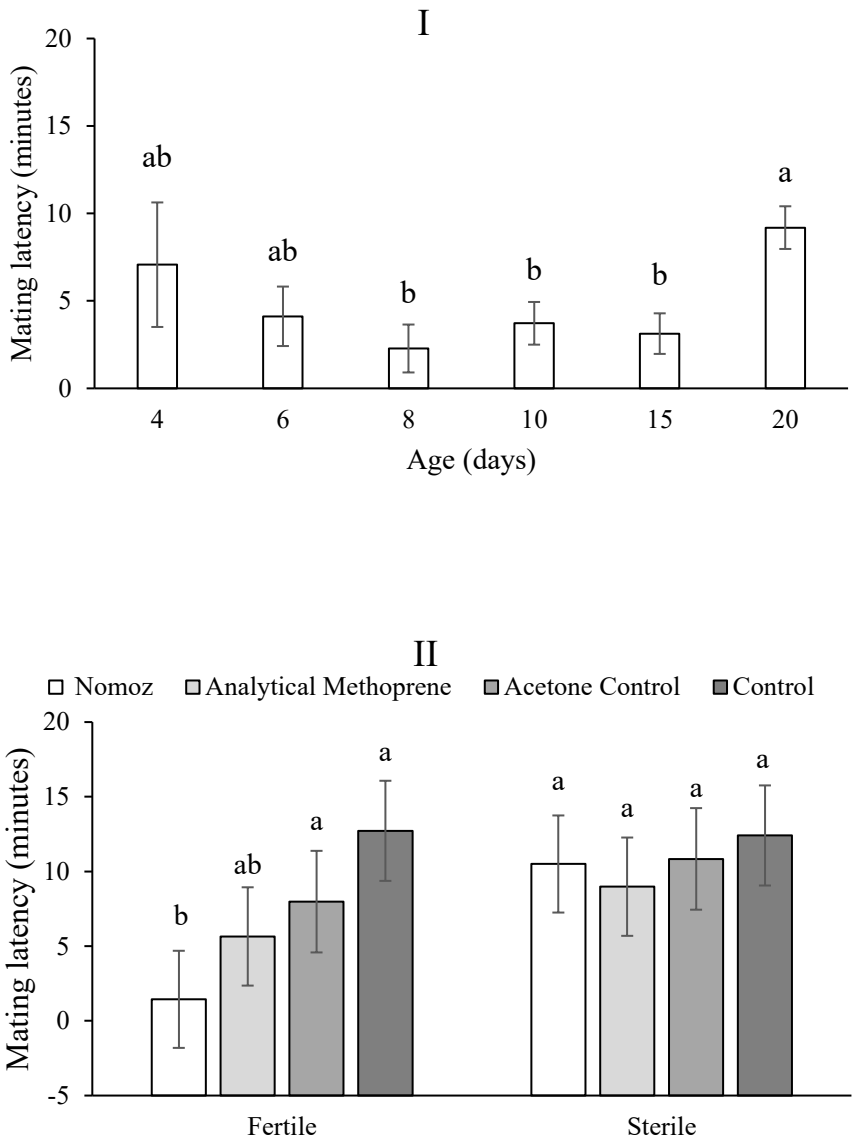
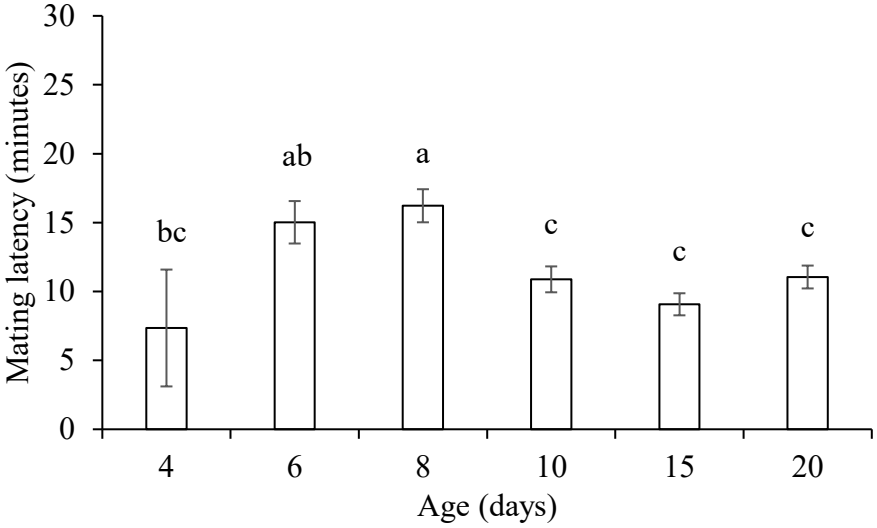
673 **Figure 1.** Relationship between age (4, 6, 8, 10, 15, and 20 days post emergence) and
674 proportion of male and female Q-flies mating (mean± SE) after being fed one of four diet
675 treatments (Nomoz, Analytical methoprene, Control, Acetone control) for two days after
676 emergence and then sugar only.



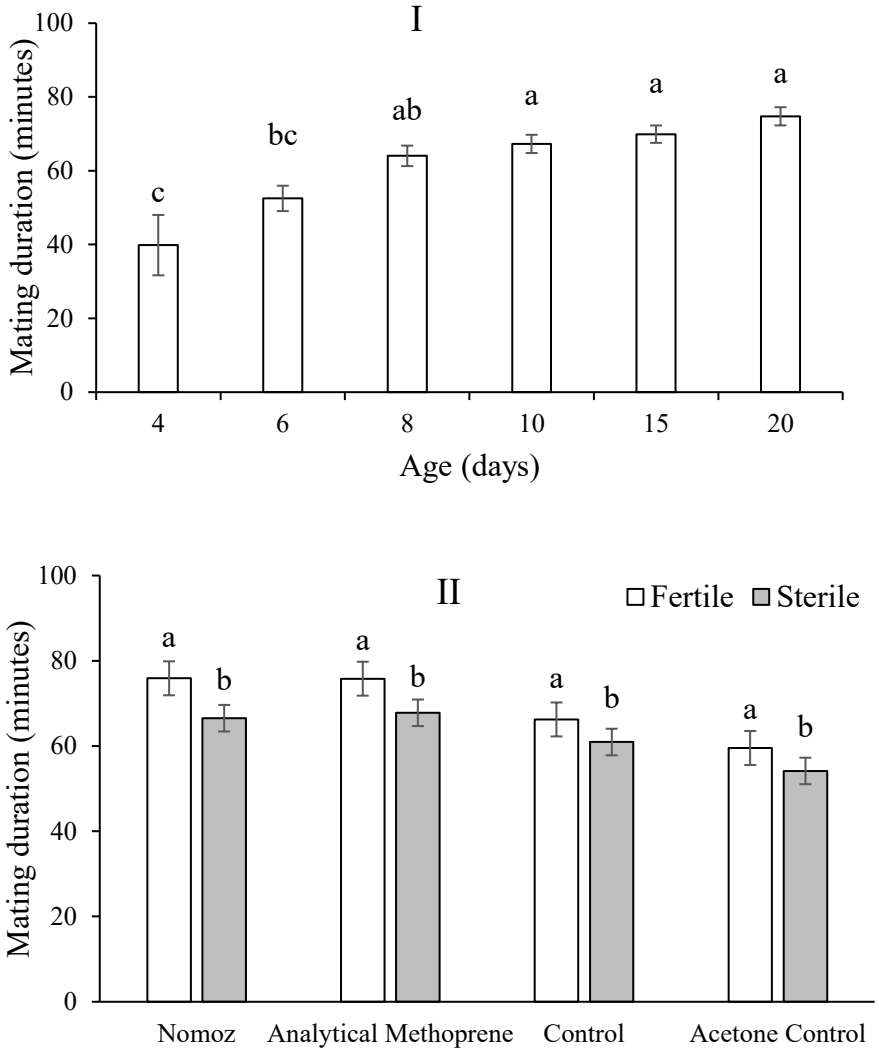
677 **Figure 2.** Effects of age (4, 6, 8, 10, 15, and 20 days post emergence), Sterility (fertile or
678 sterile) and Treatment (Nomoz, Analytical methoprene, Control, and Acetone control) on
679 male Q-fly mating latency (mean± SE). Means separated (Tukey test) by different letters
680 indicates significant differences among ages (I) and differences among treatments (II) (P <
681 0.05).



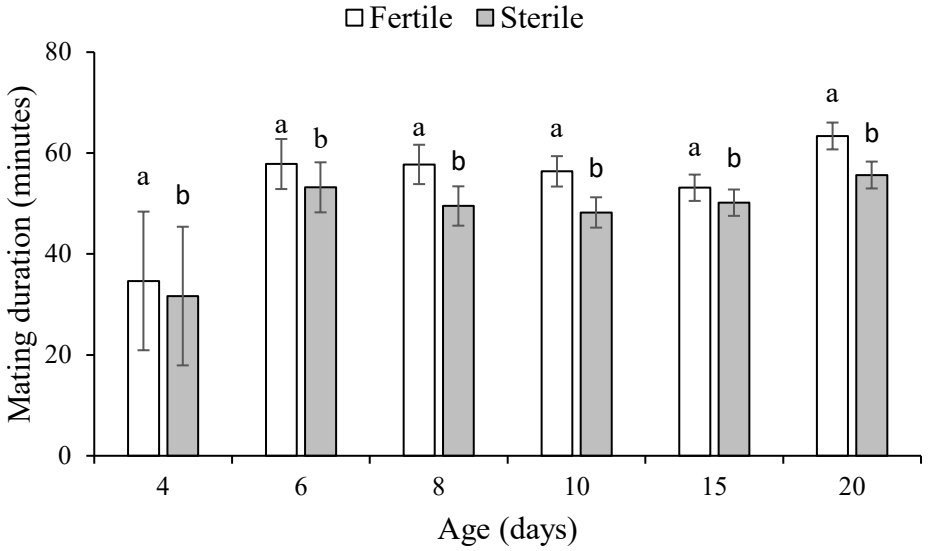
682 **Figure 3.** Effects of age (4, 6, 8, 10, 15, and 20 days post emergence) on female Q-fly mating
683 latency (mean± SE). Means separated (Tukey test) by different letters indicates significant
684 differences among ages.



685 **Figure 4.** Effects of age (4, 6, 8, 10, 15, and 20 days post emergence), Sterility (fertile or
686 sterile) and Treatment (Nomoz, Analytical methoprene, Control, and Acetone control) on
687 male Q-fly mating duration (mean± SE). Means separated (Tukey test) by lowercase letters
688 indicates significant differences among ages (I) and differences between fertile and sterile
689 males (II) (P < 0.05).



690 **Figure 5.** Effects of age (4, 6, 8, 10, 15, and 20 days post emergence) on female Q-fly mating
691 duration (mean± SE). Means separated (Tukey test) by separate letters present differences
692 between fertile and sterile females (P < 0.05).



List of Tables

Table 1: GLM analysis testing fixed effects of Age (4, 6, 8, 10, 15, and 20 days post emergence), Sterility (fertile or sterile) and Treatment (Nomoz, Analytical methoprene, Control, and Acetone control) on male and female Q-fly mating probability.

	Source	*df*	χ^2	*P*
Male	Sterility	1	2.942	0.086
	Treatment	3	2.158	0.540
	Age	5	422.903	<0.001
	Age×Treatment	15	27.876	0.022
Female	Sterility	2	0.559	0.455
	Treatment	3	1.797	0.616
	Age	5	417.274	<0.001

Table 2. Linear analysis testing fixed effects of Age (4, 6, 8, 10, 15, and 20 days post emergence), Sterility (fertile or sterile) and Treatment (Nomoz, Analytical methoprene, Control, and Acetone control) on male and female Q-fly mating latency.

	Source	*df*	F	*P*
Male	Sterility	1, 777	18.426	<0.001
	Treatment	3, 777	4.737	0.003
	Age	5, 777	3.210	0.007
	Sterility×Treatment	3, 777	3.002	0.030
Female	Sterility	1, 565	0.770	0.381
	Treatment	3, 565	2.185	0.089
	Age	5, 565	7.518	<0.001

Table 3. Linear analysis testing fixed effects of Age (4, 6, 8, 10, 15, and 20 days post emergence), Sterility (fertile or sterile) and Treatment (Nomoz, Analytical methoprene, Control, and Acetone control) on male and female Q-fly mating duration.

	Source	*df*	F	*P*
Male	Sterility	1, 780	6.087	0.014
	Treatment	3, 780	5.033	0.002
	Age	5, 780	9.399	<0.001
Female	Sterility	1, 565	5.112	0.024
	Treatment	3, 565	0.143	0.934
	Age	5, 565	2.811	0.016

Chapter Four

Image captured by Author only

This chapter has been formatted in accordance with style of *Journal of Insect Physiology*.

Declaration

Chapter four of my PhD includes and expand upon findings presented in my MRes thesis (http://minerva.mq.edu.au:8080/vital/access/manager/Repository/mq:44536). As for Chapter two, while similarity remains between Chapter four of my PhD and my MRes thesis, several additional experiments were carried out during PhD to investigate the effect of Methoprene and Yeast hydrolysate on reproductive organ development. The collection of additional data for Chapters four of my PhD comprised the three months of data collection in my PhD, followed by several months of re-analysis and writing. Novelty and development of research reported in Chapter four of my PhD thesis is outlined below:

1. Experiments and data collection: I conducted two replicates of new experiments to assess the effect of Methoprene and Yeast hydrolysate on accessory gland maturation during my PhD. In addition, I conducted one additional replicate to assess the effect of Methoprene and Yeast hydrolysate on testis and ovarian maturation during my PhD while data on apodeme development were obtained during MRes.

2. Data analysis and visualization: Data obtained during my MRes and from experiments conducted in the first six months of my PhD have been subjected to revised statistical analysis (For changes, see Tables 2, 3, 4 in Chapter Four and Tables 2, 3 in MRes thesis; In Table 1 analysis did not change, During PhD I ran revised analysis for apodeme, but finally retained the analysis of my MRes). In addition, I created new graphical presentations for Chapter four for better visualisation of analysed data and these are completely different from those reported in my MRes thesis (For changes, see Figures 1, 2, 3, 4 in Chapter Four and Figure 4 in MRes thesis).

3. Writing and conceptualisation: While there is similarity between the concept addressed in Chapter four of my PhD and my MRes thesis, I added substantial new data to Chapter four. Finally, I made substantial revisions of the content presented in MRes thesis and re-wrote most of the content (See abstract, graphical abstract, highlights, introduction, results, discussion, references in Chapter four and respective sections in my MRes thesis).

Overall, despite inclusion of data collected in my MRes, Chapter four of my PhD thesis includes substantial distinctiveness in terms of data collection, analysis, visualisation, and writing that goes well beyond the material examined in my MRes thesis.

Overview

It has been evident from chapter two and three that dietary incorporation of methoprene promotes early expression of sexual behaviour and mating in male Queensland fruit fly (*Bactrocera tryoni*; 'Q-fly'). However, it is not known, whether this increase in mating propensity is accompanied by maturation of reproductive organs as rate of sexual maturation and reproductive organ development in Q-fly do not follow identical trajectory. Accordingly, this chapter investigates whether provision of methoprene into diets of yeast hydrolysate and sugar (1:3) or sugar only accelerate development of testes, ejaculatory apodeme, and accessory glands in male Q-fly and ovaries in females. Based on promising findings of chapter two, highest (0.5%) and lowest (0.05%) concentrations of methoprene have been studied in this chapter.

Dietary methoprene treatment promotes rapid development of reproductive organs in male Queensland fruit fly

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16 **Highlights**

17

18 - Male Q-flies provided dietary methoprene supplements for 48 h after emergence had
19 faster development of ejaculatory apodeme and testis, matching effects on sexual
20 activity in previous studies.
21 - Male accessory glands and female ovaries were not affected by dietary methoprene
22 supplements.
23 - Access to a protein rich diet enhanced reproductive organ development in both Q-fly
24 sexes

Abstract

Methoprene supplements added to diets of yeast hydrolysate and sugar promote early expression of sexual behaviour and mating in male Queensland fruit fly (*Bactrocera tryoni*; 'Q-fly') and show promise as a pre-release treatment for sterile insect technique programs. Currently it is not known whether the early mating behaviour of methoprene-treated male Q-flies is only behavioural or is coupled with accelerated development of reproductive organs. Accordingly, the present study investigates whether incorporation of methoprene into diets of yeast hydrolysate and sugar (1:3) or sugar alone, accelerate development of testes, ejaculatory apodeme, and accessory glands in male Q-fly and ovaries in females. All organs increased in size as the flies aged and matured, and development rate of all organs was far greater when the flies were provided yeast hydrolysate in addition to sugar. Incorporation of methoprene into diets containing yeast hydrolysate was found to strongly accelerate development of testes and ejaculatory apodeme, but not accessory glands, in males. In the absence of yeast hydrolysate, methoprene treatment had only a modest effect on male organ development. In contrast to males, development of ovaries in female Q-flies did not respond to dietary methoprene supplements, regardless of whether they were fed yeast hydrolysate and sugar or sugar alone. These findings of diet-dependent effects of methoprene supplements on reproductive organs are a close match to previous studies investigating effects of methoprene supplements on mating behaviour. Overall, methoprene supplements substantially enhance the positive effects of protein rich adult diet on the early expression of sexual behaviour and accelerate development of reproductive organs in male, but not female, Q-flies. Methoprene supplements added to pre-release diets of yeast hydrolysate and sugar show promise as a means of accelerating reproductive development of Q-flies released in sterile insect technique programs.

Keywords: *Bactrocera tryoni*, methoprene, diet, testis, apodeme, accessory gland, ovary.

Graphical Abstract

Newly emerged flies

Methoprene in sugar + yeast (3:1)
Methoprene in sugar only
No Methoprene, sugar+yeast (3:1)
No Methoprene, sugar only

Male

- Yeast promotes development of ejaculatory apodeme, testes and accessory glands
- Methoprene accelerates ejaculatory apodeme development regardless of diet
- Methoprene accelerates testis development but only for flies that are fed yeast

Female

- Yeast promotes development of ovaries
- Methoprene does not affect development of ovaries

1. Introduction

Juvenile hormone (JH) regulates numerous critical physiological processes in insects, affecting larval development, metamorphosis, diapause, sexual maturation and development of reproductive organs (Vogel et al., 1979; Riddiford and Ashburner, 1991; Happ, 1992; Wyatt and Davey, 1996; Gilbert et al., 2000; Wilson et al., 2003; Dubrovsky, 2005). In females, JH regulates sexual receptivity, oocyte maturation, egg deposition, and ovarian vitellogenin (Shemshedini and Wilson, 1993; Ringo, 2002; Dubrovsky, 2005). In males, JH promotes the development and maturation of reproductive accessory glands, enhancing their growth and the production of glandular secretions (Yin et al., 1999; Wilson et al., 2003) and the synthesis of sex pheromones (Rantala et al., 2003). Because of the effects of JH on reproductive development in insects, treatment with methoprene, an analogue of JH (Klowden, 2013), has been investigated as a means of accelerating reproductive development of adult fruit flies for use in sterile insect technique (SIT) programs (Teal et al., 2000, 2013).

The rate of sexual maturation is of practical importance for fruit flies that are controlled through SIT. SIT involves the mass rearing, sterilization and release of vast numbers of insects (Enkerlin, 2005). The released sterile males mate with wild females, transferring nonviable sperm and accessory gland fluids to inhibit further sexual activity (Jang, 1995; Jang et al., 1999; Radhakrishnan and Taylor, 2007, 2008). SIT is currently used to control the Queensland fruit fly, *Bactrocera tryoni* Froggatt ('Q-fly'), which is the most economically damaging fruit fly in eastern Australia (Dominiak and Daniels, 2012). *Bactrocera tryoni* infests more than 100 native and introduced hosts (Hancock et al., 2000). In SIT programs, sterile *B. tryoni* have usually been released as sexually immature adults at 2 - 3 days of age (Reynolds and van der Rijt, 2011) and may take up to an additional week to mature (Weldon et al., 2008; Pérez-Staples et al. 2011). Development of pre-release diets and treatments to promote sexual development and performance of sterile male fruit flies has been a priority in *Bactrocera* SIT programs.

Tephritid fruit flies are mostly anautogenous, needing to acquire nutritional resources as adults to complete reproductive development (Drew and Yuval, 2000). Protein acquisition is particularly important, and in nature can be obtained from diverse sources including faeces, bacteria, yeast, pollen and plant leachates (Vijaysegaran et al., 1997; Drew et al., 1983; Drew and Lloyd, 1987; Aluja et al., 2001a). However, sources of protein can be variable and

limiting in nature, and so access to protein can constrain development of wild populations (Courtice and Drew, 1984). Yeast hydrolysate (YH) mixed with sugar is a standard adult diet used to maintain fruit fly colonies, providing a rich source of amino acids, micronutrients and carbohydrates (Fanson and Taylor, 2012) that is effective at sustaining reproductive development and production (Vijaysegaran et al., 2002; Meats and Leighton, 2004; Pérez-Staples et al., 2007). Given the importance of rapid sexual development for SIT programs, YH and sugar has also been investigated as a pre-release diet to ensure that flies are released with reproductive development already well underway and with stored resources to sustain development after release (Pérez-Staples et al., 2011; Weldon and Taylor, 2011). While ensuring that released flies have the nutritional resources they need for development is important, additional treatments may further accelerate maturation, and these are of particular interest as opportunities to increase efficacy of SIT programs.

Methoprene treatment of fruit flies receiving a diet of sugar and YH has been found to substantially accelerate the emergence of sexual behaviour and mating in some fruit flies, including *B. tryoni* (Collins et al., 2014; Adnan et al., 2018; Adnan et al., in press a, b), *B. cucurbitae* (Haq et al., 2010), *Anastrepha fraterculus* (Segura et al., 2009, 2013), *A. ludens* (Gómez-Simuta and Teal, 2010; Gómez et al., 2013; Pereira et al., 2013) and *A. suspensa* (Pereira et al., 2009). While many studies have used topical treatment of adults or pupae with solutions of methoprene dissolved in acetone, this approach has limited practical application owing to the hazardous nature of this solvent, and more recent efforts have focused on methods that obviate the need for solvent. In *B. tryoni*, Adnan et al. (2018) incorporated methoprene in the adult diet and found effects on male mating behaviour that were very similar to those of Collins et al. (2014) who treated pupae and adults with topical applications of methoprene in acetone.

Despite the numerous studies showing early emergence of mating behaviour in fruit flies treated with methoprene, there has been very little investigation of how methoprene affects development of reproductive organs. *Bactrocera tryoni* can be sexually active and start mating before their reproductive organs are at full size (Pérez-Staples et al., 2011). This raises the questions of whether methoprene treatment promotes the maturation of reproductive tissues or simply induces the expression of mating behaviour at earlier stages of reproductive development, which in turn might result in ineffective copulations. Though methoprene incorporated into YH and sucrose diet is known to accelerate sexual maturation

in male Q-fly (Adnan et al., 2018), the effects on reproductive organ development have not been investigated. Building on our understanding of how methoprene treatment accelerates the emergence of sexual behaviour in *B. tryoni* (Collins et al., 2014; Adnan et al., 2018, 2019, 2020), the present study investigates whether methoprene supplements mixed in adult diet accelerate development of reproductive organs, including ejaculatory apodemes, testes and accessory gland in males and ovaries in females.

2. Materials and Methods

2.1. Insects

Fertile Q-fly pupae were obtained from the Fruit Fly Production Facility at the Elizabeth Macarthur Agricultural Institute, New South Wales, Australia. Experimental flies were reared on a Lucerne Chaff larval diet. All pupae and flies were maintained, and experiments were conducted, at Macquarie University, Sydney, in controlled environment rooms (25.0±0.5ºC, 65±5% RH) on a 14:10 h light: dark cycle in which the first and last hour of the light phase simulated dawn and dusk by gradually ramping the light levels up and down, respectively.

2.2. Treatments

Adult males and females were treated with the following treatments as food for the first 2 days following emergence:

(1) M0.05 S+YH: Methoprene (0.05%) in sugar + YH (3:1)
(2) M0.5 S+YH: Methoprene (0.5%) in sugar + YH (3:1)
(3) M0.05 S: Methoprene (0.05%) in sugar only
(4) M0.5 S: Methoprene (0.5%) in sugar only
(5) M- S+YH: No Methoprene, sugar+YH (3:1)
(6) M- S: No Methoprene, sugar only

For the incorporation of methoprene into the adult diet, we selected NOMOZ® pellets (a trademark of Pacific Biologists, Brisbane, Queensland), a locally available mosquito larvicide. NOMOZ® pellets have as active ingredient PROLINK®, which contains 40 g/kg

(S)-methoprene. NOMOZ pellets were finely powdered using mortar and pestle. Then the powdered methoprene (as required on a dry weight basis) was mixed into the sugar and YH diet (3:1), or sugar only, using a blender.

2.3. Effect of methoprene and diet on reproductive organ development

Approximately 2400 pupae were placed in each of 6 mesh cages (Megaview Bugdorm 44545, 47.5 x 47.5 x 47.5 cm) for adult emergence. After 24 hours of emergence, non-emerged pupae were removed, and experimental diets were provided *ad libitum* for the next 48 hours. After 48 hours, the treated food was replaced with sugar only in a 90 mm Petri dish. The flies were then sorted according to sex the following day by collecting and transferring individual flies in glass tubes into 12.5 L cages. A dish containing water-soaked cotton wool was present in the cages at all times. Approximately 160 sorted flies were housed in each 12.5 L cage and provided sugar only in a 90 mm Petri dish.

To assess the effect of methoprene and YH on reproductive organ development, we measured testis, apodeme, and accessory gland development in males and ovarian development in females. Ten males and females were collected from each treatment at 4, 6, 8, 10, 12, 15, 20, 25, and 30 days after emergence. The flies were dissected in phosphate-buffered saline (PBS; pH 7.4) using fine forceps on a microscope slide under a Leica MZ6 stereomicroscope. In males, the ejaculatory apodeme, testis, and accessory glands were photographed using a 1.3-megapixel Dino eye camera through the phototube of the stereomicroscope. Images were calibrated and measured using Image J v.1.49. The length of the ejaculatory apodeme was measured from its lower end, where the ejaculatory sac joins the apodeme, to the farthest point of its upper rounded end (supplementary fig 1). The width was taken at its widest point, and area was measured by tracing the outline of the apodeme following Radhakrishnan and Taylor (2008). Length of the testis was measured by tracing a midline through the centre of the organ from the base to the curved tip, and the area of testis was measured by tracing the outline (supplementary fig 1) (Radhakrishnan and Taylor, 2008). Like medflies (Marchini et al., 2003) and olive fruit flies (*Bactrocera oleae*) (Marchini and Bene, 2006), Q-flies have two types of accessory glands: the short paired mesodermal glands and three pairs of sometimes branched, long and convoluted ectodermal glands (Radhakrishnan et al., 2009). Because it was not possible to reliably measure variation in the fragile ectodermal glands, we focused here on the mesodermal accessory glands. Accessory

gland length was measured by tracing a midline through the entire length of a single mesodermal gland and area was measured for the mesodermal accessory glands by tracing the outline of the gland (supplementary fig 1) (Radhakrishnan and Taylor, 2008). For females, the ovaries were photographed, and ovarian development was classified according to categories of Raghu et al. (2003), where stages 1 and 2 are previtellogenic, and stages 3–4 represent the vitellogenic phase. Flies were assigned to stage 5 if the most advanced ovarian follicles had mature eggs. We did not observe stage 6, which was defined by a yellow residual follicular relic, the corpus luteum. The trials were repeated twice using different batches of pupae.

2.4. Statistical analysis

All analyses were performed using JMP Statistical Software v. 14.1.0 (SAS Institute, Cary, NC, USA). In all analyses, initial models considered all interaction terms. Non-significant interaction terms were removed from the final models. Size of male reproductive organs (testes, apodeme, and accessory gland) was analyzed for each treatment using least squares regression. Main effects in ANOVA included in the model were age (ordinal), diet (categorical), and methoprene dose (categorical). Post hoc analysis was performed using Tukeys LSD tests on least square means. Model parameter estimates were inspected to identify effects. Ovarian development (ordinal) was analyzed for each treatment using ordinal logistic regression with significance test using likelihood ratio tests (*G*). Main effects in ANOVA included in the model were age of the flies (ordinal), diet (categorical) and methoprene dose (categorical).

3. Results

3.1. Ejaculatory apodeme

Ejaculatory apodeme length, width and area all increased as the flies aged (Table 1, Fig 1). There was also a significant effect of diet on ejaculatory apodeme size (Table 1). Males that had access to YH had significantly greater length and width of the ejaculatory apodeme compared to those provided sugar only (ejaculatory apodeme width least square mean ± SE, YH+ S= 0.328 ± 0.006 mm, S= 0.233 ± 0.006 mm; ejaculatory apodeme length least square

mean ± SE, YH+ S= 0.521 ± 0.006 mm, S= 0.407 ± 0.006 mm), and there was no evidence of variation in this tendency as the flies aged (i.e., no significant age × diet interaction). However, there was a significant age × diet interaction for ejaculatory apodeme area; ejaculatory apodeme area increased with age for both diets, but this increase was steeper in YH fed males compared to males fed on sugar only (Fig 1c).

Dietary methoprene treatment significantly accelerated ejaculatory apodeme development (Table 1). Males that were treated with either level of methoprene had significantly greater apodeme length, width and area compared to untreated males (apodeme length least square mean ± SE, methoprene 0.5 = 0.481 ± 0.006 mm, methoprene 0.05 = 0.479 ± 0.006 mm, control = 0.464 ± 0.006 mm; apodeme width, methoprene 0.5 = 0.295 ± 0.005 mm, methoprene 0.05 = 0.292 ± 0.005 mm, control = 0.280 ± 0.005 mm; apodeme area, methoprene 0.5 = 0.119 ± 0.002 mm^2, methoprene 0.05 = 0.121 ± 0.002 mm^2, control = 0.111 ± 0.002 mm^2)(Fig 1a, b).

Table 1. Analysis of variance for apodeme length, width, and area in relation to fly age (4, 6, 8, 10, 12, 15, 20, 25, and 30-days post emergence), diet (sugar or sugar+YH) and methoprene dose (0, 0.05 and 0.5%).

	Source	d.f.	F	P
Apodeme length	Age	8, 539	36.1033	0.0001*
	Dose	2, 539	7.6594	0.0005*
	Diet	1, 539	862.1145	0.0001*
Apodeme width	Age	8, 539	26.0481	0.0001*
	Dose	2, 539	6.4900	0.0016*
	Diet	1, 539	748.3590	0.0001*
Apodeme area	Age	8, 539	55.3256	0.0001*
	Dose	2, 539	15.6152	0.0001*
	Diet	1, 539	96.6794	0.0001*
	Age × diet	8, 539	2.6348	0.0078*

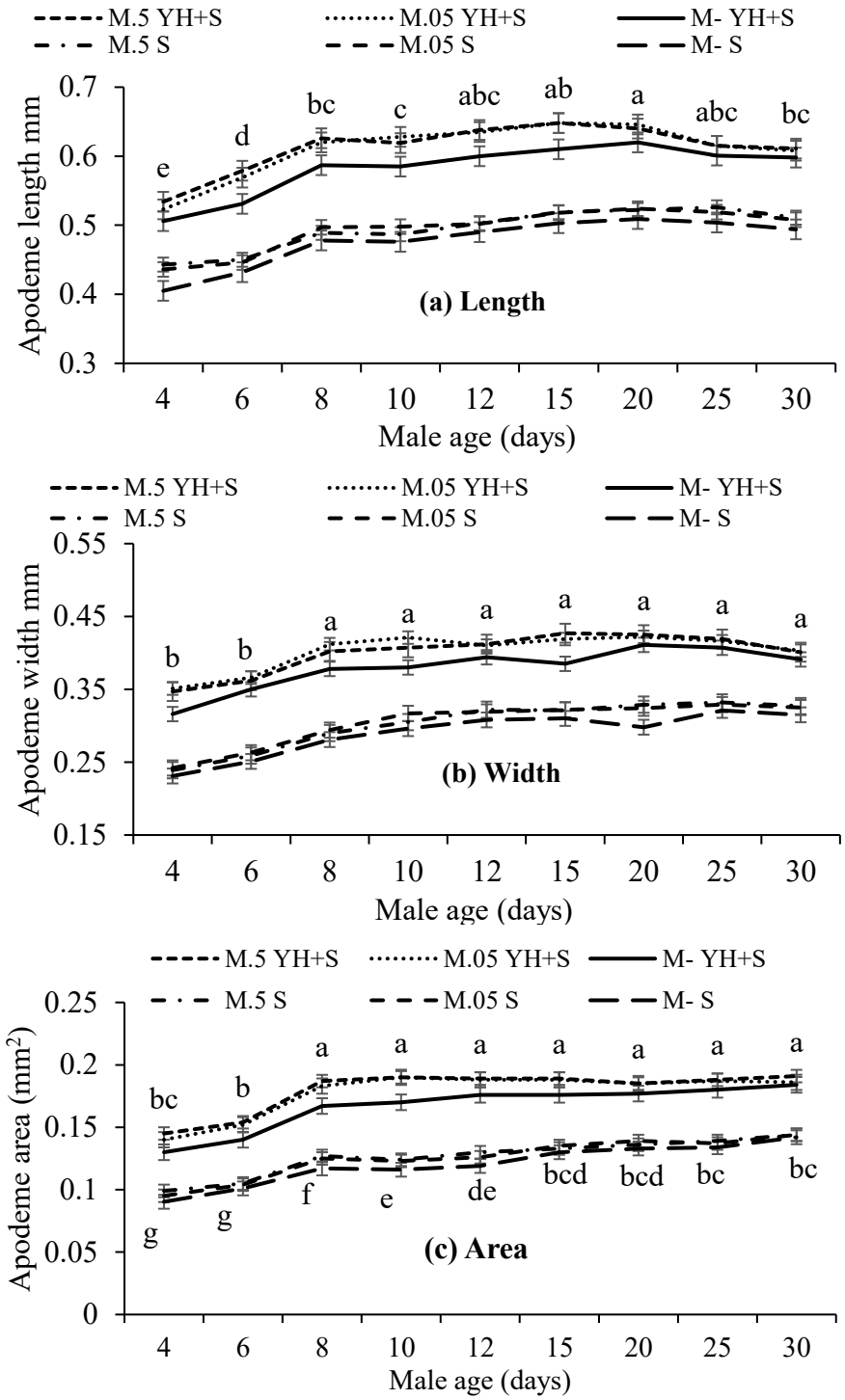


Fig 1. Effect of diet (Sugar or Yeast hydrolysate + Sugar), age (4, 6, 8, 10, 12, 15, 20, 25, and 30 days post emergence) and methoprene treatments (0, 0.05%, and 0.5%) on male ejaculatory apodeme development (mean ± SE). For ejaculatory apodeme length and width, means separated by different letters indicates significant differences between ages and for apodeme area, means separated by different letters indicates significant differences between combinations of age and diet (age * diet interaction) ($p<0.05$).

