Raspberry ketone as a promising pre-release supplement for the Sterile Insect Technique (SIT) of Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae)

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Declaration of originality

This is to certify that this thesis paper, entitled '**Raspberry ketone as a promising pre-release supplement for the Sterile Insect Technique (SIT) of Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae)**' includes all of my original research work that I did during my PhD candidature and has not been submitted previously to fulfill other degree award; it is only being submitted to Macquarie University.

I also certify that this thesis is written by me and to accomplish this PhD research all experimental designs were developed by me with the support of my primary supervisor Professor Phillip W. Taylor. I collected all data by myself and any help and assistance that I received in my research work have been acknowledged appropriately.

I also certify that all literature and other information sources used in this thesis are acknowledged properly. All photos used in this thesis were captured by me except Qfly distribution map which is acknowledged properly.

This thesis did not need any ethical approval by Macquarie University as organism that was studied is an insect, Queensland fruit fly, *Bactrocera tryoni*.

Humayra Akter (Student ID 43393489)

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General Abstract

Queensland fruit fly, *Bactrocera tryoni* Froggatt (Qfly) (Tephritidae), Australia's most economically damaging fruit fly, infests more than 100 hosts including many commercially important crops. Control of this pest is based on insecticides, bait sprays, male annihilation technique (MAT), and in some regions sterile insect technique (SIT). SIT can be a highly effective control method, and is used around the world to control some of the worst fruit fly pests, but high field mortality of the sterile flies can constrain the success of Qfly SIT. Improved quality of released males and acceleration of sexual maturation can help to overcome this problem. In this thesis I investigated potential beneficial effects of supplementing immature Qflies with raspberry ketone (RK), a metabolic enhancer. RK was mixed with the diet for 48 hours, a duration consistent with standard pre-release holding periods. When RK was provided together with yeast hydrolysate (YH), RK-fed males exhibited accelerated development of reproductive organs and increased mating propensity when 6 - 9 days of age. Positive effects of RK were not evident in males that were fed only sugar, and no positive effects of RK were evident for female development regardless of diet. As sex pheromone plays important role in calling and courtship, I investigated effects of RK on pheromone production. RK-fed males produced significantly more pheromone, and RK was detected in the pheromone blend. Increased pheromone production and presence of RK in pheromone blend may be an important factor in elevated mating propensity of RK-fed male Qflies. While mating is an important step in SIT, female remating behaviour is also important as females that mate with a released male and then a wild male will likely retain fertility. Females that mated with RK-fed males, including during the period of accelerated development, exhibited remating tendency that was similar to females mated by control males. That is, not only does RK feeding increase development rate and early mating propensity of male Qflies, the early matings of RK-fed males are of undiminished effectiveness. SIT could potentially be greatly enhanced if combined with MAT, and my studies have identified a potential new way to enable this. In some other *Bactrocera*, mature males that feed on lures are then unresponsive to lures for substantial periods, and so would not be attracted to MAT devices. However, combination of SIT and MAT has been

considered unsuitable for Qfly owing to long adult maturation period and high mortality in attempts to hold the flies to maturity before release. I found that RK-fed males exhibit a persistent reduction in responsiveness to cuelure traps, opening the possibility of simultaneous MAT and SIT application in Qfly control programmes. In addition to considering potential beneficial effects of pre-release RK supplements it is also important to consider potential drawbacks for survival. When a source of protein + sugar was available throughout life, RK supplements did not affect survival. However, RK supplements did increase susceptibility to desiccation and food deprivation in the period immediately following pre-release treatment. This thesis comprises the first series of studies to consider RK as a potential pre-release treatment for SIT in the context of a standard 2 - 3 day pre-release holding period. The substantial beneficial effects of RK supplements on development, mating, and lure response support a strong case for further investigation and consideration for trial deployment on operational SIT programs.

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List of Contributors

Co-authors contributed to each section of the thesis, and my own percent contribution in brackets. Where HA= Humayra Akter, PWT=Phillip W. Taylor, RM=Renata Morelli, JP=Jeanneth Perez, PR=Polychronis Rempoulakis, VM=Vivian Mendez, SJP=Soo Jean Park, SMA=Saleh Mohammad Adnan, JI=Jess Inskeep.

	Chapter One	**Chapter Two**	**Chapter Three**	**Chapter Four**	**Chapter Five**	**Chapter Six**	**Chapter Seven**	**Chapter Eight**
Conception and Planning	HA (100%)	HA (80%), PWT, RM	HA (90%), PWT	HA (90%), PWT	HA (90%), PWT, JP	HA (90%), PWT, PR	HA (80%), PWT, RM	HA (100%)
Data Collection	-	HA (80%), RM, JP,VM	HA (100%)	HA (100%)	HA (70%), JP, SJP	HA (85%), PR, JI	HA (70%), RM, SMA, PR	-
Data Analysis	-	HA(70%), PWT	HA (70%), PWT	HA (80%)	HA (100%)	HA (100%)	HA (70%), PWT	-
Interpretation and Writing	HA (100%)	HA(80%), PWT	HA (90%), PWT	HA (90%), PWT	HA (100%)	HA (100%)	HA (80%), PWT	HA (100%)

Chapter One

General Introduction

Photo: Humayra Akter

1.1. Introduction

Fruit flies (Diptera: Tephritidae) are important pest of fruits and vegetables in most tropical and subtropical regions, and some temperate regions, of the world (Christenson & Foote 1960; Hardy 1969, 1983; Leblanc et al. 2013). Being mainly polyphagous, adult female causes direct damage by laying eggs under the skin of fruits and vegetables. Eggs hatch into larvae that feed on the decaying flesh making fruits and vegetables unmarketable. Among more than 4,000 fruit fly species, 70 species are considered as destructive pests that cause enormous damage and threat to fruit and vegetable production throughout the world. Fruit flies cause significant losses in both amount and quality of marketable produce, impacting both domestic and international trade (Bateman 1972; Fletcher 1987). More than 100 species of fruit flies are endemic to Australia, and among these at least 10 species are economically significant pests (Drew et al. 1982). Queensland fruit fly *Bactrocera tryoni* (Froggatt) ('Qfly') is a major pest of horticultural crops in eastern Australia (Dominiak & Daniels 2012) in terms of loss of yield and international trade, and costs of monitoring, quarantine regulation, and control (Bateman 1991; Sutherst et al. 2000). The value of affected crops has been estimated at ca. $7.00 billion annually (Hyam 2007). Qfly infests more than 100 native and introduced hosts (Drew 1989; Hancock et al. 2000).

Developing Qfly larvae not only damage fruit, but also pose a serious biosecurity risk. Queensland fruit fly is currently found in Northern Territory, Queensland, New South Wales and Victoria (Clarke et al. 2011) (Figure 1), New Caledonia (White & Elson-Harris 1994), and Tahiti (Leblanc et al. 2011), and occasional outbreaks of Qfly have occurred in Western Australia, South Australia, and New Zealand (Ministry of Primary Industries report 2015). For decades the organophosphate insecticides, especially dimethoate and fenthion, have provided a convenient and widely accepted tool for suppressing pest fruit fly populations, with limited use of other techniques such as cultural practices (e.g., field sanitation), male annihilation technique (MAT)/mass trapping (MT), community awareness, regulation of host produce, chemical applications and the sterile insect technique (SIT) (Bateman 1991). However, owing to concerns about environmental safety as well as effects of residues on human health, pre-harvest use has been suspended or greatly

reduced, while postharvest application is currently restricted (APVMA 2011, 2012; Dominiak & Ekman 2013). These restrictions have prompted a rethink about cntrol strategies for Qfly, and especially a significant effort to develop an effective SIT program to protect Australia's major southern growing regions.

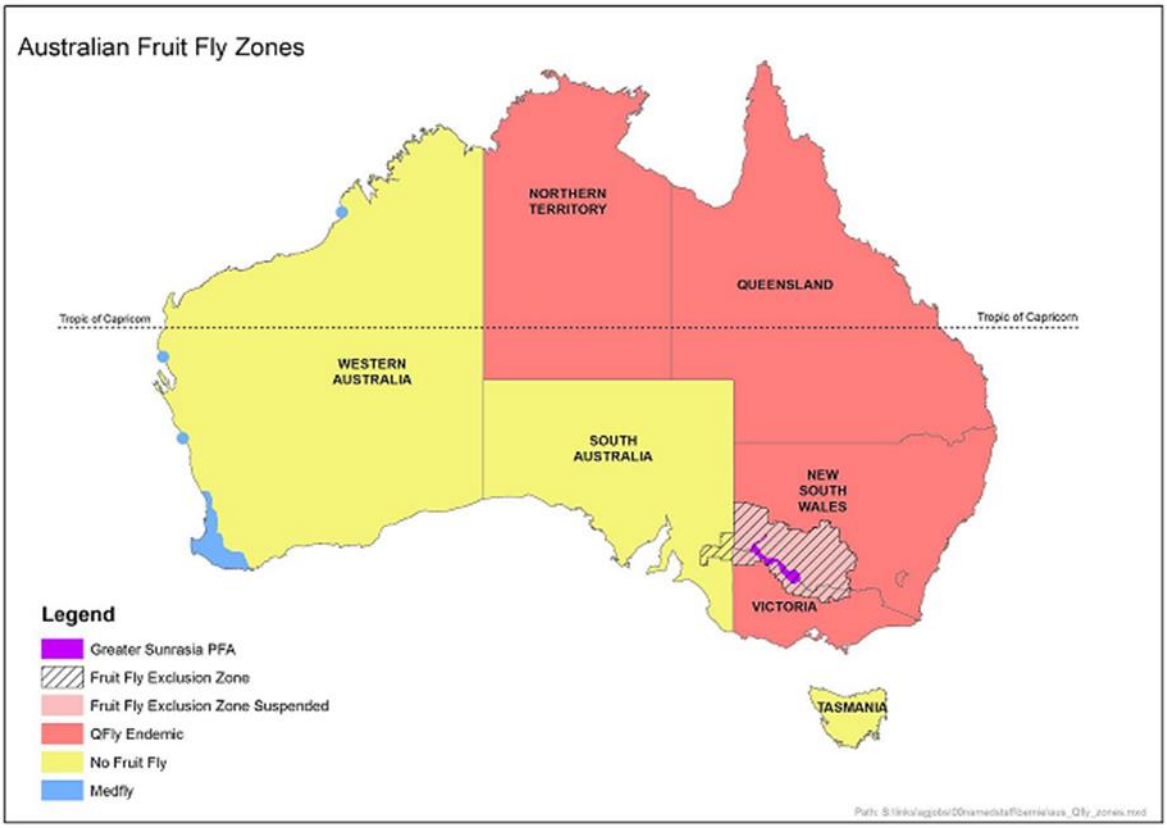

Figure 1: Present distribution of Queensland fruit fly in Australia (Courtesy: Dr. Bernard C. Dominiak 2016).

1.2. Biology of Queensland fruit fly

Biology of target species needs to be considered seriously when developing insect control strategies, especially when two or more independent techniques are to be deployed. Queensland fruit fly is highly polyphagous and multivoltine, and while it has limited ability to disperse by flight, especially when temperatures drop below 16°C (Meats & Fay 2000), it has proven highly amenable to anthropogenic dispersal through the movement of infested produce (Dominiak et al. 2011; Meats et al. 2003; Meats & Edgerton 2008). Feeding, ovipositing, and dispersing are all performed during the day, and mating takes place at dusk. The time spent in each

type of activity depends on many factors including age, sex, availability of mates and hosts, and weather (Fletcher 1987). Ovarian development is inhibited if temperature remains below 13.5°C for several days and sperm in the spermatheca also not retained over winter, so remating is thought to be common in spring (Fletcher 1975). Adult females oviposit by piercing fruits and vegetables with their ovipositor. Egg hatching and larval development occurs inside the flesh of fruits. After passing through three instars, mature larvae exit their host and drop to the ground to pupate under 2-3 cm of soil, emerging as sexually immature adults after 8-10 days in favourable conditions (Bateman 1972; Meats 1981). After emergence, flies of wild populations become sexually mature after 11-12 days (Tychsen 1977). Like other fruit fly species, Qfly needs water and carbohydrate to survive and protein for reproductive development (Christenson & Foote 1960; Bateman 1972; Leighton 1979). Qflies can lay up to 100 eggs per week over several months (Fitt 1984). For mating usually they prefer the foliage of host or nonhost plants (Bateman 1972; Tychsen 1977) where males aggregate and take up individual territories on leaves, which they aggressively defend from incursions by other males, when light intensity drops near dusk (Fletcher 1987). Sexually active males produce a buzzing sound (calling) by rapid wing fanning, and simultaneously release pheromone from their rectal glands (Giannakakis 1976) to attract and court females (Bellas & Fletcher 1979, Fitt 1981a). Males can mate frequently and females sometimes remate but more often exhibit high levels of sexual inhibition following their first mating (Harmer et al. 2006; Tychsen & Fletcher 1971), using the sperm of first male to fertilize their eggs (Bateman et al. 1976, Fay & Meats 1983). Qfly can complete a life cycle in 3-4 weeks under favourable conditions, and can complete 8 or more generations per year (Bateman 1972; Meats 1981).

1.3. Sterile Insect Technique and sexual maturation

In typical SIT programs, flies are mass reared, irradiated to induce sterility, and released into the field to mate with wild females after which wild females lay unfertile eggs and, the target species is suppressed (Knippling 1955). Success of SIT depends on many factors such as the quality of male flies, diet and production methods, irradiation dose, handling and transport stress, release methods, and

field conditions (Chambers 1977; Teal et al. 2007; Enkerlin 2007; Meats and Edgerton, 2008; Collins et al., 2009; Dominiak et al., 2011; Weldon and Taylor, 2011). However, high mortality and poor performance of mass reared sterile flies in the field are commonly considered to be important constraints for SIT (Monro & Osborn 1967; Dominiak et al. 2003; Meats et al. 2003). To overcome these constraints, massive over flooding ratios of sterile:wild (commonly ca. 100:1) are released (Bateman 1991; Meats 1996) and this increases the cost of SIT application. Because success of SIT is very much related to early maturation and sexual performance of released males, significant emphasis is now placed on improving the sexual performance of released sterile flies (Lance & McInnis 2005; Yuval et al. 2007). The rate of sexual maturation is very much reliant on access to key nutritional elements such as protein and carbohydrates (Meats et al. 2004; Pérez- Staples et al. 2007; 2008); post teneral protein supplementation increases mating probability of Qfly (Pérez -Staples et al. 2008; 2009). In addition, the juvenile hormone analogue methoprene can significantly improve sexual development and performance of melon flies and Qflies (Haq et al. 2010; Collins et al. 2014). In addition to these known supplements, phytochemicals also have potential to enhance mating performance of some fruit fly species although have not been considered within the context of the typical 2 - 3 day pre-release holding period.

1.4. Phytochemical and sexual maturation

Sexually mature tephritid males are commonly attracted to compounds that either occur naturally in plants or are synthetic analogues of plant-borne substances (Metcalf 1979; Vargas et al. 2010; Metcalf & Metcalf 1992; Nishida et al. 1988; Mazomenos & Haniotakis 1985; Fitt 1981b; Fletcher et al. 1975; Fletcher 1974; Kawano et al. 1968). One of the widely recognised compounds is methyl eugenol (ME), 4-allyl-1,2-dimethoxybenzene, which is found in at least 450 plant species in 80 different plant families (Tan & Nishida 2012; Metcalf et al.1979). Cuelure (CL), 4-(4-acetoxyphenyl)-2-butanone, has not been isolated as a natural product, but it is rapidly hydrolyzed to form 4-(4-hydroxyphenyl)-2-butanone, also known as raspberry ketone (RK), a natural plant constituent found in orchids, raspberries and cranberries (White 2000). No species are effectively attracted to both ME and

CL (Drew 1974; Vargas et al 2010), however, zingerone, a major component of the fruity odour of the orchid *Bulbophyllum baileyi*, is attractive to males of several *Bactrocera* species those are either ME or CL responsive (Tan & Nishida 2007). Interestingly, *B. dorsalis* males feeding on *B. baileyi* flowers sequester zingerol (a reduced form of zingerone) in the body, suggesting its role as a possible component of sex pheromones used to attract females during courtship (Tan & Nishida 2007). Being highly attractive, lures are commonly used in detection and eradication efforts of tephritid pests; ME has been known to fruit fly workers as an attractant since the 1910s (Qureshi et al. 1976) and CL from the 1960s (Beroza et al. 1960; Bateman et al. 1966; Fletcher 1987). CL is widely recognized as a strong male-specific lure for Qfly and has been deployed as the standard lure in surveillance programs and MAT (Monro & Richardson 1969; Drew 1974; Cowley et al. 1990; Meats et al. 2002).

The response of male flies to lures is influenced by their age, their physiological maturity, and the time of day (Chiu 1984; Fitt 1981c). The attraction of male Qflies to CL is closely associated with sexual maturation (Drew 1987; Weldon et al. 2008). Lure compunds have an important role in the reproductive biology of male tephritids; firstly lures can act as 'rendezvous' stimuli that bring the sexes together in proximity to suitable host plants to initiate mating (Metcalf et al. 1979; Raghu & Clarke, 2003), and secondly lures serve as male pheromone precursors or stimulants that play important role in sexual selection (Raghu 2004). Lure feeding of adult males is correlated with higher mating success, both in ME- (Shelly & Dewire 1994; Wee et al. 2007) and CL/RK-responding species (Shelly & Dewire 1994; Shelly & Villalobos 1995; Shelly 2000; Wee et al. 2007). For instance, in *B. dorsalis*, larval and adult feeding on ME promotes mating success (Shelly & Nishida 2004); males allowed to feed on ME for only 30s obtained significantly more matings than unexposed males for at least 35 d after feeding (Shelly & Dewire 1994). In addition, males of the Mediterranean fruit fly (medfly), *Ceratitis capitata*, exposed to trimedlure gain a mating advantage immediately after exposure to trimedlure (Shelly et al. 1996a). Access to orange peel substance or ginger root oil can enhance sexual performance of male medflies (Papadopoulos et al., 2001; Shelly et al., 2004; Shelly et al., 2007). Female *B. cucurbitae* are more attracted to CL-fed males than CL-deprived males (Khoo & Tan 2000). Exposure

to CL confers a mating advantage of 1–2 days in *B*. *cucurbitae* (Shelly & Villalobos 1995) and Qfly (Kumaran et al. 2013). Enhanced mating success of phytochemical-treated males seems to be due in part to increased sexual advertisement such as wing vibrations (Shelly & Dewire 1994), pheromone calling (Kuba & Sokei 1988; Papadopoulos et al. 1998; Kaspi et al. 2000) that promote female visitation and receptivity (Shelly & Dewire 1994; Khoo & Tan 2000; Shelly 2001).

1.5. Raspberry ketone and sexual maturation

RK is a natural male attractant occurs in a variety of plant species and is highly attractive to males of some *Bactrocera* species (Drew 1974; Drew & Hooper 1981). Male *B. cucurbitae* feed on *Dendrobium superbum* orchids and sequester RK in their rectal gland (Nishida et al. 1990; 1993). CL is a synthetic derivative of RK and is converted to RK within 6 hours in the rectal gland of Qfly males (Tan & Nishida 1995).

Mature male Qflies are attracted to CL and show enhanced mating success, shorter mating latency, and more attractive pheromone after feeding on CL (Kumaran et al. 2013). In addition to pheromonal effects, female Qflies mated with CL-fed males show increased fecundity, decreased remating receptivity, and reduced longevity (Kumaran & Clarke 2014). These changes in female reproductive phenotype suggest that RK analogue feeding may change the male ejaculate, as post-copulatory changes in female behaviour have been attributed to male accessory gland proteins in *Drosophila* and *Bactrocera* (Chapman & Davies 2004; Radhakrishnan & Taylor 2007). In addition, flight activity and wing fanning of mature Qfly males increases in the presence of CL (Dalby-Ball & Meats 2000). RK is known, across a diverse range organisms to be involved in increasing energy metabolism (Park 2010; Morimoto et al. 2005). If this also occurs in *Bactrocera*, then this may represent an additional benefit to young male flies in accelerating their sexual activity and courtship. Considering all these facts, RK has potential to enhance sexual performance and to accelerate sexual maturation of Qfly males.

1.6. Research objectives

To date phytochemicals have only been exploited for enhancing sexual performance of mature males (Section 1.4 and 1.5). However, the possible use of these metabolic enhancers to accelerate sexual maturation of immature adults has not been examined yet. Shortening the pre-copulatory period of males is important in species that have long adult maturation phases as sterile males are normally released into the field soon after emergence where they are exposed to predators and adverse environmental conditions that cause mortality before they reach sexual maturity (Hendrichs et al., 2007). The aim of my PhD project is to examine the potential exploitation of RK to accelerate sexual maturation and reproductive development of immature Qflies. To determine the broader consequences of pre-release RK, I also investigated related biological and physiological consequences.

1.7. Thesis at a glance

This thesis comprises 8 chapters; Chapter 1 is a General Introduction that briefly outlines the relevant literature and Chapter 8 is a General Discussion that briefly reviews findings as a whole and future direction on the basis of these findings. Chapters 2 - 7 comprise research dealing with separate but linked issues associated with pre-release RK supplements as potentially deployed in Qfly SIT programs.

Chapter 2 investigates effects of RK-feeding by Qfly males on the developmental emergence of mating behaviour. This is the first study to consider RK as a means of accelerating sexual maturity. Along with mating activity I also observed the effect of RK on longevity to test whether RK poses any positive or negative impact on Qfly life span. This chapter has been published in *Pest Management Science*.

Chapter 3 focuses on the morphological variation in reproductive organs of both males and females after feeding on RK as immature adults. Sexual and reproductive development are often discussed as synonymous, but it was possible that the elevated mating performance of RK supplemented males in Chapter 2 reflected a behavioural effect on mating behaviour only without promotion of underlying development of reproductive organs. Here I investigated whether this is

the case. This chapter is co-authored with Phil Taylor and formatted for *Journal of Insect Physiology*.

Chapter 4 explores whether matings by RK-fed males are effective at inducing remating inhibition in females. If females mated with RK-fed males later remate then the precocious mating by RK-fed male will be of little value in SIT as females could lay fertile eggs. This chapter is co-authored with Phil Taylor and formatted for *Insect Science*.

Chapter 5 focuses on the quantity and quality of pheromone produced and released by Qfly males after feeding on RK as immature adults. In this chapter I also show the effect of RK on total pheromone amount and ratio of constituent compounds in response to diet, age and RK dose. This study provides some insight into whether early mating by young RK-fed Qfly males is related to pheromone production and release. This chapter is co-authored with Phil Taylor, Jeanneth Perez, Soo Jean Park and formatted for *Journal of Chemical Ecology*.

Chapter 6 is on how RK-fed males and females cope when they face adverse environmental conditions such as lack of food and water and desiccating atmosphere. This chapter is co-authored with Phil Taylor, Polychronis Rempoulakis, Jess Inskeep and formatted for *Journal of Insect Physiology*.

Chapter 7 focuses on the attraction of RK-fed males to CL at different ages. This finding has great implications in practical application of SIT as MAT and SIT might be used simultaneously to significantly reduce control cost. This chapter is co-authored with Phil Taylor, Renata Morelli, Saleh Mohammad Adnan, Polychronis Rempoulakis and formatted for the *PLoS One.*

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Chapter Two

Raspberry ketone supplement promotes early sexual maturation in male Queensland fruit fly, *Bactrocera tryoni* (Diptera: Tephritidae)

Photo: Humayra Akter

Vignette

Raspberry ketone (RK) occurs in a variety of plant species and is highly attractive to males of many *Bactrocera* species (Tan & Nishida 1995; Tan & Nishida 2005; Nishida et al. 1993). Exposure of RK enhance mating in mature melon fly *Bactrocera cucurbitae* (Shelly 2000). The RK-responsive species are also attracted to cuelure, a synthetic analogue of RK, and sexually mature males that feed on cuelure sequester RK in their rectal gland and have increased mating success (Shelly & Villalobos 1995; Kumaran et al. 2013; Kumaran et al. 2014a). However, effects of RK on immature males are unknown. Physiological effects of phytochemical consumption are linked to energy metabolism in zingerone-fed sexually mature Qfly males (Kumaran et al. 2014b) and zingerone showed enhanced mating in Qfly (Kumaran et al. 2013). Dietary RK also known to promote energy metabolism in animals (Park 2010) which may promote courtship activity. Therefore, this chapter investigated the possibility of RK as an accelerator of sexual maturation of immature Qfly.

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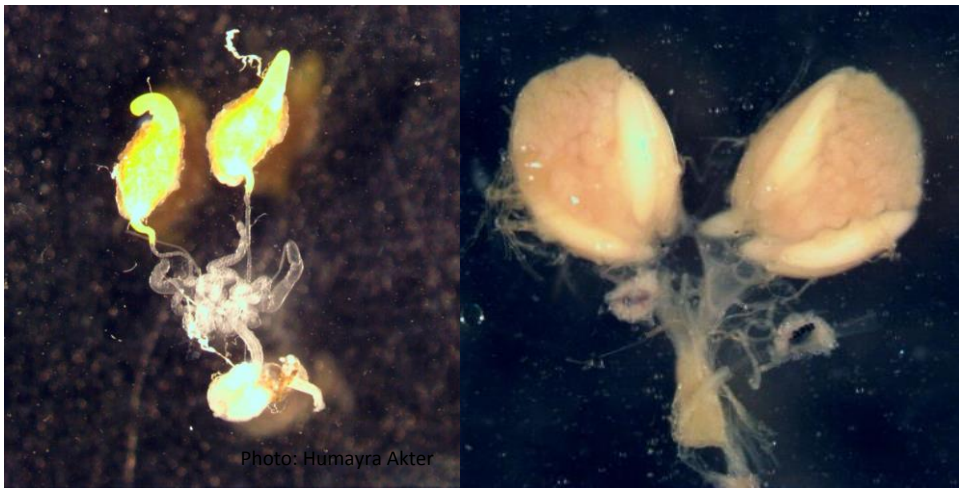
Chapter Three

Raspberry ketone supplement accelerates reproductive organ development of Queensland fruit fly, *Bactrocera tryoni* (Froggatt)



Abstract

Raspberry ketone (RK), 4-(4-Hydroxyphenyl)-2-butanone, is highly attractive to mature males of some *Bactrocera* and *Zeugodacus* fruit flies (Tephritidae), including Queensland fruit fly *Bactrocera tryoni* (Froggatt) ('Qfly'). . By mixing RK in food, a recent study has exploited these metabolic effects of RK to accelerate the emergence of sexual activity in developing male Qflies. RK shows potential as a maturation-enhancing pre-release supplement for sterile insect technique programs, with effects paralleling those of methoprene treatment. Previous studies only considered effects of RK on mating, but the possibility exists that RK only effects behaviour without accelerating development of reproductive organs such that the early matings of RK supplemented flies may be ineffective. The present study assesses the effects of RK supplements on reproductive organ development in both sexes of Qfly. Recently emerged flies were treated for 48 hours with RK (0% control, 1.25%, 5%) in a diet of sugar only or sugar+yeast hydrolysate (YH)(3:1), and were provided only sugar thereafter. For males fed sugar+YH, RK accelerated growth of ejaculatory apodeme area and testes length, indicating that the early matings reported previously were supported by corresponding development of reproductive organs. For males fed sugar only, however, RK showed no effect on testes development and suppressed development of ejaculatory apodemes. For females fed sugar+YH there was no evidence that RK affected ovarian development but for females fed sugar only RK significantly suppressed ovarian development. Findings of the present study confirm that the supplementation of recently emerged Qflies with RK induces accelerated development of reproductive organs that matches the accelerated emergence of mating activity in male Qflies that has been reported previously, but only if flies are fed a diet that includes YH. In the absence of the nutritional support of YH, RK supplements appear to impose metabolic costs that result in suppressed development of some reproductive organs.

1. Introduction

Raspberry ketone (RK), 4-(4-Hydroxyphenyl)-2-butanone, is a phenolic compound that is found naturally in plants ('phytochemical'), and has an important role in the life history of some *Bactrocera* and *Zeugodacus* fruit flies (Tephritidae). RK is found at high concentrations in red raspberries, in which it provides the characteristic aroma (Aprea et al., 2015), and has also been reported in a variety of other plants (e.g., raspberry jam orchids Nishida et al., 1993; kiwifruit Garcia et al., 2011). Mature males of some *Bactrocera* and *Zeugodacus* fruit flies, including Queensland fruit fly (Qfly), *Bactrocera tryoni* (Froggatt), are attracted to and feed on RK and some have been found to gain sexual benefits over the following 1 - 3 days (Shelly and Villalobos, 1995; Shelly, 2010; Kumaran et al., 2013). 'Cuelure', a stable synthetic analogue of RK (Park et al., 2016a), is much more volatile (Metcalf and Metcalf 1992; Park et al., 2016b) and since its introduction in the early 1960s has been the standard lure used for monitoring and male annihilation technique management of RK-responsive fruit flies (Beroza et al., 1960; Drew, 1974; Shelly et al., 2012).

RK has been used commercially for many years in the food industry as a flavour, and in the cosmetics industry as a perfume. Recently, however, attention has focused much more on how RK ingestion affects animal physiology, especially when used at high concentrations. RK promotes lipid metabolism and can reduce obesity and fatty liver in animals (Morimoto et al., 2005; Wang et al., 2012), and has become a common, albeit controversial, component of commercial weight-loss products for humans (Bredsdorff et al., 2015; Lee, 2016). Recent studies also point to metabolic effects as an important driver underlying the attraction of fruit flies to RK and related compounds.

Mature male melon flies *Zeugodacus* (previously *Bactrocera*) *cucurbitae* (Virgilio et al. 2015) exhibit elevated sexual activity after feeding on RK, or on the closely related compounds cuelure and zingerone (Khoo and Tan, 2000; Shelly, 2000) and mature male Qflies exhibit enhanced mating performance after feeding on cuelure or zingerone (Kumaran et al., 2013, 2014a). Ingestion of zingerone

results in extensive enrichment of transcripts linked to energy metabolism in mature male Qflies, and ingestion of either zingerone or cuelure results in increased locomotor activity and rapid weight loss over three days, a period that matches the persistence of elevated mating activity (Kumaran et al., 2014b). Physiological effects of phytochemicals in Qflies have been referred to colloquially as a 'Red Bull® effect' (Kumaran et al., 2014b).

Recently, recognising the potential implications of RK effects on Qfly metabolism, Akter et al. (2017) extended the application of RK beyond the scope of this compound's natural relationship with sexually mature *Bactrocera* and *Zeugodacus* males. Although attraction, feeding and physiological response to phytochemicals had only previously been associated with sexually mature *Bactrocera* and *Zeugodacus* males, Akter et al. (2017) considered the possibility that, as a metabolic enhancer, RK mixed in the diet of recently emerged male Qfly might accelerate sexual maturation. Indeed, while no effects of RK were observed when mixed with sugar only diets, when mixed in a sugar+YH diet RK advanced the emergence of male Qfly sexual activity by two to three days (Akter et al., 2017), an effect of magnitude comparable to what has been reported previously for methoprene treatment of Qfly pupae or recently emerged adults (Collins et al., 2014).

Acceleration in the emergence of sexual activity has important implications for management of Qfly populations. Qfly is a major fruit pest in Australia (Clark et al., 2011), and sterile insect technique (SIT) has been deployed to suppress outbreaks in some regions (Meats et al., 2003; Fanson et al., 2014; Reynolds et al., 2014). In SIT programs, millions of sterile male flies are released to mate with females of pest populations. Females mated by sterile males suffer reproductive failure and, consequently, pest populations are reduced in the next generation (Knipling, 1955; Lance and McInnis, 2005). For species in which there is a substantial period of maturation required in the adult stage before flies can engage in sexual activity and contribute to SIT, the success of SIT is linked to development rate. Although they usually do not mature in laboratory conditions until 7-10 days of age even when provided an unlimited and rich diet (Vijaysegaran

et al., 2002; Pérez-Staples et al., 2007), in SIT programs Qflies are usually released one to three days after emerging as adults (Dominiak et al., 2003; Reynolds et al., 2014), when still immature, and then need to forage in the field for nutrition required to complete reproductive development. Provision of yeast hydrolysate during the pre-release holding period has been found to promote development of Qfly reproductive organs and mating activity at young ages (Pérez-Staples et al., 2008, 2009, 2011), and to increase prevalence of mature cuelure-attracted adults in the field (Reynolds et al., 2014). Recent findings of Akter et al. (2017) indicate that these positive effects of yeast hydrolysate can be substantially improved upon by the addition of RK into the pre-release diet.

While the findings of Akter et al. (2017) are promising, further investigation is required to more fully understand the effects of RK on immature Qflies and the subsequent performance of supplemented flies once mature. Akter et al. (2017) focused solely on the expression of male sexual behaviour as flies aged and found substantially elevated mating levels in young RK-fed flies. The emergence of sexual behaviour and other aspects of reproductive development, such as morphological development of reproductive organs, usually occur in concert. However, it is possible that these effects of RK on immature male Qflies are only behavioural, as RK might dissociate the emergence of sexual behaviour from other aspects of reproductive development. That is, RK might do no more than render the treated flies sexually precocious, without promoting development of reproductive organs. As such, it is possible that the early matings of RK-treated flies might not be effective if at these ages the males lack adequate physical development. In the present study, we investigate whether accelerated emergence of sexual behaviour in RK-supplemented Qflies is associated with accelerated development of reproductive organs.

2. Materials and methods

2.1. Source and treatment of flies

Qfly pupae were obtained from the Fruit Fly Production Facility at Elizabeth Macarthur Agricultural Institute at Camden, New South Wales, Australia, and had been in culture for approximately 70 generations (for production details, see Dominiak et al., 2008). Fertile flies were used because the gamma-irradiation used to sterilise flies in historical Qfly SIT programs will not be used in future programs. X-irradiation procedures are under development for future programs, and ultimately may be an interim measure as new technologies may dispense with irradiation altogether. All pupae and flies were maintained, and experiments 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 30 min of the light phase simulated dawn and dusk by gradually ramping the light levels up and down, respectively. Upon arrival at Macquarie University, ca. 2000 pupae were transferred to each of six open Petri dishes that were placed in separate 47.5×47.5×47.5 cm mesh cages (Bugdorm 44545, Megaview, Taichung, Taiwan). Adult flies emerged in these cages, where they were provided only water-soaked sponge for sustenance. Usually only a small number of flies emerge on the first day of emergence, and these were discarded. Flies from the following 24 hours of emergence were used in experiments.

After adult emergence, each cage of 0-24 hour old adult flies was provided one of six combinations of diet and RK 4-(4-Hydroxyphenyl)-2-butanone (≥98%, Sigma–Aldrich®) . Three cages were provided a mixture of sugar and yeast hydrolysate (Sugar+YH) and three cages were provided only sugar. For each of the two diet groups, one cage was with 5% RK (‘high dose’), one cage was with 1.25% RK (‘low dose’), and one cage received no RK (‘control’). Because male Qflies of this age are not attracted to and do not feed voluntarily on RK, and female flies rarely respond to RK at any age, RK was mixed with food (sugar only or sugar+YH) in a blender so that flies received RK as a consequence of feeding. Because phytochemicals can exert effects on fruit fly physiology through olfaction

as well as through feeding (Shelly 2001; Vera et al., 2013; Haq et al., 2015), RK-treated and control flies were kept in separate rooms to avoid olfactory contamination.

After 48 hours food was removed and flies of each treatment group were separated by sex, with males and females transferred to separate 12.5L plastic cages (150 flies/cage) that had three 12 cm mesh-covered openings for ventilation. Laboratory-adapted, mass-reared flies rarely mate before 5 days of age even when provided both YH and RK (Meats et al., 2004; Pérez-Staples et al., 2007; Akter et al., 2017). After separating by sex, flies were provided only water-soaked sponge and sugar for sustenance. At 4, 6, 8, 10, 15, 20, 25 and 30 days after emergence, male and female Qflies were preserved in 70% of ethanol (99.5%, Fisher Scientific, UK) for later dissection and assessment of reproductive organ development. All procedures were repeated using two batches of pupae that were obtained two months apart.

2.2. Measures of development

Measurements of reproductive organs generally followed methods of previous studies (Drew, 1969; Vijaysegaran et al., 2002; Raghu et al., 2003; Radhakrishnan and Taylor, 2008; Pérez-Staples et al., 2011; Weldon and Taylor, 2011). Flies were rinsed in phosphate-buffered saline (PBS, pH=7.2-7.6, Sigma-Aldrich®) in Petri dishes before being dissected in a drop of PBS on a microscopic slide under a stereoscope. Images were captured using a TOUP 3.7 digital camera through a stereo-microscope (SZX12; Olympus, Tokyo, Japan) and then calibrated and measured using ImageJ 1.46r (US National Institutes of Health, Bethesda, MD, USA). Five males and females were assessed for each treatment group on each day in each replicate.

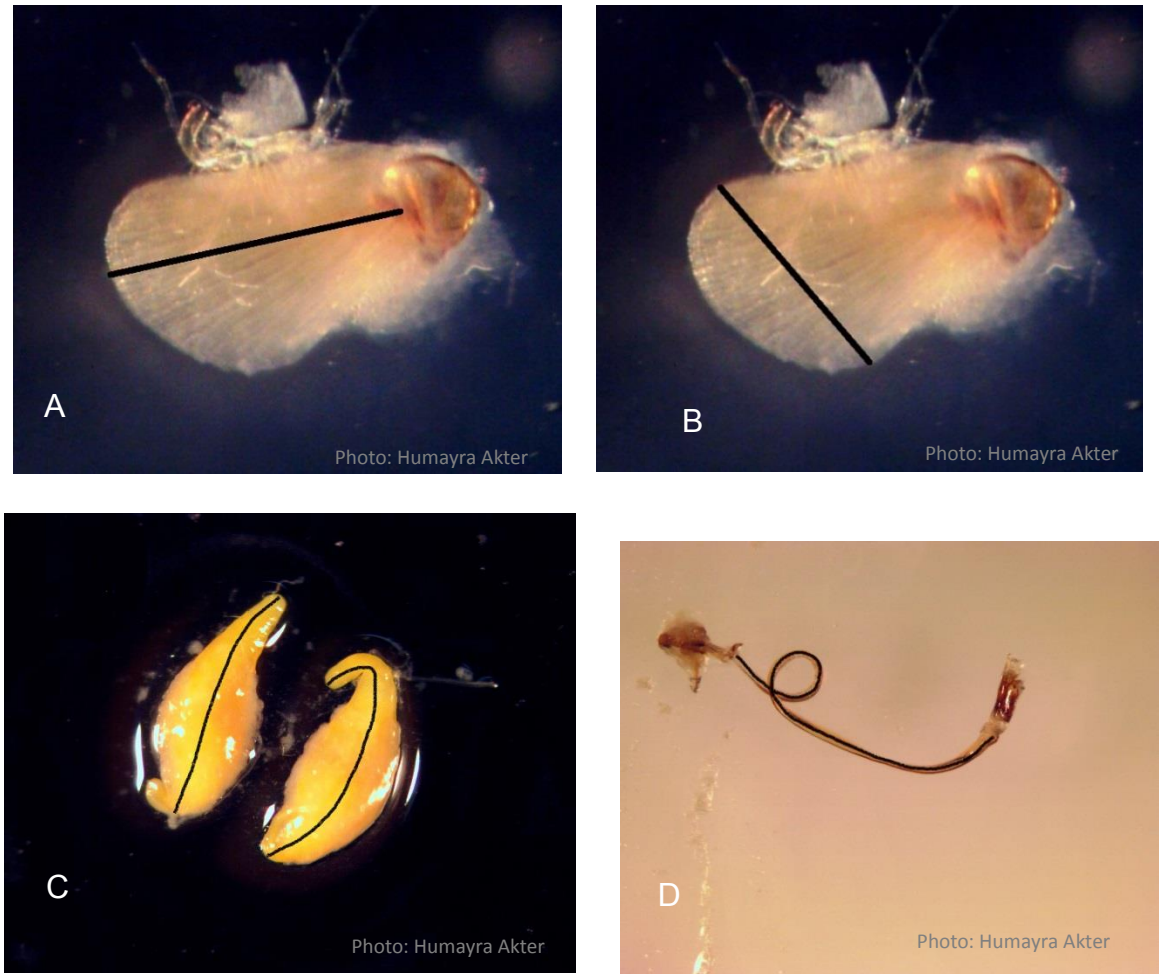


Figure 1: Male reproductive organs of *Bactrocera tryoni*. Black line is showing measurements points; (A) Apodeme length (B) Apodeme width (C)Testis length (D) Aedeagus length. Area of apodeme was measured by tracing a line around the organ.

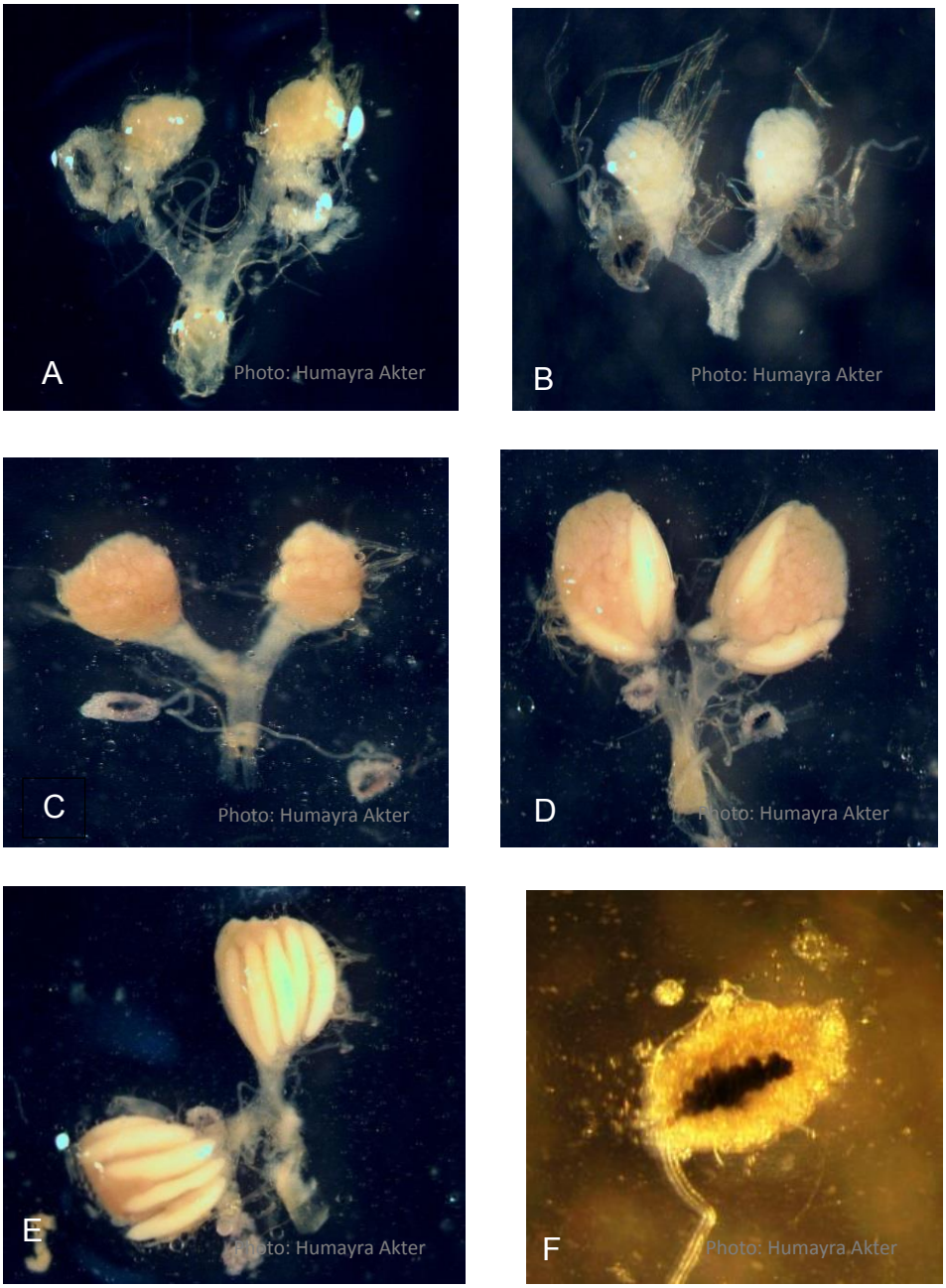


Figure 2: Female reproductive organs of *Bactrocera tryoni* showing ovarian developmental stages (A-E) and spermatheca (F).

(A) stage 1, ovaries with no visible follicle cells ; (B) stage 2, visible follicle cells ; (C) stage 3, vitellogenesis started, nurse cells occupy most of the ovary; (D) stage 4, mature oocytes visible but still nurse cells are visible; (E) stage 5, appearance of mature oocytes without the presence of any nurse cells. (F) Spermathecal area was measured by tracing a line around the organ.

For males, measurements were male of ejaculatory apodeme length, width and area, testis length, and aedeagus length. Ejaculatory apodeme length was measured from the ejaculatory sac at its base to the most distal point (Figure 1A). Ejaculatory apodeme width was measured at the widest point (Figure 1B). Ejaculatory apodeme area was measured by tracing the outline of each organ and using ImageJ software to calculate the area. Testis length was measured by tracing a midline through the centre (Figure 1C), the layer of fat surrounding the testes was carefully removed using entomological pins before photographing. Aedeagus length was measured by tracing a line from the top of the basiphallus to its tip, excluding the distiphallus (Figure 1D).

For females, measurements were made of ovary development stage and spermathecal gland area. Ovarian development was based on a classification used previously by Fletcher et al. (1978) for *B. oleae* and Raghu et al. (2003) for *B. cacuminata* (Hering) in which the ovarian maturation process was divided into five stages (Figure 2 A-E): (1) previtellogenic, ovaries with no visible follicle cells; (2) previtellogenic, developing oocytes entered the vitellarium region; (3) early vitellogenic, nurse cells occupy most of the ovary; (4) late vitellogenic, mature oocytes visible but nurse cells still located at the anterior end of the follicle; and (5) fully mature ovary, appearance of mature oocytes without the presence of any nurse cells and the follicular epithelium (see also Anderson and Lyford, 1965; Meats and Khoo, 1976). Spermathecal gland area was measured by tracing an outline using Image J. (Figure 2 F)

2.3. Data analysis

Reproductive organ development was assessed by standard least square models. In all analyses, Diet (sugar only, sugar+yeast hydrolysate,) and RK Dose (0% control, 1.25%, 5%) were treated as nominal fixed effects and Age (4, 6, 8, 10, 15, 20, 25 and 30 days) was treated as a continuous effect. The experiment was repeated for two batches of pupae and so Batch was included as a nominal

random effect in all models. All interaction terms were assessed, but non-significant interactions terms were excluded from final models.

3. Results

3.1. Development of male reproductive organs

3.1.1. Ejaculatory apodeme size

Ejaculatory apodeme length (log transformed), width (log transformed) and area all varied with Age (log transformed) x RK Dose x Diet interactions (Table 1). Overall, flies fed sugar+YH had larger ejaculatory apodemes than those fed sugar only (Figure 1, 2, 3), but the extent of this difference varied with both age and RK Dose in each measure. To understand this interaction, models were re-run separately for the two diet groups.

Ejaculatory apodeme length of flies that had been fed sugar only varied with Age x RK Dose interaction (Age $F_{1,233}$=201.21, P<0.001, RK Dose $F_{2,233}$=8.07, P<0.001, Age x RK Dose $F_{2,233}$=14.89, P<0.001); growth of ejaculatory apodeme length was suppressed in flies that received either dose of RK (Figure 3). Ejaculatory apodeme length of flies that had been fed sugar+YH did not vary significantly with RK Dose (Age $F_{1,233}$=99.47, P<0.001, RK Dose $F_{2,233}$=1.52, P=0.22, Age x RK Dose $F_{2,233}$=1.32, P=0.27) (Figure 3).

Results for ejaculatory apodeme width very closely resembled results for ejaculatory apodeme length. Ejaculatory apodeme width of flies that had been fed sugar only varied with Age x RK Dose interaction (Age $F_{1,233}$=290.96, P<0.001, RK Dose $F_{2,233}$=3.07, P=0.048, Age x RK Dose $F_{2,233}$=6.64, P=0.002); growth of ejaculatory apodeme width was suppressed in flies that received either dose of RK (Figure 4). Ejaculatory apodeme width of flies that had been fed sugar+YH did not

vary significantly with RK Dose (Age $F_{1,233}$=201.77, P<0.001, RK Dose $F_{2,233}$=0.92, P=0.40, Age x RK Dose $F_{2,233}$=1.02, P=0.36) (Figure 4).

Results for ejaculatory apodeme area were similar to those for ejaculatory apodeme length and width for flies that had been fed sugar only but differed for flies that had been fed sugar+YH. Ejaculatory apodeme area of flies that had been fed sugar only varied with Age x RK Dose interaction (Age $F_{1,233}$=352.19, P<0.001, RK Dose $F_{2,233}$=9.40, P<0.001, Age x RK Dose $F_{2,233}$=15.70, P<0.001); growth of ejaculatory apodeme area was suppressed in flies that received either dose of RK (Figure 5). Unlike ejaculatory apodeme length and width, however, ejaculatory apodeme area of flies that had been fed sugar+YH also varied with Age x RK Dose interaction (Age $F_{1,233}$=257.05, P<0.001, RK Dose $F_{2,233}$=9.20, P<0.001, Age x RK Dose $F_{2,233}$=5.66, P=0.004); growth of ejaculatory apodeme length was accelerated in flies that received the higher dose of RK (Figure 5).

3.1.2. Testis length

Testis length (log transformed) varied with Age (log transformed) x RK Dose x Diet interactions (Table 2). Overall, flies fed sugar+YH had longer testes than those fed sugar only (Figure 6), but the extent of this difference varied with both age and RK Dose in each measure. To understand this interaction, models were re-run separately for the two diet groups. In flies that had been fed sugar only testes length increased as the flies aged but did not vary with RK Dose (Testis length Age $F_{1,233}$=117.75, P<0.001, RK Dose $F_{2,233}$=0.18, P=0.838, Age x RK Dose $F_{2,233}$=0.46, P=0.63; Figure 6). In flies that had been fed sugar +YH testes length varied with Age x RK Dose interaction (Age $F_{1,233}$=228.845, P<0.001, RK Dose $F_{2,233}$=11.382, P<0.001, Age x RK Dose $F_{2,233}$=8.213, P<0.001); like ejaculatory apodeme area, testis length was greater in young flies that received the high dose of RK (Figure 6).

Table 1. Effects of Age (4, 6, 8, 10, 15, 20, 25, 30 days), RK dose (0%, 1.25%, 5%) and Diet (sugar only, sugar+YH) on ejaculatory apodeme length (R^2=0.64), width (R^2=0.71), and area (R^2=0.77) (for all, N=480).

Source	*d.f.*	Length		Width		Area	
		F	*P*	*F*	*P*	*F*	*P*
Age	1,467	308.73	**<0.001**	492.66	**<0.001**	585.22	**<0.001**
RK dose	2,467	8.53	**<0.001**	2.72	0.067	11.68	**<0.001**
Diet	1,467	104.51	**<0.001**	112.98	**<0.001**	71.44	**<0.001**
RK Dose*Diet	2,467	2.57	0.078	1.84	0.160	6.42	**0.002**
RK Dose*Age	2,467	12.51	**<0.001**	5.31	**0.005**	14.96	**<0.001**
Diet*Age	1,467	26.59	**<0.001**	24.08	**<0.001**	0.97	0.325
RK Dose*Diet*Age	2,467	6.81	**0.001**	3.83	**0.023**	5.11	**0.033**

Table 2: Effects of Age (4, 6, 8, 10, 15, 20, 25, 30 days), RK dose (0%, 1.25%, 5%) and Diet (sugar only, sugar+YH) on mean testis length (R^2=0.54) (N=480).