264 3.2. Testes

265

266 Age, diet, and methoprene treatment significantly affected testes length and area (Table 2).
267 Both length and area of the testis increased with age, but the rate of growth was much steeper
268 for flies with access to YH (significant age × diet interaction) (Fig 2a, 2b).

269

270 Both doses of methoprene significantly increased testis length, and there was no
271 evidence of this effect varying with diet (i.e., no dose × diet interaction). Males provided both
272 doses of methoprene had significantly greater testis length compared to untreated males
273 (Testis length least square mean ± SE, methoprene 0.5 = 1.160 ± 0.014, methoprene 0.05 =
274 1.150 ± 0.014, control = 1.100 ± 0.014). However, effects of methoprene on testis area did
275 vary with diet (i.e., significant dose × diet interaction). For males that had been provided
276 access to YH, both doses of methoprene resulted in significantly larger testes compared with
277 untreated controls.

278

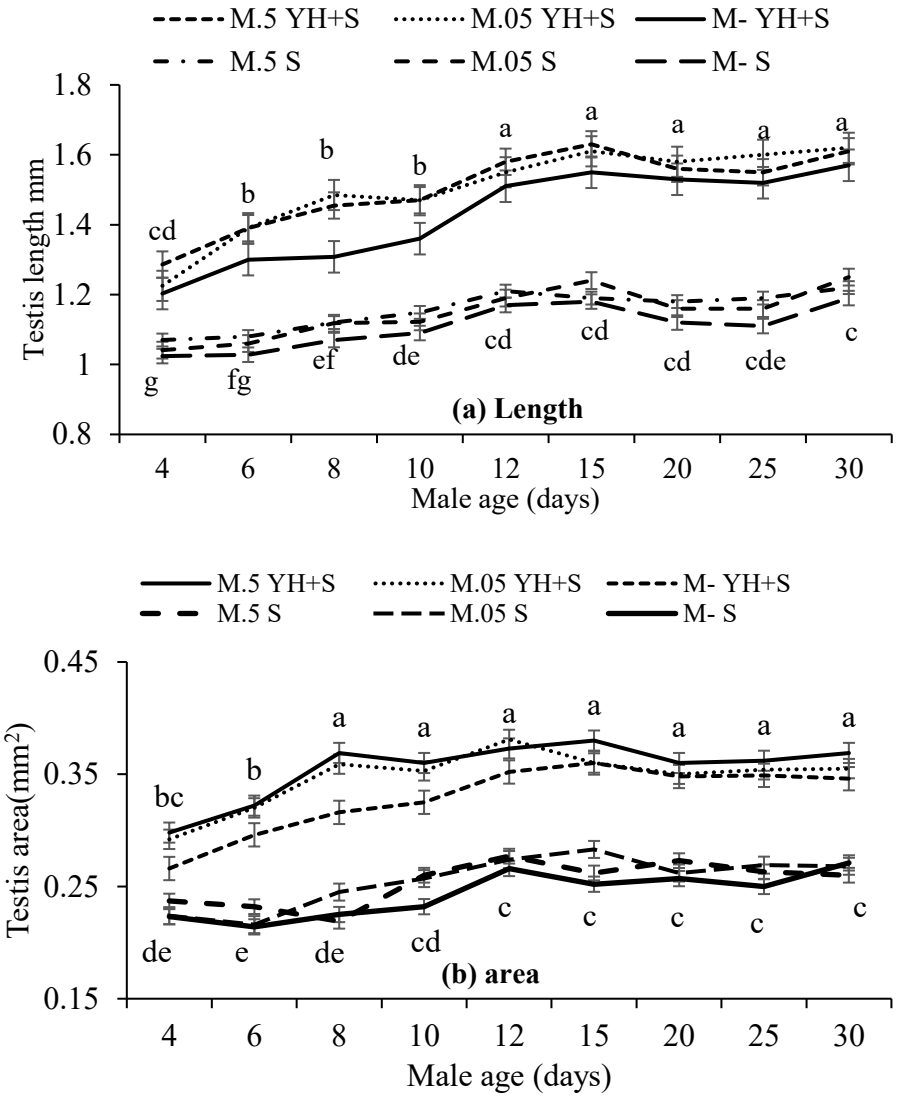
279 **Table 2.** Analysis of variance for testes length and area in relation to fly age (4, 6, 8, 10, 12,
280 15, 20, 25, and 30-days post emergence), diet (sugar or sugar+YH) and methoprene doses (0,
281 0.05 and 0.5%).

	Source	d.f.	F	P
Testes length	Age	8, 1079	42.2596	0.0001*
	Dose	2, 1079	15.0250	0.0001*
	Diet	1, 1079	50.8084	0.0001*
	Age × diet	8, 1079	8.0670	0.0001*
Testes area	Age	8, 1079	41.5644	0.0006*
	Dose	2, 1079	11.5645	0.0001*
	Diet	1, 1079	29.7690	0.0001*
	Age × Diet	8, 1079	12.4536	0.0007*
	Dose × Diet	2, 1079	3.2209	0.0403*

282

283 In contrast, for males that had been provided access to sugar only there was no evidence of
284 significant differences between methoprene-treated and untreated controls (for YH+S diet,
285 Testis area least square mean ± SE, methoprene 0.5 = 0.355 ± 0.004, methoprene 0.05 =

286 0.347 ± 0.004, control = 0.328 ± 0.004; for Sugar only diet Testis area least square mean ±
287 SE, methoprene 0.5 = 0.255 ± 0.004, methoprene 0.05 = 0.253 ± 0.004, control = 0.244 ±
288 0.004).
289



290 **Fig 2.** Effect of diet (Sugar or Yeast hydrolysate + Sugar), age (4, 6, 8, 10, 12, 15, 20, 25, and
291 30 days post emergence) and methoprene treatments (0, 0.05%, and 0.5%) on male testis
292 length (mean ± SE) (a) and area (mean ± SE) (b). Means separated by different letters
293 indicates significant differences between combinations of age and diet (age * diet interaction)
294 ($p<0.05$).

295 3.3. Accessory glands

296

297 Accessory gland length and area varied significantly with age and diet but not with
298 methoprene treatment (Table 3). Effects of age on growth in accessory gland length and area
299 varied with diet (i.e., significant age × diet interaction). Males that had been provided with
300 access to YH exhibited rapid development of accessory gland length and area, peaking at 15
301 days of age, whereas males provided access to sugar exhibited slower growth of accessory
302 gland length and area (Fig 3a, 3b).

303

304 **Table 3.** Analysis of variance for accessory gland (length and area) in relation to fly age (4, 6,
305 8, 10, 12, 15, 20, 25, and 30 days post emergence), diet (sugar or sugar+YH) and methoprene
306 doses (0, 0.05 and 0.5%).

	Source	d.f.	F	P
Length	Age	8, 1079	80.7753	0.0001*
	Dose	2, 1079	2.2072	0.1105
	Diet	1, 1079	49.1007	0.0001*
	Age × Diet	8, 1079	7.7772	0.0001*
Area	Age	8, 1079	63.7063	0.0001*
	Dose	2, 1079	0.5474	0.5686
	Diet	1, 1079	36.7631	0.0001*
	Age × Diet	8, 1079	10.6400	0.0001*

307 3.4. Female ovarian development

308

309 Ovarian development progressed as females aged, and the rate of development differed
310 greatly between the two diets (i.e., significant diet × age interaction) (Table 4, Fig 4). Ovaries
311 of females with access to YH continued developing until ca. 20 days of age, when the
312 majority of ovarioles contained mature eggs. In contrast, ovaries of females with access to
313 only sugar were still at pre-vitellogenesis stage even at 30 days of age. Ovarian development
314 was not affected by methoprene treatment (Table 4, Fig 4).

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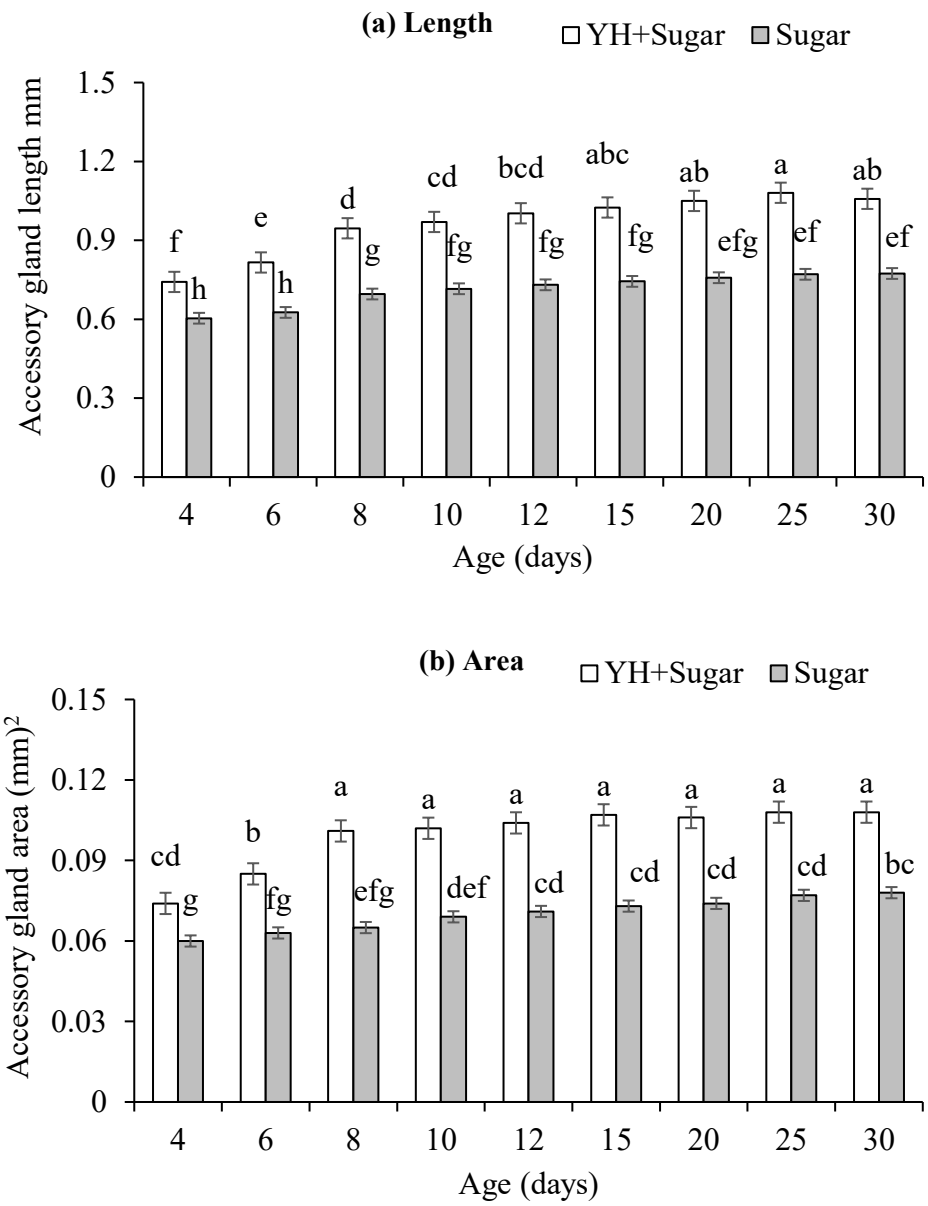
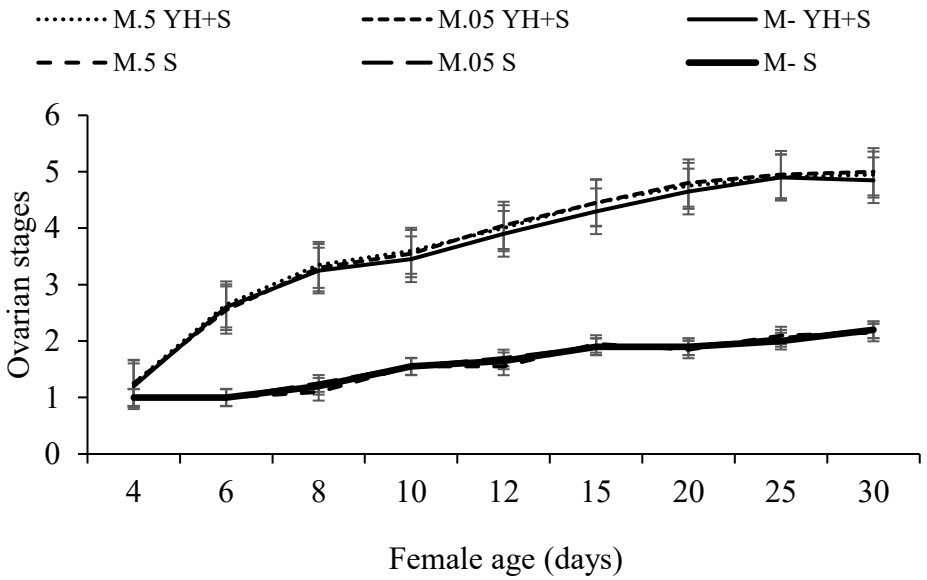


Fig 3. Effect of diet (Sugar or Yeast hydrolysate + Sugar), age (4, 6, 8, 10, 12, 15, 20, 25, and 30 days post emergence) on male accessory gland length (mean ± SE) (a) and area (mean ± SE) (b). Means separated by different letters indicates significant differences between combinations of age and diet (age * diet interaction) ($p<0.05$).

320 **Table 4.** Analysis of variance for ovarian stage in relation to fly age (4, 6, 8, 10, 12, 15, 20,
321 25, and 30 days post emergence), diet (sugar or sugar+YH) and methoprene doses (0, 0.05
322 and 0.5%).

	Source	d.f.	G	P
Ovary stage	Age	8, 1079	1491.7041	0.0001*
	Dose	2, 1079	3.4105	0.1817
	Diet	1, 1079	21.2854	0.0001*
	Age × Diet	8, 1079	27.0511	0.0007*



323 **Fig 4.** Effect of diet (Sugar or Yeast hydrolysate + Sugar), age (4, 6, 8, 10, 12, 15, 20, 25, and
324 30 days post emergence) and methoprene treatments (0, 0.05%, and 0.5%) on Q-fly female
325 ovarian maturation (mean ± SE).

4. Discussion

Yeast hydrolysate (YH) promoted development of reproductive organs in male *B. tryoni*; growth of the testes, ejaculatory apodeme, and accessory glands was substantially faster in males that received YH than in males that received only sugar. The response to dietary YH is expected, as YH provides a rich source of amino acids, sterols, vitamins and minerals to support development (Meats and Leighton, 2004). This finding closely resembles Pérez-Staples et al. (2011), who found substantial increases in growth of testes, accessory glands, ejaculatory apodeme, and ejaculatory duct in male *B. tryoni* that were provided 48 hours access to YH rather than sugar only. Similarly, Weldon and Taylor (2011) found more rapid growth of the ejaculatory apodeme in male *B. tryoni* that were provided 48 hours access to YH rather than sugar only. Such positive effects of YH on development of reproductive organs have also been reported in other fruit flies (e.g., *B. dorsalis,* Reyes-Hernández et al., 2018). Previous studies investigating effects of YH on development have indicated that sexual behaviour is first expressed in *B. tryoni* males around the same age that the maximum ejaculatory apodeme size is reached (Drew, 1969; Vijaysegaran et al., 2002), and a similar pattern has been reported in *B. cacuminata* (Raghu et al., 2003).

Methoprene supplements accelerated growth of the ejaculatory apodeme regardless of dose and diet and also accelerated growth of testis development in males that were provided dietary YH. This is, together with unpublished studies on *A. ludens* by Macías-Díaz del Castillo (2017), the first study to demonstrate effects of methoprene treatment on development of reproductive organs in any fruit fly. While positive effects of methoprene treatment on the age of mating have been reported in several fruit fly species, including Q-fly (Segura et al., 2009; Haq et al., 2010; Collins et al., 2014; Adnan et al., 2018; Adnan et al., in press a, b), we here show that in Q-fly this is not only a behavioural effect, but is also accompanied by increased development of reproductive organs. The lack of effect of methoprene on testis development of males that were provided only sugar is likely related to insufficient resources. The role of JH in the maturation of testis is not well understood. Spermatogenesis proceeds in the testis before and after adult emergence, and JH does not appear to mediate this process (Wyatt and Davey, 1996). Although testes size was not measured, no effect of methoprene has been found in sperm transfer in *A. fraterculus* (Abraham et al., 2013).

Despite strong effects on development of ejaculatory apodeme and testis, methoprene did not significantly affect the growth of male accessory gland length or area. The absence of an effect of methoprene on accessory gland development in *B. tryoni* males seems inconsistent with the effects reported in other insects, in which JH stimulates maturation of the accessory gland (Wigglesworth, 1936; Loher, 1961; Blaine and Dixon, 1973; Herman, 1975; Herman and Bennett, 1975; Ramalingam and Craig, 1977). Beyond effects of endogenous JH, topical application of JH-III induced male accessory gland growth in *Plautia stahli* bugs (Kotaki and Yagi, 1989). In a taxonomically closer example, methoprene mixed into a diet including YH for five days increased the growth of male accessory gland area in sterile *A. ludens* (Macías Diaz del Castillo, 2017). Given these examples in other insects, it is not clear why no effects of methoprene on accessory gland development were detected in the present study of *B. tryoni*. Perhaps a longer pre-release exposure period beyond 48 hrs is needed for an effect of methoprene to be evident in this reproductive organ.

In parallel to the effects on development of male reproductive organs, inclusion of YH in the early adult diet induced a substantial increase in development of ovaries. These effects of diet on ovarian maturation were in general agreement with previous studies that describe very low ovarian development for females fed only sucrose in contrast to rapid development of ovaries in females fed YH (Vijaysegaran et al., 2002; Yap et al., 2015). *Bactrocera tryoni* females have been estimated to need at least 0.1 mg of protein per day to develop their ovaries (Drew, 1987; Meats and Leighton, 2004). However, the strong positive effects of 48 hours access to YH on ovarian development in the present study contrasts with findings of Pérez-Staples et al. (2011), who found that females fed yeast hydrolysate for 48 or 24 h exhibited low levels of ovarian development, similar to females fed sucrose only. The reason behind the difference in results between the two studies is likely due to differences in the methods used to assess ovarian development. While Pérez-Staples et al. (2011) assessed ovary size as a measure of development, in the present study we focused on presence of mature eggs. Females provided access to YH for 48 hrs after emergence might not have larger ovaries but still have a greater number of mature eggs compared to sugar fed females. Similar effects of YH on ovarian development have also been reported in *A. obliqua* and *A. ludens* (Aluja et al., 2001b).

JH released from the corpora allata plays a key role in reproduction in many insects (Handler and Postlethwait, 1977; Wyatt and Davey, 1996), regulating vitellogenin synthesis

and ovarian development, and so effects of methoprene on ovarian development might be anticipated in *B. tryoni*. However, in contrast to the clear effects of methoprene on development of ejaculatory apodeme and testes in males, methoprene treatment did not promote ovarian maturation in females regardless of diet. This finding parallels behavioural observations of Adnan et al. (2018, in press a, b) who found no evidence that methoprene promotes early sexual activity in female *B. tryoni*. JH also does not mediate ovarian development in queens and workers of *Apis mellifera* (Robinson, 1985; Robinson et al., 1992; O'Donnell and Jeanne, 1993; Robinson and Vargo, 1997; Amdam et al., 2006). On the other hand, in female apple maggot flies, *Rhagoletis pomonella,* topical applications of a juvenile hormone analogue, pyriproxyfen, significantly enhanced ovarian development (number of eggs in ovaries and length of egg follicles) (Duan et al., 1995). In *Polistes* wasps, large workers respond to JH with increased ovarian development, while small workers do not (Tibbetts and Sheehan, 2012). There appears to be substantial taxonomic variation in how JH, and the application of analogues, affects ovarian development.

The lack of effects of methoprene on ovarian development in *B. tryoni* may be related to concentration of methoprene. Juvenile hormone is required in high doses to initiate ovarian maturation and then in lower doses to maintain vitellogenesis in *D. melanogaster* (Bownes and Rembold, 1987; Bownes, 1989). It may be that higher effective doses than in the present study are required to induce detectable effects on ovarian development of *B. tryoni*. While the results of the present study are a good match to those of Adnan et al. (2018), who found no effects of methoprene treatment on mating behaviour of female *B. tryoni*, they are not a good match to results of Collins et al. (2014) who found positive effects of methoprene treatment on female mating behaviour. These differences may be due to methodological differences in methoprene application. While in the present study methoprene treatment was mixed in the diet, in Collins et al. (2014) methoprene treatment was applied topically to pupae and adults in an acetone solution. It may be that the methods of Collins et al. (2014) provided a more direct incorporation of methoprene to the treated insects and that this was sufficient to induce effects on female mating behaviour.

In terms of practical applications, the strong positive effects of dietary methoprene treatment on development of ejaculatory apodemes and testes in males but no detected effects on ovarian development in females, support previous studies suggesting that methoprene treatment could potentially bias operational sex ratio of flies in favour of males in SIT

releases (Segura et al., 2009; Adnan et al., 2018). Because *B. tryoni* are currently only available as a bisexual strain, with both males and females released in SIT programs, procedures that bias sex ratio in favour of males are valuable for improving SIT efficacy (Vreyson et al., 2006).

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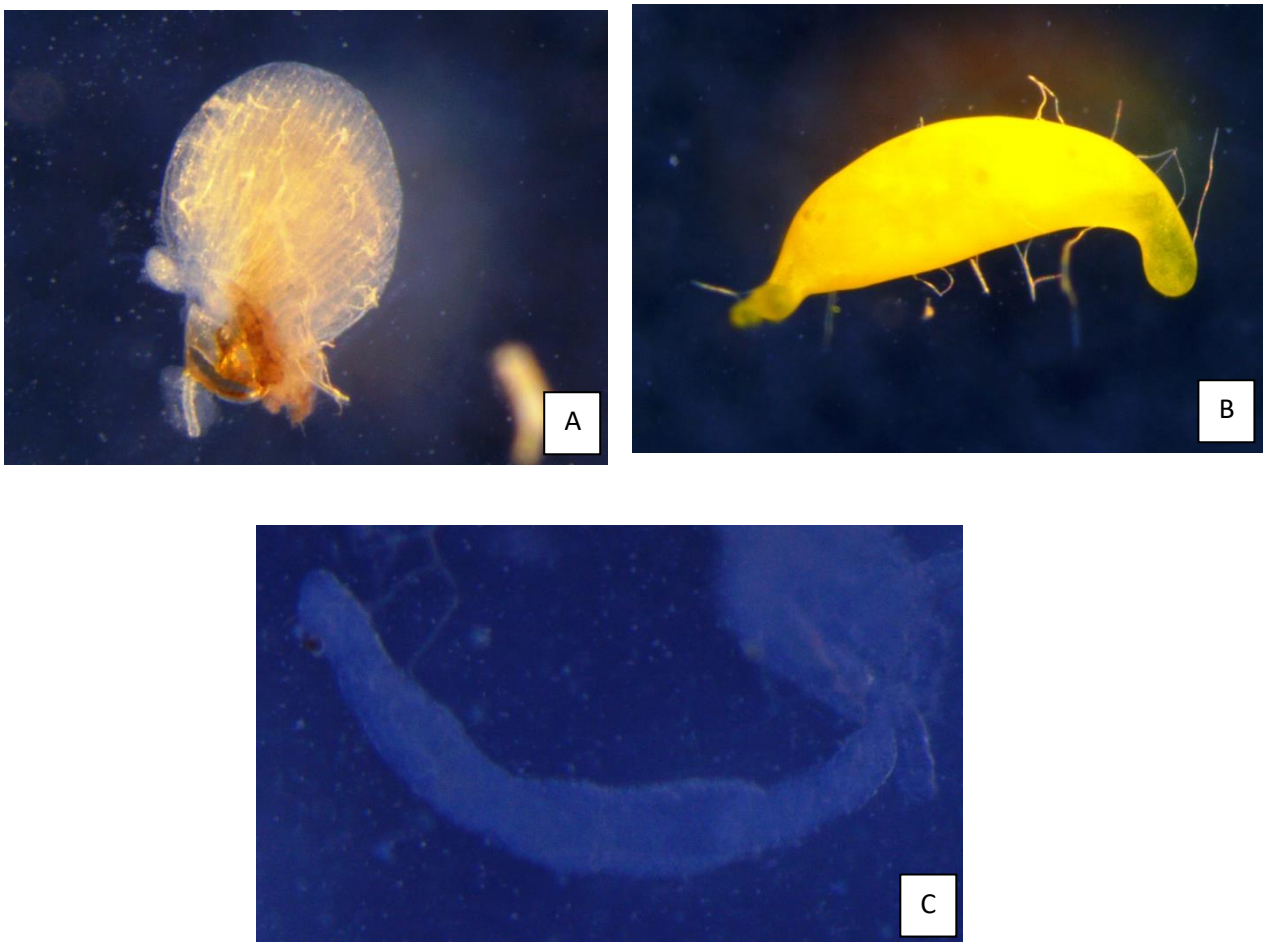

Supplementary Fig 1. Morphological traits assessed for ejaculatory apodeme (a), testis (b) and male accessory gland (c) development measurement.

Chapter Five

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Overview

Pronounced effects of methoprene diet supplements on sexual maturation and reproductive development of male Q-flies in laboratory trials (chapter two, three, and four) needs further demonstration in fields before deploying methoprene as pre-release supplements in operational SIT. Therefore, there is a priority to move beyond the laboratory context to investigate the ability of mass-reared sterile Q-fly males to compete sexually with mature wild males under more natural conditions. Accordingly, in chapter five, I investigated mating competitiveness of young methoprene-treated males (5 and 7 days old) against mature laboratory or wild males for matings with untreated mature laboratory or wild females respectively under field cage conditions. Because Q-fly are being currently released as bisex strains in SIT programs, sexual compatibility between treated and untreated sterile flies with mature flies from laboratory strains or from wild populations were also investigated. In this study, Mating trials using laboratory flies were undertaken to understand the advancement of sexual maturation in outdoor conditions because of methoprene ingestion, while trials using wild flies were performed to understand the effect of methoprene feeding on the sexual competitiveness of mass reared sterile flies.

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Chapter Six

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Overview

It has become apparent from the results of chapter two, three, and five that methoprene treatment accelerates sexual maturation in Q-fly. However, it is not known how effective the matings of methoprene-treated males are at inducing sexual inhibition in their mates. Ability of males to inhibit sexual receptivity in females is crucial for successful SIT. While methoprene-treated males might secure more mates, this would be of little value if these matings fail to induce sexual inhibition in their mates. Based on this understanding, in the chapter I asked if these additional matings by young methoprene-treated male Q-flies are effective at inducing sexual inhibition in their mates. Also, I assessed whether methoprene supplementation to male Q-flies influences other post mating events like female remating latency and remating duration.

Methoprene-induced matings of young Queensland fruit fly males are effective at inducing sexual inhibition in females

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Short title: Effective matings of methoprene-treated male Q-fly

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ABSTRACT

Pre-release dietary treatment with methoprene, a juvenile hormone analogue, decreases the age at which male Queensland fruit flies mature and hence may decrease the post-release delay until released sterile flies participate in sterile insect technique (SIT) programs. However, if matings of young methoprene-treated males are not effective at inducing sexual inhibition in their mates, then this treatment may not enhance SIT. The present study investigates efficacy of matings of methoprene-treated males at inducing sexual inhibition in their mates. Methoprene incorporated into a diet of sugar and yeast hydrolysate (w/w 3:1) for 48 hrs after emergence resulted in significantly increased male mating propensity when flies were less then 10 days of age, but not when older, and longer copulations. Copula latency did not vary with methoprene treatment but did decrease with age. The matings of young methoprene-treated males were effective at inducing overall 30 % sexual inhibition in their mates, matching the efficacy of untreated mature males. Regardless of treatment, females had reduced tendency to remate if their first mate was 15 days of age than if their first mate was younger (6, 8 days) or older (20, 25, 30 days). Females mated by methoprene-treated males that did remate tended to remate later in the day than females mated by untreated males. Also, second copula durations of females first mated by a 6 - 10 day old male were shorter if the male was methoprene treated. These patterns in remating females may indicate greater efficacy of the initial mating of methoprene-treated males. Overall, we find that the additional matings of young methoprene-treated male Queensland fruit flies are effective at inducing sexual inhibition in their mates. This finding supports the incorporation of methoprene into pre-release diet for SIT.

Keywords: Juvenile hormone, development, sterile insect technique, *Bactrocera tryoni*.

1 Introduction

Sexual success of male insects is determined not only by their ability to secure matings but also depends on the subsequent sexual receptivity of mated females (Eberhard, 1996; Simmons, 2001). In some insects, females remate frequently, and may remate almost immediately after their first mating, whereas females of other species have reduced sexual receptivity following their first mating (Thornhill & Alcock, 1983). Mating-induced sexual inhibition has been reported in females of numerous tephritid fruit flies, including melonfly [*Zeugodacus cucurbitae* (Coquillett)] (Kuba & Itô, 1993), cucumber fruit fly [*Bactrocera cucumis* (French)] (Chinajariyawong, Drew, Meats, Balagawi, & Vijaysegaran, 2010), wild tobacco fly [*Bactrocera cacuminata* (Hering)] (Chinajariyawong et al., 2010), olive fruit fly [*Bactrocera oleae* (Rossi)] (Zouros & Krimbas, 1970), Caribbean fruit fly [*Anastrepha suspensa* (Loew)] (Sivinksi & Heath, 1998), Mexican fruit fly [*Anastrepha ludens* (Loew)], West Indian fruit fly [*Anastrepha obliqua* (Macquart)] (Aluja, Rull, Sivinski, Trujillo, & Pérez-Staples, 2009), South American fruit fly [*Anastrepha fraterculus* (Wiedemann)] (Abraham, Goane, Cladera, & Vera, 2011; Abraham, Cladera, Goane, & Vera, 2012), Mediterranean fruit fly, or medfly [*Ceratitis capitata* (Wiedemann)] (Mossinson & Yuval, 2003; Vera, Cladera, Calcagno, Vilardi, & McInnis, 2003; Bertin et al., 2010), apple maggot fly [*Rhagoletis pomonella* (Walsh)] (Opp & Prokopy, 2000), as well as in Queensland fruit fly, or Q-fly [*Bactrocera tryoni* (Froggatt)] (Barton-Brown, 1957; Harmer, Radhakrishnan, & Taylor, 2006; Pérez-Staples, Harmer, Collins, & Taylor, 2008; Radhakrishnan, Pérez- Staples, Weldon, & Taylor, 2009). Accessory gland fluids transferred in the semen have been found to be key to mating-induced sexual inhibition in female Q-flies (Radhakrishnan & Taylor, 2007, 2008; Radhakrishnan, Raftos, Nair, & Taylor, 2008; Radhakrishnan, et al., 2009), and in some other fruit flies (Jang, 1995; Gabrielli et al., 2016), although sperm and other attributes of copulation or the ejaculate may also be important in some species (Miyatake et al., 1999; Mossinson & Yuval, 2003; Lentz, Miller, Spencer, & Keller, 2009; Abraham et al., 2012, Abraham et al., 2016).

The incidence of mating induced sexual inhibition is important for understanding the overall sexual performance of male fruit flies, but is also important for understanding the efficacy of matings in sterile insect technique (SIT) programs that have been deployed to manage some of the world's most economically important tephritid fruit fly pests, including *C. capitata* (Reyes et al., 2007), *Z. cucurbitae* (Kakinohana, 1993; Itô, Kakinohana,

Yamagishi, & Kohama, 2003), Oriental fruit fly [*Bactrocera dorsalis* (Hendel)] (Orankanok et al., 2007), and *A. ludens* (Orozco-Dávila, de Lourdes Adriano-Anaya, Quintero-Fong, & Salvador-Figueroa, 2015). In SIT, sterile males are released to mate with females of pest populations, inducing reproductive failure (Knipling, 1955; Krafsur, 1998). As a result, reproductive potential of pest populations is reduced and, over generations, pest abundance declines. If released males fail to induce sexual inhibition in their mates then effectiveness of SIT can be greatly reduced (Robinson, Cayol, & Hendrichs, 2002; Kraaijeveld & Chapman, 2004; Collins, Pérez-Staples, & Taylor, 2012).

Q-flies infest a vast diversity of commercial and non-commercial crops in eastern Australian states of Queensland, New South Wales and Victoria (Dominiak & Daniels, 2012) and significant outbreaks have also occurred in South Australia, Western Australia, Tasmania and New Zealand, as well as becoming established on some Pacific Islands. SIT is increasingly relied on to manage Q-fly populations, as well as to eradicate outbreaks in non-endemic regions. SIT programs depend on survivorship, maturation, and high levels of pre- and post-copulatory sexual performance of released sterile males, and there has been substantial research effort directed at developing pre-release treatments that improve post-release performance of Q-flies and other fruit flies. Like most fruit flies, Q-flies are anautogenous, relying on nutritional resources acquired as adults, especially protein, in order to complete reproductive development (Vijaysegaran, Walter, & Drew, 2002). In Q-fly, provision of yeast hydrolysate (YH) together with sucrose has been found to promote development (Meats & Leighton, 2004; Pérez- Staples, Prabhu, & Taylor, 2007; Pérez-Staples, Weldon, & Taylor, 2011) and increase longevity (Prabhu, Pérez-Staples, & Taylor, 2008; Pérez-Staples et al., 2007; Fanson, Weldon, Pérez-Staples, Simpson, & Taylor, 2009) (for a review, see Taylor et al., 2013). Sterile Q-flies have usually been released as sexually immature adults and inclusion of YH in pre-release diet can increase the proportion of released flies that survive to maturity and participate in mating activity (Weldon, Pérez-Staples, & Taylor, 2008; Reynolds, Orchard, Collins, & Taylor, 2014). Adding to the beneficial effects of YH as a component of pre-release diets, there is now increasing interest in additional treatments that might further increase the post-release performance of sterile male Q-flies.

Juvenile hormone (JH) is an important mediator of sexual maturation, reproduction, and metamorphosis in insects (Gilbert, Granger, & Roe, 2000; Wilson, DeMoor, & Lei, 2003;

Dubrovsky, 2005). Pre-release treatment with methoprene, an analogue of JH, has been explored as a way to accelerate development of male fruit flies for use in SIT programs (Teal, Gómez-Simuta, & Proveaux, 2000; Teal et al., 2013). Methoprene treatment has been found to substantially accelerate sexual maturation in *Z. cucurbitae* (Haq et al., 2010a, b), *A. fraterculus* (Bachmann et al., 2017; Liendo et al., 2013; Segura et al., 2009, 2013), *A. ludens* (Gómez, Teal, & Periera, 2013; Periera, Teal, Conway, & Sivinski, 2013), *A. suspensa* (Pereira, Sivinski, & Teal, 2009; Pereira, Sivinski, Teal, & Brockmann, 2010), and Q-fly (Collins, Reynolds, & Taylor, 2014; Adnan et al., 2018, Adnan, Farhana, Inskeep, Rempoulakis, & Taylor, in press a, b). While many studies have treated pupae or recently emerged adults topically with methoprene dissolved in acetone (e.g., Teal et al., 2000, 2013; Pereira et al., 2013), recent studies have increasingly incorporated methoprene into the diet as an approach that is more readily implemented in operational SIT programs. This approach has been found to be highly effective at promoting sexual development of male Q-fly and to have no effect on sexual development of females (Adnan et al., 2018, in press a, b). However, are the additional matings of young methoprene-treated male Q-flies effective at inducing sexual inhibition in their mates?

Findings for sexual inhibition in mates of methoprene-treated male fruit flies are mixed. In *A. fraterculus*, methoprene treatment elevates the mating propensity of young males but young methoprene-treated males exhibit lower ability to induce sexual inhibition in their mates than older untreated males (Abraham et al., 2013). On the other hand, in *A. ludens* and *Z. cucurbitae* young methoprene-treated males have been found to be as effective as older untreated males at inducing sexual inhibition (Haq, Vreysen, Teal, & Hendrichs, 2014; Gómez-Simuta, Díaz-Fleisher, Arredondo, Díaz-Santiz, & Pérez-Staples, 2017). Having found substantially elevated mating propensity in young Q-flies following dietary methoprene treatment for two days following emergence (Adnan et al., 2018), we here investigate whether these additional matings by young methoprene-treated male Q-flies are effective at inducing sexual inhibition in their mates.

2 Materials and Methods

2.1 General methods and diet treatments

Q-fly pupae were obtained from the Fruit Fly Production Facility at The Elizabeth Macarthur Agricultural Institute, New South Wales, Australia. Experimental flies were reared on a Lucerne Chaff larval diet (for production details see Fanson, Sundaralingam, Jiang, Dominiak, & D'arcy, 2014.; Dominiak, Sundaralingam, Jiang, Jessup, & Barchia, 2008.). All pupae and flies were maintained, and experiments were conducted, at Macquarie University, Sydney, in controlled environment rooms (25±0.5ºC, 65±5% RH) on a 14:10 h light: dark cycle in which the first and last 0.5 hour of the light phase simulated dawn and dusk by gradually ramping the light levels up and down, respectively.

Approximately 6500 pupae from a single day of pupation were placed in each of three mesh cages (Megaview Bugdorm Taiwan 44545, 47.5 x 47.5 x 47.5 cm) for adult emergence. Few flies usually emerge on the first day of emergence, and these were discarded. After the following 24 hours of emergence, non-emerged pupae were discarded and food (sugar and YH [MP Biomedicals LLC], 3:1) containing one of three doses of methoprene (0% control, 0.05%, and 0.5%) was provided on a 90 mm Petri dish *ad libitum* to each cage of flies for the following 48 hours. Cages were also supplied with water-soaked cotton wool in a 70-mL sample container. This period of exposure to methoprene treatment matches the pre-release holding period that has commonly been used in Q-fly SIT (Reynolds & van der Rijt, 2011). For the incorporation of methoprene into the adult diet, we selected NOMOZ® pellets (a trademark of Pacific Biologists, Brisbane, Queensland). These have as active ingredient PROLINK®, which contains 40 g/kg (S)-methoprene. This product was locally available, being marketed as a mosquito larvicide. NOMOZ pellets were finely powdered using mortar and pestle. Then the powdered methoprene (as required on dry weight basis) was mixed into the diet using a blender. After 48 hours of *ad libitum* feeding, the treated food was replaced with sugar only in a 90 mm Petri dish. The flies were then sorted according to sex within 3 days after emerging by collecting and transferring 200 male flies in glass tubes to 12-L cages where they were provided sugar in a 90 mm Petri dish and water-soaked cotton wool. The 12-L cages were of clear plastic with a mesh-covered window (ca. 80cm^2) for ventilation.

As mature flies to pair with treated males and mated females, ca. 1200 pupae from four different weekly batches were placed in mesh cages (Megaview Bugdorm 44545) for adult emergence. Cages were supplied with water soaked cotton wool in a 70-mL sample container and sugar and YH (3:1) as food on a 90 mm Petri dish; this diet is effective at

supporting development of both male and female Q-flies (Vijaysegaran et al., 2002; Pérez-Staples et al., 2007, 2011). Adult flies were sorted according to sex within 3 days after emerging by collecting and transferring individual flies in glass tubes to 12-L cage where flies were provided sugar and YH (3:1) and water-soaked cotton wool. Approximately 200 male or female flies were housed in each 12-L cage. No calling, courting, or mating were observed in cages prior to separating the sexes.

2.2 Effect of methoprene on male sexual maturation

Mating trials were conducted at 6, 8, 10, 15, 20, 25, and 30 days post emergence. Q-flies usually initiate calling and mating over a period of 30 - 40 minutes at dusk (Barton-Browne, 1957; Tyschen, 1977), but sometimes start calling and mating in the laboratory before the onset of simulated dusk (Adnan et al., 2018). Accordingly, observations started 90 minutes prior to the onset of dusk. On each day of mating trials, at least four hours before the onset of dusk, fifty males from each treatment group were placed individually in clear plastic 1.125 L containers that had a mesh-covered window (ca. 28 cm^2) for ventilation. Each male fly was paired with a sexually mature (12 - 17 days old) female fly; virgin Q-flies of this age fed with sugar and YH diet exhibit high levels of sexual receptivity (Pérez-Staples et al., 2007; Prabhu et al., 2008). All cages were provided with water-soaked cotton wool. To assess mating latency (elapsed time from the start of dusk till the onset of mating), the time of onset of copulation was recorded for each mating pair. To assess mating duration, observations continued until the last pair had separated. 150 flies were paired for each test day, providing a total of 1050 test pairs. The experiments were repeated three times using pupae from different production batches over a period of five months (a total of 3,150 test pairs).

2.3 Effect of methoprene on female sexual inhibition

The morning after the first mating, we removed all pairs that failed to copulate and also removed the mated males from the mated pairs. Mated females were kept in the 1.125 L mating cages and were provided continuous access to sugar and YH along with water-soaked cotton wool until their remating trial. To test remating tendency, females were randomly divided into three groups for remating trials at 1, 7, and 15 days after their first mating.

In each remating trial, a female was given opportunity to mate with a sexually mature (12 - 17 days old) virgin male that had been provided continuous access to sugar and YH. Similar to mating trials, at least four hours before the onset of dusk flies were paired individually in clear plastic 1.125 L containers that had a mesh-covered window (ca. 28cm^2) for ventilation. Food and water were removed from the mating cages when pairing the flies. We recorded whether mating occurred, as well as the start and end of mating for assessment of mating latency and duration.

2.4 Statistical analysis

All analyses were conducted using JMP 14.1.0 (SAS Institute Inc 2018). For variables with binary outcomes including mating and remating probability, a generalised linear model was fitted with a binomial distribution. Main effects included age of the male and methoprene treatment for both mating and remating probability, and additionally included number of days since mating for remating. Batch was included as a random effect.

Continuous variables of mating latency and mating duration were analysed using least squares regression. Main effects included age and methoprene treatment for both mating and remating, and additionally included number of days since mating for remating. Initially all interactions were included, and a backward model selection process was performed by removing non-significant interactions. Post-hoc comparisons were performed using Tukey's Honestly Significant Differences (HSD) tests to compare estimated least-square means for main effects, and test slices of significant interactions.

3 Results

3.1 Male mating performance

3.1.1 Male mating probability

Male mating probability varied significantly with age ($G_1 = 11.905$, $p<0.001$) and methoprene treatment ($G_2 = 17.059$, $p<0.001$), and age x methoprene treatment interaction ($G_2 = 10.302$, $p = 0.006$) (Fig 1). To explore this interaction, the effects of age were analysed

separately for each of the methoprene treatments. There was no significant effect of age on mating propensity of flies that received either 0.05% methoprene ($G_1 = 0.595$, $p = 0.441$) or 0.5% methoprene ($G_1 = 0.389$, $p = 0.533$), as flies receiving these treatments exhibited high mating propensity from the youngest age tested. In contrast, there was a significant effect of log age on mating propensity of the controls ($G_1 = 22.666$, $p < 0.001$) which had comparatively low mating propensity at younger ages. Subsequent analysis of mating propensity at each test day detected significant differences between both methoprene treatments and the untreated control at 6, 8 and 10 days of age but not at older ages (Fig 1).

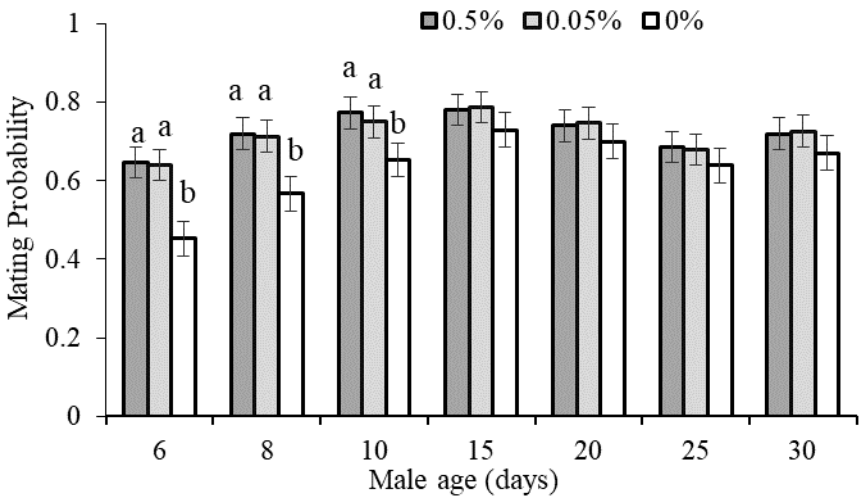

Figure 1. Relationship between age (6, 8, 10, 15, 20, 25, and 30 days post emergence) and mating probability (mean ± SE) of male Q-flies after being fed sugar plus yeast hydrolysate combined with one of two levels of methoprene supplementation (0.5%, 0.05%) or untreated control (0%) for two days after emergence and then sugar only. Different letters denote significant differences within that day by slice contrast.

3.1.2 Mating latency

Of 2195 mated pairs, 279 pairs (12.71%) started mating before the onset of the dusk. Mating latency varied significantly with male age ($F_{6, 2174} = 3.066$, $p = 0.006$), but did not vary significantly with methoprene treatment ($F_{2, 2174} = 1.578$, $p = 0.207$). Tukeys HSD tests found significant difference only between 10 days and 30 days, with latencies on all other days being intermediate between these values and not significantly different from either (Fig 2).

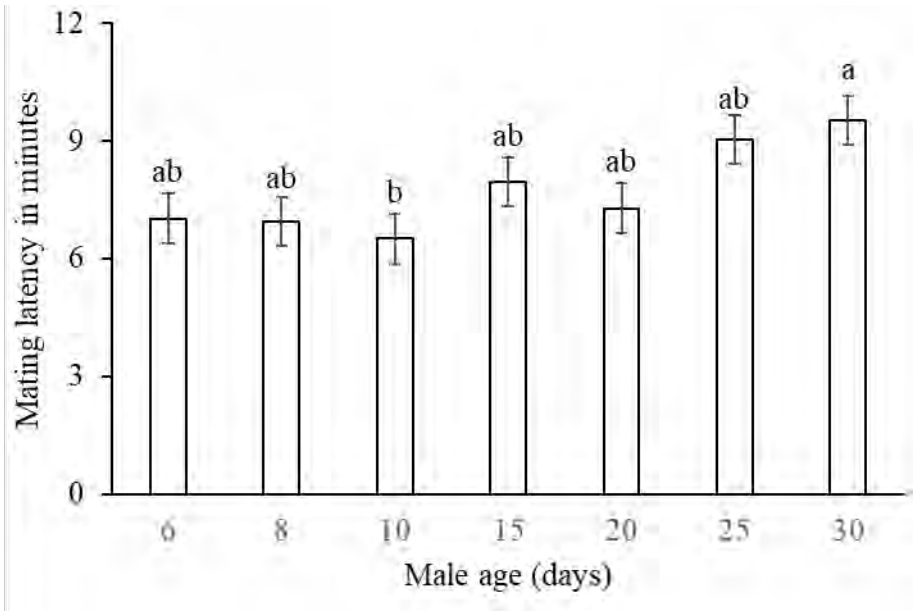

Figure 2. Relationship between age (6, 8, 10, 15, 20, 25, and 30 days post emergence) and mating latency (mean ± SE) of male Q-flies after being fed sugar plus yeast hydrolysate combined with one of two levels of methoprene supplementation (0.5%, 0.05%) or untreated control (0%) for two days after emergence and then sugar only. Means separated by different letters indicates significant differences between ages ($p<0.05$).

3.1.3 Mating Duration

Mating duration did not vary significantly with age ($F_{6, 2174} = 1.565$, $p = 0.153$) but did vary significantly with methoprene treatment ($F_{2, 2174} = 4.825$, $p = 0.008$). Males treated with either dose of methoprene had significantly longer copulations than males that received no methoprene, and there was no significant difference between the methoprene treatments in mating duration (least square mean±SE: control = 64.12 ± 3.87b min, 0.05% = 74.37 ± 3.24a min, 0.5% = 79.75 ± 3.24a min; Means separated by different letters indicates significant differences between ages ($p<0.05$).

3.2 Female sexual inhibition

3.2.1 Remating probability

Female remating probability did not vary significantly with methoprene treatment of the first mate ($G_2 = 3.982$, $p = 0.137$) or with number of days that had passed since the first mating.

(G_2 = 5.491, p = 0.064) but did vary significantly with age of the first mate (G_6 = 23.882, p < 0.001). Females mated with 15-day old males exhibited remating tendency that was significantly lower than that of females mated with younger (6, 8 days) or older (20, 25, 30 days) males but was not different from those mated with 10-day old males (Fig 3).

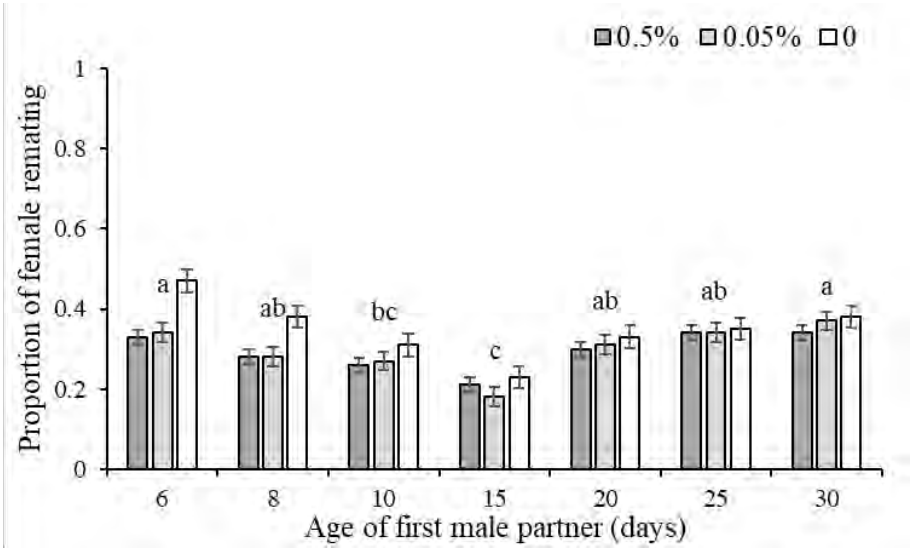

Figure 3. Remating probability (mean ± SE) of female Q-flies in relation to the age of their first mate (6, 8, 10, 15, 20, 25, and 30 days post emergence). Means separated by different letters indicates significant differences between ages (p<0.05).

3.2.2 Remating latency

Female remating latency did not vary significantly with age of the first mate ($F_{6, 529}$ = 0.634, p = 0.703) or with number of days that had passed since the first mating. ($F_{2, 529}$ = 1.218, p = 0.297), but did vary significantly with methoprene treatment ($F_{2, 529}$ = 6.076, p < 0.001). Tukeys HSD tests reveal that females mated by methoprene-treated males of either dose had significantly longer remating latency (i.e., mated later in the evening) than females mated by untreated males, and there was no significant difference between the methoprene treatments in female remating latency (least square mean±SE: control, 0 = 6.3 ± 1.13 min, 0.05% = 8.75 ± 1.14 min, 0.5% = 9.05 ± 1.14 min) (Fig 4).

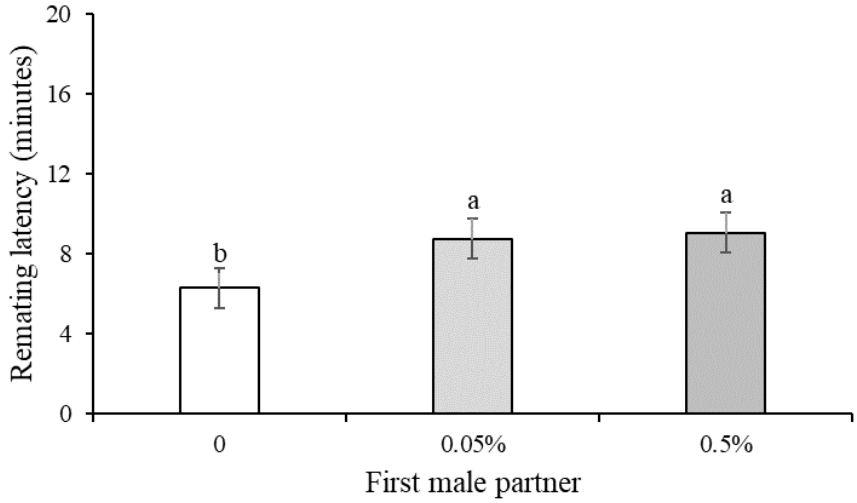

Figure 4. Female remating latency (least square mean ± SE) in relation to methoprene treatment of the first mate (0 %, 0.5 % and 0.05 %). Different letters denote significant differences among the treatments.

3.2.3 Remating duration

Remating duration did not vary with number of days since the female's first mating ($F_{2, 508}$ = 0.569, p = 0.567), but did vary significantly with both age and methoprene treatment of the first mate along with significant age × methoprene dose interaction (Age $F_{6, 507}$ = 4.885, p < 0.001, Dose $F_{2, 507}$ = 10.684, p < 0.001, Age × Dose $F_{12, 507}$ = 1.951, p = 0.027). Remating duration did not vary with age of first mate if the first mate was methoprene treated (0.05% $F_{6, 508}$=1.200, p = 0.305; 0.5% $F_{6, 508}$ = 1.231, p = 0.389), but decreased with age of the first mate if the first mate was untreated ($F_{6, 508}$ = 6.654, p <0.001). Consequently, remating duration was significantly shorter for mates of males receiving either methoprene treatments than for mates of controls when the first mate was 6, 8, or 10 days of age (Fig 5). Effects of methoprene treatment on remating duration were not evident when the first mate was 15 days old, or older (Fig 5).

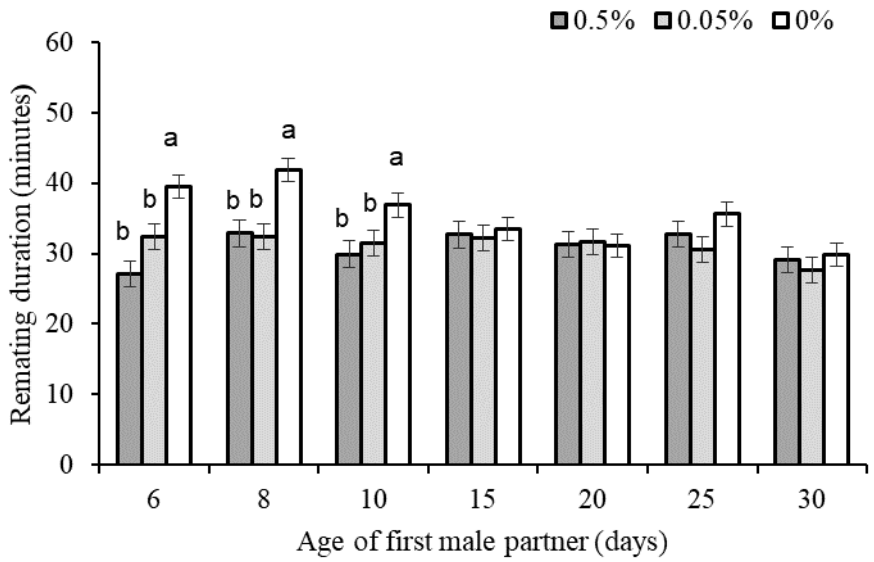

Figure 5. Female remating duration (least square mean ± SE) in relation to ages of first mate (6, 8, 10, 15, 20, 25, and 30 days post emergence) and methoprene treatment of the first mate (0 %, 0.5 % and 0.05 %). Different letters denote significant differences within that day by slice contrast.

4 Discussion

Dietary supplementation with methoprene has previously been found to substantially decrease the age at which male Q-flies mate and to increase copula duration without affecting copula latency (Adnan et al., 2018, in press a, b), and these patterns are reconfirmed and expanded upon in the present study. If the additional matings secured by young methoprene-treated males are not effective at inducing sexual inhibition in their mates, such accelerated sexual development may be of little benefit to SIT programs. The present study finds that, like *Z. cucurbitae* (Haq et al., 2014) and *A. ludens* (Gómez-Simuta et al., 2017), the additional matings of young methoprene-treated Q-fly males are as effective as matings of same aged untreated males at inducing sexual inhibition in their mates. Similar to the present study, Q-fly males provided raspberry ketone for two days following emergence exhibit accelerated sexual development (Akter et al., 2017) and the matings of young treated males are as effective as those of untreated control males at inducing sexual inhibition in females (Akter & Taylor, 2018). The consistent patterns across methoprene and raspberry ketone in both accelerating development and maintaining efficacy of matings at inducing sexual

inhibition in mates suggests a general pattern whereby traits relevant to pre- and post-copulatory sexual performance develop in concert.

The mechanisms mediating mating-induced sexual inhibition vary across fruit fly species, and it is currently unknown how methoprene treatment affects these mechanisms. In Q-flies male accessory gland fluids transferred with the ejaculate are the primary agents responsible for mating-induced sexual inhibition (Radhakrishnan & Taylor, 2007, 2008; Radhakrishnan et al., 2008, 2009). JH regulates male accessory gland activity in some insects (Parthasarathy et al., 2009), and so there was a possibility that application of methoprene might increase the ability of male Q-flies to suppress female remating even beyond the effects on sexual maturation. That is, females mated by methoprene-treated males might have expressed even higher levels of sexual inhibition than those mated by untreated control males. The present study found no evidence for such direct effects of methoprene on accessory glands, as remating tendency of females was not diminished by methoprene treatment of their mates. Instead, sexual inhibition was very similar for mates of treated and untreated males. This suggests a very broad effect of methoprene in male Q-flies, such that ability to mate and ability to induce sexual inhibition in mates both reflect a general effect on sexual maturation. However, a quite different pattern is indicated in *A. fraterculus*. *Anastrepha fraterculus* is similar to Q-flies in that male accessory gland products transferred with the ejaculate are potent mediators of mating-induced sexual inhibition (Abraham et al., 2012) and that methoprene treatment accelerates development (Segura et al., 2009, 2013). However, unlike Q-flies, the mates of young methoprene-treated *A. fraterculus* males remate more often and sooner than the mates of older untreated males (Abraham et al., 2013).

While female remating tendency was not affected by methoprene treatment, significant effects of male age were found. There is some inconsistency in reports of how male Q-fly age affects remating propensity of mates. In the present study, females mated with 10 or 15 day old males exhibited reduced remating tendency compared to those mated with younger or older males. This finding is reminiscent of patterns reported in *C. capitata* in which 10 - 11 day old males have been found to be more effective at inducing sexual inhibition in their mates than younger or older males (Shelly, Edu, & Pahio, 2007; Gavriel, Gazit, & Yuval, 2009). While Akter & Taylor (2018) did not find evidence of higher remating propensity of females mated by young male Q-flies, they did find (as in the present study) that females mated by old males were more likely to remate. However, this effect was

evident only in trials conducted on the day after their first mating, being no longer evident at 7 days or 15 days after their first mating (Akter & Taylor, 2018). Pérez-Staples et al. (2008) found no evidence of links between male age and female remating tendency in Q-flies, as has also been reported in *A. ludens* (Abraham et al., 2016). Given that male accessory glands are key mediators of sexual inhibition in Q-flies, age-related variation in ability of males to induce sexual inhibition in their mates in some studies likely reflects variation in quality or titre of accessory gland fluids. At young ages, the accessory glands may not yet be active to their full capacity, while at older ages the accessory glands might lose function through senescence. If ejaculate quality is reduced as males age, then increased remating propensity of females mated by old males may serve to replace a poor quality ejaculate (Radwan, 2003; Gasparini, Marino, Boschetto, & Pilastro, 2010.). However, this would require a means for females to assess either the age and quality of the male, or the quality of the ejaculate.

In the present study female remating propensity did not vary with time since the first mating (1d, 7 d, or 15d). This result differs from Akter & Taylor (2018), who found higher remating tendency of Q-fly females on the day after their first mating than they did 7 or 15 days after mating. Barton Brown (1957) also reported that number of mating attempts of Q-fly males was similar when females were virgin or one-day post-mating but were significantly reduced at seven days after mating. However, matching the patterns reported for Q-fly by Akter & Taylor (2018) and Barton Brown (1957), Gavriel et al., (2009) reported relatively high levels of remating in female *C. capitata* on the days following the first mating, followed by a significant decline two weeks after mating. Also in *A. serpentine,* female remating receptivity varied with sexual refractory periods as higher remating tendency was evident at 5 and 20 days after first mating, with a decrease in the intermediate period (10, and 15 days after first mating) (Landeta-Escamilla, Hernández, Arredondo, Díaz-Fleischer, & Pérez-Staples, 2016). The reasons for these differences amongst studies are not apparent. Since the inhibition of female receptivity after copulation is usually related to the quality of the first mating, differences in physiological state of test insects might provide the possible explanation. Also, this inconsistency among the findings may relate to the origins, rearing or handling of the insects, or details of experimental design.

While methoprene treatment of a female's first mate did not affect female remating tendency, it did affect temporal dynamics of rematings when they did occur; females mated by methoprene-treated males remated later in the day and had shorter second copulations than

did females mated by un-treated males. These patterns are consistent with interpretation as greater reluctance to re-mate in females mated by methoprene-treated males. In laboratory cages females have quite limited capacity to escape from persistent males and so may sometimes be coerced into matings that might not have occurred in a larger cage or in the field where females have more control over interactions with males. In the field, unreceptive females may not respond to male pheromones and so would be unlikely to encounter males during the mating period (as is the case in Medflies; Jang 1995), and any interactions could be readily avoided or terminated by females. In cases where rematings do occur, shorter second matings of females mated initially with methoprene males may be of value to SIT. There is little evidence of association between copula duration and sperm storage in Q-flies, and sterile males transfer few sperm owing to spermatogenesis being disrupted by irradiation (Radhakrishnan et al., 2009). Nonetheless, Collins et al. (2012) found that fertility of female Q-flies that mated with a sterile and then a fertile was significantly lower if their second copulation was short. Hence, methoprene treatment may increase the ability of sterile males to preserve high levels of sterility if their mates do accept a second mate.

5 Conclusion

Incorporation of methoprene into adult diet of sugar and YH accelerates sexual development of male Q-flies. Methoprene treatment may hence provide a means to increase the proportion of flies released in SIT programs that mature and participate in mating before numbers are reduced by high mortality rates. However, if the additional matings secured by young methoprene-treated males are not effective at inducing sexual inhibition in their mates, then few or no benefits would accrue for SIT. Here we find that the additional matings of young methoprene-treated males match the efficacy of untreated males in the induction of sexual inhibition.

Conflict of Interest Statement

There was no conflict of interest regarding the preparation and submission of this manuscript.

Author Contribution

SMA and PWT conceived research. SMA, IF, PR conducted experiments. SMA and PWT conducted statistical analyses. SMA and PWT wrote the manuscript. All authors read and approved the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Chapter Seven

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Journal of Insect Physiology

Overview

Experiments carried out in previous chapters have investigated the efficacy of dietary yeast and methoprene treatment as pre-release supplements in Q-SIT. By this time, it has been confirmed that accelerated sexual development rendered by dietary methoprene could provide a solution to overcome long adult maturation phases and high post release mortality rates so that a greater proportion of Q-fly males contribute to SIT programmes. While the effect of methoprene on sexual maturation has been explored in several tephritid fruit fly species, activity patterns of methoprene-treated flies has not been studied. Given the link of locomotor activity with other behavioural activities including courtship, foraging and dispersal, predator avoidance, and as an indication of energy reserves or metabolic condition, investigation of locomotor activity can provide useful insights to the broader effects of methoprene treatment on Q-fly physiology. Also, given the links between locomotor activity and adaptation to different types of abiotic stressors, it is imperative to investigate whether dietary provision of methoprene as pre-release diet influences the activity level in flies subjected to food-water stress and desiccation condition. Building on above key questions, this chapter assessed locomotor activity of Q-fly belonging to different treatments.

Dietary yeast hydrolysate and methoprene supplements elevate locomotor activity of mass-reared Queensland fruit flies

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Highlights

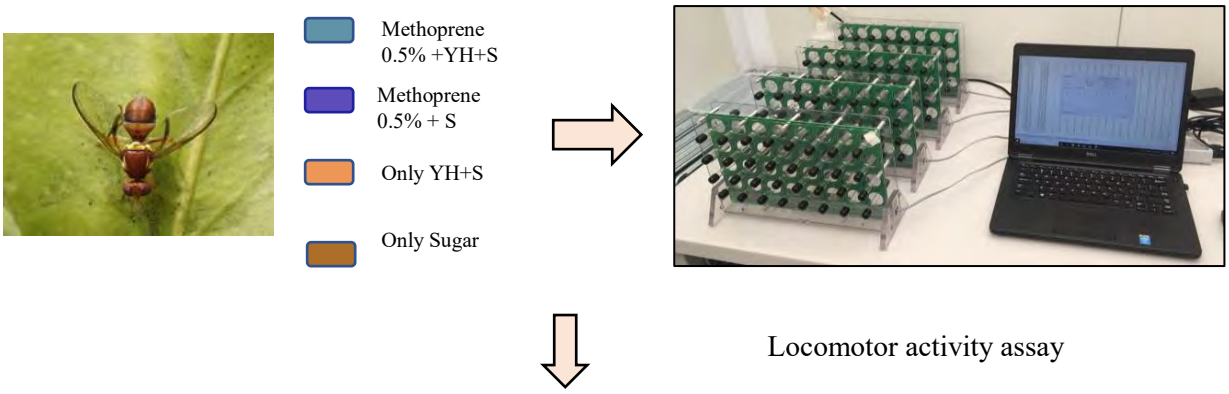
- Dietary Yeast hydrolysate (YH) and methoprene substantially increased activity in young Q-flies.
- Methoprene increased activity levels in females more than males.
- Greater activity in methoprene-fed flies was consistent throughout the trial period, whereas activity levels of untreated flies exhibited declining trend.
- Provision of methoprene and YH increased the fly activity while they were subjected to desiccation and food and water stress as well.