Source	Testis length		
	d.f.	*F*	*P*
Age	1,467	340.24	**<0.001**
RK dose	2,467	4.76	**0.009**
Diet	1,467	0.21	0.644
RK Dose*Diet	2,467	7.41	**<0.001**
RK Dose*Age	2,467	2.87	0.058
Diet*Age	1,467	12.43	**<0.001**
RK Dose*Diet*Age	2,467	6.23	**0.002**

Table 3. Effects of Age (4, 6, 8, 10, 15, 20, 25, 30 days), RK dose (0%, 1.25%, 5%) and Diet (sugar only, sugar+YH) on Ovary development stage (R^2=0.70) and Spermathecal gland area (R^2=0.31) (for both, *N*=480). Non-significant interactions were removed from final models.

Source	Ovary stage			Spermathecal gland area		
	d.f.	*F*	*P*	*d.f.*	*F*	*P*
Age	1,469	199.16	**<0.001**	1,474	165.22	**<0.001**
RK dose	2,469	3.51	**0.031**	2,474	1.64	0.195
Diet	1,469	4.79	**0.029**	1,474	19.88	**<0.001**
Diet*Age	1,469	30.17	**<0.001**	1,474	23.90	**<0.001**
RK Dose*Diet	2,469	11.45	**<0.001**			
RK Dose*Age	2,469	4.11	**0.017**			

3.1.3. Aedeagus length

Aedeagus length (log transformed) increased as flies aged (log transformed) (*b*=0.026±0.003, $F_{1,474}$=58.40, *P*<0.001) and, across ages, flies fed sugar+yeast had longer aedeagus than did flies fed sugar only (Least Square Means: Sugar Only=3.868±0.003 mm, Sugar+YH=3.916±0.003 mm, $F_{1,474}$=7.37, *P*=0.007). However, Aedeagus length was not affected by RK Dose ($F_{2,474}$=1.86, *P*=0.156) (Figure 7).

3.2. Development of female reproductive organs

3.2.1. Ovaries

Age, RK Dose and Diet all affected development of ovaries (Table 3). Ovary development of flies that had been fed sugar only varied with Age (log transformed) x RK Dose interaction (Age $F_{1,233}$=48.903, *P*<0.001, RK Dose $F_{2,233}$=2.127, *P*=0.122, Age x RK Dose $F_{2,233}$=5.000, *P*=0.008); like ejaculatory

apodeme length, width and area in males, ovary development was suppressed in flies that received either dose of RK together with a diet of sugar only (Figure 8). Like ejaculatory apodeme length and width in males, ovary development of flies that had been fed sugar+YH did not vary significantly with RK Dose (Age $F_{1,233}$=155.021, P<0.001, RK Dose $F_{2,233}$=2.539, P=0.081, Age x RK Dose $F_{2,233}$=1.417, P=0.245) (Figure 8).

3.2.2. Spermathecal gland area

Spermathecal gland area (log transformed) varied with an age (log transformed) x diet interaction, but did not vary with RK Dose (Table 3, Figure 9). Spermathecal gland area increased more rapidly in flies that received YH.

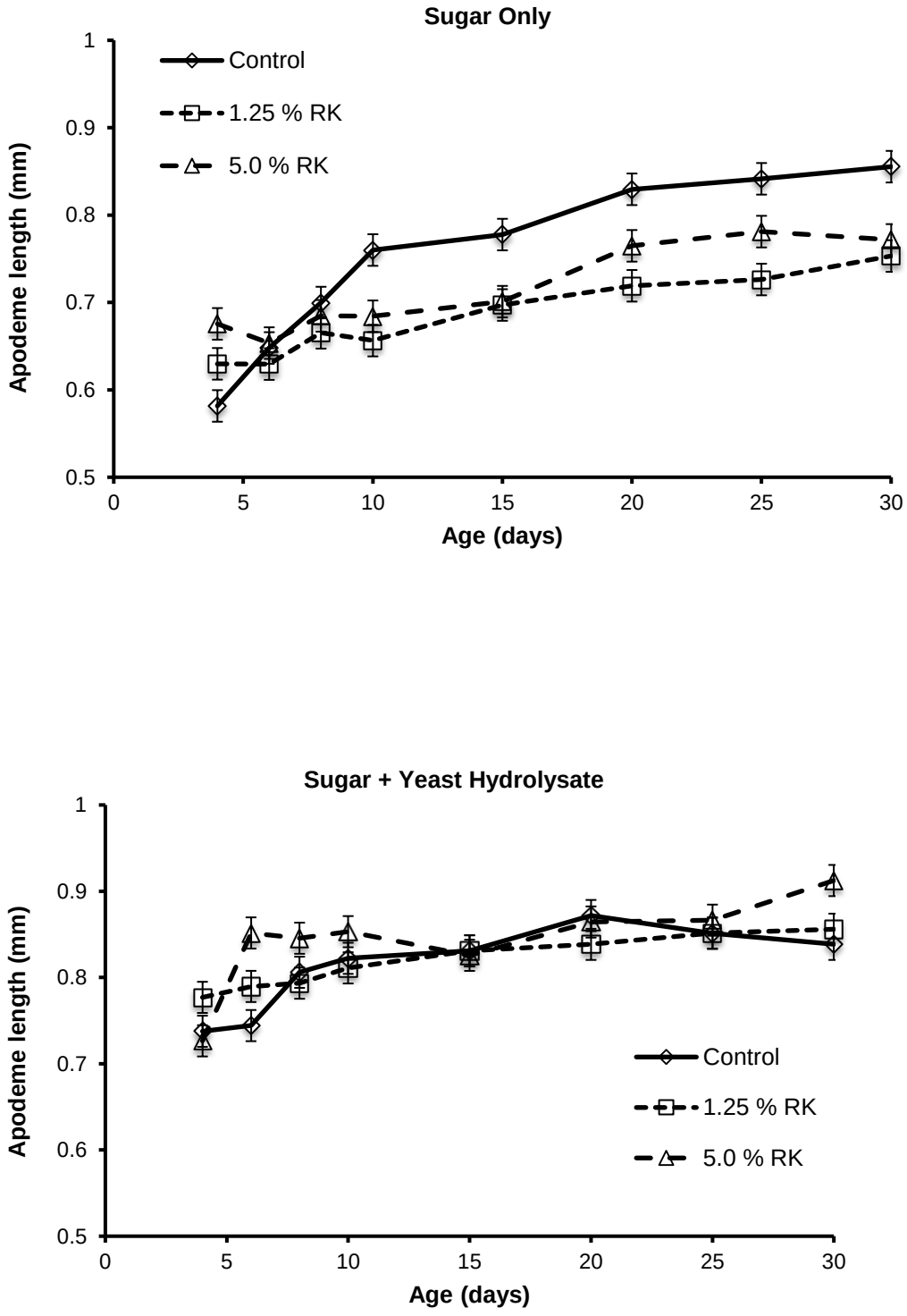
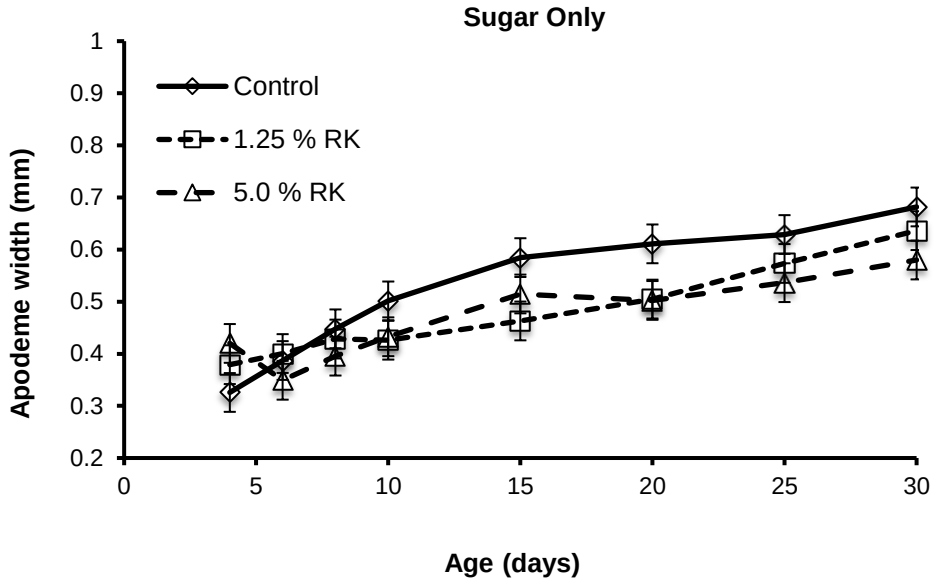


Figure 3. Effect of Age (4, 6, 8, 10, 15, 20, 25, 30 days) and RK Dose (0%, 1.25%, 5%) on ejaculatory apodeme length in Qfly males that were fed a diet of Sugar Only or Sugar + Yeast Hydrolysate for 48 hours, and sugar only thereafter. Bars represent standard errors.



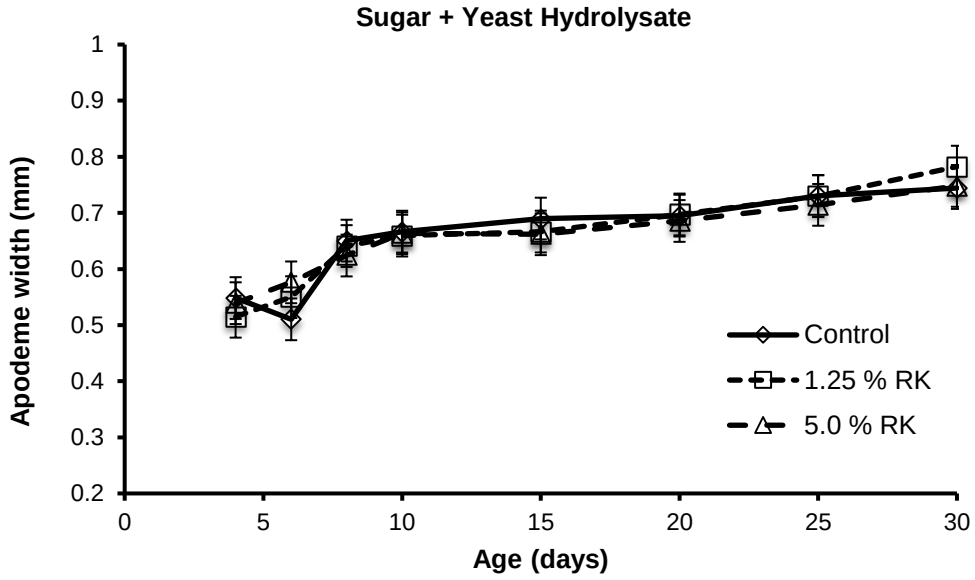


Figure 4. Effect of Age (4, 6, 8, 10, 15, 20, 25, 30 days) and RK Dose (0%, 1.25%, 5%) on ejaculatory apodeme width in Qfly males that were fed a diet of Sugar Only or Sugar + Yeast Hydrolysate for 48 hours, and sugar only thereafter. Bars represent standard errors.

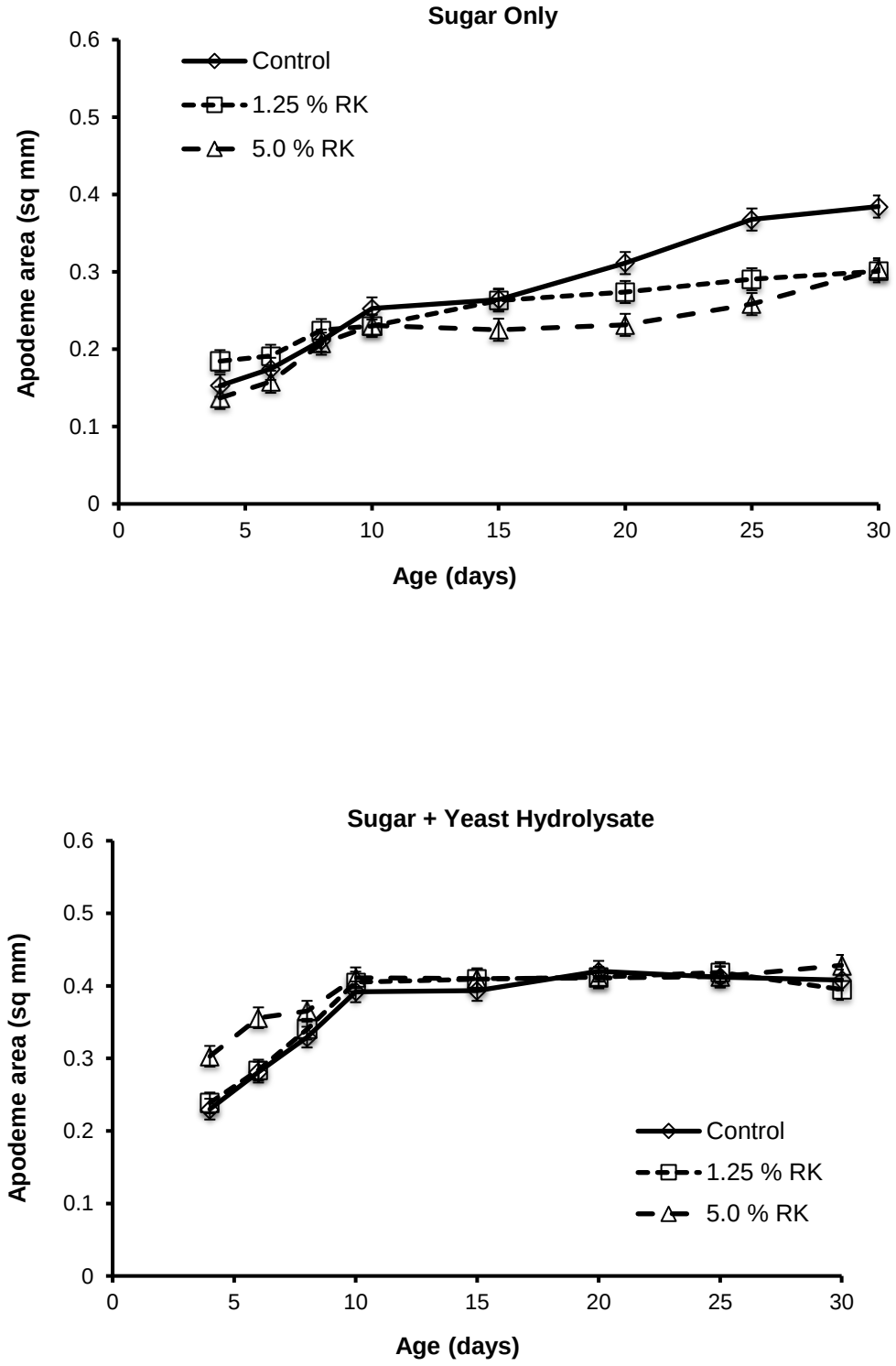


Figure 5. Effect of Age (4, 6, 8, 10, 15, 20, 25, 30 days) and RK Dose (0%, 1.25%, 5%) on ejaculatory apodeme area in Qfly males that were fed a diet of Sugar Only or Sugar + Yeast Hydrolysate for 48 hours, and sugar only thereafter. Bars represent standard errors.

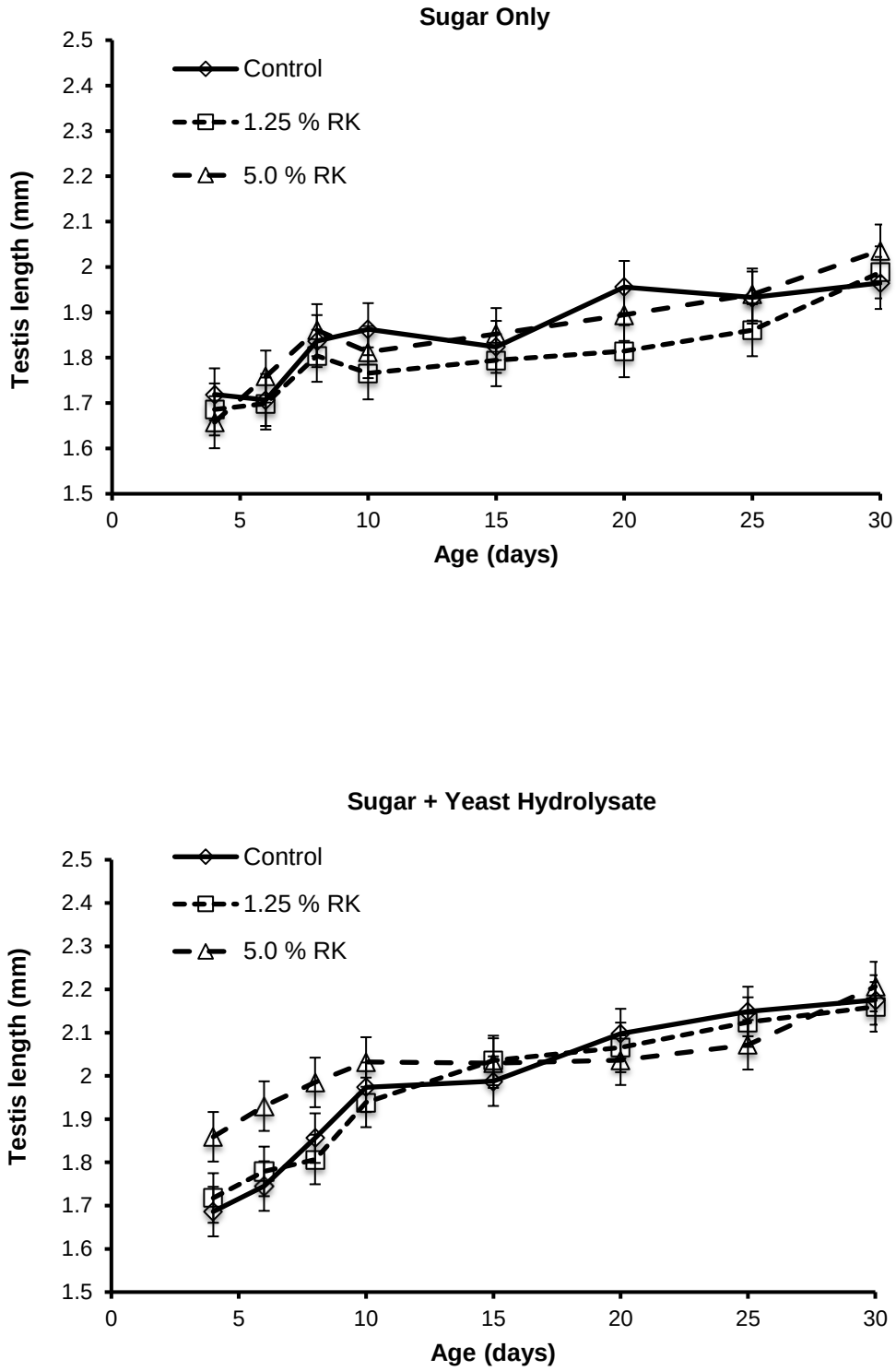


Figure 6. Effect of Age (4, 6, 8, 10, 15, 20, 25, 30 days) and RK Dose (0%, 1.25%, 5%) on testes length in Qfly males that were fed a diet of Sugar Only or Sugar + Yeast Hydrolysate for 48 hours, and sugar only thereafter. Bars represent standard errors.

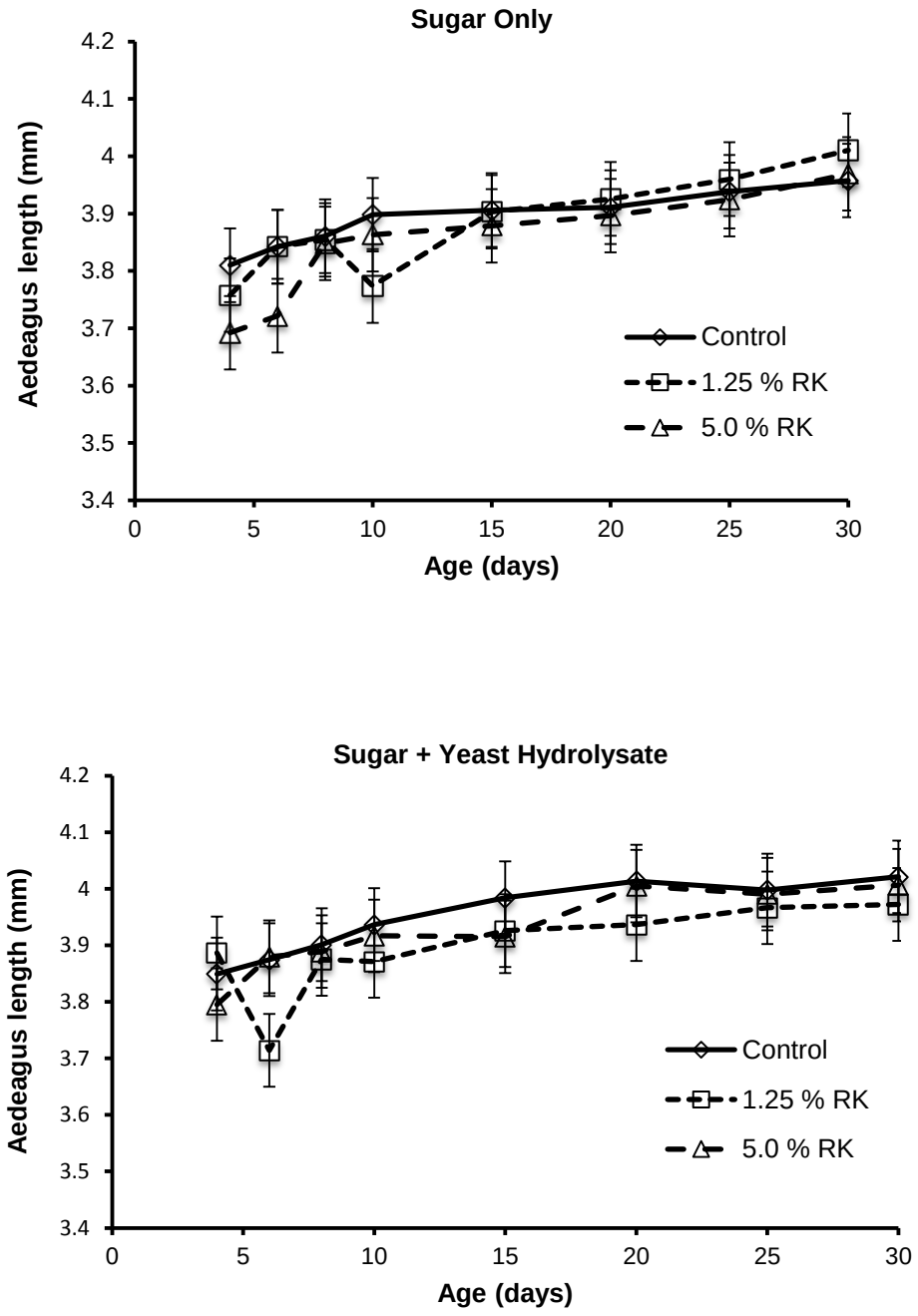


Figure 7. Effect of Age (4, 6, 8, 10, 15, 20, 25, 30 days) and RK Dose (0%, 1.25%, 5%) on aedeagus length in Qfly males that were fed a diet of Sugar Only or Sugar + Yeast Hydrolysate for 48 hours, and sugar only thereafter. Bars represent standard errors.

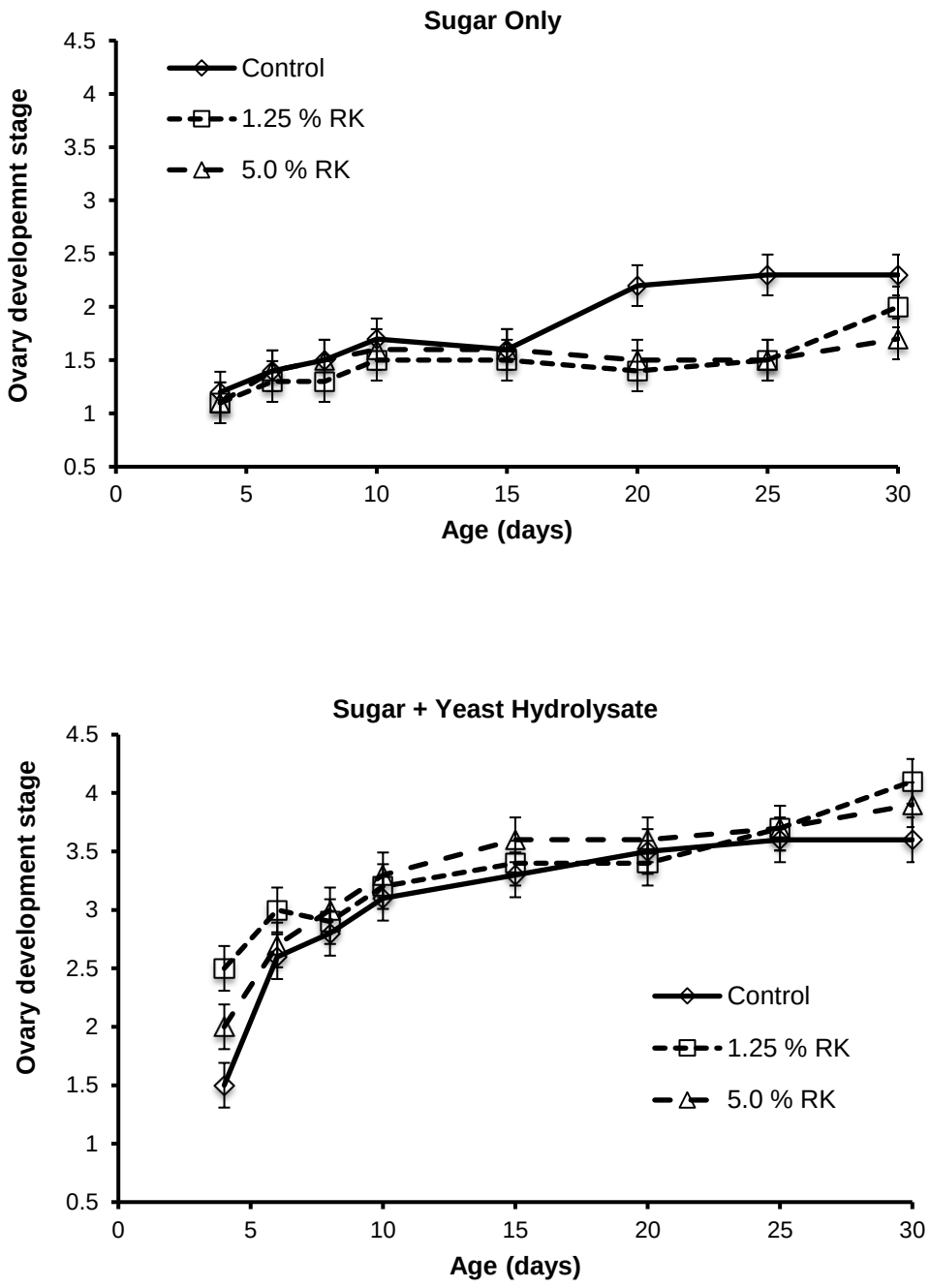


Figure 8. Effect of Age (4, 6, 8, 10, 15, 20, 25, 30 days) and RK Dose (0%, 1.25%, 5%) on ovary development in Qfly females that were fed a diet of Sugar Only or Sugar + Yeast Hydrolysate for 48 hours, and sugar only thereafter. Bars represent standard errors.

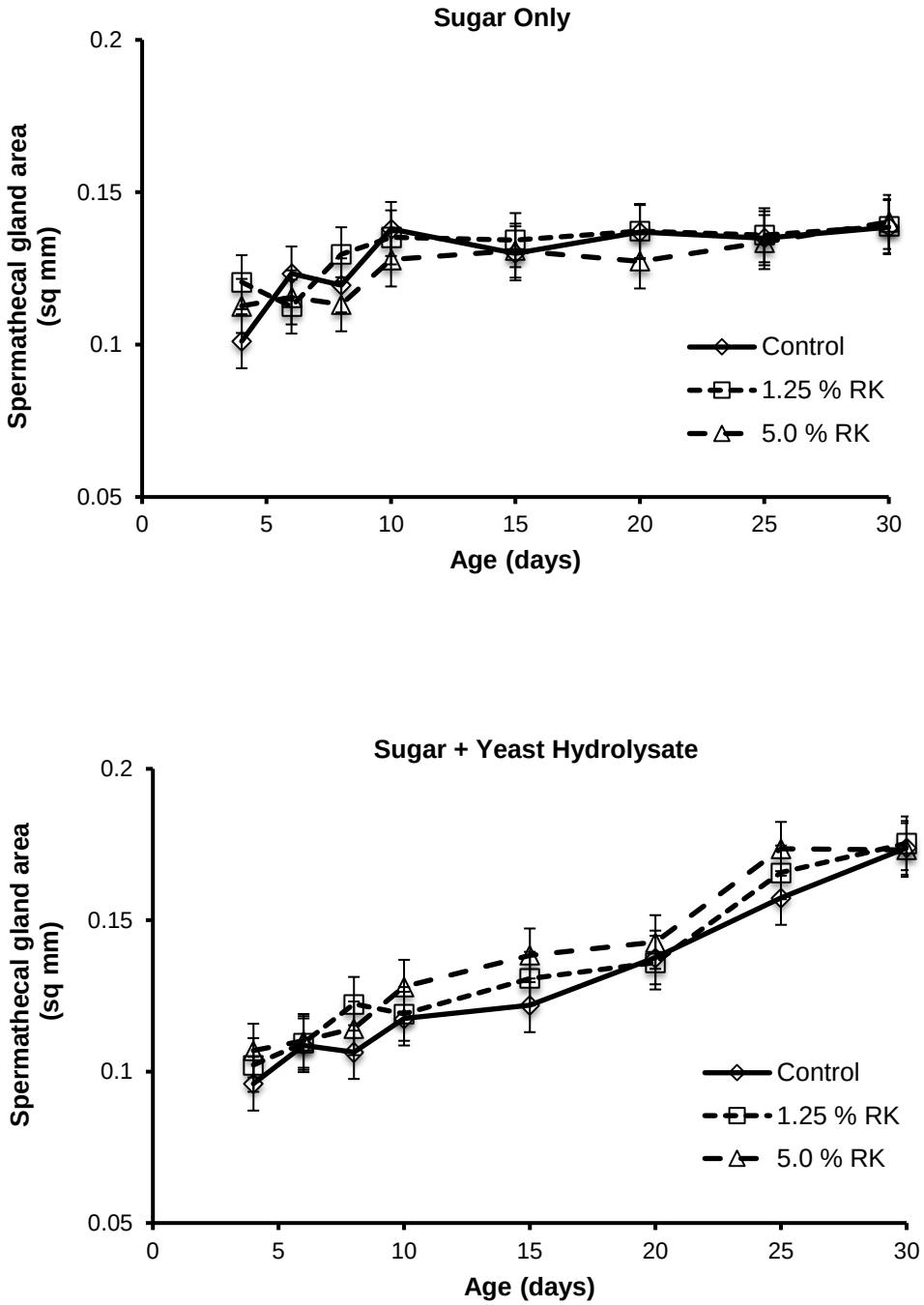


Figure 9. Effect of Age (4, 6, 8, 10, 15, 20, 25, 30 days) and RK Dose (0%, 1.25%, 5%) on spermathecal gland development in Qfly females that were fed a diet of Sugar Only or Sugar + Yeast Hydrolysate for 48 hours, and sugar only thereafter. Bars represent standard errors.

4. Discussion

4.1. Do RK supplements only affect behaviour?

SIT requires sexual competence of released sterile flies. While most assessments of fruit fly sexual competence focus on male mating ability, sexual competence is also important beyond the initiation of copulation. RK-induced accelerated emergence of sexual behaviour reported by Akter et al. (2017) may be of little value to SIT if RK supplements simply dissociate the emergence of sexual behaviour from other aspects of reproductive development. Copulations by young males would likely be ineffective if not supported by adequate physical development of reproductive organs. Findings of the present study demonstrate that the accelerated emergence of sexual behaviour in YH-fed RK-supplemented male Qflies reported by Akter et al. (2017) is matched by accelerated development of reproductive organs. Rather than causing flies to mate precociously when they lack sufficient development of reproductive organs, RK supplements provided together with YH accelerate both emergence of sexual behaviour and maturation of reproductive organs in concert.

4.2. Development of male reproductive organs

Overall, in the present study YH promoted reproductive development of both ejaculatory apodeme and testes and this pattern matches findings of Pérez-Staples et al. (2011). These results differ somewhat from those of Vijaysegaran et al. (2002) who found that YH increased development of ejaculatory apodeme size (sum of length and width) but did not detect effects of YH on development of testes. These differences in reported findings of Vijaysegaran et al. (2002) compared with Pérez-Staples et al. (2011) and the present study are likely explained by differences in how testes were measured. The present study found significant effects of YH on testes length. Vijaysegaran et al. (2002) did not assess length, but instead assessed width. While the layer of fat surrounding the testes was removed before photographing and measuring in the present study,

Vijaysegaran et al. (2002) left this fat layer in place and so their measures of testes width included both the testes themselves and the surrounding fat layer. Rather than indicating differences between ejaculatory apodeme and testes development in response to dietary YH, findings of Vijaysegaran et al. (2002) likely reflect having a much more accurate measure of ejaculatory apodeme than testes.

In addition to effects of YH, RK supplements mixed in the diet also affected development of both ejaculatory apodeme and testes in male Qflies. However, these reproductive organs were quite different in how their development responded to combined and separate provision of RK and YH.

When provided together with YH, RK did not significantly affect development of ejaculatory apodeme length or width, but the higher RK dose did induce significantly increased development of ejaculatory apodeme area. That is, the present study provides evidence that the accelerated emergence of mating activity in male Qflies fed a combination of RK and YH reported by Akter et al. (2017) is supported by corresponding acceleration in the development of at least some dimensions of ejaculatory apodeme size. Similarly, when provided together with YH, RK induced a significant increase in early development of testes length. These findings are important in that they provide a direct association between the effects of RK on emergence of sexual behaviour as flies aged and development of reproductive organs.

The present study found no evidence that RK affects testis development if male Qflies are fed a sugar only diet, and this matches Akter et al.'s (2017) finding of no evidence that RK affects mating propensity in male Qflies fed a sugar only diet. However, effects of RK on ejaculatory apodeme development of males fed a sugar only diet provide an interesting departure from patterns observed for mating activity by Akter et al (2017). While Akter et al. (2017) found no effect of RK on mating propensity of male flies fed a sugar only diet, the present study found that for males fed a sugar only diet the addition of high dose RK supplements induce a substantial suppression of ejaculatory apodeme development. That is, there

appears to be some mis-match in how RK affects emergence of mating propensity and ejaculatory apodeme development. The effect of RK on ejaculatory apodeme development in flies fed sugar only diets may reflect physiological demands on flies as a consequence of RK feeding, and the need for adequate nutrition to meet these demands. If RK feeding elevates metabolic rate in sexually immature Qflies in a manner similar to the effects of zingerone reported for mature adult males (Kumaran et al., 2014b), then RK-feeding might force flies to increase their investment in maintaining somatic tissues at the expense of developing reproductive tissues. This is an important point to bear in mind for potential application of RK in SIT contexts. There is already substantial support for the inclusion of YH as a pre-release dietary supplement in Qfly SIT programs (Pérez-Staples et al., 2008, 2009, 2011; Reynolds et al., 2014). Because the benefits of RK supplementation are only apparent when provided together with YH, RK supplements are perhaps best considered as a potential companion to YH supplements.

4.3. Development of female reproductive organs

Although significant efforts are currently under way to develop genetic sexing strains and to develop other methods to bias sex ratio in favour of males, there is currently no single sex strain of Qfly available for SIT releases and no other effective method for biasing sex ratio. Historically all Qfly SIT programs have irradiated and released males and females in approximately equal numbers (Dominiak et al., 2008; Collins et al., 2009) and bisex releases remain the only available option. Because females are released in current Qfly SIT programs, the effects of pre-release treatments such as YH and RK supplements on females also need to be considered.

Results of the present study support previous studies demonstrating a need for dietary protein for development of ovaries in *Bactrocera* fruit flies (Drew 1987). When flies were provided continuous access to a ‘full’ diet of sugar+YH, Vijaysegeran et al. (2002) found fully developed ovaries packed with mature eggs

in 7-day-old Qflies. Similar patterns have been reported in other *Bactrocera*; Chou et al. (2012) recorded fully developed ovaries in 7-day-old *B. dorsalis* and Raghu et al. (2003) reported fully developed ovaries in 9-day-old *B. cacuminata*. Contrasting these studies in which YH has been available throughout adult life, Pérez -Staples et al. (2011) found that female Qflies fed sugar+YH for only one or two days developed larger ovaries than did females fed sugar only, but did not develop ovaries as large as those fed YH continuously and also exhibited much lower mating propensity at all ages tested out to 28 days. Because two days of access to YH was far more effective at supporting sexual development in males than females Pérez -Staples et al. (2011) suggested the possibility that pre-release provisioning with YH might result in male-biased sex ratio of sexually active flies in the field. Results of the present study are a good match to those of Pérez -Staples et al. (2011) in that flies provided YH tended to have increased ovarian development but their ovaries nonetheless tended to be incompletely developed even at 30 days of age.

The effect of RK on development of ovaries in female Qflies was similar to the effect on ejaculatory apodeme length and width in males. For flies that were fed sugar+YH diets for two days following emergence there was no evidence that RK affected ovarian development but for flies that were fed sugar only diets RK supplements induced a substantial suppression of ovarian development. As has been suggested for the development of ejaculatory apodeme in males, RK supplements may impose a burden on resource allocation that can be supported by flies that have received sugar+YH diets but not by flies that have received sugar only diets. Resources that would normally be allocated to development of the ovaries may be reallocated to more urgent somatic maintenance when nutritional resources are insufficient to fully support all biological processes.

4.4. Application to SIT

Accelerated emergence of sexual activity in RK-treated male Qflies (Akter et al, 2017) accompanied by accelerated development of ejaculatory apodeme

area and testis length demonstrated in the present study, encourages further investigation of RK as a pre-release supplement for Qfly SIT programs. High post-release mortality can impose severe constraints on the efficacy of SIT, especially in species such as Qflies that have long adult development periods. In the case of Qfly, the development period decreases through domestication, dropping from 15 - 20 days in new colonies to as little as six to eight days in long-term laboratory strains maintained for >50 generations (Meats et al., 2004). Most studies report maturation in 8 - 10 days when Qflies are provided a continuous diet of sugar+YH (Vijaysegaran et al., 2002; Pérez-Staples et al., 2007, 2011). Given the substantial and compounding effects of post-release mortality in Qflies (Meats, 1998), treatments that accelerate maturation of released flies by two to three days can have a significant impact on the number of flies that attain maturity in the field and contribute to SIT.

Effects of YH and RK supplements on females do not appear to present an impediment for the use of these supplements to promote development of males. First, while males gain significant developmental advantages from two days of access to YH the developmental gains for females are comparatively modest (see also Pérez-Staples et al., 2011). Second, when flies are provided YH the additional supplementation with RK yields significant additional gains for males in terms of accelerated emergence of sexual activity and accelerated development of testes but there is currently no evidence that RK affects development of females when provided together with YH. None the less, because the presence of large numbers of sexually receptive females in bisex releases may substantially diminish the efficacy of SIT programs (Vreyson et al., 2006) there is a need now to assess effects of RK on mating propensity of female Qflies. Also, it is important to note that the present study was carried out using fertile flies. While the historical Qfly SIT programs used gamma irradiation to induce sterility (Collins et al., 2009; Reynolds et al., 2014), future programs will use x-irradiation and may later dispense with irradiation altogether, and so experiments using fertile flies provide a relevant baseline. However, for direct consideration in the context of historical or emerging SIT programs, there is a need for data on how dietary RK supplements affect development of irradiated male and female Qflies.

Like Qflies, mature males of some other *Bactrocera* and *Zeugodacus* are attracted to and feed on RK/cuelure and are known to gain sexual advantages from this 'pharmacophagy' (e.g., melon fly, Khoo and Tan 2000; Shelly 2000). Similarly, mature males of other *Bactrocera* are attracted to zingerone or methyl eugenol, and gain sexual advantages by feeding, or by exposure to aroma (e.g., oriental fruit fly, Shelly et al., 2010; Haq et al., 2015). While phytochemicals have long been known to promote sexual performance of mature adult male fruit flies, the recent application of these compounds to immature flies also shows great promise and is more compatible with a short pre-release holding period in SIT programs. The possibility that development of other *Bactrocera* and *Zeugodacus* species, or even other fruit flies, can be accelerated through supplements of RK, zingerone, methyl eugenol, or related phytochemicals, in pre-release diets warrants investigation as a potential means of enhancing SIT programs across a broad range of species.

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Chapter Four

Sexual inhibition of female Queensland fruit fly *Bactrocera tryoni* (Froggatt) mated by young raspberry ketone supplemented males

Photo: Humayra Akter

Abstract Raspberry ketone (RK) shows promise as a pre-release supplement for use in sterile insect technique (SIT) programs. Sexual maturation of Queensland fruit fly (Qfly) males is accelerated when their yeast and sugar diet is supplemented with RK for two days following emergence. Ability of males to induce sexual inhibition in their mates is key to SIT, as females that remate might acquire fertile sperm. The value of RK supplements would be greatly reduced if the early matings of young RK-treated males are ineffective at inducing sexual inhibition in mated females. Male Q-flies that received RK supplements (1.25% or 5% RK in 1:3 diet of yeast hydrolysate and sugar) and untreated control males of 6, 8, 10, 20 and 30 days old were mated with mature virgin females. Mated females were tested for re-mating at 1, 7 or 15 days after their first mating. RK supplemented males tended to mate earlier in the day than controls, but did not differ from control males in copula duration. Females mated by RK supplemented and control males did not differ in remating probability, second copula latency or second copula duration. RK-supplemented Qfly males not only mate at younger ages but these early matings are as effective as those of untreated controls at inducing sexual inhibition in mates.

Introduction

Queensland fruit fly *Bactrocera tryoni* Froggatt ('Qfly') is Australia's most damaging insect pest of commercial fruit. Originally found in the tropical and warm subtropical regions of eastern Australia, Qfly has expanded its distribution southward (Clarke *et al.*, 2011). Over the past decade Qfly has become substantially more prevalent in Australia's major southern horticultural regions and has increased in threat posed to the 'fruit fly free' regions of South Australia and Tasmania. Synthetic insecticides have for many decades provided high levels of crop protection in endemic areas, but the most effective and economical compounds are now highly restricted owing to risks posed to consumers and the environment (Dominiak and Ekman, 2013). Alternative, sustainable, approaches are needed to defend the remaining fruit fly free regions and as part of an overall management strategy for endemic regions.

Sterile insect technique (SIT) is used to manage some of the world's most damaging fruit fly pests (e.g., *Ceratitis capitata* 'medfly' Krafsur, 1998; Enkerlin *et al*., 2015, *Anastrepha ludens* 'mexfly' Orozco-Dávila *et al.*, 2007; *Bactrocera dorsalis* 'oriental fruit fly' Vargas *et al*., 2010). In SIT, millions of flies are reared, reproductively sterilised and released into the field. Females of pest populations that mate with released sterile males suffer reproductive failure (Knipling, 1955; Hendrichs *et al*., 1995). As a consequence, pest population size is reduced in the next generation. For several decades, SIT has been used to combat Qfly outbreaks in regions that are usually free of this species (Sproul *et al*., 1992; Dominiak *et al*., 2008).

Sexual performance of released males is central to the efficacy of fruit fly SIT programs, and significant research programs have been invested in understanding factors that influence male sexual performance and interventions that can yield improvements (Hendrichs *et al*., 2002; Shelly, 2010). Fruit flies are usually held in 'rearing out' facilities for several days before release and this period provides opportunity for the pre-release treatments that can improve the survival,

development, or sexual performance of released flies (Yuval *et al.*, 2007; Pereira *et al*., 2013).

Fruit flies commonly suffer high mortality rates after release in SIT programs (Meats, 1998; Hendrichs *et al*., 2007). Treatments that accelerate development of species with long adult maturation phases can increase the proportion of released flies that attain sexual maturity and contribute to SIT programs. Fruit flies are typically anautogenous, requiring a source of protein to complete reproductive maturation after emerging as adults. Addition of protein, most often as hydrolysed yeast, to the adult diet has been suggested as a useful pre-release supplement in some *Anastrepha* (Aluja *et al.*, 2001), medfly (Taylor & Yuval, 1999; Yuval *et al*., 2002, 2007; Gavriel *et al*., 2009) and Qfly (Pérez-Staples *et al*., 2008, 2009, 2011; Reynolds *et al.*, 2014). Juvenile hormone analogues such as methoprene have also proven very effective for accelerating sexual maturation of males in some *Anastrepha* and *Bactrocera* species (Teal *et al.*, 2000, 2007; Pereira *et al.*, 2009; Haq *et al.*, 2010), including Qfly (Collins *et al.*, 2014). Recently, Akter *et al.* (2017) found that raspberry ketone (RK) supplements mixed in diet of recently emerged male Q-flies is also very effective at accelerating maturation (Akter *et al.*, 2017). The effects of RK on Qfly maturation observed by Akter *et al*. (2017) closely resemble those reported previously for methoprene treatment (Collins *et al.*, 2014).

Mature males of many *Bactrocera* species are strongly attracted to plant produced ‘phytochemicals’ such as methyl eugenol and RK; these compounds and their analogues (e.g., cuelure, melolure) have served for decades as effective lures for monitoring and in some cases for male annihilation technique (Metcalf & Metcalf, 1992). Phytochemicals have an important role in the reproductive biology of male *Bactrocera*, imparting sexual advantages to mature males (Shelly & Dewire, 1994; Wee *et al.*, 2007). Mature Qfly males hydrolyse consumed cuelure to RK, which is released with the pheromone blend (Tan & Nishida, 1995), and gain enhanced mating success (Kumaran *et al.*, 2013, 2014ab). However the need to wait for the flies to mature before applying pre-release phytochemical treatments (Shelly *et al*., 2008) has greatly constrained practical application in SIT

programs. Findings of Akter et al. (2017) are transformational in that they demonstrate that RK supplements provided only during short periods, within the typical pre-release holding period, generate substantial beneficial effects in terms of male reproductive development.

Most research on pre-release treatments has focused on male mating propensity, as this is an obvious and essential element of SIT. However, mating is not the final step required to induce reproductive failure in females of pest populations. After mating, females of many fruit flies show greatly reduced sexual receptivity including medflies (Nakagawa *et al.*, 1971; Jang 1995; Chapman *et al.*, 1998; Mossinson & Yuval, 2003; Vera *et al.*, 2003; Kraaijeveld & Chapman, 2004), melon flies (Kuba & Itô, 1993; Miyatake *et al.*, 1999), olive fruit flies (Tsiropoulos & Tzanakakis, 1970; Cavalloro & Delrio, 1974), oriental fruit flies (Landolt, 1994), and Q-flies (Barton-Browne, 1957; Harmer *et al.*, 2006; Radhakrishnan & Taylor, 2007, 2008; Radhakrishnan *et al*., 2009). SIT can be compromised if females of a pest population that mate with sterile males remate with fertile males and are hence able to produce offspring (Kraaijeveld & Chapman, 2004; Collins *et al*., 2012).

Pre-release treatments that accelerate the emergence of sexual behaviour have limited value if the precocious matings of treated males fail to induce sexual inhibition in mates. Effects of cuelure feeding on post-copulatory processes have been reported in Qfly; female Q-flies mated by males that have recently fed on cuelure as mature adults exhibit increased fecundity and decreased remating receptivity (Kumaran & Clarke, 2014; Kumaran *et al.*, 2014b). In the present study we are interested in whether early matings of young male Q-flies that feed on RK as immature adults are effective at inducing sexual inhibition, and whether any effects of RK-treatment vary with age of a female's first mate or with the passage of time after mating.

Materials and methods

Origin and treatment of flies

Qfly pupae were obtained from the Fruit Fly Production Facility at Elizabeth Macarthur Agricultural Institute, New South Wales, Australia (F70-72, for production details, see Dominiak *et al.*, 2008). All flies were maintained in a controlled environment laboratory (25±0.5^{o}C, 65±5% RH) on a 0.5 dusk:11.5 light:0.5 dawn:11.5 dark cycle in which the lights turned on gradually during dawn and turned off gradually during dusk.

Pupae were sorted into three 47.5×47.5×47.5 cm mesh cages (Bugdorm 44545, Megaview, Taichung, Taiwan). Usually few flies emerge on the first day of emergence and so these flies were discarded. Emerging flies were collected over a period of 24 hours on the second day of emergence with only water for sustenance. Flies in one cage were then provided a 1:3 blend of yeast hydrolysate and sugar (control, 0% RK), thoroughly mixed in a blender. The flies in the other two cages were then provided one of two doses of raspberry ketone (RK) 4-(4-Hydroxyphenyl)-2-butanone (≥98%, Sigma–Aldrich®) ('RK-supplemented': 'high dose' of 5% RK; 'low dose' of 1.25% RK) blended together with the 1:3 blend of yeast hydrolysate and sugar. After 48 hours the food was removed. Male flies were transferred to 12.5L plastic cages and supplied with sugar and water-soaked cotton wool as sustenance until testing. RK-supplemented and control males were maintained in separate rooms during treatment and after sorting to avoid exposure of control flies to RK odours. Sorting flies immediately after treatment ensured virginity of males. Even when offered sexually mature virgin females, less than 5% of yeast hydrolystae and RK-supplemented Q-flies mate at four days (Akter et al., 2017). Two days of access to yeast hydrolysate is not sufficient to complete reproductive maturation in most female Q-flies (Pérez-Staples et al., 2011).

Mature virgin males and females used in this experiment as partners for tested flies were also obtained as pupae from the Fruit Fly Production Facility at

Elizabeth Macarthur Agricultural Institute, New South Wales, Australia, and were maintained under the same laboratory conditions as experimental males. Until used in experiments all flies were provided a 1:3 mixture of yeast hydrolysate and sugar, and water-soaked cotton wool, for sustenance. Females and males were sorted within 3 days of emerging and were kept in separate 12 L cages. These flies were kept in a separate room from the experimental male flies to avoid exposure to RK odours. These flies were used at 12-17 days of age as virgin partners for first matings (females) or to test female remating propensity (males).

First mating

When 6, 8, 10, 20 and 30 day old RK-supplemented and control males were paired individually with a 12-17 day old sexually mature virgin female in 1-L cage at least 4 hours before dusk. Cages were of clear plastic, with a 40 cm^2 mesh-covered window for ventilation. Sugar coated paper and water-soaked cotton wool were placed in each cage as sustenance. Cages were observed periodically from set up and then constantly (using a dim red lamp for illumination) from 2 hours before dusk until the end of the copulation to assess the time between initiation of dusk and the initiation of copula ('copula latency') and the time between intromission and withdrawal of the aedeagus ('copula duration'). If copulations commenced before dusk, a negative copula latency was recorded. On each day new male flies were paired with new mature virgin females. Overall, 110 pairs of flies were tested for each treatment on each test day across the 3 treatment groups, providing a total of 1,650 test pairs over the experiment using three different batches of flies obtained at least 3 weeks apart.

Table 1. Results of logistic regression investigating the effects of Male age (6, 8, 10, 20, 30 days), RK Dose (control, low, high) and remating trial day (1, 7, 15 days) on female remating probability.

	d.f.	*G*	*P*
Male age	1	3.515	0.061
RK Dose	2	1.226	0.542
Remating day	2	21.932	**<0.001**
Male age x RK Dose	2	0.573	0.751
Male age x Remating day	2	8.881	**0.012**
RK Dose x Remating day	4	1.446	0.836
Male age x RK Dose x Remating day	4	4.289	0.368

Table 2. Results of mixed models investigating the effects of Male age (6, 8, 10, 20, 30 days), RK Dose (control, low, high) and remating trial day (1, 7, 15 days) on Latency and Duration of second matings.