Abstract

The Sterile Insect Technique (SIT) is an environmentally benign pest management technique that involves releasing millions of sterile male insects to suppress reproduction of target insect species, including Queensland fruit fly ('Q-fly'). Because of long adult maturation periods and high mortality rates, a large proportion of released sterile fruit flies may die before sexually maturing. Pre-release treatments that accelerate development, such as dietary yeast and methoprene treatment, can increase the number of released insects that survive to maturity and participate in SIT. While the effect of methoprene on sexual maturation has been investigated in several tephritid fruit fly species, activity patterns of methoprene-treated flies has not been studied. In the present study, newly emerged fertile and sterile male and female domesticated Q-flies were provided with methoprene (0.05% and 0% control) incorporated into an adult diet of sugar+yeast hydrolysate (YH) (3:1) or sugar only for 48 hours post emergence. We measured daily activity level of Q-flies using locomotor activity monitors for four consecutive days. Flies that received methoprene exhibited significant increase in locomotor activity, but this increase was greater for the flies fed sugar+YH than for those fed sugar only and was also greater for females than for males. While methoprene-fed flies had consistently high activity throughout the trial period, activity levels of untreated flies declined over the four days of testing. There was no evidence of difference between fertile and sterile flies in activity level. Furthermore, we also measured activity level of Q-flies for 24 hours that were subjected to food and water stress and desiccation. Activity level was greater for flies provided methoprene and YH rich diet, while fly activity did not vary with stress type or sex. These findings provide insights into behavioural and physiological changes associated with methoprene treatments such as have been proposed for use in fruit fly SIT programs.

Key words: *Bactrocera tryoni*, protein, sterile insect technique, Juvenile hormone analogue

Graphical Abstract



- Dietary methoprene increased activity in young YH fed Q-flies.
- Methoprene increased activity levels in females compared to males.
- Activity in methoprene-fed flies was consistent over 4 day's trial period, while untreated flies dropped their activity.
- Greater activity was found for methoprene fed flies under stress condition as well.

1 Introduction

The Sterile Insect Technique (SIT) is a sustainable approach for regional management of major insect pests, including some tephritid fruit flies (Enkerlin, 2005; Hendrichs et al., 1995; Knipling, 1955; Orozco-Dávila et al., 2017). SIT involves releasing millions of sterile male insects that mate with females of pest populations, inducing reproductive failure and suppressing subsequent generations (Benelli et al., 2014; Knipling, 1955). SIT has been used to combat some of the most damaging fruit fly species, including Mediterranean fruit fly, medfly, *Ceratitis capitata* (Wiedemann) (Reyes et al., 2007), melon fly *Zeugodacus cucurbitae* (Coquillett) (Itô et al., 2003; Kakinohana, 1994), Oriental fruit fly *Bactrocera dorsalis* (Hendel) (Orankanok et al., 2007) and Mexican fruit fly *Anastrepha ludens* (Loew) (Orozco-Dávila et al., 2015). The Queensland fruit fly, *Bactrocera tryoni* (Froggatt), or 'Q-fly', affects a vast diversity of commercial and non-commercial crops in Australia (Clarke et al., 2011; Dominiak and Daniels, 2012; Hancock et al., 2000). SIT has been used sporadically to manage outbreaks of Q-fly in and around 'fruit fly free' regions for more than 20 years (Fanson et al., 2014), and is currently undergoing a substantial renewal to improve efficacy and cost effectiveness.

Q-flies are anautogenous, needing to acquire nutritional resources as adults to complete reproductive development (Drew and Yuval, 2000). Protein acquisition is particularly important and can be obtained from diverse sources in nature including faeces, bacteria, yeast, pollen, and plant leachates (Drew and Lloyd, 1987; Drew et al., 1987; Vijaysegaran et al., 1997; Aluja et al., 2001). Q-fly SIT programs have usually released 2-3 day old sexually immature adults. However, Q-flies may take a week, or longer, to attain sexual maturity (Pérez-Staples et al., 2008, 2011; Weldon et al., 2008; Reynolds et al., 2012). During this time, predators, pathogens, and abiotic stressors can significantly diminish the abundance of released males (Meats, 1998; Weldon et al., 2008). To increase the proportion of released flies that survive to maturation, there is substantial interest in pre-release treatments that accelerate sexual development. Yeast hydrolysate (YH) mixed with sugar is a standard adult diet used to maintain fruit fly colonies, providing a rich source of amino acids, micronutrients and carbohydrates (Fanson and Taylor, 2012). In Q-fly, provision of YH together with sucrose has been reported to promote reproductive development in both sexes (Meats and Leighton, 2004; Pérez-Staples et al., 2011; Vijaysegaran et al., 2002; Weldon and Taylor, 2011), promote mating propensity and competitiveness (Pérez-Staples et al., 2007),

increase longevity (Fanson et al., 2009; Pérez-Staples et al., 2007; Prabhu et al., 2008), and increase abundance of sexually mature males in field releases (Reynolds et al., 2014) (for a review, see Taylor et al., 2013).

Juvenile hormone is a potent mediator of insect physiology, including developmental processes (Happ, 1992; Wyatt and Davey, 1996; Gilbert et al., 2000; Wilson et al., 2003). Methoprene, an analogue of juvenile hormone, has been found to accelerate reproductive development of adult fruit flies for use in SIT programs (Teal et al., 2000, 2013). So far, application of methoprene has been found to accelerate maturation and improve mating performance in *Z. cucurbitae* (Haq et al., 2010), *A. fraterculus* (Abraham et al., 2013; Liendo et al., 2013; Segura et al., 2009, 2013), *A. ludens* (Gómez-Simuta et al., 2017; Gómez et al., 2013; Pereira et al., 2013), *A. suspensa* (Pereira et al., 2009, 2010). In Q-fly, provision of methoprene in the diet for two days following emergence increased mating propensity of males but not of females (Adnan et al., 2018), and when applied topically to adults or pupae increased mating propensity in both sexes (Collins et al., 2014). Studies of methoprene treatment have focused primarily on the positive effects on reproductive development, but in order to fully assess the merits of such treatment it is important to better understand its broader effects on physiology and performance.

Locomotor activity in animals is directly or indirectly linked with most fitness-related behavioural activities, including courtship (Cobb et al., 1987; Greenspan and Ferveur, 2000), foraging and dispersal (Martin et al., 1999), and predators avoidance (Heath and Wilkin, 1970; Lang et al., 2006; Simmons, 1988). Daily activity levels may be linked to overall energy reserves, metabolic state, or health (Catterson et al., 2010). Irradiation that is used to induce reproductive sterility of fruit flies for SIT program can have substantial deleterious effects on somatic tissues and insect performance (Kim et al., 2014; Lopez-Martinez and Hahn, 2014; Qu et al., 2014). In Q-flies, irradiation at sterilizing doses has been found to reduce survival under stress assays (Collins et al., 2009; Collins et al., 2008), to reduce longevity when provided a protein-deficient diet (Pérez-Staples et al., 2007), to alter calling behaviour (Mankin et al., 2014), and to affect general activity patterns (Dominiak et al., 2014b; Weldon et al., 2010).

Given the very widespread effects of juvenile hormone on insect physiology, the strong effects of dietary methoprene on sexual development in Q-flies (Adnan et al., 2018) are likely

to be just one dimension in a constellation of physiological effects, some of which will be positive for SIT and others negative. Given the links between locomotor activity and overall behaviour and health, the effects of methoprene treatment on locomotor activity are a good place to start exploring the broader physiological implications of methoprene treatment for accelerated development. To date, there are no studies of how methoprene treatment affects locomotor activity in any tephritid fly. In the present study, we investigate the combined and separate effects of dietary methoprene and dietary YH on locomotor activity of Q-flies. Because irradiation used to sterilise Q-flies for SIT is known to also affect behaviour, we compare effects of dietary methoprene and YH in fertile and sterile flies. Because current SIT releases rely on bisexual strains, and sexually active females can impede SIT efficacy, we investigate effects of methoprene, YH and sterility in both males and females. Understanding the general effects of methoprene treatment on locomotor activity of Q-flies will help to guide the direction of future studies that seek to understand the more specific effects on key fitness-related traits. Also, given the links between locomotor activity and adaptation to different types of abiotic stressors, it is imperative to investigate whether dietary provision of methoprene as pre-release diet influences the activity level in flies subjected to food-water stress and desiccation condition.

2 Materials and Methods

2.1 Insects

Mass-reared Q-flies were obtained as pupae from a colony maintained at Macquarie University, New South Wales, Australia (25 generations from wild). Domesticated flies were reared on a gel larval diet (Moadeli et al., 2017) that was based on the liquid diet formulation of Chang et al. (2006). The colony and experiments were maintained in separate controlled environment rooms of 25±0.5ºC and 65±5% RH. The photoperiod was 13:11 (L:D), with a simulated dawn and dusk as lights gradually increased and decreased for 0.5 h at the beginning and end of the light phase. To obtain sterile flies, 4000 pupae were sealed in zip lock plastic bags (100 x 150 mm) two days prior to emergence and maintained at 18ºC for 18 hours to induce hypoxia. The pupae were then sterilised by exposure to 65 Gy of gamma radiation using a ^{60}Co irradiator at Macquarie University, New South Wales, Australia (Collins et al., 2009; Dominiak et al., 2014a).

2.2 Treatments

Fertile and sterile Q-fly males and females were treated with the following combinations of diet and methoprene for first 48 hours following emergence:

1. Sugar+YH (3:1) with Methoprene (0.05%)
2. Sugar only with Methoprene (0.05%)
3. Sugar+YH (3:1) without Methoprene
4. Sugar only without Methoprene

For the incorporation of methoprene into the adult diet, we used NOMOZ® pellets, which contain the active ingredient PROLINK® (40 g/kg (S)-methoprene). This product is locally available, being marketed as a mosquito larvicide. NOMOZ pellets were finely powdered using mortar and pestle. Then, using a blender, the powdered methoprene (as required on dry weight basis) was mixed into diet of sugar+YH or sugar only.

To provide the treatments, approximately 1000 fertile and 1000 sterile pupae were divided amongst eight mesh cages (Megaview Bugdorm 44545) for adult emergence (ca. 250 fertile or sterile pupae per cage). Few flies emerge on the first day of emergence, and these were discarded. After 24 hours of emergence, non-emerged pupae were discarded and one of the diets (sugar+YH or sugar only) containing methoprene (0.05%) or control (0%) was provided to each cage for next two days. As a source of water, cages of flies were provided water-soaked cotton wool in a 70-ml sample container. After 48 hours of treatment, flies were tested for activity monitoring.

2.3 Activity monitoring

2.3.1 under ad libitum access to food water

Daily activity levels were assessed using a Locomotor Activity Monitor (LAM10, TriKinetics, MA, USA) (see Dominiak et al 2014b; Fanson et al. 2013). A total of seven males and seven females from each treatment of domesticated flies (112 in total) were randomly placed in the locomotor activity monitors in individual glass tubes (1 cm internal

diameter and 10 cm long). Each glass tube was plugged at one end with agar diet containing sugar (300 g white sugar, 1 L water, 20 g agar, 1.5 g Nipagin) to a depth of 1 cm and loose cotton wool at the other end to a depth of 2 cm, allowing ventilation and leaving 7 cm for the fly to move. Activity was quantified as the number of times each fly broke an infrared beam projected through the tube midpoint. Four monitor units were used, each containing 32 tubes (four rows by eight columns). For each monitor, four glass vials were kept empty to check any errors in readings provided by the LAM devices. Flies were transferred to activity monitors ca. 4 hours before recording started. Activity was assessed over four consecutive days. The experiments were repeated six times using batches of pupae, with a total of 672 flies recorded.

2.3.2 under stress condition

Daily activity levels under stress condition were assessed following experimental procedure similar to that of *ad libitum* access to food and water. Since pupal irradiation did not influence activity level in earlier trials, only fertile flies were used in the present trial. Flies were placed under two different conditions i.e. a. food and water stress: flies were given no access to food and water inside the glass tubes plugged at both ends b. desiccation: flies were exposed to four to six large granules of silica gel desiccant (Sigma-Aldrich, USA) and were also withheld from food and water inside the glass tubes plugged at both ends. A loose barrier of cotton wool separated the flies from the desiccant. For each stress condition, a total of 15 males and 15 female flies from each dietary treatment were randomly placed in the locomotor activity monitors in glass tubes. For each monitor, two glass vials were kept empty to check any errors in readings provided by the LAM devices. Flies were transferred to activity monitors ca. 4 hours before recording started. Activity was assessed over next 24 hours. The experiments were repeated two times using two separate batches of pupae, with a total of 480 flies recorded.

2.4 Statistical Methods

All analyses were conducted using R v3.4.1 (R Core Team). To address the main questions, data were summarized by calculating the total activity (sum of all movements) for each fly during the light period in laboratory (07:30 to 19:30) of each day. A general linear mixed model was performed with total daily activity during the light phase as response variable. As

total activity values were sufficiently large, a Gaussian distribution was assumed for the model and the response variable was log-transformed. The model included the following main effects as fixed effects: irradiation (sterile or fertile), methoprene treatment (treated or untreated), diet (sugar+YH or sugar only), and sex (male or female). All possible interactions between main effects were initially included. Day effect was included as covariate to control for potential monitor effects as this was the only treatment across all monitors. Finally, fly identity and replicate were included as random effects to control for correlations due to the hierarchical design. A backward model selection process was then undertaken in which the highest-level interactions were removed. The simpler models were compared to the more complex models using a log-likelihood ratio test. For instance, a 4th order model was compared to 3rd order model and then the 3rd order versus the 2nd order model. For any significant effects and/or interactions, post-hoc least square mean comparisons were ran and Tukey p-value corrections were used. Model assumptions (e.g. homoscedasticity, normality) were assessed through graphical analyses of the residuals.

A general linear model (GLM) was performed for total activity under stress assuming a gaussian distribution. Total activity was square-root transformed to stabilize the variance. The model included the following main effects as fixed effects: methoprene treatment (treated or untreated), diet (YH-sugar or Sugar), stress condition (desiccation or water+food stress), and sex (male or female). Post-hoc pairwise comparisons were performed with a Tukey's correction. Initially all interactions were included in the model, and a backward model selection process was performed by removing non-significant interactions. Model assumptions was assessed and justified through graphical analyses of the residuals.

3 Results

3.1 Effect of methoprene on locomotor activity in flies with ad libtum access to food/water

The backward model selection resulted in a two-way model being selected. The two-way model was significantly better than the one-way model (χ^2 = 15.4, df= 6, p = 0.017) but was not significantly different from higher order models. In the final model, fly activity level during the light phase varied significantly with a diet × methoprene treatment interaction (Table 1). Flies that were provided access to methoprene exhibited an increase in locomotor

activity, but this increase was greater for flies fed sugar+YH (log-difference $\Delta\beta = 0.32 \pm 0.05$, $p < 0.001$) than for flies fed sugar only ($\Delta\beta = 0.15 \pm 0.04$, $p < 0.001$; Fig 1). A significant sex × methoprene treatment interaction (Table 1) indicates that dietary methoprene resulted in a greater increase in activity of females ($\Delta\beta = 0.30 \pm 0.04$, $p < 0.001$) than males ($\Delta\beta = 0.17 \pm 0.04$, $p < 0.001$; Fig 1). A significant methoprene treatment × day interaction (Table 1) reflects a substantial difference in the way that activity of methoprene treated and control flies changed over the days of testing. Activity levels of methoprene treated and control flies did not vary significantly on the first day (log-difference $\Delta\beta = 0.06 \pm 0.04$, $p= 0.11$). However, while activity levels of methoprene treated flies remained high over the following three days, the activity levels of control flies dropped substantially after the first day, resulting in a significant effect of methoprene on days 2, 3, and 4 (Log difference Day 2 = 0.25 ± 0.04; $p < 0.001$; Day 3 = 0.28 ± 0.04; $p < 0.001$; Day 4 = 0.34 ± 0.04; $p < 0.001$; Fig 1). Pupal sterilsation did not influence activity level in flies (Table 1). Sterile and fertile flies did not differ in activity levels ($\Delta\beta = 0.04 \pm 0.11$, $p= 0.93$). In addition, sex alone did not influence activity level as well (Table 1).

Table 1: General linear mixed model testing the effect of irradiation (sterile, fertile), methoprene treatment (treated or untreated), diet (YH + sugar or sugar only), sex (male or female), and day (1, 2, 3, and 4) on total activity level during light phase.

Variable	**df**	**F**	**P**
Sex	1, 558.2	0.60	0.46
Diet	1, 549.5	4.20	0.041
Sterility	1, 558.9	0.01	0.97
Methoprene treatment	1, 765.7	0.70	0.42
Day	3, 1713.2	1.40	0.24
Sex × Methoprene treatment	1, 558.8	4.90	0.027
Diet × Methoprene treatment	1, 525.4	6.60	0.011
Day × Methoprene treatment	3, 1712.8	17.60	<0.001

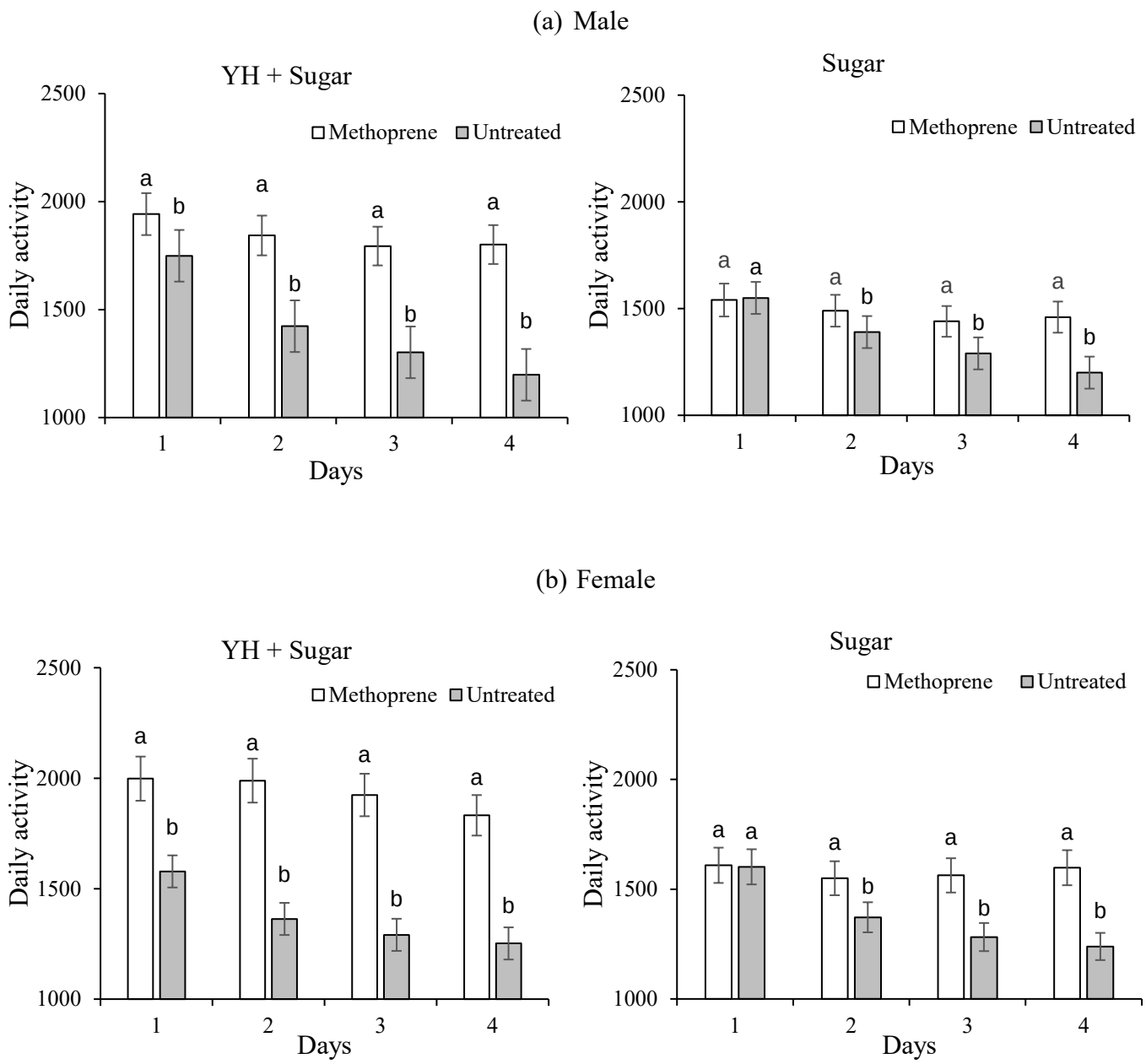


Fig 1. Effect of methoprene treatment (treated or untreated), diet (Yeast hydrolysate + sugar or sugar only), sex (male or female), and day (1, 2, 3, and 4) on total fly activity level (mean ± SE) during light phase. Means separated by lower case letters indicates significant differences between treatments.

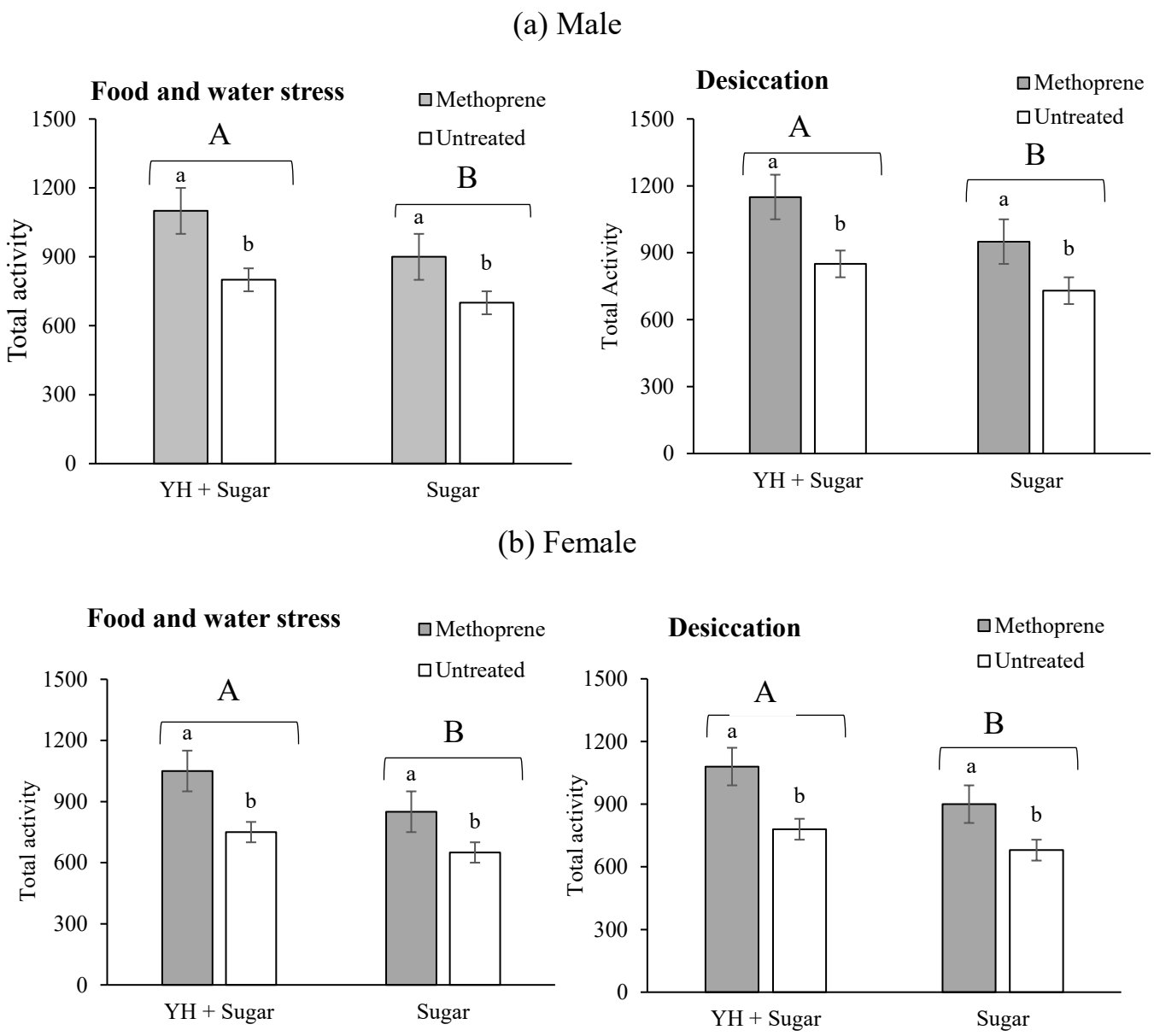


Fig 2. The effect of methoprene treatment (treated or untreated), diet (YH + sugar or sugar only), sex (male or female), and stress type (Food and water stress or desiccation) on total activity level (mean ± SE). Means separated by lower case letters indicates significant differences between treatments, whereas upper case letter presents differences between diets ($p<0.05$).

Table 2: General linear mixed model testing the effect of methoprene treatment (treated or untreated), diet (YH + sugar or sugar only), sex (male or female), and stress type (Food and water stress or desiccation) on total activity level.

Variable	df	F	P
Methoprene treatment	1, 467	17.1	<0.001
Diet	1, 467	5.2	0.023
Stress	1, 467	0.7	0.41
Sex	1, 467	2.2	0.13

3.2 Effect of methoprene on locomotor activity in flies under stress condition

Fly activity level varied significantly with methoprene treatment and diet (Table 2; Fig 2). Activity level was significantly greater for flies provided with methoprene than for the flies provided no methoprene (Fig 2). Moreover, flies that were given access to yeast-sugar diet had significantly higher activity compared to those fed on sugar only (Fig 2). However, there was no evidence of significant effect of sex or stress conditions on fly activity level (Table 2; Fig 2).

4 Discussion

Our study showed that provision of dietary juvenile hormone analogue methoprene for the first two days of emergence increased locomotor activity of Q-fly over the following four days, which would be the period immediately following release in SIT programs that release flies when 2 - 3 days old. Similar findings were reported by Kumaran et al. (2014) where feeding on raspberry ketone and zingerone increased activity levels in Q-fly. In addition, there was significant increase in activity level of flies supplemented with methoprene and dietary yeast hydrolysate under desiccation and nutritional stress. In previous studies, methoprene treatment of immature fruit flies has been found to promote reproductive development (Adnan et al., 2018; Pereira et al., 2013; Segura et al., 2009; Haq et al., 2010), but little is known about the broader physiological implications of this treatment. Methoprene is known to increase locomotor activity in codling moth, *Cydia pomonella* (Keil et al., 2001), as well as to promote flight performance in large carpenter bee *Xylocopa appendiculata* and

in honey bee *Apis mellifera* (Sasaki and Nagao, 2013; Tozetto et al., 1997), there was no previous study of how methoprene treatment affects locomotor activity in any fruit fly species.

The extent to which methoprene elevated activity levels varied with diet, being much greater for flies that were provided sugar+YH than for flies provided sugar only. This pattern contrasts with observations of Fanson et al. (2013), who found that activity levels were greater for Q-flies provided a carbohydrate rich diet than for those provided a protein rich diet. Similarly, in *Drosophila melanogaster* consumption of higher sugar concentrations has been associated with increased locomotor activity (Catterson et al., 2010). The differences in results of the present study and Fanson et al. (2013) can likely be attributed to differences in the age of flies used; in the present study the flies were 3-6 days old and were hence at a stage of rapid reproductive development whereas in Fanson et al. (2013), the flies were 14 days old, and hence were already sexually mature. Reproductive development is demanding for nutritional resources, such that the accelerated development that results from methoprene treatment (Adnan et al., 2018), is expected to increase nutritional demand. Such nutritional demand may be met in flies that received sugar+YH to a far greater extent that those that received sugar only. Accelerated development is likely associated with accelerated metabolism more generally, which might translate into increased activity. Flies receiving sugar only exhibit little reproductive development (Pérez-Staples et al., 2007), remaining in an immature state (Yap et al., 2015), and so are expected to be less metabolically active and may exhibited lower locomotor activity as a consequence.

Dietary provision of methoprene resulted in increased activity when 2 days old flies were exposed to nutritional and desiccation stress. Side by side, activity levels were greater for flies provided an YH rich diet than for those provided a sugar only diet. Increased activity of methoprene and YH fed flies under stress condition might be linked to simply avoiding or escaping from unfavourable condition. It is well documented that nutritionally stressed flies exhibit greater activity in drosophila and mosquitoes (Kim et al., 2014; Bross et al., 2005; Hagan et al., 2018), which might happen because of searching behavior to seek out nutrients the fly requires to tackle the stress (Williams et al., 2004; Ye et al., 1994). While increased activity under stress might result from several behavioural events, this greater activity in YH fed flies may also lead to increased vulnerability of the flies when they are subjected to nutritional deprivation (Kaspi and Yuval, 2000; Yuval et al., 2007; Gavriel et al., 2010;

Fanson and Taylor, 2012; Taylor et al., 2013b). There is a risk that greater activity in methoprene treated flies might exacerbate this effect beyond the vulnerability protein fed flies demostrated.

The effect of methoprene on Q-fly activity level was more pronounced in females than males. Sex specific patterns in locomotor activity are quite well known in *D. melanogaster* (Belgacem and Martin, 2002; Gatti et al., 2000), but are not well studied in tephritid flies. Weldon et al. (2010) studied the general activity patterns of Q-flies in cages, investigating frequency and duration of episodes of flying, walking, grooming and inactivity, and found no evidence of sex differences. Using methods very similar to those of the present study, with 3-days old flies studued over a period of 5 days, Dominiak et al. (2014b) found that male Q-flies were more active in LAM devices than were females. While these previous studies provide information about sex differences in Q-fly general activity patterns and locomotor activity, no previous studies have considered effects of methoprene treatment on the activity of male and female Q-flies. Given that dietary methoprene treatment yields a very substantial increase in sexual development of males, but not females (Adnan et al., 2018), the relatively greater increase in activity levels of females seems counter-intuitive. Further research is necessary to elucidate why methoprene affected female more than male activity despite having no significant effect on reproductive development of female.

Locomotor activity of sterile flies did not vary from fertile flies in the present study, and this finding contrasts several previous studies. Weldon et al. (2010) found that sterile Q-flies tended to spend more time inactive in cages than did fertile Q-flies. However, there were no differences in other aspects of measured activity, as sterile and fertile Q-flies were not different in frequency of flying, walking, grooming and inactivity, and in duration of flying, walking and grooming. Locomotor assays such as were used in the present study are not very sensitive to amount of time spent inactive, as the assessment is of number of locomotory events (passing the tube midpoint) and not duration. Weldon et al. (2010) and the present study are consistent in lack of significant difference in frequency of walking and number of midpoint crossings, respectively, these being the most comparable measures in these studies. Findings of Dominiak et al. (2014b) were similar to the present study in that sterile and fertile females did not differ in locomotor activity. However, while the present study found no evidence of differences in locomotor activity of sterile and fertile males, Dominiak et al. (2014b) found that sterile males had lower activity than fertile males. Experimental

procedures and insect handling were very similar in the two studies. However, there were differences in the flies used for these studies; in the present study the domesticated flies were from a colony maintained at the research laboratory at quite low density and reared on gel diet whereas in the study of Dominiak et al. (2014b) the flies came from a mass-reared (factory) colony reared on lucerne chaff diet, and were transported to the research laboratory. These differences in fly source may have rendered the male flies more sensitive to effects of irradiation in the study of Dominiak et al. (2014b).

In the context of SIT, greater increase in activity for methoprene treated sterile flies might have positive effects on sexual activity, foraging, predator evasion, and dispersal. However, it is also possible that elevated locomotor activity imposes a substantial physiological burden that exposes sterile Q-flies to elevated energetic and nutritional demands that might render them less able to tolerate adverse environmental conditions. A combination of laboratory studies of behaviour and physiology, and field studies of dispersal and survival is needed to assess the merits of dietary methoprene as a pre-release treatment for Q-fly SIT.

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Chapter Eight

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Overview

Dietary methoprene treatment significantly promotes sexual development and accelerates activity level of Queensland fruit flies (Q-fly). However, there is possibility that elevated metabolism following methoprene treatment, might render treated flies more vulnerable to food deprivation and desiccation stress, which might counter the benefits of methoprene provided accelerated maturation. While previous studies have focused very much on the potential positive aspects of methoprene, on mating performance of sterile flies targeted for SIT, potential negative effects such as increased vulnerability to environmental stress has been largely unnoticed. Accordingly, the present study investigates whether dietary methoprene treatment influences Q-fly survival under nutritional and desiccation stress. The survival assays have been undertaken in group and individual conditions to assess if experimental housing and fly density influence the survival. Furthermore, to gain insight into the role of lipid and water reserves on ability to resist food and water stress and desiccation stress, metabolic reserves in stressed flies have been estimated in this study as well.