	Latency			Duration		
	d.f.	*F*	*P*	*d.f.*	*F*	*P*
Male age	1,224.2	5.622	**0.019**	1,562.7	0.654	0.419
RK Dose	2,542.8	0.453	0.636	2,562.2	1.482	0.228
Remating day	2,386.9	2.284	0.103	2,563.1	0.977	0.377
Male age x RK Dose	2,556.9	0.666	0.514	2,562.1	0.791	0.454
Male age x Remating day	2,564.6	4.582	**0.011**	2,562.2	0.244	0.784
RK Dose x Remating day	4,563.6	0.738	0.566	4,562.1	1.385	0.238
Male age x RK Dose x Remating day	4,563.7	0.841	0.499	4,562.1	0.163	0.957

Remating

On the day after the first mating, all mated males were removed from the cages and mated females were divided equally into three groups to test for remating 1, 7 and 15 days after their first mating. All unmated males and females were discarded.

For female flies to be tested for remating 1 day after their first mating, the sugar-soaked paper and water soaked cotton wool was left in place from the first day. For flies mated 7 and 15 days after their first mating, the female was left in the cage and provided with water soaked cotton wool, granulated sucrose, and hydrolysed yeast in a 30 mm diameter Petri dish (sucrose: yeast = 3:1). Water soaked cotton was replaced every day and yeast was replaced if mould was observed. On the day of remating trials, food and water was removed and the mated female was paired with new 12-17 day old virgin male in same cage where the female had mated on the first day and been maintained since. Incidence of mating, copula latency and copula duration were recorded as for the first mating.

Data analysis

Copula latency and copula duration of first matings and rematings were assessed using least squares linear mixed models and female remating probability was assessed using logistic regression. Models included batch as a random effect, male age as a continuous fixed factor, and RK Dose and remating day as nominal fixed factors. All analyses were performed in JMP version 10 (SAS Corp).

Results

First mating latency

Mating latency (Figure 1) tended to increase with Male age (b=0.29±0.09, $F_{1,1358}$=9.481, P=0.002) and varied with RK Dose ($F_{2,1357}$=41.310, P<0.001) and there was no evidence of interaction between effects of Male age and RK Dose ($F_{2,1357}$=0.451, P=0.637). Pairings with control males tended to mate shortly after the onset of dusk, but pairings with RK-supplemented males tended to mate before the onset of dusk (Control 3.18±3.27 min > low RK dose -8.67±3.27 min > high RK dose -14.41±3.27 min, all significantly different by Tukey's HSD).

First mating copula duration

Copula duration did not show significant variation with Male age or RK Dose (Male age $F_{1,1358}$=1.895, P=0.169, RK Dose $F_{2,1357}$=0.479, P=0.620, Male age x RK Dose $F_{2,1357}$=1.796, P=0.166).

Remating probability

Remating probability of mated females was not affected by RK treatment, but did vary with an interaction between Male age and Remating day (Table 1; Figure 2). Females were more likely to remate on the day after their first mating than at 7 or 15 days after their first mating (Table 1; 1 day 49% of 566, 7 days 38.9% of 486, 15 days 36.6% of 314). Females that mated first with an old male were more likely to remate on the day after their first mating than were females that mated first with a young male (G_1=13.168, P<0.001), but no effect of Male age was evident for females tested seven days after their first mating (G_1=0.214, P=0.643) or fifteen days after their first mating (G_1=0.202, P=0.653).

Remating latency and Remating copula duration

Remating latency varied with male age, although this tendency differed amongst the Remating days (Table 2; Figure 3). For females tested seven days after their first mating there was a significant positive relationship between Male age (i.e., age of her first mate) and Remating latency (b=0.41±0.11, $F_{1,184.9}$=13.019, P<0.001), but no effect of Male age was evident for females tested on the day after their first mating ($F_{1,273.6}$=0.044, P=0.834) or fifteen days after their first mating ($F_{1,111}$=1.552, P=0.216). RK treatment of the female's first mate did not affect female Remating latency on any of the remating days tested. Remating copula duration did not vary with age or RK treatment of the female's first mate, or with number of days since the female's first mating (Table 2).

Figure 1. Effects of male age (6, 8, 10, 20, 30 days) and RK Dose (control, low, high) on mean first mating latency at each male age tested for each of the RK treatments. Error bars are standard errors.

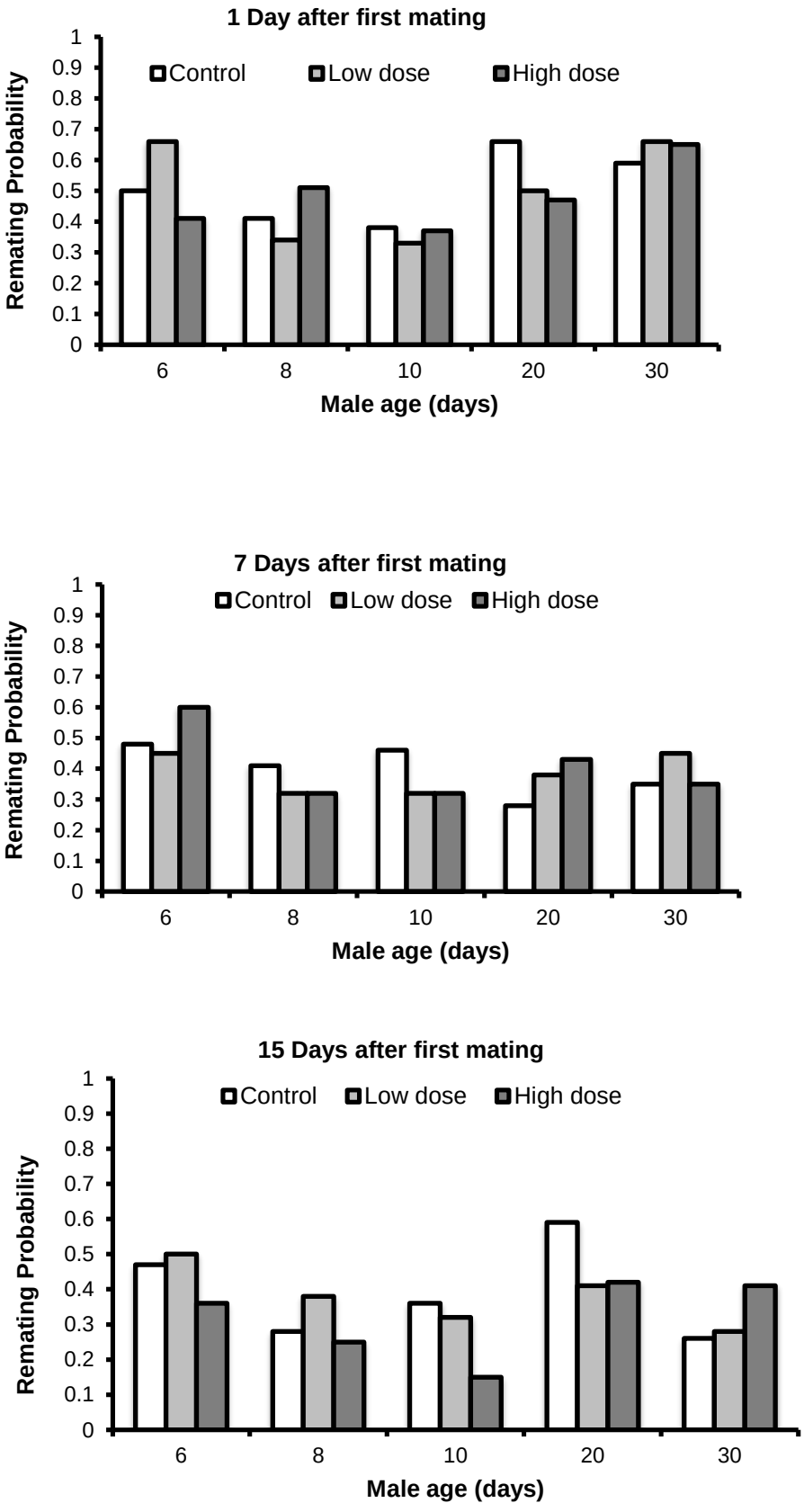

Figure 2. Effects of male age (6, 8, 10, 20, 30 days), RK Dose (control, low, high), and number of days since first mating (1, 7, 15 days) on female remating probability. Data presented are pooled totals for all batches tested.

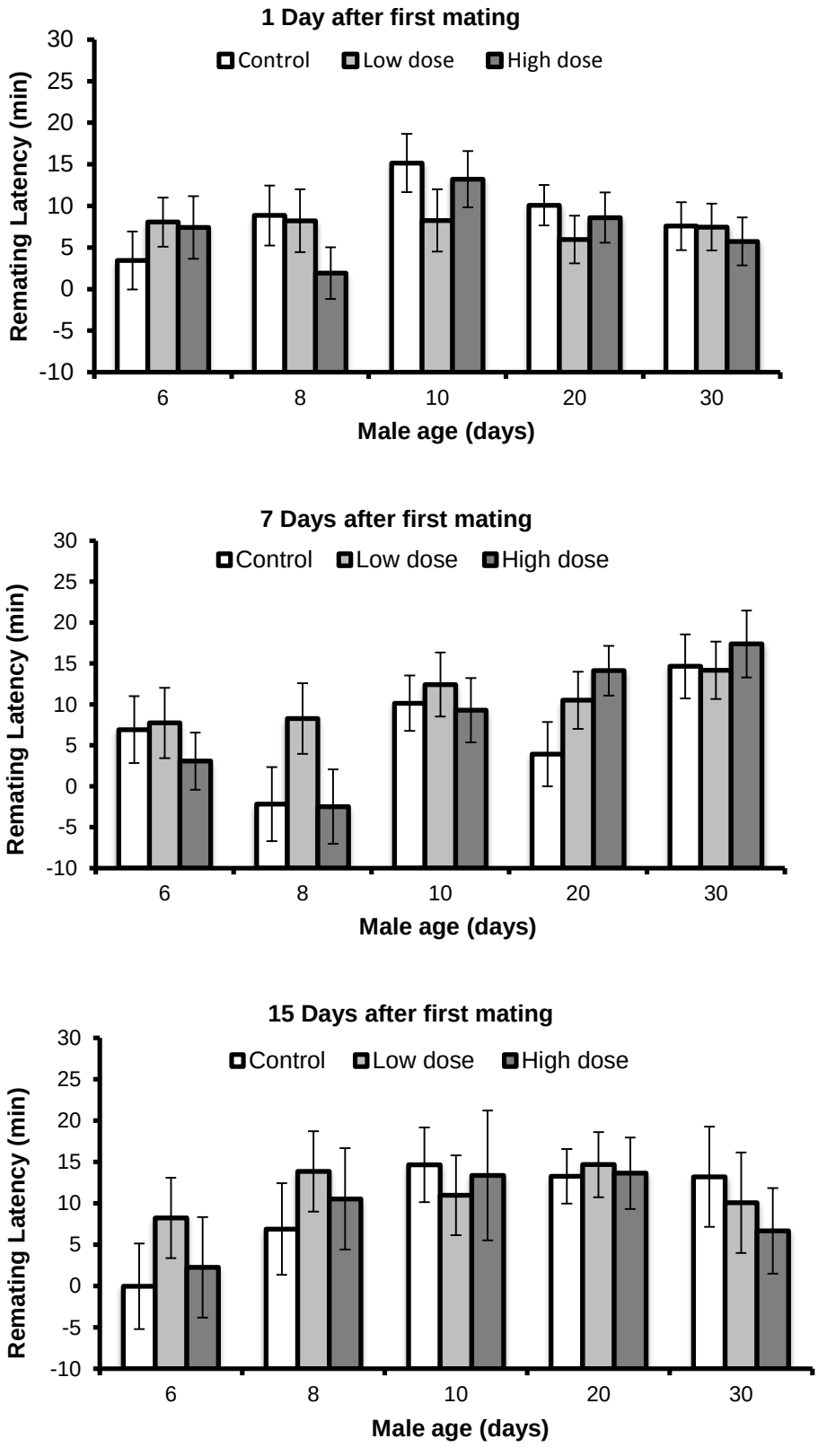

Figure 3. Effects of male age (6, 8, 10, 20, 30 days), RK Dose (control, low, high), and number of days since first mating (1, 7, 15 days) on female remating latency. Error bars are standard errors.

Discussion

No effect of male RK supplements on female remating

This is the first study to assess remating propensity of female fruit flies after mating with males that have been provided RK supplements as immature adults. The purpose of RK supplementation of immature adult males has some similarities to the conventional approach of treating mature adults in that enhanced mating performance of released flies is a sought outcome. However, the purpose is also different in that benefits accrue largely from accelerated development, and in this regard RK supplementation of immature males has similarity to methoprene treatment of pupae and young adults (e.g., Collins *et al.*, 2014). RK supplements provided to males did affect the time of onset of their copulations, with pairings of RK-supplemented males mating earlier in the day than controls, but did not affect copula duration. Although RK-supplemented males mate at younger ages than controls (Akter et al., 2017), there was no evidence that copulations of RK-supplemented males differed from controls in remating propensity of their mates.

Several previous studies have considered remating propensity of female *Bactrocera* mated by males that have had access to natural phytochemicals and their analogues such as RK, cuelure, or methyl eugenol as mature adults rather than as immature adults (see below). While such treatments of mature flies do not have a purpose of accelerating development, by considering the differences and similarities of biological responses we can gain some insight to differences in mechanisms responsible for the effects observed. It is also possible that RK fed to immature males persists in the body through development and be present to act directly on physiology after the flies mature.

Contrasting findings of the present study in which RK was fed to immature males, Kumaran *et al.* (2013) found that females mated by males provided access to cuelure or zingerone as mature adults exhibited approximately half the remating propensity of females mated by untreated males. Other aspects of female

reproduction were also affected, as females mated by treated males exhibited elevated fecundity, especially over the three weeks after mating, and also exhibited reduction in longevity. While effects of mating with males fed RK as immature adults on female fecundity and longevity have not yet been assessed, the marked differences in female remating propensity in the present study and Kumaran et al. (2013) suggest that RK fed to immatures does not persist in the body to later act directly on mature adults. This is not unexpected, given that even when fed to mature adult males the effects of cuelure and zingerone only last 1 - 3 days (Kumaran *et al.*, 2014ab). In a close parallel to the findings of Kumaran *et al.* (2013), Morelli *et al.* (2013) exposed mature male medflies to ginger root oil and found that the remating propensity of their mates was halved in comparison to mates of untreated males. While there has been substantial exploration of pre-release treatments targeting mature adult male fruit flies in terms of mating potential, much remains to be learned about the effects of these mating enhancers on post-copulatory processes.

Treatments for immature adults differ from those for mature stages in that benefits are generally sought in terms of reproductive development. While additional benefits might accrue from enhanced sexual performance (e.g., more vigorous courtship, more attractive pheromone) compared with males that mature without RK supplements, the primary value of RK supplements as deployed with Qfly in the present study is in substantial acceleration in the emergence of sexual behaviour (Akter *et al.*, 2017). Methoprene has also been used as a pre-release treatment to accelerate emergence of sexual behaviour in Mexican fruit fly (Gómez *et al.*, 2013; Periera *et al.*, 2013), Caribbean fruit fly (Teal *et al.*, 2000), medfly (Faria *et al.*, 2008) and melon fly (Haq *et al.*, 2010), and so to some extent may be considered a parallel approach. In Q-flies, the acceleration of mating found with RK supplements is very similar to what has been observed for methoprene treatment of adults and pupae (Collins *et al.*, 2014). Of concern, Abraham *et al.* (2013) found that females of South American fruit fly that mated with 6-day-old methoprene-treated males, the age at which elevated mating propensity and competitiveness is expressed (Segura *et al.*, 2009, 2013; Liendo *et al.*, 2013), remated more often and sooner than females mated with mature males that had not received methoprene treatment. These results point to a decoupling of sexual

maturation in terms of mating behaviour and reproductive development in terms of underlying morphology and physiology. That is, to some extent, the effects of methoprene may be largely behavioural, promoting relatively ineffective matings by young, reproductively immature, males. While RK has an effect on the emergence of sexual behaviour in a manner that closely parallels effects of methoprene, in the present study the matings of young RK-supplemented male Q-flies were effective at inducing sexual inhibition in females.

Male age-linked changes in female remating

Female remating propensity changed with time since the female's first mating in the present study, and this tendency was similar for mates of RK supplemented and control males. On the day after their first mating, females were more likely to remate than was the case if when other females were tested seven or fifteen days after mating. However, this elevated remating tendency of one-day post-mating females was closely linked to male age; older males more often failed to induce sexual inhibition in their mates over this time frame but over longer periods were as effective as younger males. This is the first report of male age-dependent ability to induce sexual inhibition in Qfly.

As in medflies (Jang, 1995, 2002; Marchini *et al.*, 2003) and South American fruit fly (Abraham *et al.*, 2012), accessory gland fluids transferred with the first mate's ejaculate are key to the induction of sexual inhibition of mated female Q-flies (Harmer *et al.*, 2006; Radhakrishnan & Taylor, 2007, 2008; Radhakrishnan *et al.*, 2009). It seems likely that the ejaculate of older males differed from that of younger males in composition or titre and that this has diminished their ability to induce sexual inhibition in their mates on the following day. Alternatively, while there are currently no studies linking male Qfly behaviour to the induction of sexual inhibition in mates it is conceivable that older males were deficient in some aspect of pre- or post-copulatory courtship and that this has resulted in a diminished ability to induce sexual inhibition.

It is interesting to note that no effects of first mate age were apparent when the females were tested for remating seven or fifteen days after their first mating. That is, females that mated with old males had high remating probability the next day but by seven days these females exhibited the same level of sexual inhibition as females mated by young males. Such a delay in the onset of sexual inhibition of females mated by older males may comprise a post-copulatory expression of female mating preference. At the time of their first mating, all females were virgin and had never before encountered a sexually mature male. Accordingly, these females may estimate males as very rare and predict a long delay until encountering a second male. Under these conditions, females might be highly inclined to mate with the first male they encounter for reproductive security. Then, having securing an ejaculate to fertilize their eggs females that mate with non-preferred male types might then retain an elevated sexual receptivity to enable an ejaculate 'upgrade' should an opportunity become available soon after.

What is the significant of male age-dependent ability to induce sexual inhibition in mates to SIT? First, the effect was only expressed on the day after the first mating and was no longer evident seven days after mating. It would be useful to know the time course followed by the induction of sexual inhibition between these two time points and how this relates to male age or other aspects of mate quality; a shorter period of retained receptivity after a first mating with older males would mean a lower prevalence of remating overall and less reason for concern. Second, with high daily mortality rates in the field following release it is likely that the large majority of matings are by younger males that have ample capacity to induce sexual inhibition in their mates.

Summary

Treatments that accelerate development of fruit flies released in SIT programs have potential to substantially improve efficacy and cost effectiveness. Recently, Akter *et al.* (2017) developed a novel approach to accelerate development of Q-flies - incorporation of RK in the diet for two days shortly after adult emergence.

RK-supplemented male Q-flies typically mate two to three days earlier than untreated males, but the question of whether these precocious matings are effective had not been resolved. Here we find that the matings of young RK-supplemented male Q-flies show no reduction in success at inducing sexual inhibition in mates, a key measure of success in this species.

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Chapter Five

Do raspberry ketone supplements provided to immature Queensland fruit fly *Bactrocera tryoni* (Froggatt) change sex pheromone quantity or composition?

Abstract

Raspberry ketone (RK) supplements provided together with sugar and yeast hydrolysate accelerate sexual maturation of Queensland fruit fly (Qfly) males. However, the mechanisms underlying this precocious mating are currently unknown. Pheromones are an important element of Qfly sexual calling and courtship and so changes in pheromone quantity or quality may be involved, and the present study investigated this possibility. Flies were fed a diet of sugar only or yeast hydrolysate mixed with sugar (YH+S) (1:3) that contained 0% RK (control), 1.25% RK (low dose), or 5% RK (high dose) for two days after emergence. Pheromone was collected from rectal glands and headspace when flies were 6, 8, 10, 20, and 30 days old. Two endogenously produced pheromonal compounds *N*-(3-methylbutyl)propanamide and *N*-(3-methylbutyl)acetamide were dominant in RK-fed and RK-unfed male Qfly in both rectal gland and released volatiles. High dose RK had strong effect on the production of total pheromone (endogenous + RK) and endogenous pheromone in rectal glands; males fed high dose RK exhibited a significant increase in pheromone production in rectal glands compared to control. Low dose RK had no effect on production or release of total pheromone or endogenous pheromone compounds. Unaltered RK was found in rectal gland until 30 days in both diet groups at both high and low RK doses, however, in released volatiles RK was found inconsistently. Age had a significant effect on production and release of total pheromone and endogenous pheromone components in low dose treated males. In high dose treated male age had significant effect on pheromone production in rectal glands when interacted with dose and diet; age also affected released pheromone significantly. Overall, YH+S fed flies stored and released more pheromone than sugar-fed flies. Ratio of *N*-(3-methylbutyl)propanamide to *N*-(3-methylbutyl)acetamide in YH+S fed males was higher than sugar-fed males regardless of RK-fed or RK-unfed and it changes with age. The results were discussed in the context of mating advantage and sterile insect technique.

Introduction

Insects commonly maintain close relationships with plant allelochemicals which they co-opt for defences or/and sexual communication (Boppré, 1984; Krasnoff and Dussourd, 1989; Landolt and Phillips, 1997; Amano et al., 1999; Tillman et al., 1999; Reddy and Guerrero, 2004; Lucas-Barbosa et al., 2011; Beyaert and Hilker, 2014). For instance, volatile chemicals from corn silk triggers the production and release of sex pheromone in *Helicoverpa zea* (Raina et al., 1992), and the arctiid moths *Creatonotos gangis* and *Creatonotos stransiens* release significantly altered pheromones after feeding as larvae on plants containing pyrolizidine alkaloids (Schneider et al., 1975). Mature males of many tephritid fruit flies are strongly attracted to certain non-host phytochemicals that can affect their sexual communication (Renou and Guerrero, 2000). Raspberry ketone (RK),4-(4-hydroxyphenyl)-2-butanone and methyl eugenol (ME) 1,2-dimethoxy-4-(2-propenyl)-benzene are found in a wide range of plants and are highly attractive to mature males of some *Bactrocera* and *Zeugodacus* fruit flies (Metcalf and Metcalf, 1992). Because of this attraction, some phytochemicals (or their synthetic analogues) are widely used as lures for monitoring and control of fruit fly populations (Jessup et al., 2007; Vargas et al., 2010; Dominiak et al., 2011; Dominiak and Ekman, 2013).

Attraction of adult male fruit flies to particular plants, and specifically to particular phytochemicals emitted by these plants, is related to sexual performance as males exposed to these compounds can gain substantial mating advantages (Shelly and Dewire, 1994; Shelly and Villalobos, 1995; Shelly, 2000a; Shelly and Nishida, 2004; Shelly, 2010; Shelly et al., 2010). In part, these mating advantages may be related to pheromone production. Ingested phytochemicals may be transported via the haemolymph to the rectal gland, which is the site of pheromone storage (Hee and Tan, 2005, 2006; Wee and Tan, 2007). Phytochemicals may either be transported to the rectal gland in an unaltered state as is the case for raspberry ketone (Nishida et al., 1993; Tan and Nishida, 1995), as hydrolysed analogues (Nishida et al., 1990; Kumaran et al., 2014), or as conversion products such as methyl eugenol converted to coniferyl alcohol (CF) (Hee and Tan, 2004, 2006; Wee and Tan, 2007). Oriental fruit fly *B. dorsalis* males

are attracted to methyl eugenol-containing flowers and accumulate metabolites of methyl eugenol in their rectal glands for release as pheromone components (Nishida et al., 1988; Nishida and Fukami, 1990; Tan and Nishida, 2012). Male *B. dorsalis* that fed on a *Bulbophyllum baileyi* orchid flowers sequester zingerol (a reduced form of zingerone) (Tan and Nishida, 2007). Males of *B. cucurbitae* are attracted to the flower surface of *Dendrobium superbum orchids* and sequester RK in their rectal glands (Nishida et al., 1993). Males of *B. caudatus* similarly sequester RK in their bodies after feeding on *Bulbophyllum apertum* (Tan and Nishida, 2005). Females of some species respond more strongly to odours released from males that have fed on phytochemicals (Hee and Tan, 1998; Khoo and Tan, 2000; Wee and Tan, 2005, 2007; Wee et al., 2007).

Queensland fruit fly (Qfly), *Bactrocera tryoni* (Froggatt), an economically important pest in Australia, responds strongly to cuelure/raspberry-ketone (Meats and Hartland, 1999). Cuelure is used in monitoring (Brieze-Stegeman et al., 1978; Osborne et al., 1997; Meats et al., 2002; Dominiak et al., 2003; Jessup et al., 2007; Dominiak et al., 2011; Dominiak and Ekman, 2013) and for control by attracting and killing males (male annihilation technique, MAT) (Bateman, 1982; Dominiak et al., 2009). Chemical insecticides have historically been the mainstay of Qfly control but recent restrictions on the use of the most effective insecticides have presented substantial challenges for management of this devastating pest (Dominiak and Ekman, 2013). Environmentally friendly as well as effective control methods are in demand for Qfly control, and Sterile Insect Technique (SIT) is an important element of this (Dominiak et al., 2011; Meats et al., 2003; Reynolds et al., 2010). In SIT, millions of sterile males are released in the field to mate with wild females, curtailing their reproduction. High mortality and poor performance of mass reared sterile flies in the field can be an important constraint on the success of SIT (Monro and Osborn, 1967; Dominiak, 2003; Meats, 2003). Pre-release treatments that can accelerate sexual maturation or increase performance of males are of particular interest.

In a recent study, Akter et al. (2017) found that raspberry ketone (RK) combined with a protein-rich diet accelerated sexual development of immature

Qfly adults, potentially offering a solution to counter high pre-maturation mortality in the field. However, the mechanisms underpinning the mating advantages of young RK-fed males are unknown. Male Qflies emit pheromones to attract mates at dusk (Fletcher, 1968; Bellas and Fletcher, 1979). Given studies of other fruit fly species for which mature adult males are thought to gain mating advantages owing to presence of RK in pheromones (Tan and Nishida, 1995, 1996, 2000; Tan, 2000; Hee and Tan, 2004; Tan et al., 2011, 2014), it is possible that changes in pheromone quantity and/or composition released by RK-fed males could be in part responsible for the elevated mating performance of young RK-treated males. Considering these possibilities, this chapter assesses the quantity and quality of pheromone produced by male Qfly after feeding as immature adults on diets with or without RK.

Materials and methods

General methodology

Biological material. Qfly pupae were obtained from the Fruit Fly Production Facility at the Elizabeth Macarthur Agricultural Institute at Camden, New South Wales, Australia (for production details, see Dominiak et al., 2008). All pupae and flies were maintained, and experiments 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 hour of the light phase simulated dawn and dusk by gradually ramping the light levels up and down, respectively. Upon arrival at Macquarie University, ca. 2000 pupae were transferred to each of six open Petri dishes that were placed in separate 47.5×47.5×47.5 cm mesh cages (Bugdorm 44545, Megaview, Taichung, Taiwan). Adult flies emerged in these cages, where they were provided only water-soaked sponge for sustenance. Usually only a small number of flies emerge on the first day of emergence, and these were discarded. Flies from the second 24 hours of emergence were used in experiments.

Fly treatment. Overall, fly treatment matched those of Akter et al (2017). After emergence 0-24 hr old flies were provided raspberry ketone (RK), 4-(4-

Hydroxyphenyl)-2-butanone (≥98%, Sigma–Aldrich®) in one of two diets; a 1:3 mixture of yeast hydrolysate and sugar (YH+S) or sugar alone. Two doses of RK were used in two experiments; in one experiment a high dose of 5% RK was used along with control group (0% RK) and in the other experiment a low dose of 1.25% RK was used along with a control group. After 48 hours of feeding, the treated food was removed. Flies were sorted by sex at 3 days of age that ensure virginity. Laboratory-adapted, mass-reared Q-flies do not begin mating before 5 DAE (Meats et al., 2004). After sorting, males were kept in 12.5L clear plastic cages (with three 10 cm diameter mesh-covered openings for ventilation) with sugar and water available ad libitum until testing. Treated and control males were maintained in separate rooms after sorting to avoid exposure of the control males to RK odors.

Pheromone collection

Rectal gland extraction. Rectal gland extracts were collected from 6, 8, 10, 20 and 30 day old RK-fed and RK-unfed adults. On each day, male flies were collected with 5 mL clear plastic vials and were anesthetized by chilling in dry ice before dissection. Dissection was performed under a stereomicroscope (SZX12; Olympus, Tokyo, Japan) and forceps were cleaned with acetone between dissections to avoid contamination. During dissection the abdomen (dorsal side up) was squeezed carefully towards the anal end with one set of fine forceps and with another set of fine forceps the rectal glands were pulled gently and excised with fine scissor. After excising 10 intact glands were collected in teardrop tubes (micro screw neck vial 1.1 mL, 32X11.6mm clear glass conical, Global Science) which were imbedded in dry ice. After all glands had been harvested the teardrop tubes were removed from the dry ice and 100 µL of hexane (≥98.5%, HPLC grade, Fisher Scientific, UK) was added. Glands were left in hexane for 10 minutes to extract the contents of the glands.After 10 minutes the extracted sample was transferred to 1.5 mL screw cap clear vial (Grace Discovery Sciences) using 100 µl disposable capillary tubes (Drummond Microcaps® Lambdas) and stored at -20ºC until GC-MS analysis. All dissection and extraction were finished 2 hours before dusk to ensure full contents in rectal glands. Such 7 replicates were done at high RK dose along with control in one set and 8 replicates were done at low RK dose along with control in another set. Flies of each replicates were from different batches of pupae that were obtained two weeks apart.

*Head space collection.*To assess the pheromone components released by calling males 30 virgin males from each treatment (RK-fed and RK-unfed) were tested on 6, 8, 10, 20 and 30 days of age. Males were placed into a cylindrical glass chamber half an hour before dusk to allow acclimatization. Each replicate of head space sampling was accompanied by an air sampling using one empty chamber. An air sampling was to take account of impurities in head space of a particular replicate. The glass chamber was equipped with an inlet and an outlet (150 mm long and 40 mm diameter, CBG, Australia) and closed with Cone/Screw-thread Adapters (Quickfit®, UK, Sigma-Aldrich®). The outlet of chamber was connected to a tenax tube (100/50 mg Tenax® TA, Sigma-Aldrich®) and the inlet of the chamber was connected to charcoal filter (Sigma-Aldrich®) with tygon tubes (Tygon® formula E-3603, USA, Sigma-Aldrich®). The down stream of tenax tube was connected to the flowmeter, which was connected to a pump as a pulling system. Upon the commencement of an experiment the pump pulled laboratory air through a charcoal filter to purify air, over the males at a flow rate of 800 mL/min and the duration of head space collection was 1.5 hours. Volatile compounds were trapped in a tenax tube that were eluted with 1000 µL of hexane (HPLC grade) into 1.5 mL screw cap clear vials. The samples were stored at -20ºC until GC-MS analysis. Such 7 replicates were done at high RK dose along with control in one set and 8 replicates were done at low RK dose along with control in another set which resulted in 210 flies/treatment/testing day at high RK dose and 240 flies/treatment/testing day at low RK dose.

GC-MS analysis and data processing. GC-MS analysis was performed on a Shimadzu GC QP2010 equipped with a split/splitless injector, a Restek Rxi-35 fused silica capillary column (30 m × 0.25 mm, 0.25 µm film) and integrated MS that has a NIST library (NIST21 and NIST107). Helium gas (BOC, North Ryde, NSW, Australia) (>99.999%) was used as a carrier gas with a constant flow of 1 mL/min. The temperatures of injector and detector were set at 270 and 290 °C respectively. The initial column temperature was set to 50 °C and held for 4 minutes, then increased to 250 °C at a rate of 10 °C /min and held at 250 °C for 6 minutes. The data acquisition rate was 100 Hz/scan. Obtained GC-MS data were processed using the GC-MS Postrun Analysis software. Six pheromone components, *N*-(2-methylbutyl)acetamide, *N*-(3-methylbutyl)acetamide, *N*-(2-

methylbutyl)propanamide,*N*-(3-methylbutyl)propanamide,*N*-(2-methylbutyl)-2-methylpropanamide, and *N*-(3-methylbutyl)-2-methylpropanamide*,* and raspberry ketone were confirmed by comparing retention time and MS fragmentation patterns of the samples with that of the authentic samples. Impurities in head space samples were identified and eliminated by comparing with the air samples if any.

Calculating amount of pheromone per fly. Standard solutions of *N*-(2-methylbutyl)acetamide,*N*-(3-methylbutyl)acetamide,*N*-(2-methylbutyl)propanamide,*N*-(3-methylbutyl)propanamide,*N-(2-methylbutyl)-2-*methylpropanamide,*N*-(3-methylbutyl)-2-methylpropanamide and raspberry ketone were prepared by the serial dilutions of a stock solution. As an internal standard, hexadecane (2.73 µg mL^{-1}) was also incorporated into the standard solutions for both the headspace and rectal gland extract samples. GCMS responses of the standard solutions were obtained and the ratio of the GC peak area of a component to that of the internal standard was used to generate standard curves. The equations from linear regression of standard curves were used to calculate the amount of each endogenous pheromone component and RK.

Statistical analysis. Raspberry ketone dose and diet were considered as nominal variables and age was considered as continuous variable. RK dose and diet were considered as fixed factor while age considered as covariate. Effect of dose, diet and age on the production of pheromone in rectal glands and the pheromone release were analysed, including all possible interaction terms. The ratio of two main pheromone compounds was Log transformed and analysed by General Linear Model (GLM-Univariate)*.* All data were analysed in IBM SPSS Statistics 22.

Results

Pheromonal compounds in rectal gland and released volatiles

As reported previously by Bellas and Fletcher (1979) in Queensland fruit fly, six endogenous compounds were found in rectal gland and in released

pheromone although these compounds were not present in all samples. Two compounds, *N*-(3-methylbutyl)acetamide and *N*-(3-methylbutyl)propanamide, were mostly common and were detected in large quantities in both RK-fed and RK-unfed males which are presented in Table 1 and Table 2. In addition to the endogenously produced compound, males fed RK accumulated the ingested compounds in their rectal glands and released RK volatiles as an unaltered state (Table 1 and 2, Figure 1) until 30 days in rectal glands and until 10 days in released volatiles in both high and low RK dose treated groups (Table 3).

Effect of RK dose, age and diet on the amount of total pheromone (endogenous + RK) in rectal glands and headspace

High dose treated group. High dose RK had significant effect on pheromone production in rectal glands, RK-fed males produced more pheromone than RK-unfed males (Table 4, Figure 2). Males that had access to YH+S produced more pheromone than those that had access only to sugar. The effects of diet and RK on total amount of pheromone in rectal gland varied with age significantly (Table 4). However, unlike rectal glands, RK did not show any effect on total pheromone in released volatiles. Both age and diet showed significant effect on total amount of pheromone in released volatiles (Table 4, Figure 3).

Low dose treated group. Age and diet had significant effect on pheromone production in rectal glands as well as in released pheromone. Both RK-fed and RK-unfed males showed increased amount of pheromone at 6, 8 and 10 days of age and YH+S fed males produced and released more pheromone than sugar fed males (Figure 4 and 5). Diet and age interaction also affected the release of pheromone at low dose, however, RK did not have any significant effect on pheromone production or pheromone release at low dose (Table 5)

Table 1: Amount (ng/fly, mean±SD) of endogenous (main 2 compounds) and exogenous compounds in rectal glands and released pheromone of RK-unfed and RK-fed male Q-flies that received high RK dose in diet of sugar or YH+sugar. Data for all tested ages are pooled. Where compound was found in only 1 sample, standard deviation (SD) is not presented.

Age of males tested (days)	Pheromone compound*	Rectal gland				Headspace			
		Control		High RK		Control		High RK	
		YH+S	Sugar	YH+S	Sugar	YH+S	Sugar	YH+S	Sugar
6	1	22.46827 ±35.9326	3.97262 ±5.16297	136.56430 ±136.30491	3.8734 ±5.7443	17.80027 ±25.4820	6.2271 ±9.7441	31.32214 ±52.27067	14.33910 ±25.88977
	2	124.7085 ±237.6612	7.45259 ±8.09039	279.0702 ±346.82868	5.9934 ±2.56163	30.5802 ±44.01507	5.0480 ±8.32080	23.0360 ±38.10304	11.72482 ±25.14344
	3			1385.33974 ±1661.7938	1.1601 ±1.1089				
8	1	71.6842 ±64.7431	8.54538 ±12.42685	227.94804 ±251.37351	19.46374 ±26.4608	89.31942 ±162.3497	10.62451 ±18.99930	127.56402 ±141.7753	20.21886 ±30.62540
	2	167.14284 ±131.5737	27.50834 ±45.35990	383.80510 ±407.0609	27.4083 ±26.4429	98.704271 ±177.2060	8.49698 ±14.8331	211.30544 ±296.76847	12.54024 ±21.4049
	3			300.3227 ±527.2842	297.40381 ±641.0868			0.16388453	
10	1	37.43052 ±56.89252	18.68738 ±21.180602	154.6032 ±224.3747	71.7581 ±106.6819	32.86445 ±38.46246	8.54730 ±8.840368	61.0464 ±82.54629	5.73080 ±4.722086
	2	100.39215 ±120.4372	17.8647 ±14.31393	373.6382 ±707.87676	79.1532 ±163.992	61.40986 ±56.08203	9.60054 ±8.79252	95.15178 ±112.08731	3.74347 ±3.12897

	3			348.03398 ±668.4661	555.97665 ±778.2464				
20	1	20.35092 ±34.77044	6.68680 ±10.210487	174.17446 ±131.5165	25.14489 ±31.0711	6.05901 ±7.53551	1.970548 ±2.00999	8.16395 ±3.910128	8.74958 ±12.61609
	2	32.33694 ±51.8597	4.72262 ±6.282389	364.30547 ±273.8410	5.76192 ±7.1908	6.264354 ±7.33996	1.38226 ±2.38426	12.27398 ±5.5052	1.46016 ±2.58350
	3			509.27046 ±631.7922	456.2882 ±320.3843				122.88774
30	1	14.56658 ±11.53447	296.52273 ±715.967938	71.73823 ±99.16930	6.14013 ±9.8352	5.18607 ±6.1866	3.97033 ±7.2225	7.54271 ±7.8185	3.51229 ±2.35499
	2	31.9084 ±21.73564	566.8627 ±1373.2245	104.10187 ±162.98201	15.80156 ±31.9384	3.11605 ±4.59606	2.64178 ±4.59606	10.26311 ±11.0003	0.446962 ±0.4595
	3			81.29662 ±179.6464	10.20353 ±179.6464				

*Compound 1= *N*-(3-methylbutyl)acetamide, compound 2= *N*-(3-methylbutyl)propanamide, compound 3=4-(4-hydroxyphenyl)-2-butanone.

Table 2: Amount (ng/fly, mean±SD) of endogenous (main 2 compounds) and exogenous compounds in rectal glands and released pheromone of RK-unfed and RK-fed male Q-flies that received low RK dose in diet of sugar or YH+sugar. Data for all tested ages are pooled. Where compound was found in only 1 sample, standard deviation (SD) is not presented.

Age of males tested (days)	Pheromone compound*	Rectal gland				Headspace			
		Control		Low dose		Control		Low dose	
		YH+S	Sugar	YH+S	Sugar	YH+S	Sugar	YH+S	Sugar
6	1	60.50500 ±59.1895	8.00929 ±8.8091927	65.68476 ±99.0060	8.192746 ±9.19657	36.13261 ±18.3445	5.77606 ±4.9415	26.9135 ±13.3942	10.30427 ±10.3018
	2	192.79674 ±197.9225	13.45053 ±18.32507	207.1597 ±299.868	16.5962 ±30.8463	68.20813 ±35.2275	5.96564 ±4.27826	34.942 ±10.822	6.5075 ±6.419
	3			62.04095 ±151.0647	31.8497 ±47.9040			4.3602 ±7.11403	7.1507 ±9.7080
8	1	76.93401 ±89.4322	18.64327 ±34.94718	53.94618 ±28.83418	14.6207 ±9.0019	41.2832 ±38.8395	5.80734 ±5.67802	51.892 ±40.0771	7.0426 ±8.4075
	2	293.81294 ±334.1557	28.16926 ±51.64117	186.1526 ±82.4900	17.5528 ±15.0084	62.27932 ±56.69098	4.81200 ±4.64056	69.6250 ±27.837	4.7308 ±5.3576
	3			42.13149 ±26.3592	64.6043 ±84.38254			4.7799 ±5.3891	
10	1	62.9600 ±56.95732	30.1564 ±30.78076	78.7623 ±98.38276	16.513172	20.8780 ±23.26895	11.9076 ±12.862117	34.2780 ±25.9289	5.37426 ±6.6681
	2	134.73933 ±82.92817	47.7197 ±51.27172	273.5345 ±390.74301	26.590872	26.8350 ±25.47067	9.093031 ±6.0384	58.92133 ±31.175	3.3752 ±4.7509

	3			14.6061 ±21.6522	27.864955			1.888 ±3.10826	1.13963
20	1	36.00418 ±59.12749	5.16634 ±5.52400	40.2392 ±45.59716	5.25933 ±5.4868	19.45293 ±20.72368	5.27085 ±3.81533	11.0952 ±10.7301	4.7288 ±5.09502
	2	106.53420 ±197.8148	6.3172 ±7.41365	99.3963 ±100.7799	3.45479 ±4.19745	24.51063 ±26.76929	2.39516 ±26.7692	12.9971 ±12.0180	
	3			4.0657 ±6.0498					1.8104362
30	1	6.6328 ±5.60504	2.54078 ±1.4575	21.6740 ±24.6425	8.01098 ±12.6475	7.71281 ±5.83941	3.9512 ±3.1194	11.75143 ±12.5711	4.67932 ±5.4059
	2	22.70089 ±27.6881	1.5319 ±1.62492	33.86113 ±39.027359	2.13240 ±2.43954	8.5555 ±8.13690	1.6372 ±1.00152	11.5116 ±11.0647	1.8175 ±2.1342
	3			0.75458					

*Compound 1= *N*-(3-methylbutyl)acetamide, compound 2= *N*-(3-methylbutyl)propanamide, compound 3=4-(4-hydroxyphenyl)-2-butanone.

Table 3: The amount of RK (ng/fly, mean±SD) in rectal gland and head space of male Qfly at different age, diet and RK dose (where N=1, SD is not given, empty cells represent absence of RK).

Age (days)		Sugar only		YH + Sugar	
		High RK dose	**Low RK dose**	**High RK dose**	**Low RK dose**
Rectal gland	**6**	1.16±1.11	31.85±47.90	1385.34±1661.80	62.04±151.06
	8	297.40±641.09	64.60±84.38	300.32±527.28	42.13±26.36
	10	555.98±778.25	27.86±43.84	348.03±668.47	14.61±21.65
	20	456.29	4.07	509.27±320.38	4.07±6.05
	30	10.20	0.75	81.30±631.79	0.75
Head space	**6**		7.15±9.71		4.36±7.11
	8			0.16	4.78±5.39
	10		1.14		1.89±3.11

Table 4: Effect of age (6, 8, 10, 20 and 30 days), RK dose (0% control and 5% high) and diet (YH+S and sugar) on total pheromone production (rectal gland) (N= 473, R^2 = 0.048) and release (head space) (N=384, R^2 = 0.051) in male Qflies at high dose RK treated group.

Source	Rectal gland			Head space		
	df	F	*P*	df	F	*P*
Age	1	0.639	0.425	1	6.289	**0.013**
Dose	1	11.036	**0.001**	1	1.634	0.202
Diet	1	11.039	**0.001**	1	11.363	**0.001**
Dose * Age	1	6.579	**0.011**	1	0.417	0.519
Diet * Age	1	8.811	**0.003**	1	3.742	0.054
Dose * Diet	1	0.028	0.867	1	0.702	0.403
Dose * Diet * Age	1	1.742	0.188	1	0.226	0.635

Table 5: Effect of age (6, 8, 10, 20 and 30 days), dose (0% control and 1.25% low) and diet (YH+S and sugar) on total pheromone production (rectal gland) (N=523, R^2 = 0.048) and release (head space) (N=448, R^2 = 0.122) in male Qflies at low dose RK treated group.

Source	Rectal gland			Head space		
	df	F	*P*	df	F	*P*
Age	1	9.09	**0.003**	1	12.176	**0.001**
Dose	1	0.095	0.758	1	0.201	0.655
Diet	1	13.352	**<0.001**	1	32.916	**<0.001**
Dose * Age	1	0.052	0.82	1	0.104	0.747
Diet * Age	1	2.676	0.102	1	6.612	**0.010**
Dose * Diet	1	0.195	0.659	1	0.122	0.727
Dose * Diet * Age	1	0.069	0.793	1	0.061	0.805

Table 6: Effect of age (6, 8, 10, 20 and 30 days), dose (0% control and 5% high) and diet (YH+S and sugar) on amount of endogenous pheromone production (rectal gland) (N= 431, R^2 = 0.066) and release (head space) (N=381, R^2 = 0.054) in male Qflies at high dose RK treated group.

Source	Rectal gland			Head space		
	df	F	*P*	df	F	*P*
Age	1	3.061	0.081	1	6.892	**0.009**
Dose	1	7.631	**0.006**	1	1.790	0.182
Diet	1	14.628	**<0.001**	1	11.411	**0.001**
Dose * Age	1	8.121	**0.005**	1	0.595	0.441
Diet * Age	1	11.808	**0.001**	1	3.394	0.066
Dose * Diet	1	0.240	0.625	1	0.719	0.397
Dose * Diet * Age	1	5.885	**0.016**	1	0.152	0.697

Table 7: Effect of age (6, 8, 10, 20 and 30 days), dose (0% control and 1.25% low) and diet (YH+S and sugar) on amount of endogenous pheromone production (rectal gland) (N=482, R^2 = 0.054) and release (head space) (N=435, R^2 = 0.133) in male Qflies at low dose RK treated group.

Source	Rectal gland			Head space		
	df	F	*P*	df	F	*P*
Age	1	7.832	**0.005**	1	13.243	**<0.001**
Dose	1	0.17	0.68	1	0.012	0.913
Diet	1	14.84	**<0.001**	1	35.509	**<0.001**
Dose * Age	1	0.111	0.739	1	0.017	0.896
Diet * Age	1	3.192	0.075	1	7.509	**0.006**
Dose * Diet	1	<0.001	0.999	1	<0.001	0.996
Dose * Diet * Age	1	0.002	0.963	1	0.002	0.963

Table 8. Effect of age (6, 8, 10, 20 and 30 days), RK dose (0% control and 5% high) and diet (YH+S and sugar) on ratio of *N*-(3-methylbutyl)acetamide to *N*-(3-methylbutyl)propanamide in pheromone production (rectal gland) (N=130, R^2= 0.15) and release (head space) (N=128, R^2=0.41) in male Qflies at high dose RK treated group.

Source	Rectal gland			Head space		
	df	F	*P*	df	F	*P*
Age	1	15.392	**<0.001**	1	29.611	**<0.001**
Dose	1	0.105	0.747	1	0.305	0.582
Diet	1	0.231	0.632	1	9.464	**0.003**
Dose * Age	1	0.027	0.87	1	0.548	0.461
Diet * Age	1	2.221	0.139	1	1.188	0.278
Dose * Diet	1	1.491	0.224	1	6.918	**0.010**
Dose * Diet * Age	1	0.327	0.568	1	9.654	**0.002**

Table 9. Effect of age (6, 8, 10, 20 and 30 days), RK dose (0% control and 1.25% low) and diet (YH+S and sugar) on ratio of *N*-(3-methylbutyl)acetamide to *N*-(3-methylbutyl)propanamide in pheromone production (rectal gland) (N=145, R^2=0.35) and release (head space) (N=145, R^2=0.35) in male Qflies at low dose RK treated group.

Source	Rectal gland			Head space		
	df	F	*P*	df	F	*P*
Age	1	16.19	**<0.001**	1	19.354	**<0.001**
Dose	1	0.953	0.331	1	5.781	**0.018**
Diet	1	7.764	**0.006**	1	14.744	**<0.001**
Dose * Age	1	0.118	0.732	1	2.777	0.098
Diet * Age	1	2.528	0.114	1	0.007	0.934
Dose * Diet	1	0.073	0.787	1	2.862	0.093
Dose * Diet * Age	1	0.297	0.587	1	1.367	0.244

Effect of RK dose, age and diet on endogenous pheromone production and release

High dose treated group. The endogenously produced pheromone was affected by high dose RK; in the presence of RK, endogenous pheromone production increased significantly in the rectal glands compare to control males, but RK did not show a significant effect on amount of pheromone released. Effects of diet and RK on amount of endogenous pheromone stored in the rectal gland varied with age. Age also affected release of endogenous pheromones. Increased amount of endogenous pheromone was produced and released by males that had access to diet of YH+S (Table 6) (Figure 6 and 7).

Low dose treated group. Low dose RK treatment did not significantly affect production or release of endogenously produced pheromone (Table 7). In contrast, both age and diet had strong effects on the production and release of endogenous pheromone (Table 7). Males fed on YH+S diet produced and released more of pheromone compare to sugar fed males but from 20 days of age pheromone release decreased (Figure 7 and 8).

Effect of RK dose, age and diet on the ratio of N-(3-methylbutyl)propanamide and N-(3-methylbutyl)acetamide

High dose treated group. Ratio of *N*-(3-methylbutyl)propanamide to *N*-(3-methylbutyl)acetamide was affected significantly with age in rectal gland as well as in released pheromone in both RK-fed and RK-unfed males (Table 8). No effect of dose and diet was found on the ratio of these two compound in rectal gland. Significant effect of diet, dose and diet interaction as well dose, diet and age interaction was found on ratio of these two compounds in headspace (table 8, Figure 10 and 11).

Low dose treated group. Ratio of *N*-(3-methylbutyl)propanamide to *N*-(3-methylbutyl)acetamide was affected by age and diet significantly both in rectal gland and released pheromone (head space) (Figure 12 and 13). Along with age

and diet, dose also had significant effect on the ratio of these two compounds in released pheromone. (Table 9).