Methoprene treatment increases development rate but also increases starvation and desiccation risk for Queensland fruit fly

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Short title: Dietary methoprene reduces Q-fly starvation and desiccation tolerance

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Highlights

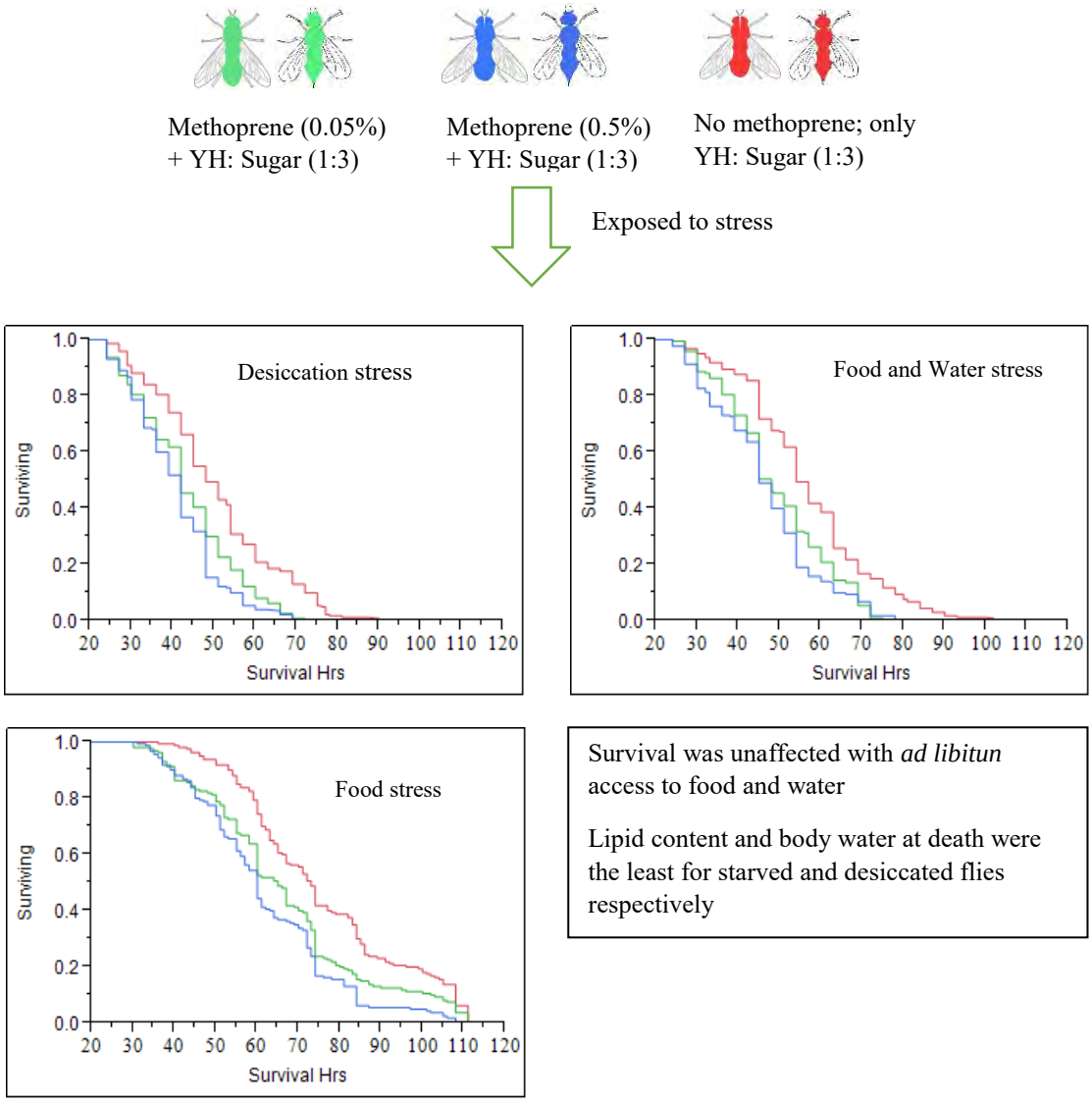
1. Dietary methoprene treatment reduced Queensland fruit fly survival under nutritional and desiccation stress.
2. Flies exposed to desiccating condition exhibited lowest survival.
3. Flies subjected to desiccation and nutritional stress had the least body water and lipid content at death respectively.

Abstract

Methoprene, a juvenile hormone analogue, incorporated into a protein rich adult diet accelerates sexual maturation of male Queensland fruit flies (Q-fly). Such accelerated development is a potentially valuable means to increase participation of released males in sterile insect technique programs. However, due to elevated metabolism following methoprene treatment, there is a risk that benefits of accelerated maturation might be countered by reduced starvation and desiccation tolerance. The present study investigates this possibility. After emergence, flies were treated with three levels of methoprene (0, 0.05%, and 0.5%) incorporated into diet of sugar and yeast hydrolysate for the first 48 hours after emergence. Survival tests of groups of flies were carried out under conditions of food stress, food and water stress, and ad libitum access to diet. Survival tests of individual flies were carried out under conditions of food stress, food and water stress, desiccation stress, and ad libitum access to diet. Most flies provided ad libitum access to diet were still alive at 7 days, whereas all stressed flies died within 4 days. Desiccation stressed flies had the shortest survival followed by food and water stress, and then food stress. Methoprene supplements increased susceptibility of flies to each stress, likely because of elevated metabolism. Flies subjected to food and water stress had the least lipids at death, whereas flies subjected to desiccation retained the least water reserves. Further investigation of how deleterious effects of methoprene treatment on stress tolerance affect flies in the field are required before deploying methoprene as a pre-release supplement for Q-fly SIT.

Keywords: Methoprene, food, water, stress, and vulnerability

Graphical Abstract



1 Introduction

Adverse environmental conditions, especially limited access to food and water, can severely affect development, reproduction and survival of insects (Koehn and Bayne, 1989). The ability of an insect to survive under prolonged food and water stress is generally linked to its nutritional status (metabolic reserves) and the rate at which stored reserves are utilised (metabolic rate) (Bennett et al., 1990). In *Drosophila melanogaster*, reduced utilisation of lipids can contribute to increased starvation resistance (Hoffmann and Parsons, 1993; Djawdan et al., 1997; Harshman and Schmid, 1998), while higher body water content and water storage in the haemolymph (Gibbs et al., 1997; Folk et al., 2001), as well as increased metabolism of stored carbohydrates that produce metabolic water, can increase desiccation resistance (Marron et al., 2003). Also, life history traits such sexual reproduction, egg production can lead to incresaed susceptibility under stress conditions (Salmon et al., 2001).

As well as being an important element of stress physiology, understanding the mechanisms underlying abiotic stress resistance in insects can be important in applied contexts, such as the Sterile Insect Technique ('SIT'). This environmentally sustainable control method has been developed to manage some of the most damaging tephritid species, including *Ceratitis capitata* (Reyes et al., 2007), *Zeugodacus* (previously *Bactrocera*) *cucurbitae* (Kakinohana, 1994; Itô et al., 2003), *Bactrocera dorsalis* (Orankanok et al., 2007) and *Anastrepha ludens* (Orozco-Dávila et al., 2015). SIT involves releasing millions of sterile male insects that mate with wild females, inducing reproductive failure (Knipling, 1955). As a result, reproductive potential of pest populations is reduced and, over generations, pest abundance declines. For successful SIT, a large proportion of the released male insects must tolerate environmental stresses to survive, mature sexually and participate in mating. Treatments provided during the pre-release holding period can provide a valuable means of ensuring rapid development of released insects and to thereby minimise the period that they must survive in the field before being effective agents of SIT. In addition to nutrients, treatment with the juvenile hormone analogue methoprene has been found to accelerate sexual maturation in some fruit fly species, including *Z. cucurbitae* (Haq et al., 2010a, b). *A. fraterculus* (Segura et al., 2009, 2013), *A. ludens* (Gómez et al., 2013; Pereira et al., 2013) and *A. suspensa* (Pereira et al., 2009, 2010). However, treatments that accelerate maturation at the cost of diminished environmental tolerance may be an overall detriment to SIT.

Queensland fruit fly (*Bactrocera tryoni* Froggatt or 'Q-fly') is the most difficult and costly challenge to market access for horticultural producers in eastern Australia, affecting a vast range of commercial and non-commercial crops (Dominiak and Daniels, 2012). SIT has been used sporadically to manage outbreaks of Q-fly for several decades, but with increasing need there is currently a significant effort underway to improve efficacy. In Q-fly, a diet containing yeast hydrolysate together with sucrose has been found to promote sexual maturation and reproductive development (Vijaysegaran et al., 2002; Meats and Leighton, 2004; Pérez-Staples et al., 2011; Weldon and Taylor, 2011), mating propensity (Pérez-Staples et al., 2007), and increased longevity (Pérez-Staples et al., 2007; Prabhu et al., 2008; Fanson et al., 2009; Taylor et al., 2013a). However, as in *C. capitata* (Yuval et al., 2007), dietary yeast hydrolysate renders Q-flies more vulnerable to food and water stress (Taylor et al., 2013b). Methoprene supplements applied topically (Collins et al., 2014) or in the adult diet (Adnan et al., 2018) can further accelerate development beyond the effects of dietary yeast hydrolysate, and this raises questions of whether methoprene further heightens the vulnerability of Q-flies to environmental stress, such as food limitation, water limitation, or desiccating conditions.

While wild Q-flies have a quite wide bioclimatic range (Sultana et al., 2017), domesticated Q-flies such as are used in SIT programs, have greatly diminished ability to tolerate desiccating conditions (Weldon et al., 2013), and there is a risk that the changes in physiological state induced by methoprene treatment might further exacerbate this potential constraint on SIT. Currently, there are no studies investigating effects of methoprene treatment on ability of Q-fly to tolerate environmental stress. Because single sex strains are not yet available for Q-fly, it is imperative to investigate the effect of pre-release treatment on both males and females. In the present study, we investigated whether dietary methoprene treatment affects the survival of male and female Q-flies challenged with food stress, food and water stress, and desiccation under laboratory conditions. In addition, the survival assays have been undertaken in group and individual conditions to assess if experimental housing and fly density influence the survival. Furthermore, to gain insight into the role of lipid and water reserves on ability to resist food and water stress and desiccation stress, we quantified remaining dry mass, water, and lipid content at death.

2 Materials and methods

2.1 Insects

Mass-reared Q-flies were obtained as pupae from a colony maintained at Macquarie University, New South Wales, Australia (20-22 generations from wild). Experimental flies were reared on a gel based larval diet of Moadeli et al. (2017) that was based on the liquid diet formulation of Chang et al. (2006). The colony and experiments were maintained in separate controlled environment rooms of 25±0.5ºC and 65±5% RH. The photoperiod was 13:11 (L:D) h, with a simulated dawn and dusk as lights gradually increased and decreased for 0.5 h at the beginning and end of each light phase.

2.2 Treatments

Newly emerged adult males and females were provided a diet of sugar and yeast hydrolysate (3:1) containing methoprene at 0.05%, 0.5%, and 0% (control). Methoprene was obtained as NOMOZ® pellets (a trademark of Pacific Biologists, Brisbane, Queensland) that have PROLINK® as active ingredient containing 40 g/kg (S)-methoprene. This product is locally available as a mosquito larvicide. NOMOZ pellets were finely powdered using mortar and pestle. Then, the powdered methoprene (as required on dry weight basis) was added into the respective diet using a blender.

Approximately 4000 pupae (100 ml of pupae, ca. 40 pupae per ml) were placed in three mesh cages (Megaview Bugdorm 44545, 47.5×47.5 × 47.5 cm) for adult emergence. Usually few flies emerge on the first day of emergence, and these were discarded. After the following 24 hours of emergence, all non-emerged pupae were removed from the cage, and a Petri dish containing diet with one of the methoprene treatments was provided to flies in each of three cages for next two days *ad libitum*.

2.3 Survival test

2.3.1 Survival in group cages

Following exposure to dietary treatments, the treated flies were immediately subjected to environmental stresses. From each treatment (0.5%, 0.05%, and 0% methoprene), two groups

of 50 males and two groups of 50 female flies were transferred to 12-L cages. The cages were of clear plastic and had mesh-covered windows for ventilation. Subsequently, flies were subjected to the following stresses:

- Food stress: Flies had access to moistened cotton wool as a source of water but no food.
- Food and water stress: Flies had no access to food or water.
- Ad libitum access to food and water: Flies had access to food (sugar) and water.

Dead flies were collected from the cage floor and counted every 6 hours (06:00, 12:00, 18:00, and 00:00 h AEST) around the clock for 7 days. The experiment was replicated two times (i.e. total of 200 flies were tested for different stress from each sex and treatment).

2.3.2 Survival in individual vials

Application of dietary treatments for assessing longevity in individual vials was similar to that of group container trial. Following 48 hrs of treatment, 40 females and 40 males from each treatment (0.5%, 0.05%, and 0% methoprene) were transferred individually to 5-mL round bottom glass labelled vials (Lab Australia Pty Ltd, Australia). Flies were subjected to four different conditions to assess their survival:

- Food stress: Flies were individually placed in glass vials with access to a small block of agar gel (prepared by boiling 10g agar in 100 ml water and cooling to set before cutting), thereby allowing access to water but not food.
- Food and water stress: Flies were provided no access to food or water.
- Desiccation: Flies were exposed to two to three large granules of desiccant (silica beads, Sigma Aldrich) to test survival under conditions food and water stress (above) with the added challenge of desiccating conditions. A loose barrier of cotton wool was used to separate the flies from the desiccant (following Weldon et al., 2013).
- Ad libitum access to food and water: The flies were placed individually placed in glass vials with 1gm of sugar-agar diet (diet composition: 30 g white sugar, 100 ml water, 10 g agar, 1.5 g Nipagin), thereby allowing fly access to water and carbohydrates.

In all treatments, the glass vials were tightly sealed with corks to maintain the similar micro condition for the flies. Mortality was recorded by visually inspecting the vials every 3 hours around the clock. Flies were considered dead when they were incapable of holding onto the inner surface of the vial, and when no movement of their legs or mouthparts was observed after the vials were gently flicked with a finger. Three replicates of the experiment were conducted.

2.4 Metabolic reserve analysis

2.4.1 Remaining body water and dry mass at death

Forty females and forty males from each methoprene dose (0.5%, 0.05%, and 0%) were transferred individually to 5-mL round bottom glass labelled vials (Lab Australia Pty Ltd, Australia). Flies were then subdued for two minutes in freezer, weighed (to 0.0001 g) using an analytical balance (Sartorius Cubis® Manual Mass Comparator, origin Germany) to measure initial weight and returned to their vials and were subjected to food stress, food and water stress, and desiccation stress. Mortality was recorded by visually inspecting the vials every 3 hours around the clock. Immediately after death, weight (wet mass) was recorded. Following weighing, we preserved the flies in a freezer at -20 °C for 2 – 4 weeks. The flies were then oven dried at 60 ° C for 48 hrs to calculate dry mass at the time of death. Dry mass was subtracted from wet mass at death to calculate remaining body water at death.

2.4.2 Remaining lipid content at death

First, the samples (flies) were dried individually in 5 mL glass vials at 60°C for 48 hrs in an oven that contained a plate with silica gel for absorbing humidity. Then we weighed the samples, keeping them dry by using silica gel. The lipids were extracted from the flies using three 24 h washes of chloroform (Sigma Aldrich). At the end of the third chloroform wash and on day four, the vials were kept open so that chloroform (Sigma Aldrich) evaporated overnight under the fume hood. Again, the samples were dried for 48 hours and reweighed. The difference between the two dry weights indicated the remaining soluble lipid content present in dead flies (Ellers, 1996).

2.5 Statistical analysis

All analyses were conducted using R v3.5.1 (R Core Team, 2018). For the individual survival data, two separate analyses were conducted. For the flies with *ad libitum access*, fly survival were censored, and a Cox-proportional hazard model was performed including dose and sex as main effect. Post-hoc pairwise comparisons were performed with a Tukey correction. For the flies subjected to stress treatments, fly survival were not censored and a linear mixed model were performed using survival times (log-transformed) including dose, sex, and stress as fixed effects. Post-hoc pairwise comparisons were performed with a Tukey correction to test for differences in doses. In both models initially all interactions were included, and a backward model selection process was performed by removing non-significant interactions.

Similarly, in-group survival analysis for the flies with ad libitum access, a frailty Cox proportional hazard model was performed including dose and sex as main effect and cage as a random effect. For the flies subjected to stress treatments, a linear mixed model was performed using including dose, sex, and stress as fixed effects and cage as a random effect. Again, post-hoc pairwise comparisons were performed with a Tukey correction. In both models initially all interactions were included, and a backward model selection process was performed by removing non-significant interactions.

For metabolic analysis, separate linear models were performed for the remaining water, dry mass, and lipid content. For each response variable sex, dose, and stress, as well as all interactions were included as main effects. Initial body mass and survival time were also included as linear covariate to control for variable initial mass as well as to control for differences in survival times. Both covariates were centered at their mean. Post-hoc pairwise comparisons were performed with a Tukey correction testing for effects of dose within sex and stress. Model assumptions was assessed and justified through graphical analyses of the residuals.

3 Results

3.1 Survival tests

3.1.1 Survival in group cages

The survival of flies subjected to stresses was significantly affected by stress and doses of methoprene, but not by sex, whereas no significant effect of methoprene dose or sex was found on the survival of flies given *ad libitum* access to food and water (Table 1, 2; Fig 1). All flies that were subjected to food and water stress and food stress died during the trial period, whereas the 99.5% of the flies provided with *ad libitum* food and water access were alive after 7 days. Furthermore, flies subjected to food stress had greater survival than those subjected to food and water stress (Food stress vs food and water stress, estimate of differences = 0.11 ± 0.02, $p < 0.001$) (Fig 1). For both stressors, flies treated with methoprene had a shorter survival compared to control flies (Fig 1). Most importantly, this vulnerability increased with the increasing dose of methoprene since flies fed on 0.5% methoprene had the shortest survival, followed by 0.05% methoprene and control flies (Methoprene 0.05 vs control estimate of differences = -0.18 ± 0.02, $p < 0.001$; Methoprene 0.5 vs control estimate of differences = -0.33 ± 0.02, $p < 0.001$; Methoprene 0.05 vs Methoprene 0.5 estimate of differences = 0.14 ± 0.020, $p < 0.001$) (Fig 1).

Table 1. Effects of methoprene supplementation (0, 0.05%, & 0.5%) on survivorship of male and female *Bactrocera tryoni* in single sex groups housed in 12-L cages and individually in 5ml glass vial with *ad libitum* access to food and water

Experiment	**Source**	**df**	**χ^2**	**P**
Individual	Sex	1	0.8	0.37
	Dose	2	0.6	0.74
Group	Sex	1	0.1	1.00
	Dose	2	0.1	1.00

3.1.2 Survival in individual vials

Survival of the flies was significantly influenced by the stress type and doses of methoprene treatment but not by sex, while the survival of flies given *ad libitum* access to food and water did not vary with sex or methoprene treatment (Table 1, 2; Fig 2). Among the stressed flies, survival of the flies that were subjected to desiccation was the shortest, whereas flies provided with food stress had the longest survival. Flies subjected to food and water stress had intermediate survival (Desiccation vs food stress difference of estimate (log-scale) = -

0.41 ± 0.017, $p < 0.001$; Desiccation vs food and water stress difference of estimate (log-scale) = -0.13 ± 0.016, $p < 0.001$ and food stress vs food and water stress, estimate of differences (log-scale) = 0.28 ± 0.076, $p < 0.001$). Survival of both sexes significantly declined with the doses of methoprene. For all stressors, flies that were given access to both doses of methoprene had shorter longevity compared to the flies provided no methoprene and also flies provided 0.5 methoprene had shorter survival than the flies fed 0.05 methoprene (Methoprene 0.05 vs control estimate of differences = -0.14 ± 0.016, $p < 0.001$; Methoprene 0.5 vs control estimate of differences = -0.20 ± 0.016, $p < 0.001$; Methoprene 0.05 vs Methoprene 0.5 estimate of differences = 0.06 ± 0.016, $p = 0.001$).

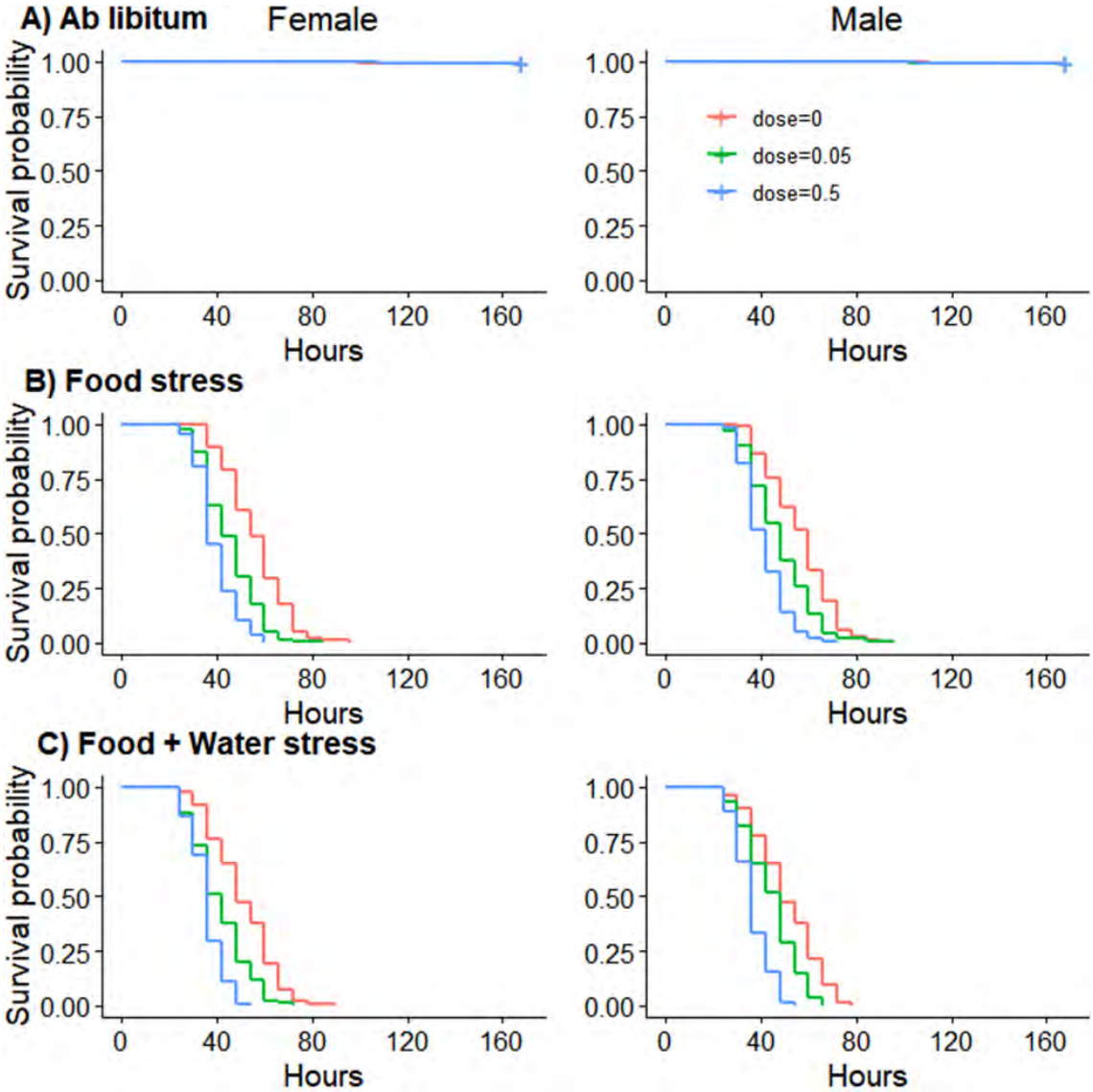


Fig 1. Relationship between types of stress (food stress, food and water stress, *ad libitum access*) and survival of male and female Q-flies after being fed a diet of sugar plus yeast hydrolysate combined with one of three levels of methoprene supplementation (0, 0.05, 0.5

%) for two days after emergence and then were subjected to group survival assay in 12-L cages.

Table 2. Effects of methoprene supplementation (0, 0.05%, & 0.5%) and different level of stress (food stress, food and water stress, desiccation) on survivorship of male and female *Bactrocera tryoni* as housed individually in 5ml glass vial and as groups in 12-L cages

Experiment	Source	df	χ2	P
Individual	Stress	2	297.4	<0.001*
	Dose	2	80.4	<0.001*
	Sex	1	0.1	0.74
Group	Stress	1	27.4	<0.001*
	Dose	2	178.3	<0.001*
	Sex	1	2.5	0.12

3.2 Remaining metabolic reserves

3.2.1 Remaining water at death

The remaining water content of the flies at death significantly varied with stress along with significant stress× doses of methoprene interaction but not with sex (Table 3, Fig 3). Flies that were exposed to desiccation conditions had the lowest remaining body water at death whereas food stressed flies had the highest remaining body water. Flies subjected to food and water stress had intermediate remaining water content at death (Desiccation vs food stress difference of estimate =2.6 ± 0.14, $p < 0.001$; Desiccation vs food and water stress difference of estimate =1.4 ± 0.12, $p < 0.001$; food stress vs food and water stress difference of estimate =1.1 ± 0.13, $p < 0.001$). However, significant stress× methoprene treatment interaction revealed that effect of methoprene doses on remaining water level are evident for only the flies that were subjected to desiccation condition. Flies provided only 0.5% methoprene retained greater amount of water during death compared to control flies under desiccation condition (Methoprene 0.5 vs control estimate of differences = 0.402 ± 0.123, p=0.0033; Methoprene 0.05 vs control estimate of differences = 0.165 ± 0.123, p=0.37).

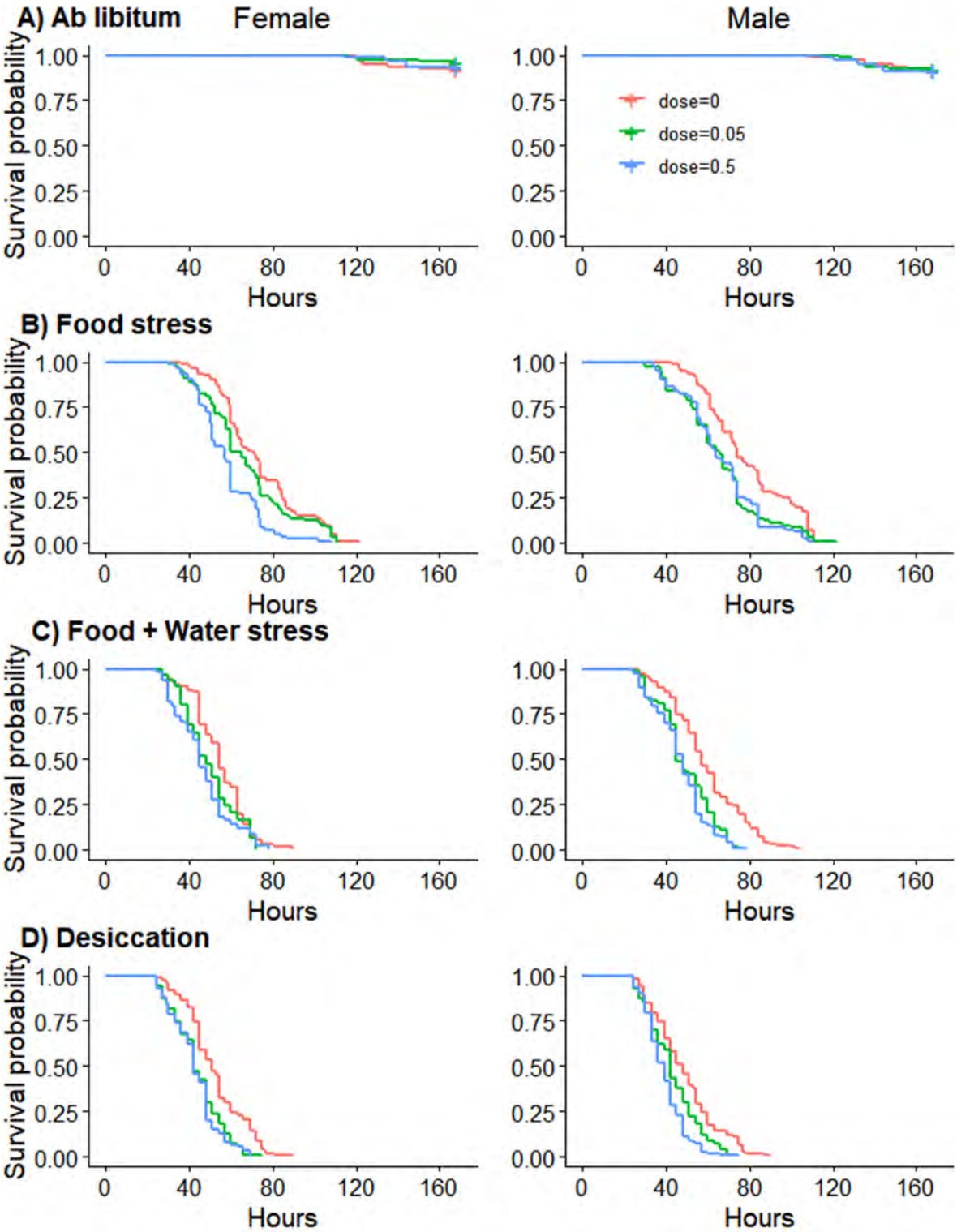


Figure 2. Relationship between types of stress (food stress, food and water stress, desiccation, and *ad libitum access*) and survival of male and female Q-flies after being fed a diet of sugar plus yeast hydrolysate combined with one of three levels of methoprene supplementation (0, 0.05, 0.5 %) for two days after emergence and then were subjected to survival assay in 5ml individual vials.

3.2.2 Remaining dry mass at death

Dry mass content at death was significantly influenced by sex, stress, and dose of methoprene treatment including all possible significant interactions (Table 3, Fig 4). Significant sex × stress × methoprene treatment interaction reveals that under desiccation stress, remaining dry mass is greater for untreated males compared to that of males treated either methoprene doses (For desiccation, methoprene 0.05 vs control estimate of differences= -0.170 ± 0.022, $p<0.001$; methoprene 0.5 vs control estimate of differences= -0.152 ± 0.022, $p<0.001$), whereas females provided 0.5% and no methoprene had greater dry mass than females fed 0.05% methoprene (methoprene 0.05 vs control estimate of differences= -0.084 ± 0.022, $p<0.001$; methoprene 0.05 vs methoprene 0.5 estimate of differences= -0.084 ± 0.022, $p<0.001$) (Fig 4). Moreover, in food stressed flies, significant effect of dose was evident only for the males that were treated with 0.5% methoprene as males provided 0.5% methoprene had lower remaining dry mass content compared to untreated males (methoprene 0.5 vs control estimate of differences= - 0.051 ± 0.022, $p=0.05$). However, in food and water stress, there was no significant effect of dose on remaining dry mass content in any sex.

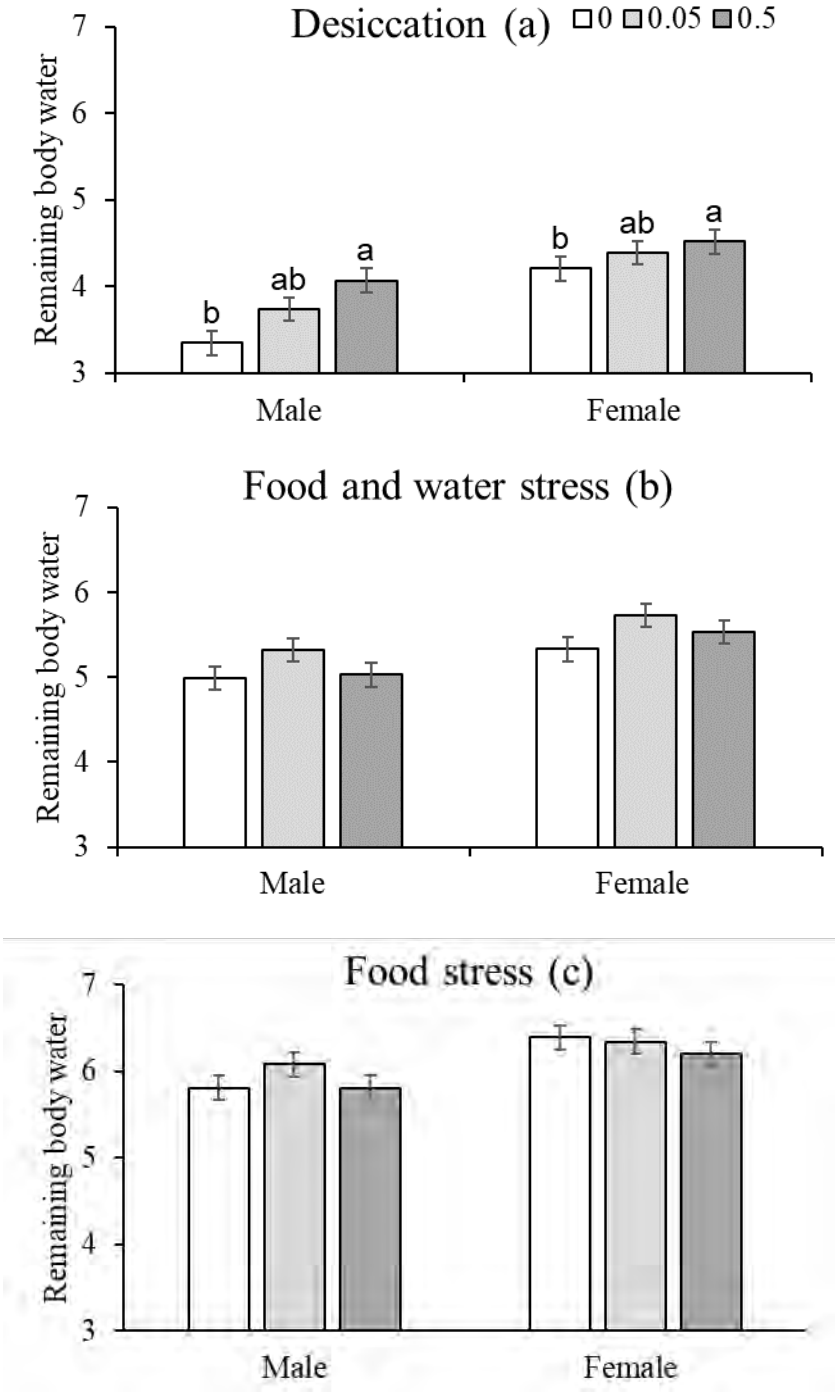
3.2.3 Lipid level at death

Remaining lipid contents at death varied significantly with sex, stress, and methoprene dose including all possible significant interactions (Table 3, Fig 5). Flies that were subjected to desiccation stress retained greater lipid level during death than those subjected to food stress (Desiccation vs food stress difference of estimate = 0.99 ± 0.07, $p < 0.001$) and food and water stress (Desiccation vs food and water stress difference of estimate = 0.93 ± 0.06, $p < 0.001$). However, there was no evidence of significant difference between remaining lipid content of flies exposed food stress and food and water stress (food stress vs food and water stress difference of estimate =0.06 ± 0.06, $p = 0.66$). Significant sex × stress × methoprene dose interaction reveals several patterns. Males that were subjected to desiccation and food stress, there was no significant effect of methoprene doses on existing lipid content, whereas under food and water stress, males supplemented with 0.5% methoprene had higher lipid level compared to those treated with 0.05% methoprene or no methoprene (methoprene 0.5 vs control estimate of differences= 0.433 ± 0.092, $p<0.001$; methoprene 0.5 vs methoprene 0.05 estimate of differences = 0.383 ± 0.090, $p<0.001$). Females provided both methoprene doses had significantly lower lipid level at death than untreated flies when exposed to desiccation

condition (methoprene 0.05 vs control estimate of differences= - 0.322 ± 0.091, p=0.0012; methoprene 0.5 vs control estimate of differences= -0.226 ± 0.090, p=0.034), whereas under food stress, females provided both methoprene doses had significantly greater body lipid at

Table 3. Linear model analysis of remaining body water, dry mass, and lipid content in relation to fly sex (male, female), stress (food stress, food and water stress, and desiccation) and methoprene treatment (0, 0.05%, & 0.5%).