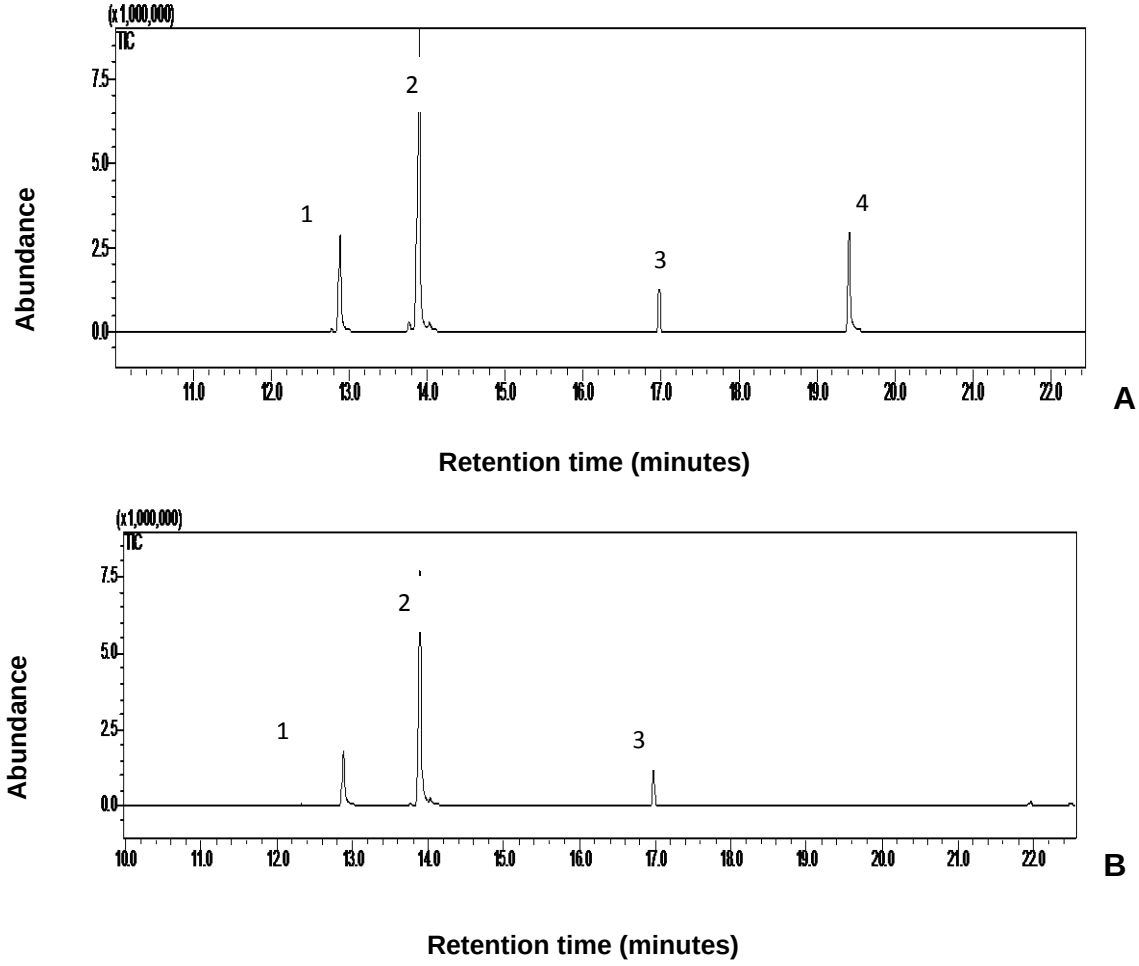

Figure 1: Chromatogram showing main compounds detected in rectal glands; A. RK-fed B. RK-unfed. (1) *N*-(3-methylbutyl)acetamide (2) *N*-(3-methylbutyl)propanamide (3) Internal standard, hexadecane (4) Raspberry ketone, 4-(4-hydroxyphenyl)-2-butanone.

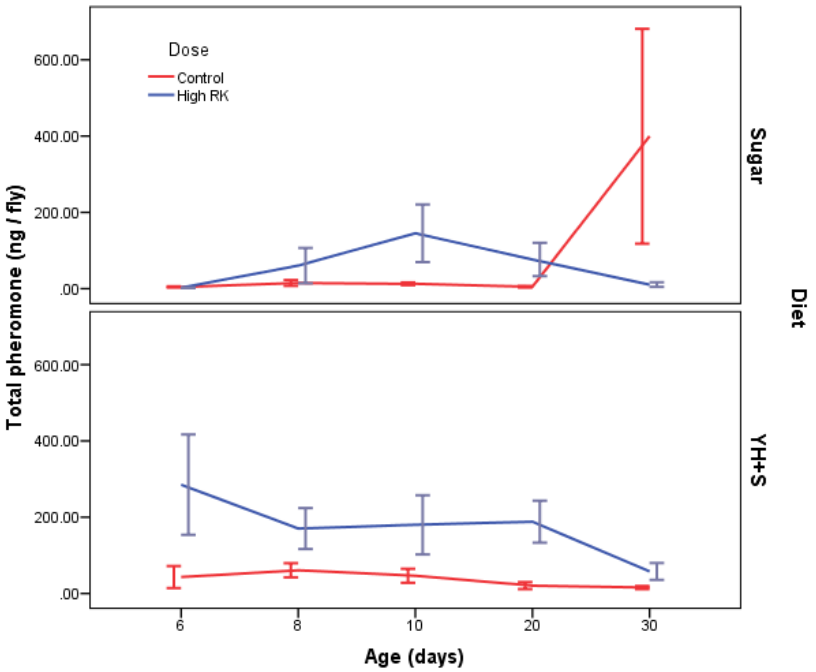

Figure 2: Amount of total pheromone (endogenous + RK) in rectal gland of RK-fed and RK-unfed Qfly males in the high RK dose group at different diet groups; sugar and yeast hydrolysate mixed with sugar (YH+S). Vertical bars represent standard error.

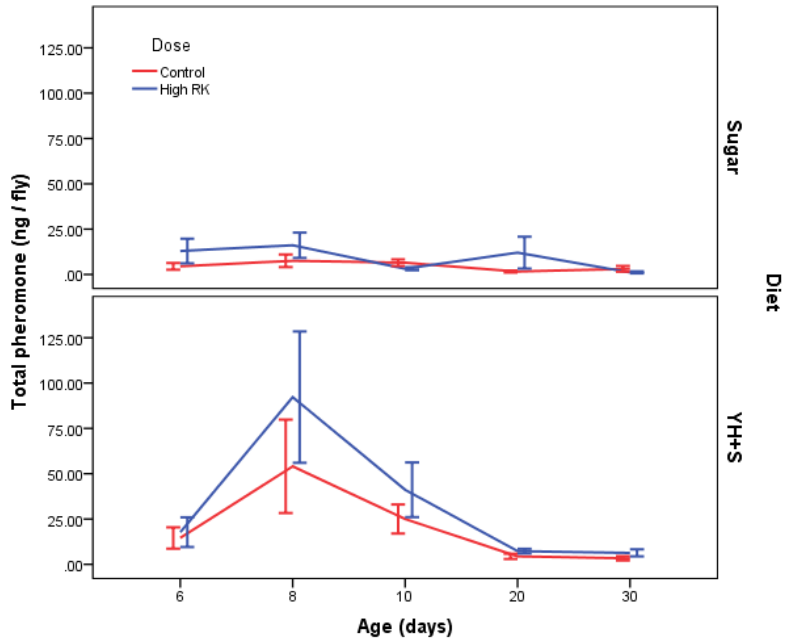

Figure 3: Amount of total pheromone (endogenous + RK) in headspace of RK-fed and RK-unfed Qfly males in high RK dose group at different diet groups; sugar and yeast hydrolysate mixed with sugar (YH+S). Vertical bars represent standard error.

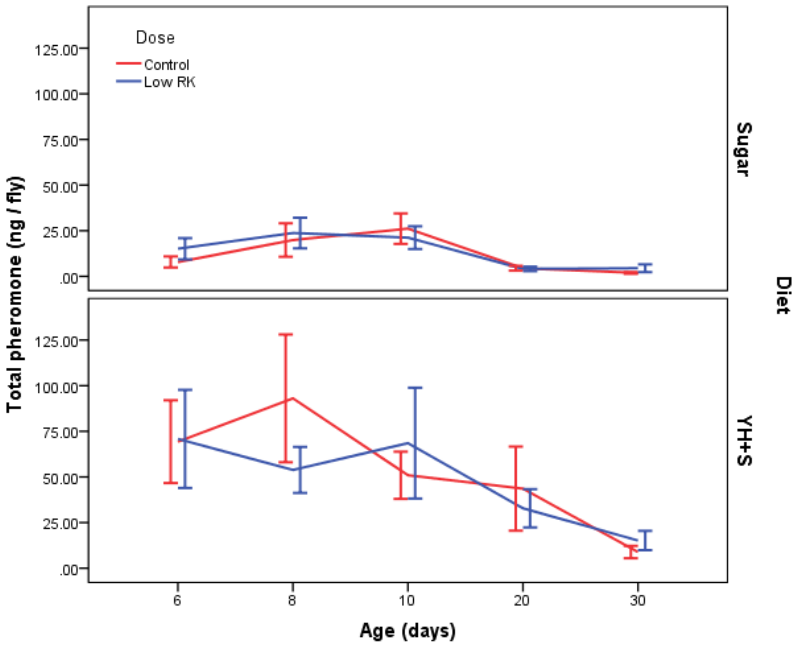

Figure 4: Amount of total pheromone (endogenous + RK) in rectal gland of RK-fed and RK-unfed Qfly males in low RK dose group at different diet groups; sugar and yeast hydrolysate mixed with sugar (YH+S). Vertical bars represent standard error.

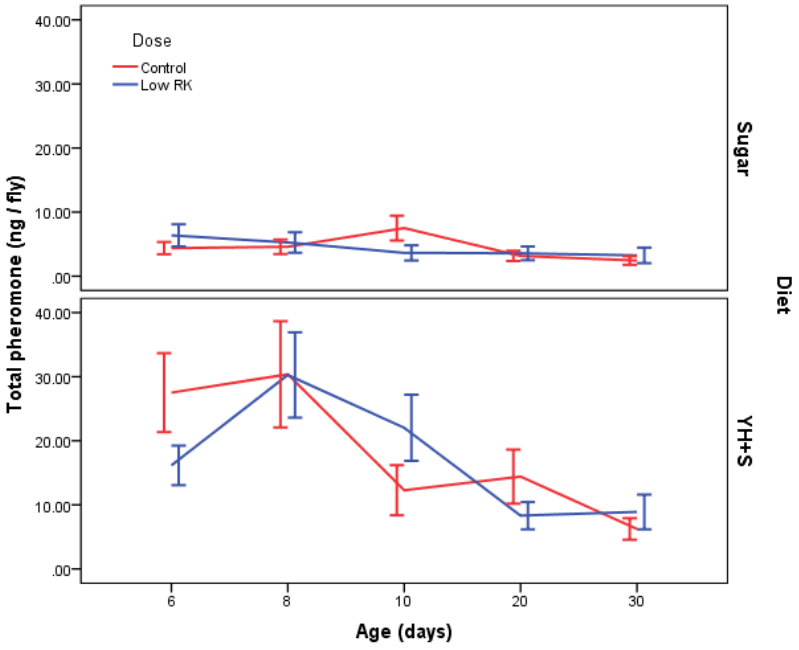

Figure 5: Amount of total pheromone (endogenous + RK) in headspace of RK-fed and RK-unfed Qfly males in low RK dose group at different diet groups; sugar and yeast hydrolysate mixed with sugar (YH+S). Vertical bars represent standard error.

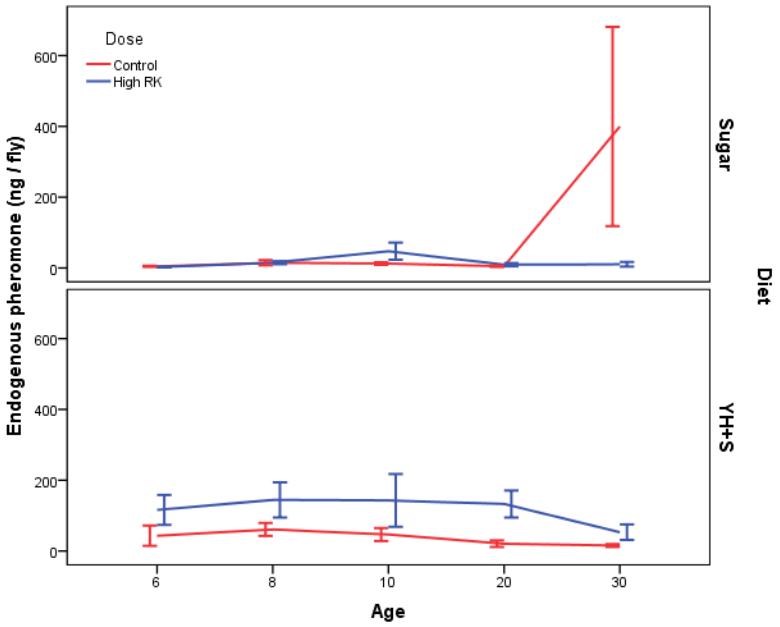

Figure 6: Amount of endogenous pheromone (all 6 compounds together) in rectal gland of RK-fed and RK-unfed Qfly males in high RK dose group at different diet groups; sugar and yeast hydrolysate mixed with sugar (YH+S). Vertical bars represent standard error.

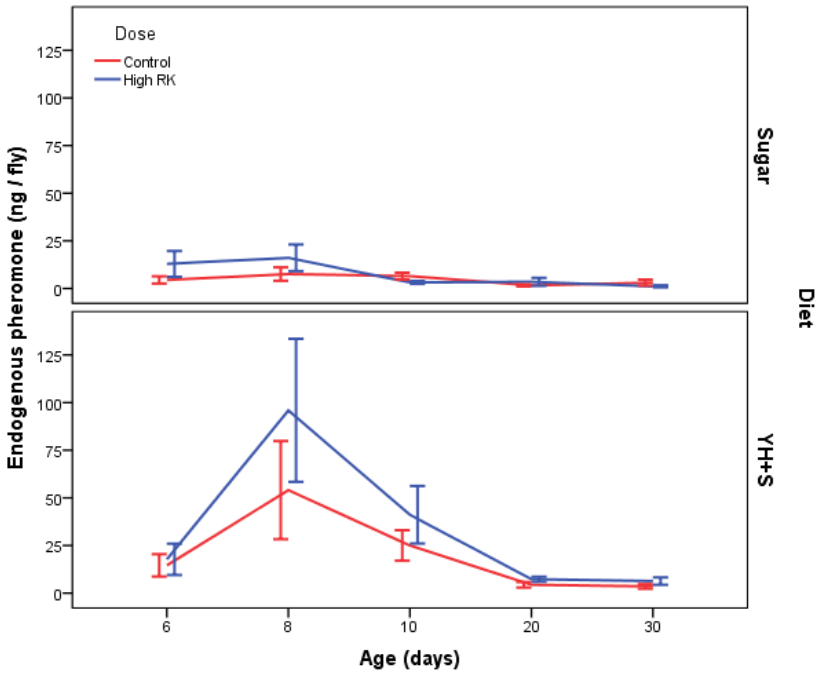

Figure 7: Amount of endogenous pheromone (all 6 compounds together) in headspace of RK-fed and RK-unfed Qfly males in high RK dose group at different diet groups; sugar and yeast hydrolysate mixed with sugar (YH+S). Vertical bars represent standard error.

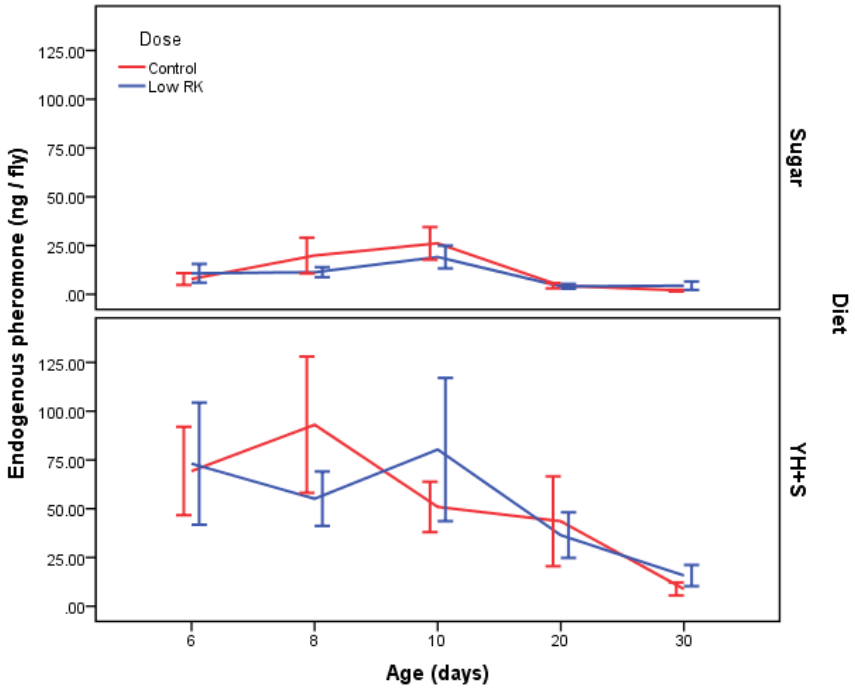

Figure 8: Amount of endogenous pheromone (all 6 compounds together) in rectal gland of RK-fed and RK-unfed Qfly males in low RK dose group at different diet groups; sugar and yeast hydrolysate mixed with sugar (YH+S). Vertical bars represent standard error.

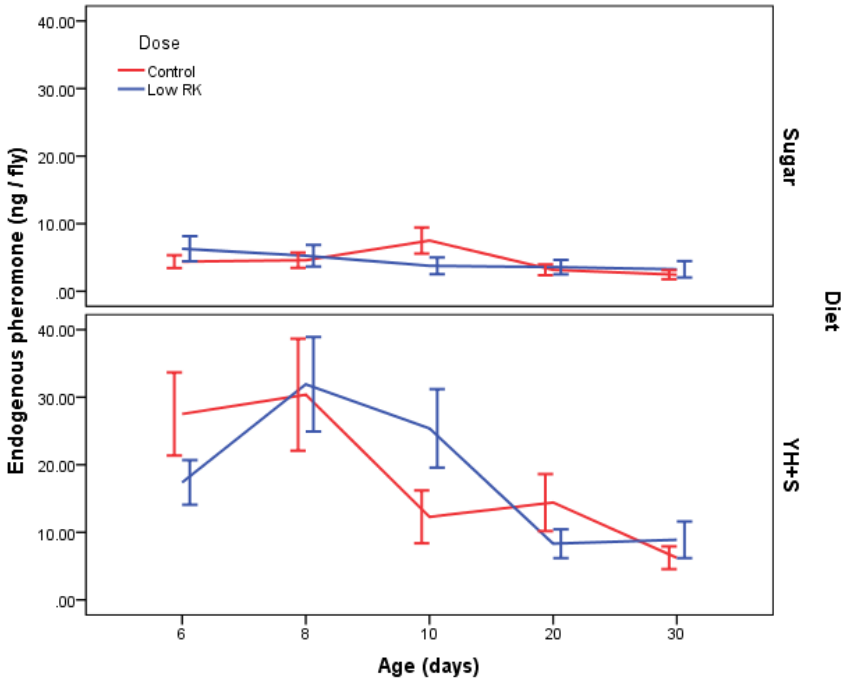

Figure 9: Amount of endogenous pheromone (all 6 compounds together) in headspace of RK-fed and RK-unfed Qfly males in low RK dose group at different diet groups; sugar and yeast hydrolysate mixed with sugar (YH+S). Vertical bars represent standard error.

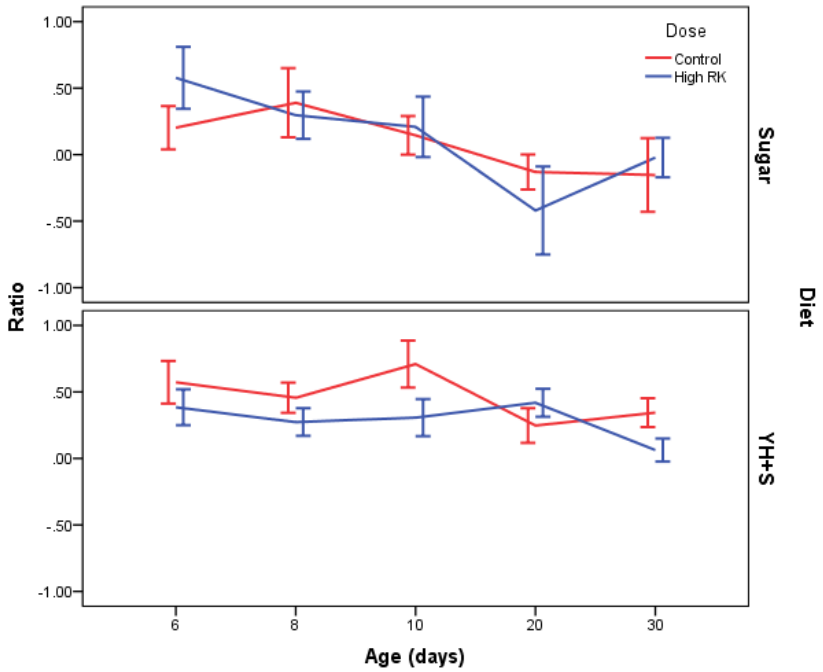

Figure 10: Comparison of ratio of *N*-(3-methylbutyl)acetamide to *N*-(3-methylbutyl)propanamide in rectal glands of RK-fed and RK-unfed males in high RK dose group at different diet groups; sugar and yeast hydrolysate mixed with sugar (YH+S). Vertical bars represent standard error.

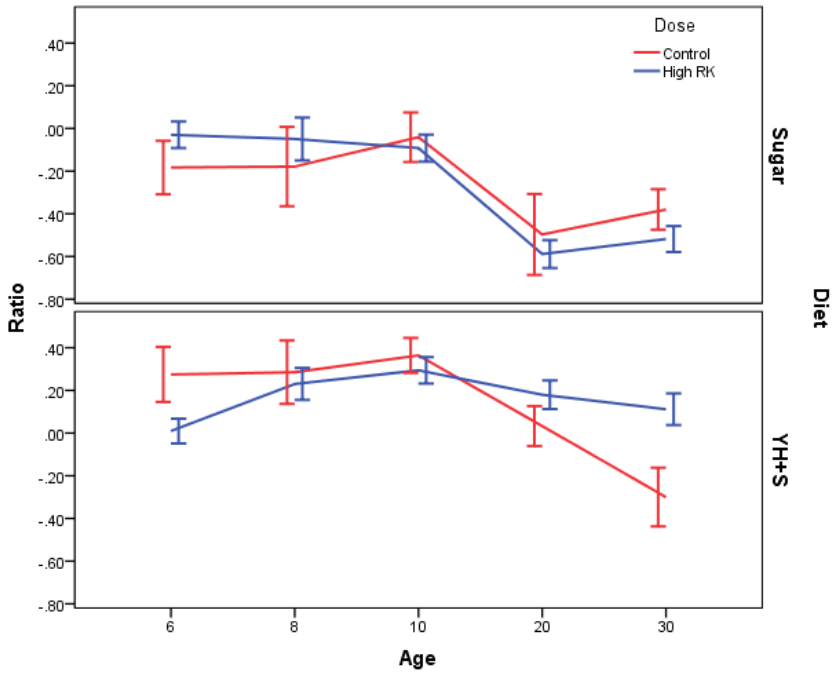

Figure 11: Comparison of ratio of *N*-(3-methylbutyl)acetamide and *N*-(3-methylbutyl)propanamide in headspace of RK-fed and RK-unfed males in high RK dose group at different diet groups; sugar and yeast hydrolysate mixed with sugar (YH+S). Vertical bars represent standard error.

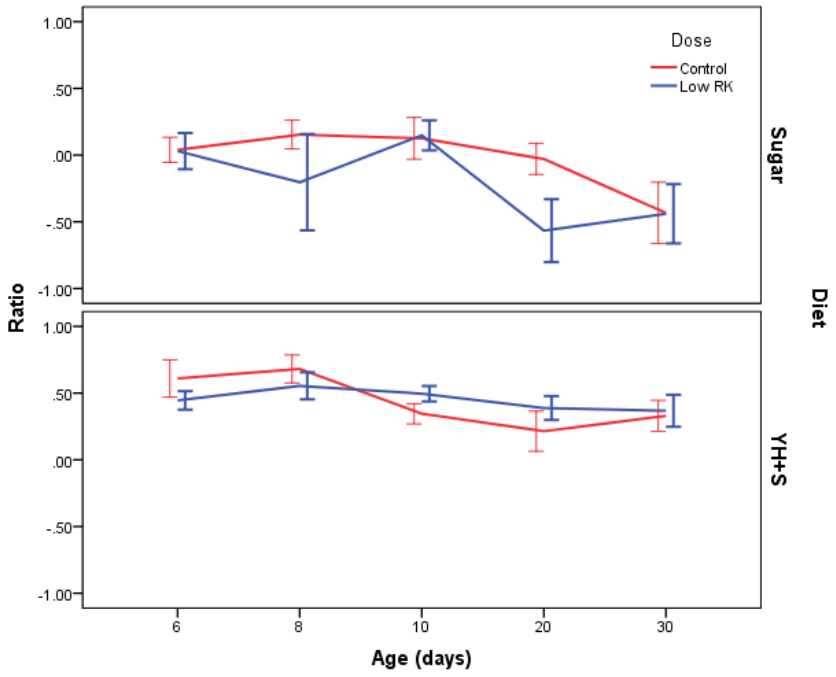

Figure 12: Comparison of ratio of *N*-(3-methylbutyl)acetamide and *N*-(3-methylbutyl)propanamide in rectal gland of RK-fed and RK-unfed males in low RK dose group at different diet groups; sugar and yeast hydrolysate mixed with sugar (YH+S). Vertical bars represent standard error.

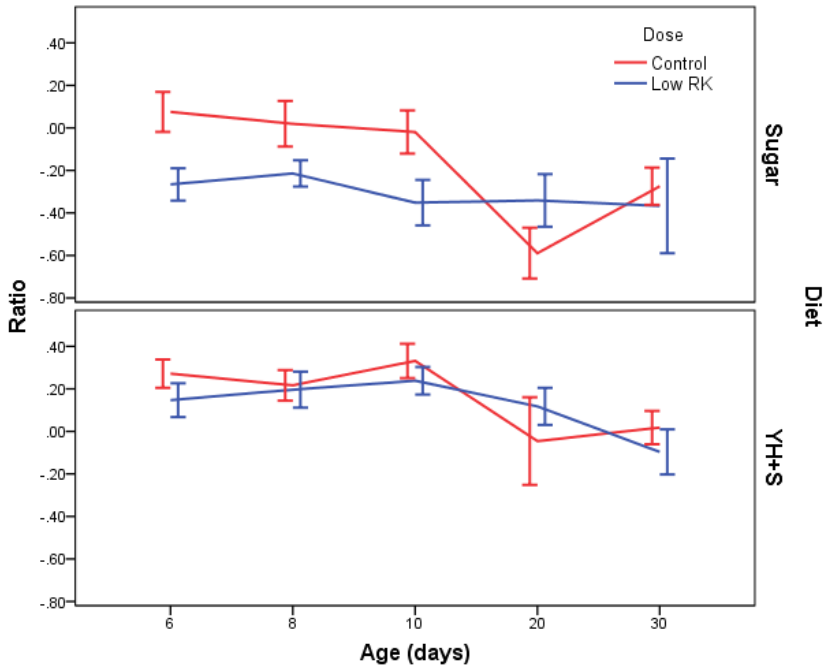

Figure 13: Comparison of ratio of *N*-(3-methylbutyl)acetamide to *N*-(3-methylbutyl)propanamide in headspace of RK-fed and RK-unfed males in low RK dose group at different diet groups; sugar and yeast hydrolysate mixed with sugar (YH+S). Vertical bars represent standard error.