Metric	Variable	df	F	P
Remaining lipid	Dose	2, 699	6.6	0.0014
	Sex	1, 699	0.1	0.77
	Stress	2, 699	127.8	<0.001*
	Dose×sex	2, 699	4.4	0.013
	Dose×stress	4, 699	23.6	<0.001*
	Sex×stress	2, 699	17.1	<0.001*
	Dose×sex×stress	4, 699	8.6	<0.001*
Remaining water	Dose	2, 704	2.5	0.08
	Sex	1, 704	7.6	0.059
	Stress	2, 704	136.4	<0.001*
	Dose×sex	2, 704	0.7	0.49
	Dose×stress	4, 704	2.9	0.022*
	Sex×stress	2, 704	1.5	0.23
Remaining dry mass	Dose	2, 700	9.9	<0.001*
	Sex	1, 700	37.9	<0.001*
	Stress	2, 700	21.7	<0.001*
	Dose×sex	2, 700	12.3	<0.001*
	Dose×stress	4, 700	4.4	0.0015*
	Sex×stress	2, 700	13.9	<0.001*
	Dose×sex×stress	4, 700	3.2	0.012*



358 **Fig 3.** Body water remained at death (Mean ± SE) in male and female Q-flies that were
359 provided a diet of sugar plus yeast hydrolysate combined with one of three levels of
360 methoprene supplementation (0, 0.05, 0.5 %) for two days after emergence and then were
361 subjected to survival assay in 5 ml glass vial under three types of stresses (food stress, food
362 and water stress, and desiccation).

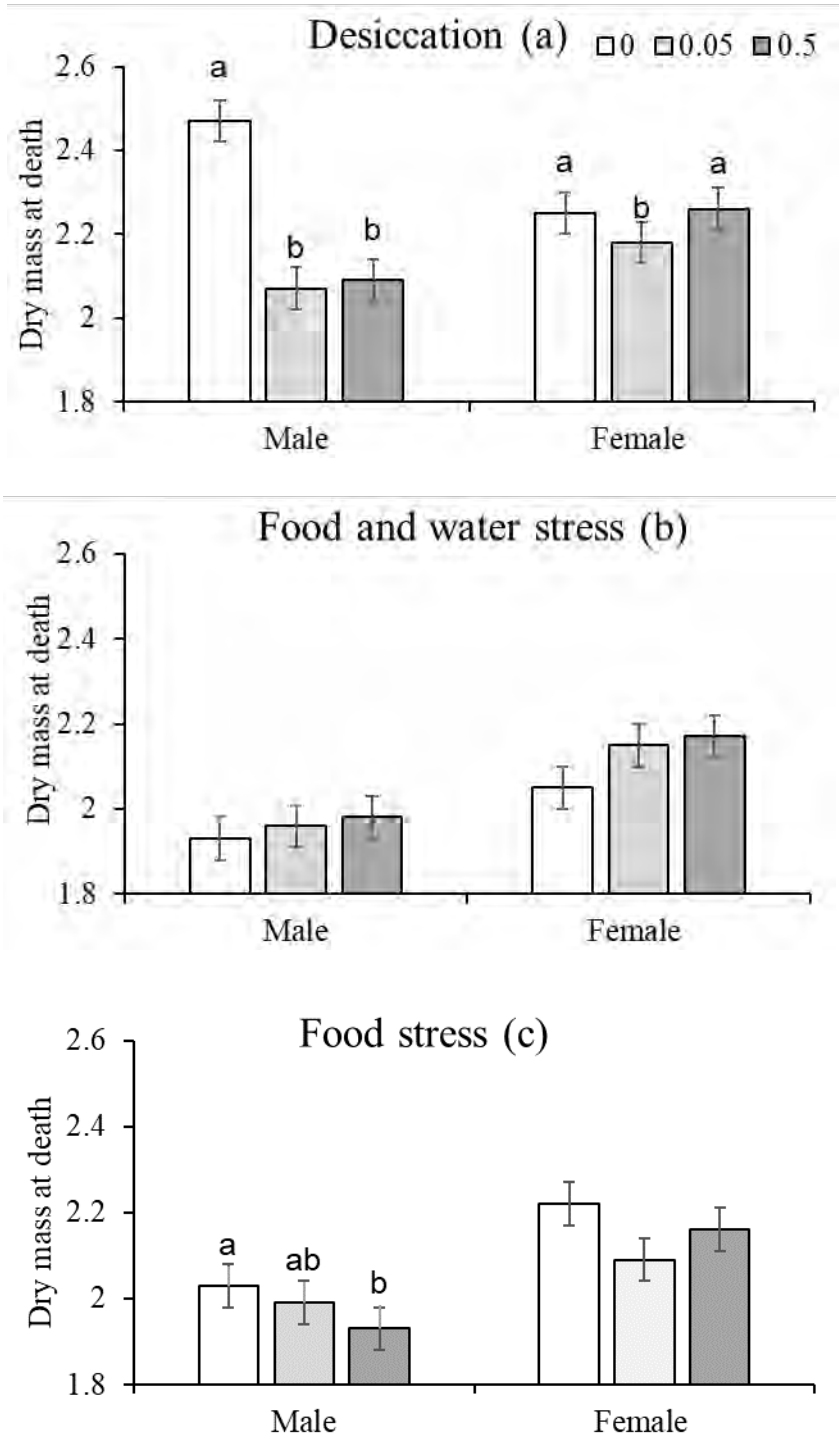


Fig 4. Dry mass at death (Mean ± SE) in male and female Q-flies that were provided a diet of sugar plus yeast hydrolysate combined with one of three levels of methoprene supplementation (0, 0.05, 0.5 %) for two days after emergence and then were subjected to survival assay in 5 ml glass vial under three types of stresses (food stress, food and water stress, and desiccation).

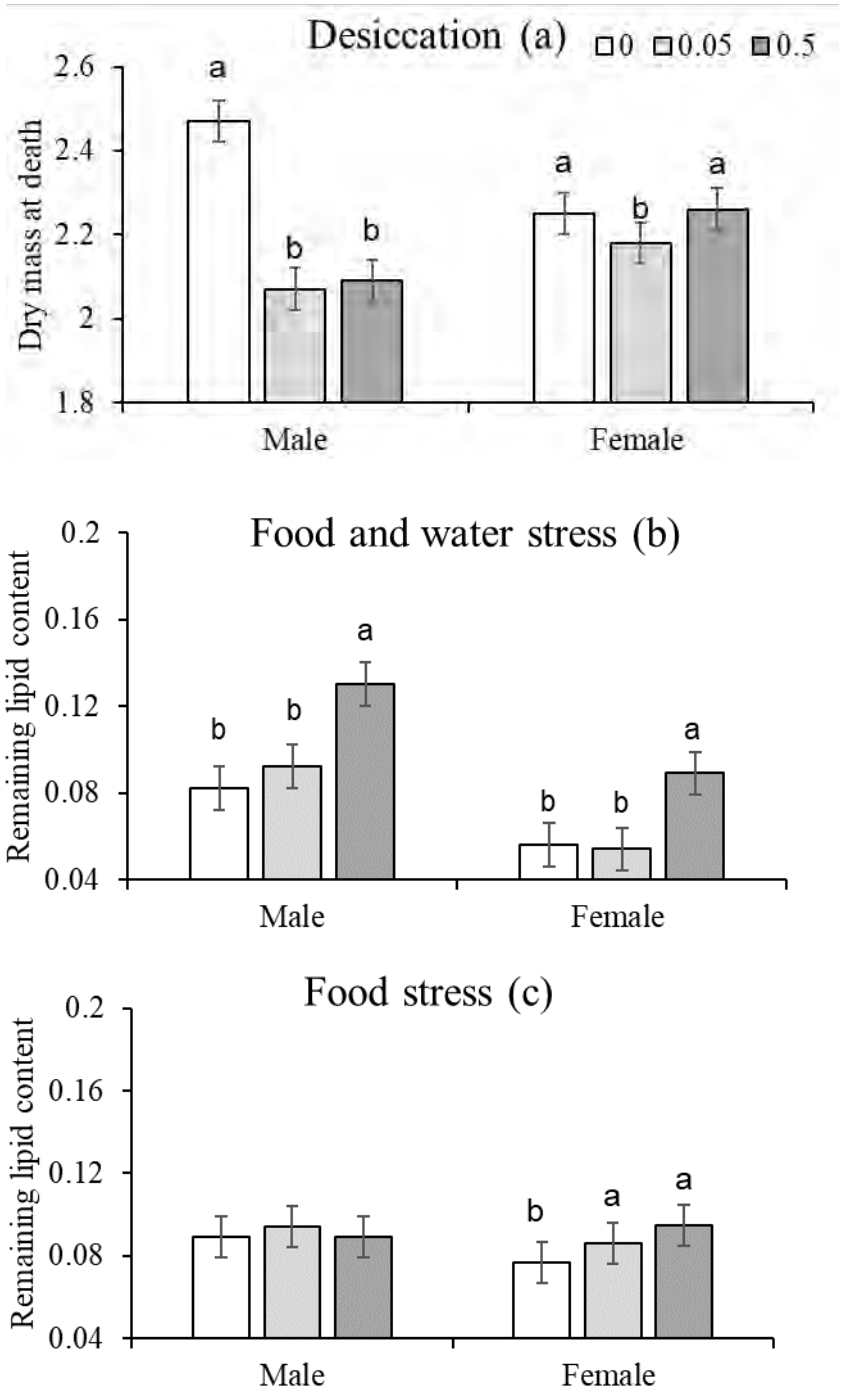


Fig 5. Lipid content at death (Mean ± SE) in male and female Q-flies that were provided a diet of sugar plus yeast hydrolysate combined with one of three levels of methoprene supplementation (0, 0.05, 0.5 %) for two days after emergence and then were subjected to survival assay in 5 ml glass vial under three types of stresses (food stress, food and water stress, and desiccation).

death than untreated females (methoprene 0.05 vs control estimate of differences= 0.604 ± 0.091, $p<0.001$; methoprene 0.5 vs control estimate of differences= 0.730 ± 0.092, $p<0.001$). However, under food and water stress, females provided with 0.5% methoprene had significantly greater lipid level compared to those treated with 0.05% methoprene or no methoprene (methoprene 0.5 vs control estimate of differences= 0.516 ± 0.090, $p<0.001$; methoprene 0.5 vs methoprene 0.05 estimate of differences = 0.635 ± 0.090, $p<0.001$).

4 Discussion

In the present study, we investigated whether incorporating methoprene into a protein-rich diet for Q-fly SIT increases the vulnerability of flies to nutritional and hydric stress. While, in previous studies, methoprene supplements significantly accelerated sexual maturation of Q-flies (Collins et al., 2014; Adnan et al., 2018), our findings indicate that the ability of Q-flies to tolerate nutritional and water stress declined sharply following methoprene treatment. To gain insights to the underlying physiological mechanisms governing the vulnerability of methoprene treated flies, we considered the specific role of water and energy reserves as related to the stress resistance of the treated flies.

Amongst stress treatments, we found that the survival was lowest for flies subjected to desiccating conditions, intermediate for flies subjected to food stress, and highest for flies subjected to food and water stress. Our findings are generally consistent with previous studies of Q-fly survival under stress (Weldon and Taylor, 2010, Weldon et al., 2013). Sensitivity to food stress and food and water stress was most likely increased by the inclusion of yeast hydrolysate in the treatment diet (Taylor et al., 2013b). Even though protein is crucial for reproductive tissues development, and methoprene treatment has little or no effect on development in the absence of a protein source, substantial metabolic demands are associated with the maturation of reproductive system and this may lead to increased vulnerability of the flies when they are subjected to nutritional deprivation (Kaspi and Yuval, 2000; Maor et al., 2004; Yuval et al., 2007; Gavriel et al., 2010; Fanson and Taylor, 2012; Taylor et al., 2013b; Utgés et al., 2013). In the present study we did not evaluate the effects of feeding the flies with sugar alone because this treatment is not an effective combination with methoprene (Adnan et al., 2018).

Methoprene treatment sharply reduced the survival of flies when they were exposed to both nutritional and hydric stress. This reduction in survival due to methoprene feeding is likely due to accelerated metabolism. Just as the development-promoting effects of dietary yeast hydrolysate increase vulnerability to starvation (Taylor et al., 2013b), the accelerated development of methoprene treated male Q-flies comes at a physiological cost of diminished ability to tolerate environmental stress. This pattern aligns with the previous study of Salmon et al (2001) who postulated that stress susceptibility might derive as a cost of reproduction. Juvenile hormone analogue methoprene which is a potential mediator of increased sexual development in several fruit fly species, can cause trade-offs with life history characters like stress resistance.

We did not find any sex related differences in survival under any of stress conditions. However, in other studies with Q-fly, we found contradictory trends in relation to the effects of sex on fly survival in starvation and desiccation resistance assays. Weldon et al. (2010, 2013) found that male Q-flies survived for longer than females in desiccation resistance assays. In *Drosophila* and in *A. ludens* female desiccation resistance tends to exceed that of males (Gibbs and Markow, 2001; Matzkin et al., 2009; Tejeda et al., 2016), while males tend to be more resistant to hydric stress than females (Matzkin et al., 2007, Tejeda et al., 2014). In the context of nutritional stress, *Drosophila melanogaster* males tend to survive longer under food and water stress than females but in other *Drosophila* species females tend to survive longer than males under food and water stress (Matzkin et al., 2009).

Resistance to both starvation and desiccation can be explained by two distinct physiological mechanisms: increasing the storage of resources (energy or water) that are being utilized during stress, or alternatively by reducing consumption rates (i.e. lower metabolic rates) (Hoffmann and Parsons, 1991). In our study, flies that died under food stress retained the least lipid. On the other hand, flies that died under desiccation stress had the least body water at death. Therefore, the effects of methoprene on vulnerability of Q-fly to stress are likely explained largely by increased utilisation of lipid and water reserves. Reduced rates of metabolism are a general explanation for stress resistance (Hoffmann and Parsons, 1989a,b, 1991; Blows and Hoffmann, 1993). Decreased metabolic rate increases the amount of time that flies can survive under food and water stress and reduces the need to open the spiracles, and consequently be exposed to loss of water, in desiccating conditions (Lighton, 1994, 1996; Zachariassen, 1996; Addo-Bediako et al., 2001). Similar to our results, in *D.*

melanogaster survival under food and water stress particularly relies on availability and use of lipid reserves (Herrewege and David, 1997; Hoffmann and Harshman, 1999; Arrese and Soulages, 2010). Similarly, *A. serpentina* and *A. ludens* deplete lipid reserves to survive under food and water stress (Jacome et al., 1995; Tejeda et al., 2014). However, accumulation of lipids and dry mass is also associated with increased starvation resistance in *Drosophila* (Chippindale et al., 1996), and this is likely applicable in Q-fly.

While resistance to food stress and food and water stress are predominantly linked to changes in lipid and dry mass content, resistance to desiccation depends on extent of water loss (Gibbs et al., 1997; Hoffmann and Harshman, 1999). Several species of *Drosophila* exhibiting increased desiccation resistance also exhibit diminished rates of water loss (Gibbs et al., 1997; Hoffmann and Parsons, 1993; Ringo and Wood, 1984). Similarly, *A. ludens* in a desiccation resistance assay made the highest use of water and the least use of lipid content (Tejeda et al., 2014). In addition to rate of water loss, amount of water present at death (dehydration tolerance) and/or exoskeleton structure (epicuticle wax) could influence desiccation resistance as reported in some desiccation-selected lines of *D. melanogaster* (Gibbs et al., 1997; Chippindale et al., 1998). In *D. melanogaster*, several studies have shown that reduced desiccation resistance is primarily caused by increased rates of water loss rather than reductions in water content or dehydration tolerance (Fairbanks and Burch, 1970; Graves et al., 1992; Nghiem et al., 2000). In addition to rate of energy consumption, stress resistance mostly depends on the types and amounts of energy flies are metabolising to tackle the stress (Marron et al., 2003). In *D. melanogaster* there was no significant difference in the rate of lipid and protein metabolism during food and water stress and desiccation stress, but carbohydrate metabolism was several times higher during desiccation (Marron et al., 2003). While lipids provide over twice as much energy per gram as carbohydrates (Withers, 1992), and are appropriate fuels to counter starvation resistance, lipids do not bind a significant amount of water, so the total amount of water becomes available after metabolism is much lower for lipids than carbohydrates (Gibbs et al., 1997). Carbohydrates reserves are more effective for countering desiccating conditions.

Methoprene treated flies exploited the least use of water and lipid stores under desiccation and nutritional stress respectively. A possible explanation is that lipid storage would provide fuel to survive under food stress only if methoprene treated flies are able to better metabolize lipids under these conditions. If methoprene treated flies have greater need

to access reserves but no greater ability to do so, then they would succumb with more resources remaining unexploited. Similarly, if methoprene treated flies could better metabolize carbohydrates under desiccation stress, water could be released to increase survival. Inability of the methoprene-treated flies to adequately utilize the available metabolic reserves under stress appears to at least partly explain the increased vulnerability of methoprene-treated flies.

In conclusion, while the incorporation of methoprene into pre-release diet significantly accelerates sexual development of Q-fly males (Adnan et al., 2018) and is very promising as an effective pre-release treatment for SIT, the present study indicates significant risks. Methoprene treated flies had increased vulnerability to food stress, food and water stress, and desiccation stress, and there is a need to consider such risks when deploying methoprene as a pre-release supplement in operational SIT programs when flies are likely to encounter challenging environments after release. It may be that methoprene treatments are suitable in areas and seasons with more benign environmental conditions, but not in other areas and seasons. By tailoring pre-release treatments to likely field conditions, pre-release treatments could be applied when advantageous by minimising the adverse effects of increased stress vulnerability. The advancement in male mating by 3-4 days (Adnan et al., 2018) would likely counteract the negative effects of methoprene treatment on environmental tolerance. Perhaps reduced doses of methoprene might be effective at accelerating development without imposing an effect on environmental tolerance as substantial as was found in the present study.

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Chapter Nine

Image captured by author only

This chapter has been formatted in accordance with style of *Journal of Pest Science*

Overview

Previous chapters of my thesis provides numerous and diverse findings to address its principal aim of investigating the viability of methoprene as pre-release treatments for Q-fly. There has been significant interest in the practical use of supplements to accelerate development of fruit flies used in SIT programs, and accordingly to date a handful number of exciting studies have been undertaken on methoprene, Rk, methyl eugenol, which have been reported as potential enhancer of sexual development in multiple fruit fly species. However, potential of caffeine, a well-known neuro-stimulator as metabolic enhancer has not been explored much in insects. So far, caffeine exhibits a hint of mediating faster sexual maturation in Med-fly. Then I decided to step forward in Q-fly. It is hypothesized that metabolic boost provided by caffeine can be translated into elevated expression of sexual effort comparable to the effects rendered by methoprene and raspberry ketone in Q-fly. Based on this possibility, in addition to investigating promise of methoprene as pre-release supplement, potential of caffeine supplements as a novel means to accelerate sexual maturation in Q-fly has been demonstrated in chapter nine.

Dietary caffeine supplements as a novel promotor of sexual development for fruit fly sterile insect technique

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Abstract

Sterile insect technique (SIT) is an environmentally benign pest management technique that involves releasing millions of sterile insects to suppress reproduction of pest populations. SIT has been used to manage outbreaks of Queensland fruit fly (*Bactrocera tryoni* Froggatt, 'Q-fly') the most difficult and costly challenge to market access for most Australian fruit growers. Q-fly has a long adult maturation period and the delay from release until maturation in the field can result in a large proportion of sterile flies dying before becoming sexually active. We here demonstrate potential of pre-release caffeine supplements as a novel means to accelerate sexual maturation in Q-fly. In longevity trials including three caffeine sources - analytical caffeine, guarana powder and instant coffee - high doses of guarana powder and instant coffee were found to reduce longevity while even the highest tested doses of analytical caffeine did not affect longevity. Using doses known to not affect longevity, each caffeine supplement was incorporated into standard adult diet of sugar and yeast hydrolysate (3:1) for two days following emergence, and mating trials were conducted from 4 to 20 days of age. While no positive effects of guarana powder and instant coffee supplements were detected, analytical caffeine was very effective at accelerating sexual maturation. Mating probability increased with age for all groups, but the flies provided analytical caffeine exhibited a steeper increase in mating probability compared with untreated flies. Flies fed analytical caffeine supplements also exhibited shorter mating latency and longer mating duration than untreated flies. Results for mating activity were reflected in morphological development of reproductive tissues, as testes and ejaculatory apodemes of flies fed analytical caffeine supplements grew faster than those of untreated flies. Pre-release caffeine supplements offer a novel method to promote rapid maturation in Q-flies released in SIT, and may provide parallel benefits for other fruit fly species that have long adult maturation periods.

Key Words: caffeine, mating, reproductive development, longevity, SIT

Key Message

- Long adult maturation stages of tephritid fruit flies can reduce efficacy of sterile insect technique (SIT) programs.
- Treatments that accelerate sexual development can enhance SIT.
- Analytical caffeine supplements added to the diets of newly emerged Queensland fruit fly males for 48 h significantly accelerated development of ejaculatory apodeme and testis and increased mating propensity.
- Pre-release caffeine supplements provide a new approach to increase the efficacy of SIT for Queensland fruit fly and other tephritid fruit flies.

Introduction

Tephritid fruit flies are amongst the world's most economically significant pests of fruit production (Qin et al. 2015; White and Elson-Harris 1992). In Australia, the Queensland fruit fly, *Bactrocera tryoni* (Froggatt), or 'Q-fly', presents a costly challenge to Australia's $9 billion horticulture industry, affecting a vast diversity of commercial and non-commercial crops (Clarke et al. 2011; Dominiak and Daniels 2012; Hancock et al. 2000). Organophosphate insecticides have been the most common solution for decades, but their use is now greatly restricted due to concerns about environmental and human health (APVMA 2015a, b). Alternative control measures are now a high priority. The Sterile Insect Technique (SIT) is a sustainable technology that is growing rapidly in favour. SIT involves releasing millions of sterile insects that reduce the reproductive capacity of pest populations by inducing reproductive failure in females (Knipling 1955; Benelli et al. 2014). SIT has been used globally to combat some of the most damaging fruit fly species, including Mediterranean fruit fly, or medfly *Ceratitis capitata* (Wiedemann) (Reyes et al. 2007), melon fly *Zeugodacus cucurbitae* (Coquillett) (Itô et al. 1993; Kakinohana 1994), Oriental fruit fly *Bactrocera dorsalis* (Hendel) (Orankanok et al. 2007) and Mexican fruit fly *Anastrepha ludens* (Loew) (Orozco-Dávila et al. 2015). While SIT has proven effective for numerous species, there is still substantial scope to increase efficacy and cost effectiveness in most programs.

Fruit flies commonly have long adult maturation phases and high mortality rates in the field, such that a quite small proportion of the released flies might survive to mature and contribute to SIT (Liedo et al. 2002; Weldon et al. 2008; Reynolds et al. 2012, Pérez-Staples et al. 2008). Most species are anautogenous, needing to acquire nutritional resources, especially protein, as adults to complete reproductive development (Drew and Yuval 2000). Yeast hydrolysate (YH) mixed with sugar is a standard adult diet used to maintain fruit fly colonies, providing a rich source of amino acids, micronutrients and carbohydrates (Fanson and Taylor 2012) that is effective at sustaining reproductive development (Vijaysegaran et al. 2002; Meats and Leighton 2004; Pérez-Staples et al. 2007). When provided for even a 1 - 2 day pre-release period, YH and sugar are effective for sustaining development of male Q-flies over the following days (Pérez-Staples et al. 2011; Weldon and Taylor 2011), increasing the prevalence of mature sterile flies in the field (Reynolds et al. 2014).

While ensuring that released flies have the nutritional resources they need for development is important, additional treatments that can further reduce the delay between release and maturity remain of particular interest. Dietary or topical application of methoprene, a juvenile hormone analogue, provides one prominent avenue, having been found to substantially accelerate adult development in Q-fly (Adnan et al. 2018; Adnan et al. 2019, in press; Collins et al. 2014), as well as in *Z. cucurbitae* (Haq et al. 2010a, b), *Anastrepha fraterculus* (Abraham et al. 2013; Liedo et al. 2013; Segura et al. 2013; Segura et al. 2009), *A. ludens* (Gómez-Simuta et al. 2017; Gómez et al. 2013; Pereira et al. 2013), *A. suspensa* (Pereira et al. 2009, 2010), and. *C. capitata* (Faria et al. 2008). Other pre-release treatments have been explored for their effects on sexual performance of already mature fruit flies, especially plant semiochemicals such as methyl eugenol to which males of some species are attracted and from which they gain substantial sexual benefits (Haq et al. 2015, 2018; Ji et al. 2013; Orankanok et al. 2013; Shelly et al. 2010a; Shelly and Edu 2008; Shelly et al. 2010b; Shelly et al. 2005; Shelly et al. 2008). While Q-flies are not attracted to methyl-eugenol, they are attracted to raspberry ketone. Dietary access to cuelure, an analogue of raspberry ketone, has been found to function as a potent simulant in sexually mature male Q-flies, significantly increasing expression of energetic pathways and significantly increasing sexual performance for 2 - 3 days (Kumaran et al. 2014). This effect has been colloquially termed 'The Red Bull Effect' (Kumaran et al. 2014). Although immature males are not attracted to raspberry ketone, Akter et al. (2017) has found that raspberry ketone mixed in the diet of immature male Q-flies yields acceleration in sexual maturation that is a close match to the effects of methoprene (see Adnan et al 2018; Collins et al. 2014). That a compound known to be effective as a stimulant in mature adult Q-flies also accelerates development of immature adult Q-flies raises the more general possibility that other stimulants might provide new products for accelerating development of fruit flies released in SIT programs.

Caffeine is a potent stimulant, affecting diverse taxa including insects (Daly 1993; Alhaider et al. 2010; Penetar et al. 1993 Andretic et al. 2008; Nall et al. 2016; Roehrs and Roth 2008; Nishi et al. 2010). There are several studies that point to possible effects of caffeine in tephritid fruit flies, including effects on mating performance. Arita and Kaneshiro (1988) compared sexual compentitioveness of male *C. capitata* from two populations, one that emerged from Jerusalem cherries (*Solanum pseudocapsicum* L.) and one that emerged from Arabian coffee, (*Coffea arabica* L.). Despite being smaller, the male flies emerging

from coffee vastly outcompeted the male flies emerging from Jerusalem cherries, regardless of which population the females came from. One possible explanation is that exposure to caffiene during the larval stage carried over to the adult stage, then either accelerated development or increased sexual effort. In a more direct study, Aquino et al. (2016) supplemented adult diet of laboratory *C. capitata* with guarana powder, which contains caffiene, and compared mating competitiveness against untreated laboratory and wild flies. Supplements were provided from emergence through to maturity, when mating competitions were staged. Males receiving guarana supplements had a substantial advantage. It remains unclear, however, whether these results are attributable to the caffiene or other compounds in the supplements, or whether the effects were through accleration of development or elevated levels of sexual effort.

In the present study we investigate whether sexual maturation of male Q-flies is accelerated by caffeine supplements from three different sources - analytical caffeine, guarana powder, and instant coffee - incorporated into the adult diet for two days following adult emergence. Because such supplements could increase mating activity without promoting development of reproductive organs (Pérez-Staples et al. 2011), we assess the effects of treatment and age on both mating success and the development of reproductive organs as separate measures of sexual maturation.

Methods

Study insects

Q-flies were obtained as pupae from the Fruit Fly Production Facility at the Elizabeth Macarthur Agricultural Institute, Menangle, New South Wales (NSW), Experimental flies were reared on a Lucerne Chaff larval diet Australia (for production details, see Dominiak et al. 2008). At Macquarie University, all pupae and flies, and experiments, were maintained in controlled environment rooms at 25±0.5°C and 65±5% RH. A L13:D11 h photoperiod was maintained, with flies experiencing a simulated dawn and dusk as the lights ramped up and down through 0.5 h at the beginning and end of the light phase, respectively. To sterilize flies, pupae were irradiated with 65 Gy gamma radiation at Department of Biological Science, Macquarie University, New South Wales, Australia (Collins et al. 2009; Dominiak

et al. 2014). For sterilisation, ca. 4000 pupae were sealed in zip lock plastic bags (100 × 150 mm) two days prior to emergence. The sealed bags were held overnight in a temperature-controlled room (18° C) to so that they were hypoxic for irradiation.

Treatments

Newly emerged adult male Q-flies were treated with the following supplements for 48 hours:

AC: Analytical caffeine + sugar and YH (3:1)
GP: Guarana powder + sugar and YH (3:1)
IC: Instant Coffee + sugar and YH (3:1)
YS: Only sugar and YH (3:1) as food

Analytical caffeine was obtained from Sigma Aldrich Australia (CAS Number 58-08-2), Guarana powder was from My Protein Australia Ltd, and instant coffee was from Moconna classic medium roast (Netherlands). To estimate caffeine content of guarana powder and instant coffee, GC-FID analysis was performed on a Shimadzu GC17A equipped with a split/split less injector, a Restek Rxi-5Ms fused silica capillary column (30 m × 0.25 mm, 0.25 µm film), flame ionization detector (FID) and an AOC-20 auto sampler. Hydrogen gas (BOC, North Ryde, NSW, Australia) (99.999%) was used as a carrier gas with a constant flow of 1.5 mL/min. Caffeine in guarana and instant coffee was extracted by dissolving in boiling water and extracting with dichloromethane (DCM). A weighed amount of guarana powder or instant coffee was added to water (15 mL) in a beaker. The solution was boiled and stirred with magnetic stirrer bar for 15 minutes. The solution was vacuum filtered, and the filtrate was allowed cool to room temperature. The filtrate was extracted with DCM (15 mL × 3) using a separator funnel. The organic layers were combined, dried over anhydrous Na_2SO_4 (Sigma Aldrich), and concentrated under reduced pressure to give 1.0 mL, which was subjected to GC-FID for quantification. A stock solution of caffeine was prepared. Standard solutions of caffeine were prepared by serial dilution of the stock solution. Tridecane (Sigma Aldrich) was used as an internal standard that was incorporated into each standard and sample solutions to give a final concentration of 8.53 µg/mL. The standard and sample solutions were run through GC-FID. The peak area ratios of GC response of caffeine to internal standard in standard solutions were plotted against the concentrations of the standard solutions to obtain a standard curve. The standard curve was used to estimate the

concentration of caffeine and hence the amount in each sample could be calculated in µg/g. Guarana powder and instant coffee used in the analysis contained 5% and 1% caffeine, respectively.

Effect of caffeine supplements on longevity

Approximately 6,000 pupae from a single day of pupation were placed in each of 22 mesh cages (Megaview Bugdorm 44545, 47.5 × 47.5 × 47.5 cm) for adult emergence. Usually few flies emerge on the first day of emergence, and these were discarded. During emergence each cage of flies was provided with water-soaked cotton wool in two 70-ml clear plastic sample container but no food. After the second day of emergence, unemerged pupae were removed from the cages and newly emerged adults (age 0–24 h) were provided a 1:3 mixture of yeast hydrolysate and sugar containing one of seven doses of analytical caffeine (0.0125, 0.025, 0.05, 0.075, 0.1, 0.125, and 0.15%), guarana powder (0.25, 0.50, 1, 1.5, 2 , 2.5, and 3%), instant coffee (1.25, 2.5,5, 7.5, 10, 12.5, and 15%) or a control (0%) for two days *ad libitum*. Based on caffeine quantification, doses from the three sources had equivalent caffeine levels. After two days, five male flies from each treatment were placed in each of five 1.125 L cages that had a mesh-covered window (ca. 28 cm^2) for ventilation (i.e., 25 flies for each treatment). Each cage was provided with water-soaked cotton wool and sugar in separate 35 mm Petri dishes. Flies were checked daily until all had died. Dead flies were removed from the cages daily. Longevity trials were repeated twice, using pupae from two different batches.

Effect of caffeine supplements on mating propensity

To assess the effect of caffeine on mating propensity, the two highest doses that were found to be non-toxic for all caffeine sources in the longevity assay were tested. Approximately 6,000 pupae from a single day of pupation were placed in each of seven mesh cages (Megaview Bugdorm 44545) for adult emergence. Each cage was provided with water-soaked cotton wool in two 70-ml clear plastic sample containers during emergence. The first day of emergence was discarded. After the second day of emergence, newly emerged adults (age 0–24 h) were provided 1:3 mixture of yeast hydrolysate and sugar containing analytical caffeine (0.075, 0.1 %), guarana powder (1.5, 2 %), instant coffee (7.5, 10%) or a control (0%) for next two days. These doses had equivalent caffeine content. After two days, the treated food was replaced with sugar only in a 90 mm Petri dish. The flies were then sorted

223 according to sex within 3 days after emerging by collecting and transferring individual flies
224 in glass tubes to transparent plastic 12-L cages (27.5 x 27.5 x 26.3 cm) that had a mesh-
225 covered ca. 80 cm^2 window for ventilation. Approximately 200 flies were maintained in
226 each 12-L cages (27.5 x 27.5 x 26.3 cm) that cage and were provided continuous access to
227 dry granular sucrose. As a source of water, cages of flies were provided water-soaked cotton
228 wool in a 70-ml clear plastic sample container.
229
230 To obtain mature flies (12-17 days old) to pair with treated flies, ca. 800 pupae from
231 numerous pupation dates were placed in separate mesh cages (Megaview Bugdorm 44545)
232 for adult emergence. Cages were supplied with water-soaked cotton wool two 70-ml sample
233 containers and dry granular sucrose along with yeast hydrolysate (3:1) as food on a 90 mm
234 Petri dish; this diet is effective at supporting Q-fly development (Pérez-Staples et al. 2007;
235 Pérez-Staples et al. 2011; Vijaysegaran et al. 2002). Adult flies were sorted according to sex
236 within 3 days after emerging by collecting and transferring individual flies in glass tubes to
237 clear plastic 12-L cages that had a mesh-covered ca. 80cm^2 window for ventilation.
238 Approximately 200 flies were maintained in each 12-L cage and were provided continuous
239 access to dry granular sucrose along with yeast hydrolysate (3:1). Cages of flies were
240 provided water-soaked cotton wool in a 70-ml clear plastic sample container. No calling,
241 courting, or mating was observed in cages prior to separating the sexes.
242
243 Mating trials were conducted at 4, 6, 8, 10, 12, 15, and 20 days post emergence.
244 Mating in Q-flies takes place only at dusk (Tychsen 1977). On each mating day, at least four
245 hours before the onset of dusk, twenty males from each treatment group were placed
246 individually in clear plastic 1.125 L containers that had a mesh-covered window (ca. 28 cm^2)
247 for ventilation. Each male fly was paired with a sexually mature virgin (12-17 days old)
248 female fly. Periodic observations were carried out from when pairs were set up, and
249 continuous observations began 90 minutes prior to the onset of dusk. To assess copula
250 latency, the time of onset of copulation was recorded for each mating pair. To assess copula
251 duration for each mating pair, observations continued until the last pair had separated.
252 Overall, 140 male flies were tested on each day, providing a total of 980 test pairs across all
253 ages. The experiment was repeated twice using batches of pupae obtained two months apart.
254
255 **Effect of caffeine supplements on reproductive organ development**
256

Application of treatments for assessment of reproductive development was similar to that for assessment of mating propensity (above). To assess the effect of caffeine on male reproductive organ development, we measured area and length of testes and ejaculatory apodeme. Ten males were collected from each treatment at 4, 6, 8, 10, 12, 15, and 20 days post emergence and were dissected in phosphate-buffered saline (PBS; pH 7.4) using fine forceps on a microscope slide under a Leica MZ6 stereomicroscope. Then the dissected ejaculatory apodeme and testes were photographed using a 1.3-megapixel camera (Model-AM4023CT C-Mount camera; Dino-Lite digital microscope, Taiwan) through the phototube of the stereomicroscope. Images were calibrated and measured using Image J (Version1.49, NIH, Maryland, USA). Ejaculatory apodeme length was measured from where the ejaculatory sac joins the apodeme to the farthest point, and area was measured by tracing the outline of the apodeme following Radhakrishnan and Taylor (2008). Length of the testes was measured by tracing a midline through the centre of the organ from the base to the curved tip, and the area of testes was measured by tracing the outline (Radhakrishnan and Taylor 2008). The experiment was repeated twice using batches of pupae obtained two months apart.

Statistical analysis

All analyses were conducted using R v3.5.1 (R Core Team, 2018). Survival of the flies (log-transformed) was assessed using a general linear mixed model (GLMM). Data were not censored and followed a Gaussian distribution (based on model residuals). Separate models were considered for each caffeine supplement with each having control as dose of zero. Dose was included as a categorical variable due to a sharp drop off in survival in two treatments. Replicate was included as a fixed effect and cage identity was included as a random effect. Dunnett's correction was used as Post-hoc.

Mating probability (binary outcome) was assessed using a general linear model with a binomial distribution. For both mating latency and mating duration (square-root transformed) a linear model (Gaussian distribution) was used. Latency was re-defined as the time from 30 minutes post-dusk as the square-root of this variable was normally distributed, but all latency results are back-transformed to the original definition (time to initiate mating). Separate models were run for each caffeine supplement. However, the same control flies were included in each model and defined as a dose of zero. The predictors were the same for each

model: dose (ordinal), age (ordinal), and replicate (nominal). Dunnett's correction was used as Post-hoc.

Area and length (both log-transformed) of ejaculatory apodeme and testis were analysed in separate general linear models (GLM, Gaussian distribution) for each caffeine supplement. As above, the control was included as dose of zero for each treatment. Replicate was also included as fixed effect. Dunnett's correction was used as Post-hoc.

Results

Effect of caffeine supplements on longevity

Analytical caffeine had no significant effect on Q-fly longevity at any tested concentration ($F_{7, 71}$=0.5, P=0.85; Fig 1), whereas the highest two doses of both guarana powder ($F_{7, 71}$=97.5, P<0.001) and instant coffee ($F_{7, 71}$=78.7, P<0.001) significantly reduced longevity (Fig 1).

Effect of caffeine supplements on mating propensity

Mating Probability

For all treatments, age had significant effect on mating probability (Table 1) as the proportion of flies mating increased over the first 8 days of adulthood, and then plateaued (Fig 2). In addition to the effects of age, mating probability was also significantly increased by both doses of analytical caffeine compared with the control (low dose log odds = 0.71±0.2, P = 0.0011; high dose log odds = 0.66±0.2, P = 0.0022; Table 1, Fig 2). No effects were detected for either guarana powder or instant coffee (Table 1, Fig 2).

Table 1. GLM analysis testing fixed effects of age (4, 6, 8, 10, 12, 15, and 20 days post emergence) and treatments (Analytical caffeine, Guarana powder, Instant coffee and Control) on male Q-fly mating probability

Mating latency

Treatment	Variable	*d.f.*	χ^2	*P*
Analytical caffeine	dose	2	12.000	0.0025
	Age	6	202.000	<0.001
	dose:age	12	5.200	0.95
Guarana powder	dose	2	0.100	0.94
	age	6	188.500	<0.001
	dose:age	12	3.500	0.99
Instant coffee	dose	2	0.400	0.84
	age	6	216.700	<0.001
	dose:age	12	3.100	1.00

For all treatments, latency to mate decreased with age until 15 days (Table 2, Fig 3). Flies that received both doses of analytical caffeine had significantly shorter mating latency than untreated flies (low dose $\Delta\beta$ = 0.45±0.12, P <0.001; high dose $\Delta\beta$ = 0.5±0.12, P <0.001; Fig 3). However, flies provided guarana powder and instant coffee had mating latency that was not significantly different from untreated flies.

Mating Duration

For all treatments, mating duration increased with age until 15 days (Table 3, Fig 4). Flies that received both doses of analytical caffeine had significantly longer mating duration than untreated flies (low dose $\Delta\beta$ = 0.69±0.17, P <0.001; high dose $\Delta\beta$ = 0.6±0.17, P <0.001; Fig 4). However, flies provided guarana powder and instant coffee had mating duration that was not significantly different from untreated flies.

Table 2. Linear model analysis testing fixed effects of age (4, 6, 8, 10, 12, 15, and 20 days post emergence) and treatments (Analytical caffeine, Guarana powder, Instant coffee and Control) on male Q-fly mating latency

Treatment	Variable	*d.f.*	*F*	*P*
Analytical caffeine	dose	2, 523	9.600	<0.001
	age	6, 523	4.000	<0.001
	dose:age	12, 523	0.300	0.99
Guarana powder	dose	2, 448	1.500	0.23
	age	6, 448	5.200	<0.001
	dose:age	12, 448	0.500	0.90
Instant coffee	dose	2, 461	2.400	0.09
	age	6, 461	3.000	0.0078
	dose:age	12, 461	1.000	0.47

Table 3. Linear model analysis testing fixed effects of age (4, 6, 8, 10, 12, 15, and 20 days post emergence) and treatments (Analytical caffeine, Guarana powder, Instant coffee and Control) on male Q-fly mating duration

Treatment	Variable	*d.f.*	*F*	*P*
Analytical caffeine	dose	2, 523	8.100	<0.001
	age	6, 523	8.600	<0.001
	dose:age	12, 523	1.400	0.18
Guarana powder	dose	2, 448	1.000	0.37
	age	6, 448	4.400	<0.001
	dose:age	12, 448	0.600	0.80
Instant coffee	dose	2, 461	0.300	0.71
	age	6, 461	3.800	<0.001
	dose:age	12, 461	1.000	0.49

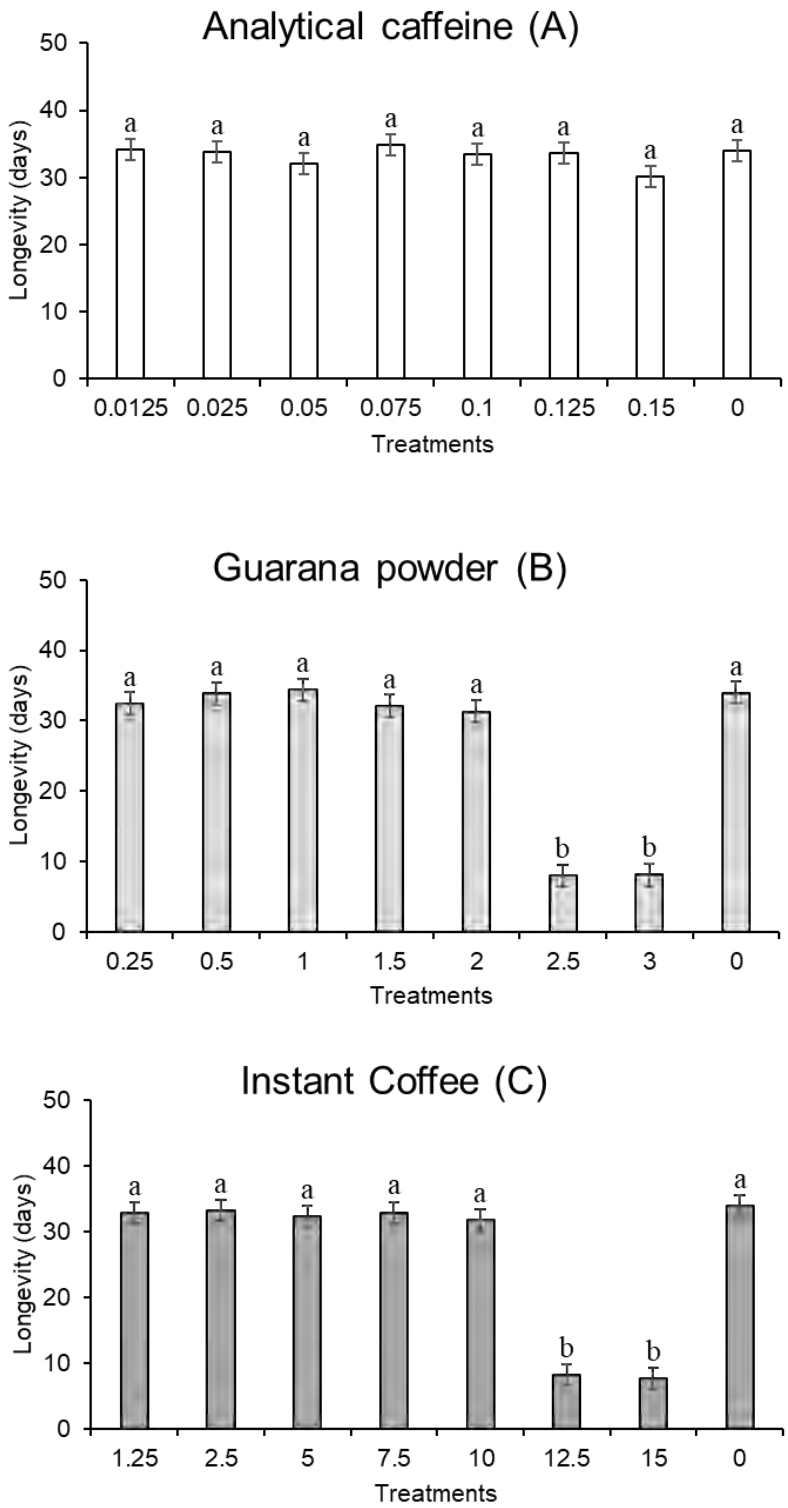


Fig 1. Effects of doses (%) of each supplement (Analytical caffeine, Guarana powder, Instant Coffee and Control) on male Q-fly longevity (mean ± SE). Means with different letter from control are significantly different from control (Dunnett's test, $P < 0.05$).

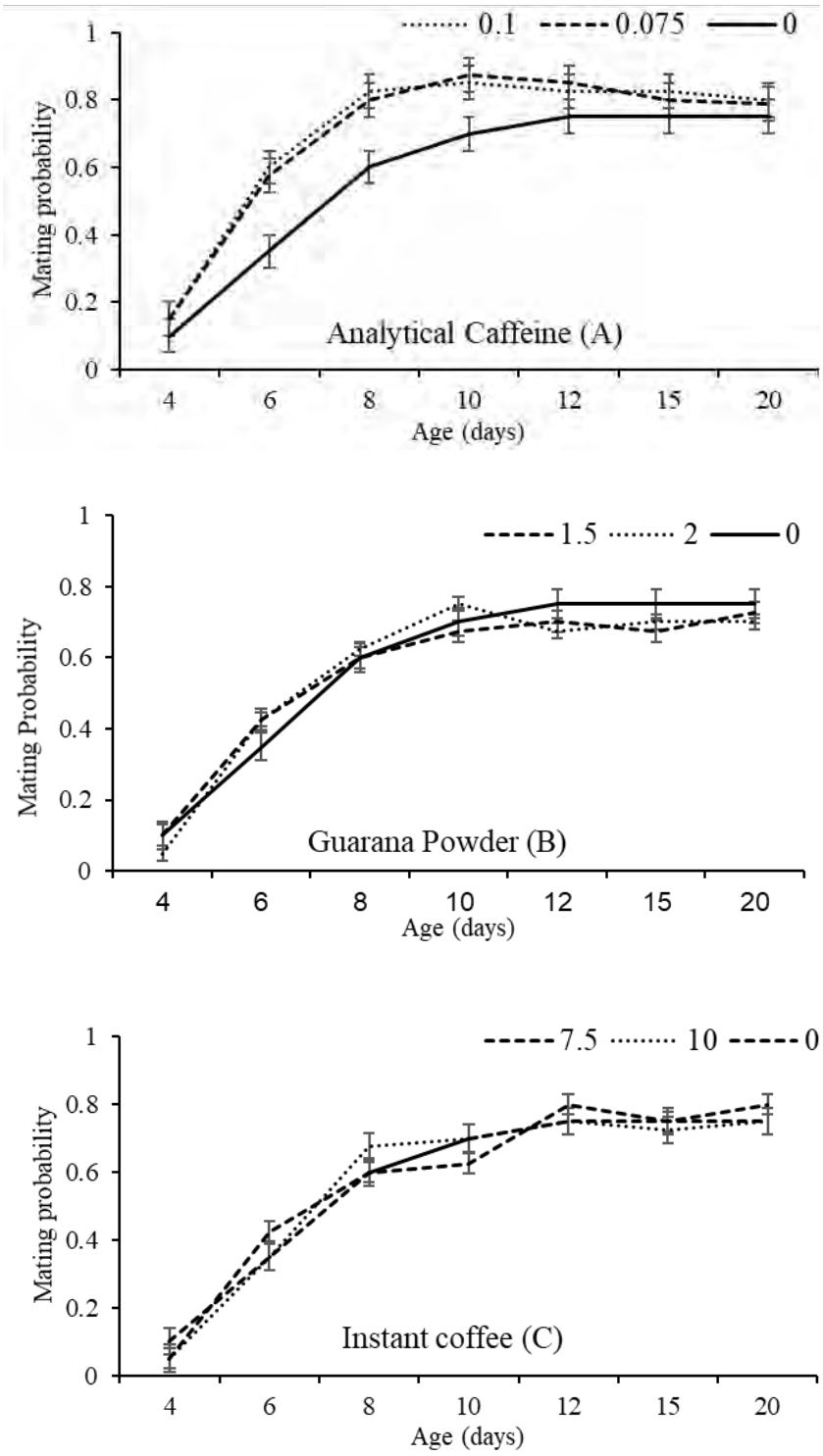


Fig 2. Relationship between age and proportion of male Q-flies mating (mean ± SE) after being fed a diet of sugar plus yeast hydrolysate combined with two doses (%) of each supplement (Analytical caffeine, Guarana powder, Instant coffee and Control) for two days after emergence and then sugar only.

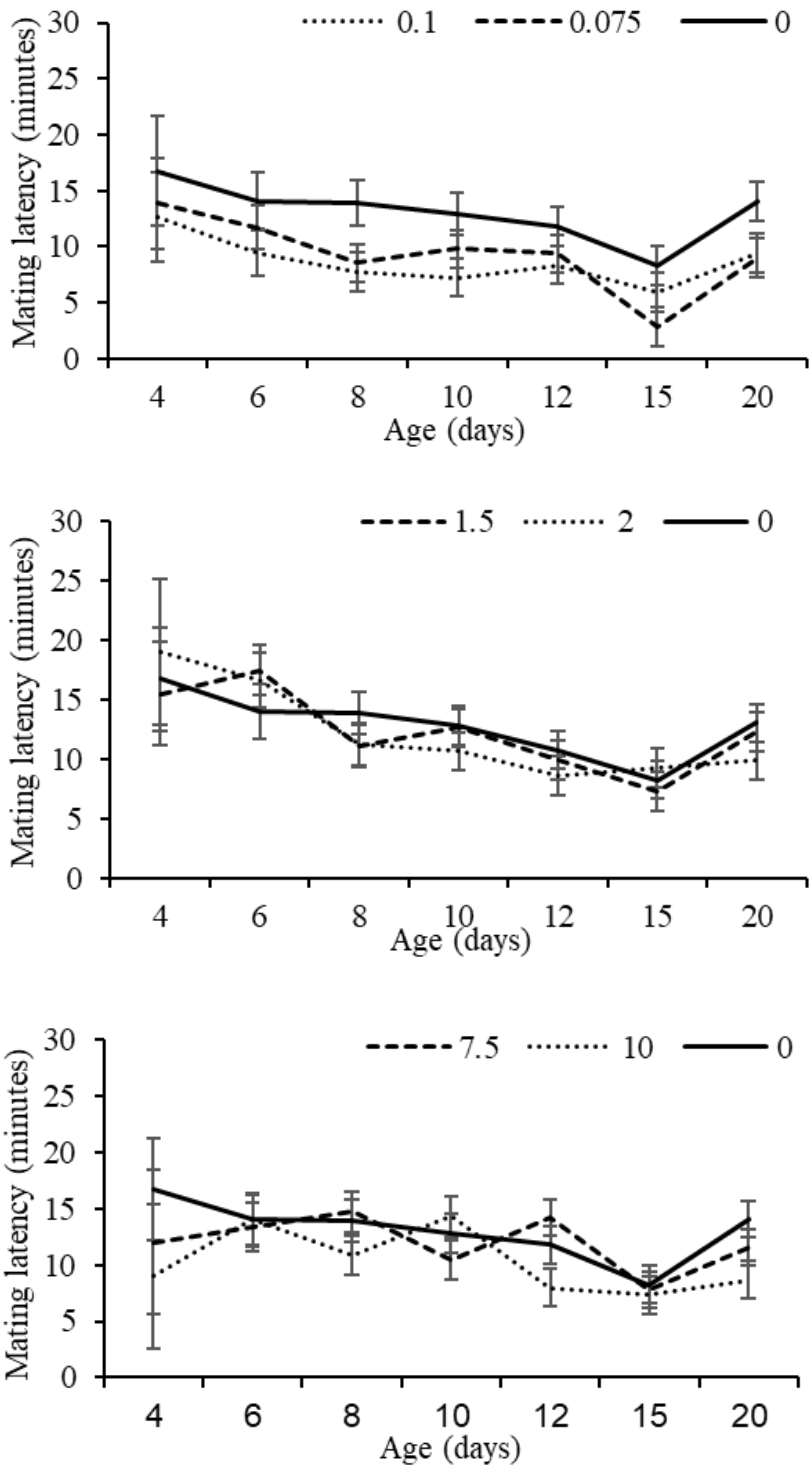


Fig 3. Relationship between age and mating latency (mean ± SE) of male Q-flies mating after being fed a diet of sugar plus yeast hydrolysate combined with two doses (%) of each supplement (Analytical caffeine, Guarana powder, Instant coffee and Control) for two days after emergence and then sugar only.

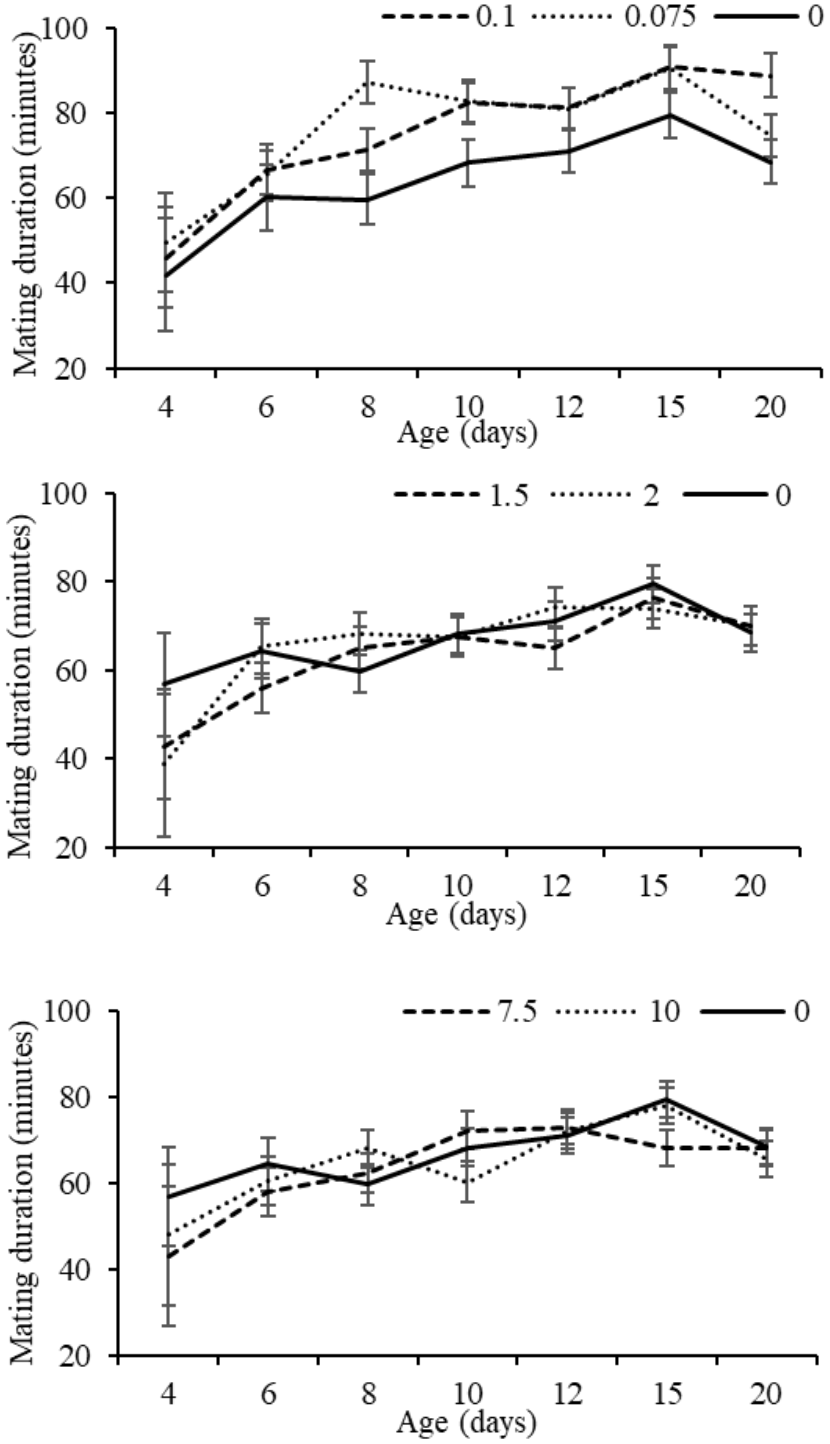


Fig 4. Relationship between age and mating duration (mean ± SE) of male Q-flies mating after being fed a diet of sugar plus yeast hydrolysate combined with two doses (%) of each supplement (Analytical caffeine, Guarana powder, Instant coffee and Control) for two days after emergence and then sugar only.

Effect of caffeine supplements on reproductive organ development

Ejaculatory apodeme

For flies provided analytical caffeine, ejaculatory apodeme length and area varied significantly with age and treatment, with a significant age × dose interaction (Table 4, Fig 5, 6). Ejaculatory apodeme length and area increased with age, but this increase was much steeper at young ages for flies that were provided the two doses of analytical caffeine compared with control flies (Fig 5, 6). For flies, provided instant coffee ejaculatory apodeme length and area increased significantly with age but was not affected by the supplements (Fig 5, 6). For flies provided guarana powder, ejaculatory apodeme length increased significantly with age but was not affected by the supplements while ejaculatory apodeme area varied with a significant age × dose interaction. Flies that were provided guarana powder had reduced apodeme area at 15 and 20 days of age compared to control flies (Fig 6).

Testis

For flies provided analytical caffeine, testis length and area varied significantly with age and treatment, with a significant age × dose interaction (Table 5, Fig 7, 8). Testis length and area increased with age, but this increase was much steeper at young ages for flies that were provided the two doses of analytical caffeine compared with control flies (Fig 7, 8). For flies provided guarana powder and instant coffee, testis length and area increased significantly with age but was not affected by the supplements (Fig 7, 8).

Discussion

Dietary supplements of analytical caffeine significantly increased mating propensity and accelerated reproductive organ development in male Q-flies. These effects were closely comparable to effects of methoprene (Adnan et al. 2018, 2019, in press) and raspberry ketone (Akter et al. 2017) supplements incorporated into the adult diet for two days following emergence. Despite having equivalent caffeine content, guarana powder and instant coffee had little effect on mating behaviour or development.

Table 4. GLM analysis testing fixed effects of age (4, 6, 8, 10, 12, 15, and 20 days post emergence) and treatments (Analytical caffeine, Guarana powder, Instant coffee and Control) on male Q-fly ejaculatory apodeme development (length and area).

Response	Treatment	Variable	*F*	*d.f.*	*P*
Length	Analytical caffeine	dose	21	2, 398	<0.001
		age	156	6, 398	<0.001
		dose:age	6.5	12, 398	<0.001
	Guarana powder	dose	1.2	2, 398	0.30
		age	164.7	6, 398	<0.001
		dose:age	0.3	12, 398	0.98
	Instant coffee	dose	0.4	2, 398	0.69
		age	163.1	6, 398	<0.001
		dose:age	0.1	12, 398	1.00
Area	Analytical caffeine	dose	34.6	2, 398	<0.001
		age	106.9	6, 398	<0.001
		dose:age	3.4	12, 398	<0.001
	Guarana powder	dose	8	2, 398	<0.001
		age	86.3	6, 398	<0.001
		dose:age	3.3	12, 398	<0.001
	Instant coffee	dose	1.6	2, 398	0.21
		age	104.5	6, 398	<0.001
		dose:age	1.3	12, 398	0.23

Table 5. GLM analysis testing fixed effects of age (4, 6, 8, 10, 12, 15, and 20 days post emergence) and treatments (Analytical caffeine, Guarana powder, Instant coffee and Control) on male Q-fly Testis development (length and area).

Response	Treatment	Variable	*F*	*d.f.*	*P*
Length	Analytical caffeine	dose	12.2	2, 398	<0.001
		age	123.4	6, 398	<0.001
		dose:age	2.9	12, 398	<0.001
	Guarana powder	dose	0.6	2, 398	0.58
		age	125.4	6, 398	<0.001
		dose:age	0.4	12, 398	0.97
	Instant coffee	dose	0	2, 398	0.99
		age	131.2	6, 398	<0.001
		dose:age	0.1	12, 398	1.00
Area	Analytical caffeine	dose	13	2, 398	<0.001
		age	36.6	6, 398	<0.001
		dose:age	2.4	12, 398	0.0058
	Guarana powder	dose	0	2, 398	0.97
		age	43.4	6, 398	<0.001
		dose:age	0.1	12, 398	1.00
	Instant coffee	dose	0.1	2, 398	0.89
		age	47	6, 398	<0.001
		dose:age	0.1	12, 398	1.00

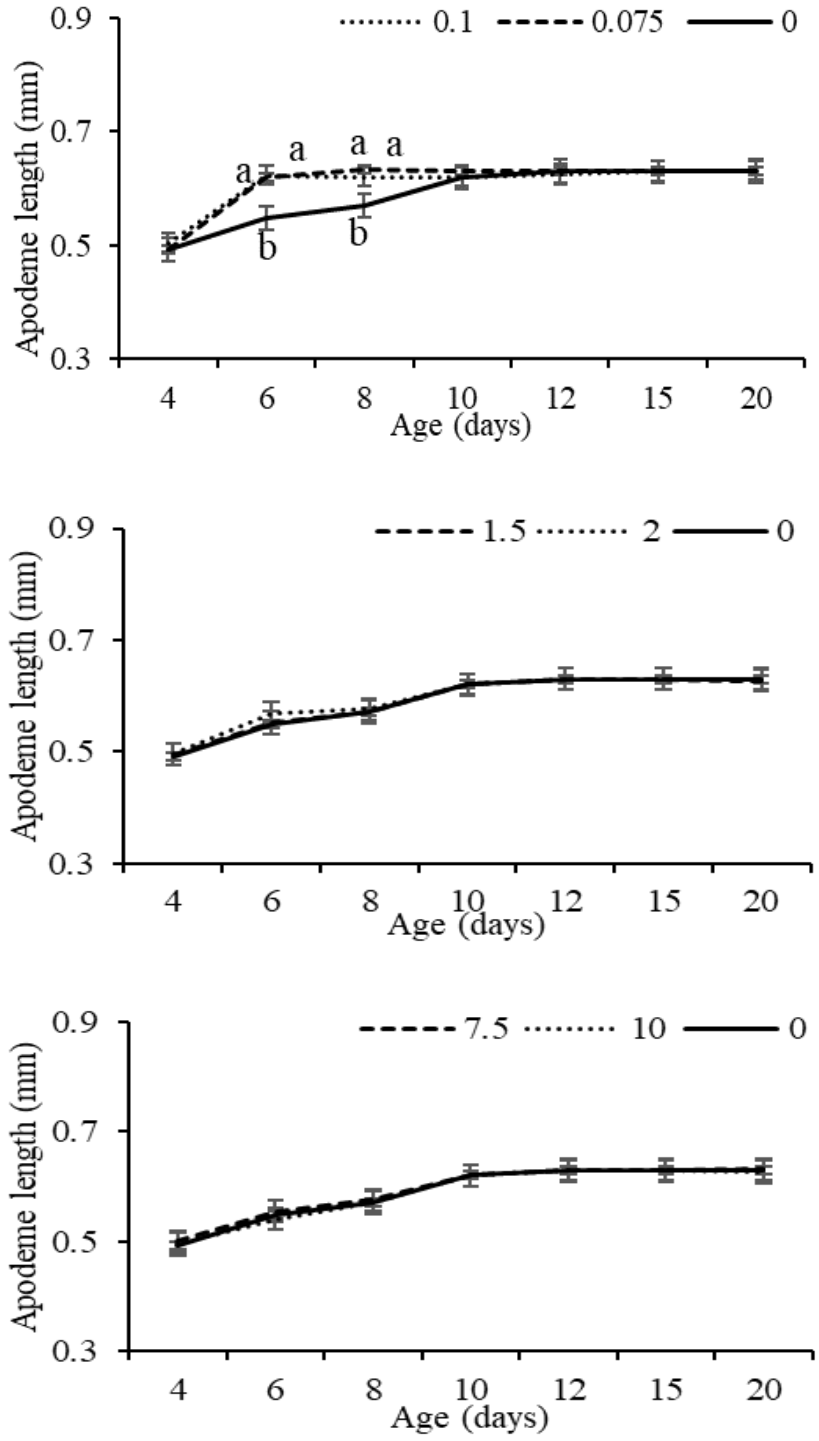
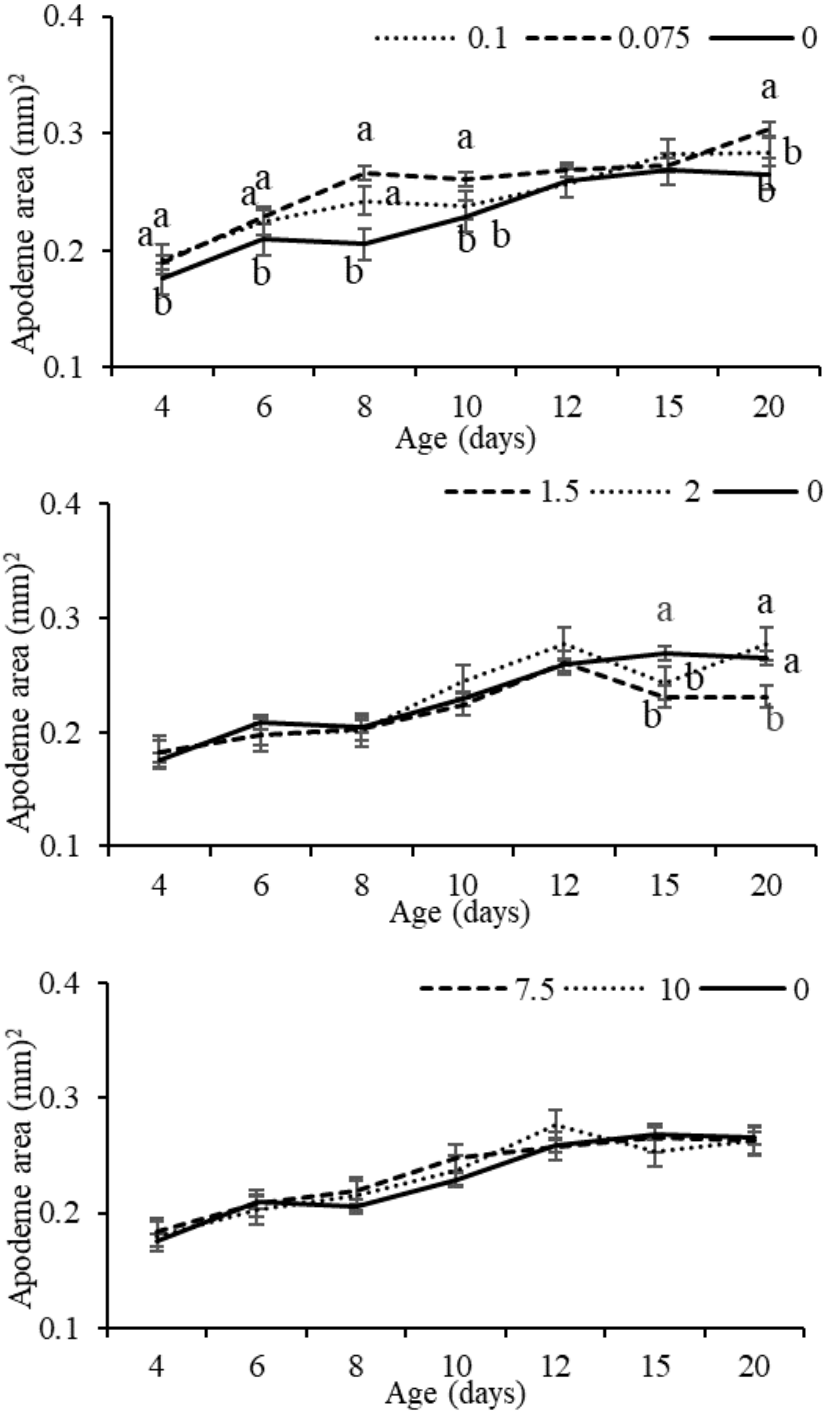
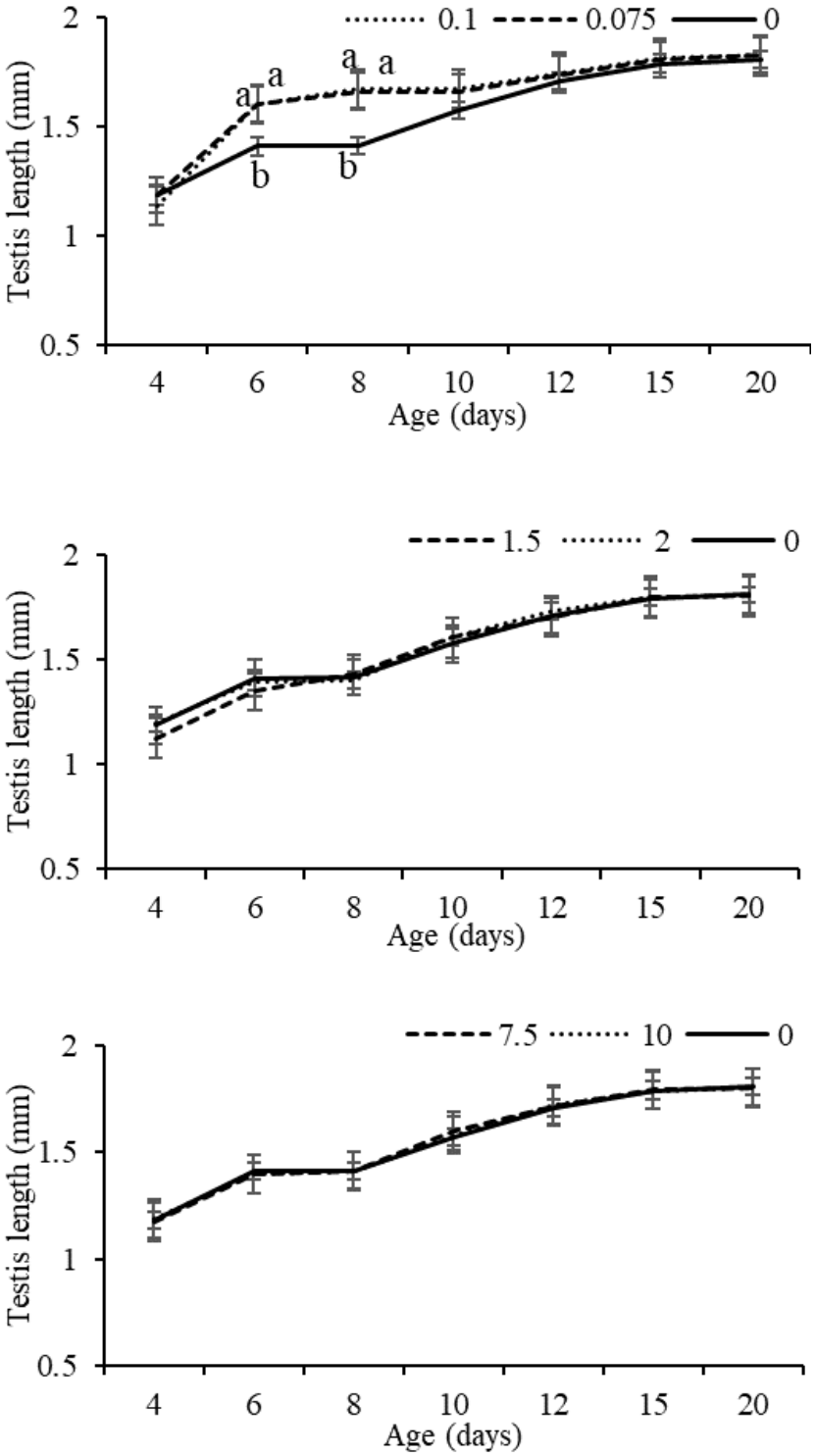


Fig 5. Relationship between age and apodeme length (mean ± SE) of male Q-flies after being fed a diet of sugar plus yeast hydrolysate combined with two doses (%) of each supplement (Analytical caffeine, Guarana powder, Instant coffee and Control) for two days after emergence and then sugar only. Means separated by different letters indicates significant differences between treatment and control at particular ages (Dunnett's test, $P < 0.05$).



402 **Fig 6.** Relationship between age and ejaculatory apodeme area (mean ± SE) of male Q-flies
403 after being fed a diet of sugar plus yeast hydrolysate combined with two doses (%) of each
404 supplement (Analytical caffeine, Guarana powder, Instant coffee and Control) for two days
405 after emergence and then sugar only. Means separated by different letters indicates
406 significant differences between treatment and control at particular ages (Dunnett's test, $P <$
407 0.05).



408 **Fig 7.** Relationship between age and testis length (mean ± SE) of male Q-flies after being fed
409 a diet of sugar plus yeast hydrolysate combined with two doses (%) of each supplement
410 (Analytical caffeine, Guarana powder, Instant coffee and Control) for two days after
411 emergence and then sugar only. Means separated by different letters indicates significant
412 differences between treatment and control at particular ages (Dunnett's test, $P < 0.05$).

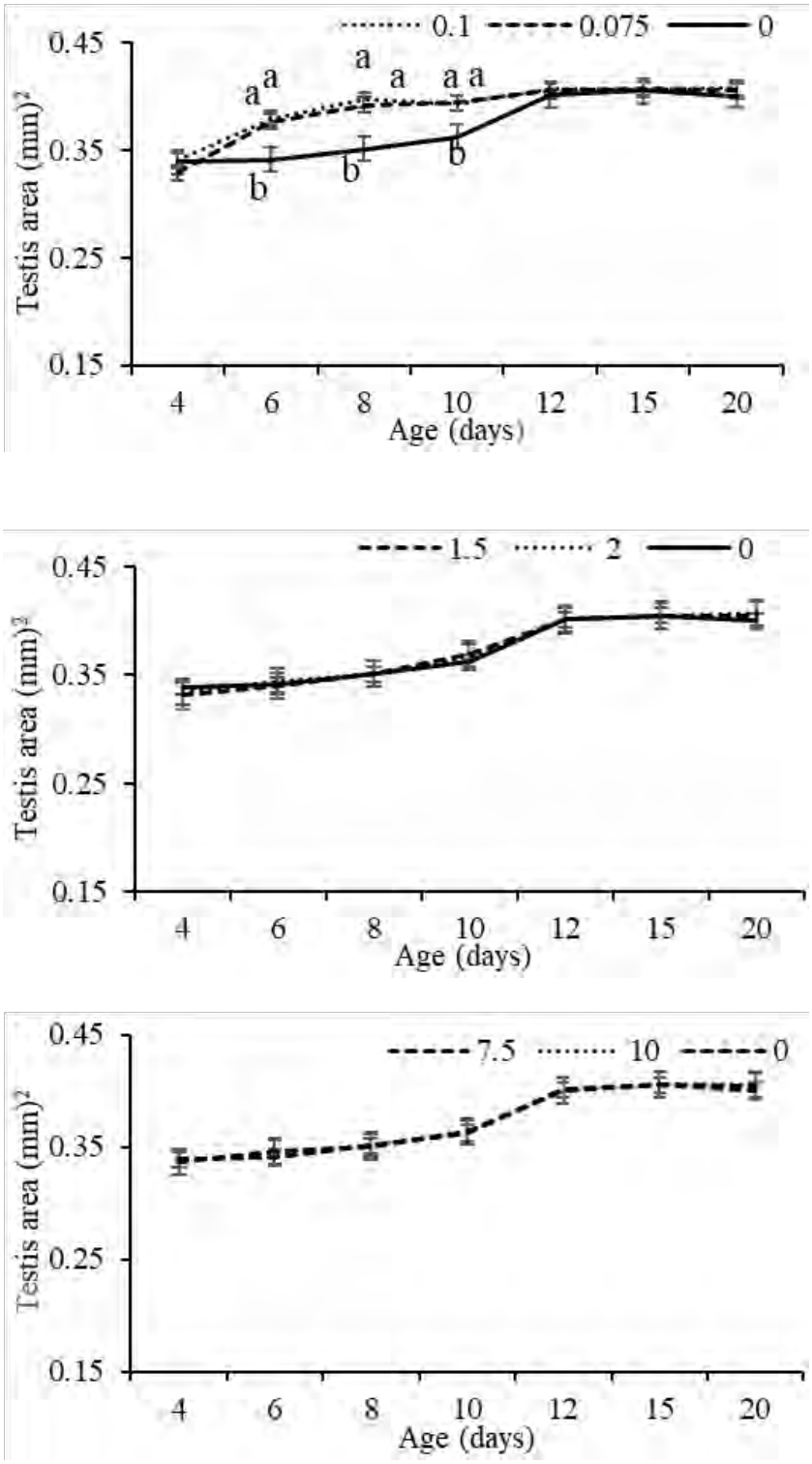


Fig 8. Relationship between age and testis area (mean ± SE) of male Q-flies after being fed a diet of sugar plus yeast hydrolysate combined with two doses (%) of each supplement (Analytical caffeine, Guarana powder, Instant coffee and Control) for two days after emergence and then sugar only. Means separated by different letters indicates significant differences between treatment and control at particular ages (Dunnett's test, $P < 0.05$).

Being a metabolic enhancer caffeine likely activates metabolic pathways in immature male Q-flies, and by this mechanism accelerates reproductive development. Caffeine is the most popular psychoactive drug in the world owing to its stimulatory properties (Alhaider et al. 2010; Penetar et al. 1993). In addition to the familiar effects on humans, caffeine also has potent effects on invertebrates such as increased spontaneous neural activity and increased locomotor activity (Kucharski and Maleszka 2002; Daly 1993). While in invertebrates caffeine influences energy reserves and metabolic activity (Cruz et al. 2016), in vertebrates caffeine mostly acts on the central nervous system (Nehlig et al. 1993). Sequentially it mobilizes intracellular calcium, inhibits specific phosphodiesterase, and most importantly it induces differential responses on the action of the methylxanthine on serotonin neurons. These responses are found to translate into altered locomotion, learning, memory, performance and coordination. Furthermore, in vertebrates caffeine promotes energy metabolism in the brain and decreases cerebral blood flow, generating a relative brain hypo perfusion. However, in *Drosophila* Nall et al. (2016) postulated that dopamine mediated the wake-promoting effect of caffeine, and that caffeine likely acts pre-synaptically to increase dopamine signalling. A cluster of neurons, the paired anterior medial (PAM) cluster of dopaminergic neurons exhibits increased activity as a result of caffeine administration. Dopamine has been reported to increase behavioural activity (locomotion, sexual behaviour, and pheromone production or secretion) in *Drosophila melanogaster* (Kume et al. 2005; Meehan and Wilson 1987; Pendleton et al. 2000; Pendleton et al. 2002; Wicker-Thomas and Hamann 2008), to mediate aggressive encounter, flying and fighting ability in crickets (Adamo et al. 1995; Stevenson et al. 2000), to increase motor response in worker bees (Božič and Woodring 1998; Menzel et al. 1999) and queen bees (Harano et al. 2005; Harano et al. 2008), and to enhance escape behaviour in cockroaches (Casagrand and Ritzmann 1992; Goldstein and Camhi 1991). Therefore, accelerated sexual maturation in caffeine-supplemented Q-fly might be related to increased dopamine signalling. Further studies are required to address the changes in biogenic amines following caffeine treatment.

In addition to increased mating propensity, young analytical caffeine-supplemented flies had shorter mating latency. It might be that shorter mating latency is simply a behavioural expression of greater sexual vigour and courtship effort. Shorter latency to initiate mating displayed by analytical caffeine-supplemented males corresponds with similar effect associated with methoprene supplements in Q-fly (Adnan et al. 2019) and also in melon fly *Bactrocera cucurbitae* (Haq et al 2010a). Additionally, analytical caffeine-

supplemented flies had longer mating duration, which also resembles the effect of dietary methoprene supplements (Adnan et al. 2018, 2019; Haq et al. 2010a). The implications of longer mating duration for SIT is not straightforward. While there is little evidence of links between between mating duration and quantity of sperm storage (Harmer et al. 2006; Pérez-Staples et al. 2007; Radhakrishnan et al. 2009; Collins et al. 2012), postcopulatory success might still be promoted by longer copulation. Longer copulations by sterile males provide higher levels of infertility in females that had previously secured mating with a fertile male or later remate with a fertile male, which is positive post-mating outcome for SIT (Collins et al. 2012).

The increase in mating propensity of analytical caffeine-supplemented flies corresponded with accelerated development of the reproductive organs (ejaculatory apodeme and testes). This pattern confirms that the effects of analytical caffeine are not solely behavioural, but that the expression of mating behaviour accords with development of the reproductive system. This is important, because if analytical caffeine only affected mating behaviour without also affecting morphological development then the matings of young caffeine treated males might be ineffective. *Bactrocera tryoni* can be sexually active and start mating before their reproductive organs are at full size (Pérez-Staples et al., 2011). Therefore, there is a possibility that expression of mating behaviour at earlier stages of reproductive development without the maturation of reproductive tissues in turn might result in nonfunctional copulations. While there is still a need to directly assess remating tendency of females mated by analytical caffeine-supplemented males, the alignment of age-dependent mating propensity with age-dependent development of reproductive organs is encouraging. Those treatments that did not result in increased mating propensity also did not result in accelerated development of reproductive organs, and this further substantiates the links between development of reproductive organs and expression of mating behaviour.

Regardless of dose, analytical caffeine did not affect the longevity of Q-flies. On the other hand, the highest two doses of guarana powder and instant coffee resulted in substantially reduced longevity. Caffeine is the most known component of roasted coffee beans, varying between 0.8% and 4.0% (w/w) depending on variety (Belitz et al. 2009). However, coffee beans also contain a large diversity of compounds, including carbohydrates (38.0-41.5%), lipids (11-17.0 %), protein (10.0%), trigonelline, niacin (1.0 %), aliphatic acids (2.4-2.5%), chlorogenic acids (2.7-3.1%), minerals (4.5-4.7%),

melanoidinsc/brown compounds (23%) (Belitz et al. 2009; Lima 2003; Trugo 2003). In addition to a high percentage (2.5–6%) of caffeine (1,3,7-tri-methylxanthine) (Heckman et al. 2010), guarana seeds also contain starch (60.88%), tannin (9.6%), protein (8.56%), Total Soluble Sugar (7.97%), reducing sugars (4.89%), as well as smaller proportions of the purine alkaloids theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine). Guarana seeds also contain a high concentration of polyphenols, particularly proanthocyanidins with a higher prevalence of catechins and epicatechins (Marx, 1990; Basile et al. 2005; Ushirobira et al. 2007; Yamaguti-Sasaki et al., 2007). Some of the diverse compounds in guarana powder and instant coffee might be toxic when applied at higher tested doses, which would explain the reduction in Q-fly longevity. Despite having similar caffeine content, guarana powder and instant coffee did not affect sexual development and this may reflect sub-lethal effects of constituents other than caffeine. Overall, the absence of negative effects of analytical caffeine supplements on longevity are promising for use as a potential pre-release supplement for Q-fly SIT. In addition, deploying analytical standard caffeine as pre-release supplement that is economical as Nomoz offers significant economic advantages in operational SIT.

Conclusion

Given the positive effect of caffeine on Q-fly reproductive development and lack of effects on longevity, caffeine has potential value as pre-release supplement to increase efficacy of SIT programs. Q-fly SIT programs commonly release immature flies and pre-release caffeine supplements might decrease the delay to maturation following release and thereby increase the number of flies that survive to maturity and participate in mating with pest populations.

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Chapter Ten

Image captured by author only

General Conclusions

10.1 General Conclusions

In response to regulatory restrictions on organophosphate insecticides (Dominiak and Ekman, 2013; PBCRC, 2015), and increasing threat to the major southern fruit-producing regions and the remaining fruit fly-free regions in Australia, there is substantial interest and investment in development of SIT as a sustainable fruit fly management practice (Clarke et al., 2011; Stringer et al., 2017). SIT has a long history for management of Q-fly in Australia following experimental trials dating back to the 1960s (Monro, 1961; Monro and Osborn, 1967) and it has been used on a limited scale for more than 20 years to eradicate populations in Q-fly-free areas (Dominiak et al., 2003; Jessup et al., 2007). Currently substantial efforts are underway to renew, refine, and upscale Q-fly SIT, so that SIT can be more widely and intensively applied to protect fruit fly free regions from Q-fly outbreaks, as well as to reduce pest populations in endemic areas. Efficacy of SIT relies on the post release performance of sterile flies (e.g., survival, maturation mating, sterility induction, dispersal). Sterile Q-flies have usually been released when sexually immature and may take a week or longer to mature (Weldon et al., 2008). Accordingly, because of high mortality during the adult maturation phase a large proportion of released sterile flies may not contribute to SIT. Under this circumstance, pre-release nutritional resources and other treatments that promote survival or development can play a crucial part in improving the efficacy of SIT (Pereira et al., 2013b; Akter et al., 2017). With the overarching goal of improving the performance of sterile Q-flies released in SIT programs, my PhD comprised a series of experimental trials in laboratory as well as in outdoor field-like conditions to investigate the potential of methoprene, a juvenile hormone analogue, and caffeine as means of accelerating sexual maturation of Q-flies released in SIT programs. To provide a balanced perspective, my focus was on both sexual performance and ecological fitness.

Because of the physiological role juvenile hormone plays in insect sexual maturation and development of reproductive tissues (Wyatt and Davey, 1996; Gilbert et al., 2000; Wilson et al., 2003), methoprene has been investigated and so far, found to promote reproductive development of adult fruit flies for use in SIT programs (Teal et al., 2000, 2013). Provision of methoprene to fruit flies receiving a diet of YH and sugar has been found to accelerate sexual maturation in numerous fruit flies (Pereira et al., 2009, 2010; Segura et al., 2009, 2013; Haq et al., 2010a, b; Abraham et al., 2013; Gómez et al., 2013; Liendo et al.,

2013; Pereira et al., 2013a; Gómez-Simuta et al., 2017), including Q-fly (Collins et al., 2014). Early studies applied methoprene in acetone solution to adults or pupae, but this approach is not practical in a factory setting (Pereira et al., 2013a; Segura et al., 2013) and may reduce fly quality (Collins et al., 2014). Several studies have found dietary administration of methoprene to be effective (Teal et al., 2007; Aluja et al., 2009; Gómez et al., 2013; Gómez-Simuta et al., 2017). Following on from these studies of other species, and also employing a much cheaper source of methoprene than has been used previously, in my second chapter we added mosquito larvicide NOMOZ® (contains 40 % S-methoprene), into Q-fly adult diet of sugar only or sugar mixed with yeast hydrolysate for two days, the usual pre-release holding period in Q-fly SIT. While dietary incorporation of methoprene was found to promote sexual maturation in males, there was no evidence of a parallel effect in females. The only common effect of methoprene treatment in both sexes was increased mating duration. Methoprene treatment did not affect longevity of Q-fly. The positive effects of dietary methoprene treatment on sexual maturation, and lack of negative effects on longevity, in chapter two (Adnan et al., 2018) provided an encouraging platform to further explore the broader effects of methoprene treatment in Q-fly.

Studies in chapter two investigated effects of dietary treatment with NOMOZ® on sexual development of fertile Q-fly, effects on sterile flies those might be deployed in SIT programs also needed to be explored. As irradiation used to induce sterility can have deleterious effects on physiology and behaviour of Q-fly (Weldon, 2005; Mankin et al., 2008; Collins et al., 2009; Weldon et al., 2010), there is a risk that the promising effects of methoprene on fertile flies may not be evident in sterile flies. While we found positive effects of dietary NOMOZ® treatment on maturation of males but not of females (Adnan et al., 2018), in previous studies topical application of analytical standard methoprene promoted sexual development in both males and females (Collins et al., 2014). This raises several key questions needed to be addressed: (1) do methoprene from NOMOZ® and analytical standard perform differently in accelerating sexual maturation in both sexes of Q-fly; and (2) are effects of methoprene similar for fertile and sterile flies. In chapter three, I carried out mating and longevity trials to investigate these questions. Fertile and sterile male Q-flies that were provided diets containing methoprene from either source exhibited substantially accelerated development and longer mating duration compared to untreated flies. Unlike males (Adnan et al., 2018), females did not respond to dietary methoprene treatment irrespective of source. Longevity was not affected by either methoprene treatments or sterilisation. Even though

analytical standard methoprene and NOMOZ® are similarly effective at accelerating sexual development of sterile males, the present study suggests that methoprene from NOMOZ® is a more suitable option for operational SIT because of both cost savings and lack of need for the use of volatile, toxic organic solvents.

Results of chapters 2 and 3 confirm the effects of dietary methoprene treatment on sexual maturation, and is consistent with studies of other species (Pereira et al., 2009, 2013; Segura et al., 2009, 2013; Haq et al., 2010a; Gómez et al., 2013; Collins et al., 2014). However, this elevation in mating propensity might not be accompanied by maturation of reproductive organs as rate of sexual maturation and reproductive organ development in Q-fly are not identical (Pérez-Staples et al., 2011). If methoprene treatment only advances expression of mating behavior at young ages without promoting maturation of reproductive tissues, earlier matings might not be effective at inducing sexual inhibition in wild populations. Also, determining the morphological development of reproductive organs provides better understanding on initiation and rate of sexual maturation in flies which has practical importance in SIT. Therefore, it is imperative to investigate if behavioural changes are supported by morphological development. In chapter four, I investigated the effect of dietary methoprene treatment on the development of testes, ejaculatory apodeme, and accessory glands in male Q-fly and ovaries in females. Methoprene treatment accelerated development of testes and ejaculatory apodeme, but not in accessory glands in males. Similar to mating trials, methoprene application did not affect ovarian maturation in females. The strong positive effect of methoprene on male reproductive development together with no effect on female development reported in chapter four further supports pre-release methoprene treatment in Q-fly SIT.

Pronounced effects of dietary methoprene treatment on sexual maturation and reproductive development of male Q-flies in laboratory trials (chapter two, three and four) indicate significant potential to increase the efficacy of SIT. However, it is also important to confirm the promising effects of laboratory trials under more natural conditions. Accordingly, in chapter five, I investigated mating competitiveness of young methoprene-treated males against mature laboratory or wild males for matings with untreated mature laboratory or wild females respectively under field cage conditions. Because Q-fly are being currently released as bisex strains in SIT programs, sexual compatibility between treated and untreated sterile flies with mature flies from laboratory strains or from wild populations were also

investigated. Five or seven days old methoprene-treated sterile males outperformed untreated mature males for copulation with mature females, while same aged untreated sterile males were far less competitive. In mating compatibility trials, methoprene treatment provided divergent effects on males and females as was also found in laboratory trials. Overall, methoprene-treated males mated irrespective of types of females, whereas methoprene-treated females tended to mate more often with untreated mature males. Untreated flies of both sexes mated more often with untreated mature flies. The male biased effects of methoprene treatment on sexual maturation may allow a bisex strain to perform functionally as a single sex release, at least in the days immediately following release (Segura et al., 2013).

The effectiveness of SIT depends not only on the ability of sterile males to secure matings with wild females but also on mating induced sexual inhibition in wild populations. Ability of males to inhibit sexual receptivity in females is crucial for successful SIT. While methoprene-treated males might secure more mates, this would be of little value if these matings are not effective at inducing remating inhibition (Abraham et al., 2013; Haq et al., 2013; Gómez et al., 2017). In chapter six, I investigated efficacy of matings by methoprene-treated males at inducing remating inhibition in their mates. Methoprene treatment again increased mating propensity of young males, and the additional matings secured by treated males were fully effective at inducing sexual inhibition in mated females. Remating tendency in females was found to be higher only if their first mate was of either young or old ages, and this did not vary with whether first male was methoprene treated. In remating trials, females mated by methoprene-treated males took longer to initiate remating and remated for shorter duration compared to females mated by untreated males. However, the present study did not directly assess the ability of methoprene-treated males in inducing sterility in terms of female egg fertility, and did not include any remating trials in outdoor conditions. These are natural extensions of the work that could be addressed in future studies. The undiminished ability of young methoprene-treated males to induce sexual inhibition in their mates encourages pre-release dietary methoprene treatment for Q-fly SIT.

Given the link of locomotor activity with other behavioural activities including courtship (Cobb et al., 1987; Greenspan and Ferveur, 2000), foraging and dispersal (Martin et al., 1999), predator avoidance (Heath and Wilkin, 1970; Simmons, 1988; Lang et al., 2006), and as an indication of energy reserves or metabolic condition (Catterson et al., 2010),

investigation of locomotor activity can provide useful insights to the broader effects of methoprene treatment on Q-fly physiology. There are no previous studies of links between methoprene treatment and locomotor activity in any tephritid fruit fly. In chapter seven, I investigated whether dietary methoprene treatment or sterilisation affects locomotor activity of mass-reared Q-flies. In addition, I tried to understand if exposure to stress condition influences the locomotor activity in methoprene treated flies. Methoprene treatment substantially increased activity in young Q-flies, and this elevation in activity level was consistent throughout the 4-day trial period. While dietary methoprene treatment does not affect sexual development of females (chapters two, three, four, and five), the effect of methoprene treatment on locomotor activity was greater in females than in males. Irradiation did not alter activity level of mass reared flies in the present study, and this result contrasts previous studies of Q-fly (Weldon et al., 2010). Greater activity in sterile flies might have positive effects in SIT, including sexual activity, foraging, predator evasion, and dispersal. Finally, locomotor activity was greater for methoprene treated flies when they were subjected to food and water stress and desiccation condition. Building on the findings of chapter six, there would be particular value in future studies of dispersal and survival in field contexts. The results of chapter six suggest that released methoprene-treated flies might disperse more rapidly but might also be more prone to exhausting nutritional reserves owing to apparent accelerated metabolism. This second possibility has been explored in the laboratory context in chapter eight.

Dietary methoprene treatment significantly accelerates sexual development and increases activity level, but an associated increase in metabolism might render treated flies more vulnerable to food deprivation and desiccation stress. This possibility was investigated in chapter eight. When water and food were available, methoprene-treated flies had survival similar to untreated flies but had significantly reduced survival when exposed to food deprivation and desiccating conditions. Previous studies have focused very much on the potential positive aspects of pre-release treatments, including methoprene, on mating performance of sterile flies targeted for SIT, potential negative effects such as increased vulnerability to environmental stress has been largely overlooked. In addition to investigating the effect of methoprene on survival under stress, I also explored the role of metabolic reserves in flies exposed to nutritional and hydric challenges. Flies that were exposed to food and water deprivation primarily relied on lipid utilization, while greater water loss was evident for flies that died from desiccation stress. Future research might seek understanding

of mechanisms underlying the environmental vulnerability of methoprene-treated flies, especially under desiccation stress. For example, the effects of methoprene treatment on cuticular hydrocarbon profile would be an interesting potential mechanism to explore, as would effects on metabolic rate and energy expenditure patterns through respirometry. Overall, vulnerability of methoprene-treated flies to environmental stress suggests that methoprene treatment might need to be avoided during the hottest and driest periods of each season and so may be more effective overall in spring and autumn releases. In all of the studies to date, no lower limit to methoprene concentrations has been identified for positive effects on development of male Q-flies. It may be that there is a lower rate that might be used to yield positive effects on sexual development without increased vulnerability to starvation and desiccation stress.

Caffeine is mostly known for its potential stimulatory effect on both invertebrates and vertebrates. In addition, caffeine mediates faster sexual maturation in tephritids (Arita and Kaneshiro, 1988; Aquino et al., 2016). It might be possible that metabolic boost provided by caffeine can be translated into elevated expression of sexual effort in insects comparable to the effects rendered by methoprene (Adnan et al., 2018) and raspberry ketone (Akter et al., 2017) in Q-fly. Based on this possibility, in addition to investigating promise of methoprene as pre-release supplement, potential of caffeine supplements as a novel means to accelerate sexual maturation in Q-fly has been demonstrated in chapter nine. First of all, longevity assays were carried out using male Q-flies provided a series of doses from three caffeine sources - analytical caffeine, guarana powder and instant coffee to find out the doses non-toxic to flies. Thereafter, I investigated whether caffeine supplements from three different sources incorporated into the adult diet for two days following adult emergence can accelerate sexual maturation and promote reproductive organ development of male Q-flies. Dietary provision of analytical caffeine to newly emerged Q-flies was associated with accelerated mating propensity, shorter mating latency and longer mating in males. Matching effects for sexual activity exhibited by analytical caffeine supplemented males were reflected in development of reproductive organs, as flies provided analytical caffeine supplements had faster growth of testis and ejaculatory apodemes than those of untreated flies. However, such positive effects of guarana powder and instant coffee supplements were not evident either on sexual maturation or on morphological development of reproductive tissues of male Q-fly. Overall, Pre-release caffeine supplements provide a new approach to promote sexual

maturation in Q-flies released in SIT, and may provide similar benefits for other fruit fly species with longer adult maturation periods.

Most of the studies in my PhD have investigated the effects of methoprene and caffeine treatment on sexual development, with mating probability as the primary index. It would be valuable in future to refine this to assess the influence of these treatments on other sexual traits such calling performance and pheromone emission. However, Further investigations are required to assess the effect of caffeine supplementation on mating competitiveness and mating compatibility under field cage condition as well as on female sexual maturation before deploying caffeine as pre-release supplement in operational SIT. Furthermore, it would be very interesting to investigate effects of methoprene and caffeine at a molecular level, such as modified expression of proteomic and transcriptomic profiles. Finally, accelerated metabolic effect of methoprene and caffeine may have consequences for nutrition and dietary preferences, as flies may need to adjust intake to compensate for changing demands. For example, the need for carbohydrates as an energy source might increase, as might need for protein as building blocks for development (as well as for energy) in treated flies. The overall effect may be an increase in dietary intake, but this may or may not be balanced between carbohydrate and protein. Dietary intake assays might provide insight to the physiological effects of methoprene and caffeine treatment on nutrition acquisition that would inform about likely post release foraging patterns of flies released in SIT as well as dietary balance that should be provided prior to release.

Overall, my thesis provides numerous and diverse findings to address its principal aim of investigating the viability of methoprene and caffeine as pre-release treatments for Q-fly SIT. Dietary methoprene treatment is a viable alternative to topical treatment in acetone or other solvents. The efficacy of a cheap insecticide NOMOZ® is comparable to analytical standard methoprene, providing an economic means of promoting sexual maturation in sterile male Q-flies. It is expected that dietary application of NOMOZ®, or similar products, would also be effective in other fruit flies and given the potential benefits to other SIT programs this is an important line of inquiry to be followed. While studies in other species are mostly limited to effect of methoprene on sexual development of sterile flies, our studies provide insight to the effects of methoprene on other aspects of Q-fly biology and this is important to assess the overall net merits of pre-release methoprene-treatment in Q-fly SIT. Studies of other fruit fly species would get benefit greatly from the broader approach that I have taken,

considering both the benefits and the risks. In summary, methoprene and caffeine treatment show significant potential as means of increasing the efficacy of Q-fly SIT by accelerating sexual development of male Q-flies, but this potential is tempered by potential risks of elevated vulnerability to environmental stress. My thesis provides numerous starting points for future detailed physiological research of mechanisms underlying the effects of methoprene and caffeine treatment in Q-flies and other tephritid fruit flies that are targeted for control using SIT.

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