Discussion

Sex pheromone is closely related to insect's mating behaviour and numerous studies have shown that the ingestion of specific phytochemicals or their analogues by mature males of various *Bactrocera* species enhances their mating success. Males of the oriental fruit fly, *B. dorsalis* and melon fly, *B. cucurbitae* feed on ME and RK respectively attained mating advantage over ME and RK-deprived males (Shelly and Dewire, 1994; Tan and Nishida, 1996, 1998; Tan, 1993, 2000; Shelly, 2000a; Shelly and Nishida, 2004). Chemical and behavioural studies have shown that ME is metabolized to 2-allyl-4,5-dimethoxyphenol (allyl-DMP) and coniferyl alcohol (CF) in *B. dorsalis* (Nishida et al., 1988; Tan and Nishida, 1996; Hee and Tan, 1998) that function as male sex pheromonal components while RK was found in unaltered condition in rectal gland of Qfly after feeding (Nishida et al., 1993; Tan and Nishida, 1995). In mature Qflies cue lure feeding initiated enhanced mating and RK was found as a hydrolysed product in pheromone (Kumaran et al., 2013, 2014). In another study, RK-feeding at immature Qflies showed accelerated and enhanced mating (Akter et al.,2017) and reproductive organs development in immature Qflies (chapter 3); as a sequel study in present observation RK was found as unaltered compound along with other endogenous compounds in rectal gland as well as in released pheromone.

Males of *B. tryoni* produce six amides as major sex pheromonal components and three of the six, namely, *N*-(3-methylbutyl)acetamide, *N*-(3-methylbutyl)propanamide, and *N*-(3-methylbutyl)-2-methylpropanamide are most abundant (Bellas and Fletcher, 1979; Tan and Nishida, 1995). However, availability of these compounds varied such as Fletcher and Kitching (1995) reported *N*-(3- methylbutyl)propanamide as a dominant pheromonal compound while Kumaran et al. (2014) detected *N*-(3-methylbutyl)acetamide in the rectal glands. In addition to the *B. tryoni*, *N*-(3-methylbutyl)acetamide was also found in *B. dorsalis* (formerly *B. papayae*) (Hee and Tan, 1998) and in the melon fly (Nishida et al., 1990). In the present study two compounds, *N*-(3-methylbutyl)acetamide and *N*-(3-methylbutyl)propanamide were mostly dominant and found abundantly in both rectal gland and released pheromone. These two compounds are reported to attract females from close range for sexual communication that increase with the sexual maturity (Tan and Nishida, 1995).

Thus, low ratio of this 2 compounds in RK-fed flies in released pheromone might play role in attraction to RK-fed males to initiate mating (Akter et al., 2017).

Age of males affected the production and release of total pheromone and endogenous pheromone in present study, increased amount of pheromone was observed within 6 to 10 days which coincide with the enhanced mating tendency of immature Qfly (Akter et al., 2017) as well as reproductive organs development in response to high RK dose in this species (chapter 3 of this thesis). Thus, this age dependent pheromone production and release might be related to the sexual maturity (Nation 1972; Tan and Nishida, 1995). Age also showed an important factor which affected the release of relative amount of pheromone compound of *A. ludens* (Bosa et al., 2016). Pheromone production and release were also found to be dependent on diet, protein-starved males showed a 40-70% reduction in pheromone release versus males that were given protein diet in *Anastrepha* (Nation, 1989). Similarly, YH+S fed Qflies produced more pheromone than sugar-fed flies regardless of RK-fed or RK-unfed in the present study. Similar to present finding males fed on a YH+S diet produced the higher amount of the three main pheromone components in *A. ludens* males and two major components in *A. obliqua* males compared to sugar-fed males (Liedo et al., 2013). In contrast, in *A. suspensa s*ugar-fed males tended to produce more (but not significantly more) pheromone than fully fed male (Epsky and Heath, 1993). Coinciding with the pheromone production in sugar-fed males, the percentage of males calling was more than fully fed males and overnight food deprivation reduced calling activity in *A. suspensa* (Landolt and Sivinski, 1992). Therefore, pheromone production and sexual behaviour like male's calling are linked both of which are affected by the diet regime.

RK was detected in rectal glands until 30 days old (27 days after ingestion) and in released volatiles until 10 days old (7 days after ingestion) in both high and low RK dose treated males. Nishida et al. (1993) detected RK in rectal glands of *Z. cucurbitae* within 6 hr after feeding on *Dendrobium superbum* orchids and presence of RK in rectal glands lasted for at least 6 days. Similarly, Tan and Nishida (1995) found that *B. tryoni* males accumulated ingested RK in the rectal gland within 6 hours of feeding in rectal glands that lasted for at least 3 days

(longer periods were not assessed) and Kumaran et al., (2014) detected RK after cue lure feeding in the rectal gland of Qfly within 3 hours. Therefore, RK does not have similar role in changing the composition of sex pheromone as ME but its presence might be responsible for mating advantage. Unlike RK, the phenylpropanoids DMP and CF that are produced in response to ME feeding by males of *B. dorsalis* accumulated in rectal gland as early as 15 min of ME ingestion and remained for almost 3 weeks (Wee and Tan, 2007). In the present study RK was present in rectal gland extraction consistently while in released pheromone was detected very inconsistently that might raise question about the role of RK presence in pheromone's attractiveness. Besides this, RK did not even show to increase the volume of endogenous pheromone in released pheromone, thus, in the present study it could not be claimed that RK presence or the volume of endogenous pheromone in released pheromone played role in precocious mating in *B. tryoni* (Akter et al., 2017) as demonstrated previously for mature *B. tryoni* (Kumaran et al., 2014). However, RK ingestion and RK presence in pheromone of rectal gland may affect the speed or rate of sexual signalling such as calling and wing fanning as evidenced in *B. dorsalis* due to accumulation of ingested phytochemicals in response to consumption of ME or methyl eugenol-containing flowers (Shelly and Dewire,1994; Shelly, 2000b) to initiate early mating. Therefore, not only the attractiveness of released pheromone but the increased level of male signalling due to the effect of pheromone could be responsible for male mating success in immature *B. tryoni* as was found in *C. capitata* and *A. suspensa* (Whittier et al., 1994, Whittier and Kaneshiro, 1995, Shelly, 2000c; Nation, 1972, 1990; Teal et al., 1999).

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Chapter Six

Raspberry ketone feeding affects the tolerance of Queensland fruit fly *Bactrocera tryoni* (Froggatt) to nutritional and desiccation stress

Abstract

Queensland fruit fly (Qfly) males exhibit accelerated sexual maturation when their full diet is supplemented with raspberry ketone (RK) for two days following emergence. However, there may be risks associated with such accelerated development, such as increased vulnerability to starvation or desiccation. The present study tests these possibilities. Flies were fed for 48 hours with a diet of sugar mixed with yeast hydrolysate (3:1) that contained 0% RK (control), 1.25% RK (low dose) or 5% RK (high dose). To test vulnerability to starvation, flies were set up in group cages under three conditions - yeast hydrolysate+sugar+water (full diet); with 'water only', and with 'no food or water' - for both control and RK-treated flies. To test vulnerability to desiccation, flies were individually housed in glass vials containing 4-5 grains of silica gel. To analyse the water and lipid storage in Qfly under different nutritional stresses another group identical to the survival in starvation study was set up. Desiccated flies were also subjected to analysis of water and lipid storage in relation to RK dose, sex and body size. To assess the RK effect on initial lipid and water level in Qfly one group of flies were separated and analysed immediately after RK treatment without exposure to any stress. Overall females were more resistant to starvation and desiccation compared to males. Raspberry ketone-fed flies were more susceptible than control flies to desiccation, however, RK-fed flies survived longer compare to control flies in starvation ('no food or water' and 'water only'). Lipid level decreased significantly in starved flies ('no food or water' and 'water only') while RK-fed flies lost significantly more lipid compared to control. However, in desiccated flies lipid did not change significantly. On the other hand water level decreased significantly in both desiccated and starved flies. Body size had effect on initial water content but no effect on initial lipid reserve. Body size did not show any significant effect on the survival, water content, water changes and lipid changes in desiccated flies but lipid reserve was affected by body size in this group of flies. In starved flies also body size affected water content ('no food or water' and 'water only'), lipid reserve ('no food or water') and water changes ('water only') but did not affect lipid changes. Results were compared with other related work and discussed in the context of the possible exploitation of RK as pre-release supplementation in SIT application.

1. Introduction

Both biotic and abiotic factors are important for the survival and reproductive performance of insects. Variation in key environmental factors, such as shortage of water and nutrition, extreme temperature, and disease, can place insects under stress. Desiccation (lack of water and humidity) and starvation (lack of food) have been studied extensively in several insects. For example, water deprivation and exposure to desiccation shortens life span and reduces reproductive ability in *Drosophila mimica* (Eckstrand and Richardson, 1980). In Qfly, a reduction in the fecundity of adult females is found during dry periods due to the greatly reduced immigration of flies from other areas and high mortality of newly emerged adults (Bateman, 1968). Along with water food (nutrition) plays very important role on the survival and development of fruit flies; both protein and carbohydrates are necessary for reproductive development of flies. The role of diet in development of reproductive organs of both male and female flies has been studied extensively in several tephritids including Qfly (Bateman, 1972; Wheeler, 1996; Aluja et al., 2001; Vijaysegaran et al., 2002; Meats and Leighton, 2004; Fanson et al., 2009, Perez-staples et al., 2011, chapter 3 of this thesis) which showed that a high nutritional status is a prerequisite for reproductive performance (Hendrichs et al., 1991; Warburg and Yuval, 1997, Romanyukha et al., 2004; Yuval et al., 2002, 2007; Pérez –Staples et al., 2007b; Liedo et al., 2013, Akter et al., 2017). In laboratory conditions yeast hydrolysate (YH) is usually provided as a protein source along with sugar for carbohydrates and water. In nature fruit flies can acquire protein from several natural sources such as honeydew, nectar, bird droppings, and bacteria (Drew and Yuval, 2000; Weldon and Taylor, 2011; Drew et al., 1983). However, nature is unpredictable in terms of availability of food. However, natural sources of protein are thought to be often scarce and poor quality (Courtice and Drew, 1984; Ben-Yosef et al., 2010).

There are some strategies through which organisms can counter adverse environment for their survival. Insects have evolved behavioral, morphological and physiological adaptations in response to adverse environmental conditions (Gibbs et al., 1997; Chown and Nicolson, 2004; Chown et al., 2011). For instance, *Anastrepha ludens* adapts to dry environments through increased desiccation

resistance which is accompanied by longer pupal development time, delayed reproduction, higher mass, and some physiological changes (Tejeda et al., 2016). Reduced cuticular permeability, reduced excretory water loss, increasing water storage, limiting water loss or by enhancing tolerance to water loss and differences in the quantity and composition of cuticular lipids can enable insects to balance water in warmer and drier environments (Chown and Nicolson, 2004; Chown et al., 2011). In addition, fruit flies that are adapted to dry environments may lose water relatively slowly (Eckstrand and Richardson, 1980; Gibbs and Matzkin, 2001).

Energy metabolism may also play a critical role in stress resistance; increased resistance to many environmental stresses is correlated with reduced energy expenditure (Hoffmann and Parsons, 1989b). Laboratory selection for stress resistance can lead to a reduction in metabolic rate (Hoffmann and Parsons,1993; Djawdan et al., 1997; Harshman and Schmid, 1998). In a genetic based study of desiccation on *Drosophila melanogaster* it was found that desiccation-selected females had relatively lower metabolic rate as they consumed less than control females (Hoffmann and Parsons, 1989a). Reduced metabolic rate increases the amount of time that flies can survive under starvation conditions and also reduces water loss under desiccating conditions (Lighton, 1994, 1996; Zachariassen, 1996; Addo-Bediako et al., 2001). In addition, flies that have lower metabolic rates tend to have lower energy requirements that leads to living longer without food (Hoffmann and Parsons, 1989b). Besides the rates of energy consumption, both the amount and form of energy storage can affect stress resistance; energy storage patterns differ between desiccation and starvation selected *Drosophila* populations (Marron et al., 2003). Starvation-selected populations of *D. melanogaster* show high lipid and carbohydrate levels (Chippindale et al., 1998) whereas desiccation-selected populations stored less lipid but much more glycogen (Djawdan et al., 1998).

Queensland fruit fly *Bactrocera tryoni* Froggatt, (Qfly) is the most economically damaging fruit fly in Australia that infests over 100 plant species, including many commercially important crops (Hancock *et al*., 2000). The developing larvae not only damage fruit, but also pose a serious biosecurity risk

(Plant Health Australia, 2008). In some regions, Qfly is managed using sterile insect technique (Jessup et al., 2007) whereby sterile mass-reared flies are released into the field 2 or 3 days after emergence and adults have traditionally been provided sugar and water during holding period (Dominiak et al., 2008). However, recent reports recommend the addition of yeast hydrolysate to the pre-release diet in Qfly (Dominiak et al., 2003; Meats et al., 2003; Reynolds et al., 2014) as well as some other fruit fly species such as *Anastrepha ludens* and *Anastrepha obliqua* (Liedo et al., 2013), *Bactrocera cucurbitae* (Haq et al., 2013), *Ceratitis capitata* (Yuval et al., 2007). The distribution and abundance of Qfly is restricted by dry conditions (Sutherst and Yonow, 1998) which suggests that the species is susceptible to desiccation. While pre-release supplements might enhance Qfly reproductive performance and longevity (Pérez –Staples et al., 2007b, 2008; Prabhu et al., 2008) they might also increase risk of starvation (Taylor et al., 2013) or desiccation. Raspberry ketone feeding has been found to accelerate sexual maturation in immature Qflies, with RK-fed males mating much earlier without any negative impact on longevity (Akter et al., 2017). This chapter investigates the effect of pre-release RK supplements on the survival of desiccation and starvation stress.

2. Materials and Methods

2.1. Biological materials

Queensland fruit fly, *Bactrocera tryoni* pupae were obtained from the eggs collected from Department of primary Industries, Ourimbah, New South Wales, Australia which were reared in gel diet (for detail please see Moadeli et al., 2017) in the Department of Biological Sciences, Macquarie University, Sydney, Australia. All pupae and flies were maintained, and experiments conducted, at Macquarie University, Sydney, in controlled environment rooms (25±0.5^{o}C, 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. Approximately 2000 pupae were transferred to each of three open Petri dishes that were placed in separate 47.5×47.5×47.5 cm mesh cages (Bugdorm 44545, Megaview, Taichung, Taiwan). Adult flies emerged in these

cages, where they were provided with only a water-soaked sponge for sustenance. The first day of emergence tends to be small and so was discarded. Flies from the second day of emergence were used in experiments.

2.2. Fly treatment with raspberry ketone (RK)

Flies (0-24 hours old) were provided raspberry ketone (RK) 4-(4-Hydroxyphenyl)-2-butanone (≥98%, Sigma–Aldrich®) with a mixture of yeast hydrolysate and sugar (YH+S) (1:3) in 47.5×47.5×47.5 cm mesh cage (Megaview Bugdorm 44545, Taichung, Taiwan) (for details see Akter et al. in press). Two doses of RK, 5% (high) and 1.25% (low), along with a control (no RK) were tested. RK-treated and control flies were maintained in separate rooms to avoid exposure of control groups to RK volatiles. After 48 hours of feeding the treated diet was removed and flies were sorted according to sex by hand (using 5mL vials). This ensured virginity of males as laboratory-adapted, mass-reared flies do not begin mating before 5 days after emergence (Meats et al., 2004).

2.3. Effect of RK and body size on initial water and lipid reserves

To observe the effect of RK on the initial water content and lipid reserves 30 males and 30 females were collected from each of 3 treatments (control, low RK and high RK dose) just after finishing RK treatment. Flies were weighed immediately and then kept at -20°C for further assessment. This group was not subjected to any stress. Body size, water content and lipid were measured described below in 2.7 and 2.8. This procedure was repeated twice using flies from different weekly production batches.

2.4. Effect of RK and nutritional stress on survival of Qfly

Flies from each treatment (Control, low RK dose and high RK dose) were sexed and transferred to 5L rectangular clear plastic cages that had a mesh-

covered opening (15×20 cm) for ventilation (Figure 1); each cage containing 30 males or 30 females. Three cages of both sexes were exposed to three different food regimes: (a) Yeast hydrolysate + sugar + water (hereafter 'full diet'), as a survivorship baseline (b) water only and (c) no food or water. Flies with 'full diet' and 'water only' were provided a 70mL plastic jar containing sponge soaked in water. RK-fed flies were kept in separate room from control flies. Cages were checked every 12 hours (10 am and 10 pm) to record dead flies for seven days. This experiment was repeated twice using flies from different weekly production batches for a total 180 flies/sex/dose/food regime and a total of 3240 flies for this experiment.

2.5. Effect of RK, sex, nutritional stress and body size on water and lipid reserves

To test the effect of different nutritional stress and body size on water and lipid reserves, flies from each RK treatment dose were transferred to 1L plastic cages (with 5 x 10 cm mesh-covered opening for ventilation), each containing 10 males or 10 females. For this test three different combinations of food identical to the survival at starvation study was offered, (a) 'full diet' (b) 'water only' and (c) 'no food or water'. Cages with full diet and water were provided, a 70mL plastic jar containing sponge soaked in water. Three cages were maintained in each food regime for each RK-fed and control group. Dead flies were collected from each cage every day for seven days, weighed immediately after death and stored at -20°C to later assess body size, water content and lipid as described in 2.7 and 2.8. This experiment was repeated twice using flies from different weekly production batches such that a total of 60 flies were tested for each sex/dose/food regime and a total of 1080 flies was tested in this experiment.

2.6. Effect of RK and sex on survival, water content and lipid reserve of Qfly in desiccated condition

For this experiment, 30 males and 30 females from each RK treatment were transferred to individual 5mL glass vials (Techno Plus Pty Ltd, Australia) that

were then loosely stoppered with cotton wool. Once all transferring was finished, 4 to 5 large granules of silica gel (Fisher Scientific, UK) were placed in between the cap and cotton so that flies were exposed to desiccating conditions without direct contact to silica gel. The commencement times of the desiccation treatment was recorded. Vials were inspected every 6 h until 20 hours and then every 3 hours after 20 hours until all the flies were dead. Flies were weighed immediately after death and stored at -20°C for later assessment of body size, water content and lipid as described in 2.7 and 2.8. This experiment was repeated twice using flies from different weekly production batches such that 60 flies were assessed for each sex and dose and a total of 360 flies was tested overall in this experiment.

2.7. Body size measurement

To correlate the body size with the survival, water content and lipid reserve the wing length of each fly was used as a standard measure of body size (Pérez-Staples *et al*., 2007a, 2008; Weldon and Taylor, 2010). The right wing of each fly was excised with fine scissors (Vannas, 8 cm, STR, World Precision Instruments Inc, USA) and mounted onto clear double-sided adhesive tape along one side of a microscope slide. A second slide was pressed onto the tape holding the wings and to protect them from dust. In this way, the wings and labels were firmly secured and held flat between two glass microscope slides. Each wing was then scanned on a flatbed scanner (Epson Perfection V300 Photo; Seiko Epson Corporation, Japan) and wing length was measured (mm) on the scanned image using ImageJ software, version 1.46r (U. S. National Institutes of Health, Bethesda, Maryland) from the intersection of the anal and median band to the margin of the costal band and the R 4+ 5 vein.

2.8. Water and lipid reserves assays

Water and lipid reserve were determined using the same methods flies that were collected just after finishing RK dose treatment, flies that were exposed to nutritional stress, and flies that exposed to desiccation stress. For first group flies

were weighed individually just after collecting from cages and for last two groups individual wet weight was taken at death to a precision of 0.001 mg on a piece of tared aluminium foil using a microbalance (Sartorius ME5, Sartorius Mechatronics Australia). After taking wet weight, flies were oven dried individually in 5 mL glass vials at 60°C for 48 hours after which flies were weighed again for dry weight. The difference between wet and dry weight is water content. After taking dry weight, the flies were washed in chloroform (≥ 99.5%, Sigma-Aldrich®) to extract lipids (following Raubenheimer et al., 2007; Ponton et al., 2011, 2015); in each 5 mL glass vial containing one fly, 1 mL of chloroform was added and kept for one day in a fume hood with a lid on to avoid evaporation. On the second day chloroform was discarded from each vial and fresh chloroform was added. On the third day again chloroform was discarded and new chloroform was added for the final wash. On the fourth day chloroform was discarded and vials were kept open for another 24 hours to evaporate remaining chloroform. All this process was carried out in a fume hood. Vials containing the flies were then oven dried at 60°C for 48 hours and the dry weight was taken again. Lipid content was determined as the difference between the dry weight before and after chloroform extraction.

2.9. Statistical analysis

To analyse RK effects RK dose, sex and stress treatment were considered as nominal variable while survival hours, water content, lipid reserves and wing length (body size) were considered as continuous variables. Effect of dose, sex and body size on the water content and lipid reserves at all condition (initial, desiccation, nutritional stress) were analysed by General Linear Model (GLM-Univariate). Effect of dose, sex and body size on survival at desiccation flies, and effect of dose, sex and nutritional stress (‘no food or water’ and ‘water only’) on survival at nutritional stress were analysed by GLM (Univariate) when both dose, sex and nutritional stress were considered as fixed factor and body size as covariate. All data were analysed in IBM SPSS Statistics 22. Survival curve at nutritional stress (‘no food or water’ and ‘water only’) has been created in JMP 13 (SW) and survival curve at desiccation has been created by Kaplan-Meier test in IBM SPSS Statistics 22. As flies of ‘full diet’ group died rarely in given 7 days

period, survival curve was not created and analysis of the data for this group was not presented.

3. Results

3.1. *Effect of RK dose, sex and nutritional stress on survival of Qfly*

Overall females survived significantly longer than males at all doses (Table 1; Figure 1) in nutritional stressed condition. Flies that had 'full diet' rarely died in given 7 days whereas flies that were given water only or no food or water all died within 7 days. There was no significant effect of interaction between dose and sex on survival of nutritional stressed flies, however, dose and stress treatment as well as sex and stress treatment interaction significantly affected the survival of Qfly (Table 1). Considering RK dose and different stress treatments, flies of low RK dose showed significantly longer survival in 'no food or water' condition (Table 1, Figure 2); average survival (±standard deviation, SD) of females in control, low and high at this condition was 45.84±13.165, 49.25±12.646 and 46.04±14.498 hours respectively and average survival (±SD) of males in control, low and high was 43.39±10.647, 45.97±13.126 and 43.37±12.843 hours respectively. Considering different stress treatments, flies of low RK dose showed significantly longer survival in 'water only' condition; average survival (±SD) of females in control, low and high was 46.53±11.602, 51.57±14.124 and 48.93±13.747 hours respectively and average survival (±SD) of males in control, low and high was 45.76±11.056, 48.85±11.569 and 45.57±11.452 hours respectively.

3.2. *Effect of RK dose, sex and body size on survival of Qflies under desiccating condition*

Control flies survived significantly more than RK-fed flies and females lived longer than males in desiccation stress (Table 1; Figure 3 and 4). The average survival (±SD) in desiccation of females in control, low and high was 43.824±14.723, 39.747±12.607 and 36.682±13.106 hours respectively and the

average survival (±SD) of males in control, low and high was 40.433±10.530, 35.350±12.327 and 34.020±10.248 hours respectively. There was no significant effect of body size and interaction of dose and sex on survival of desiccated flies (Table 1).

3.3. *Initial body size and body weight at different sex and RK dose*

Females were bigger and heavier than males; RK-fed flies were bigger than control flies and RK-fed males were lighter than control initially (just after finishing RK treatments) (Table 2, Figure 5). Initial body size (mean±SE) of female was 4.847±0.020, 4.863±0.023 and 4.859±0.021 mm and that of male was 4.503±0.027, 4.584±0.029 and 4.634±0.016 mm in control, low and high RK dose respectively. Initial body weight (mean±SE) of female was 10.899±0.281, 11.229±0.176 and 10.916± 0.222 mg and that of male was 10.176±0.241, 9.05768±0.203 and 9.67553±0.163 mg in control, low and high respectively.

3.4. *Effect of RK dose, sex and body size on water content and lipid reserves*

Raspberry ketone had significant effect on initial water content, females tended to contain significantly more water than males in RK-fed flies and a strong positive relationship was observed between body size and initial water content (Table 3 and 4). Flies with high RK dose had more initial lipid reserves than control and low dose treated flies but lipid storage was not different for males and females (Table 3 and 5). Dose had no significant effect on water contents of desiccated flies but sex had effect on water content of desiccated flies in both RK-fed and RK-unfed flies (Table 3 and 4). Body size did not affect the water content of desiccated flies but lipid level was affected significantly (Table 4 and 5). Sex and dose interaction also affected lipid storage of desiccated flies (Table 5).

Water content at death in females of ‘no food or water’ and ‘water only’ flies was significantly higher than males, dose and sex interaction had significant effect

on water content of ‘no food or water’ group (Table 3 and 4). Body size had strong effect on the water content of flies in ‘no food or water’ and ‘water only’ groups (Table 4). Low dose RK-fed fly had significantly less lipid compare to control and high dose in ‘no food or water’ and ‘water only’ groups (Table 3 and 5). Sex and dose interaction as well as body size significantly affected the lipid storage in ‘no food or water’ group of flies (Table 5).

3.5. *Effect of RK dose, sex and body size on the changes of water content and lipid level*

In addition to the water and lipid storage in flies at different stress condition, the changes of these two components at different stresses also were assessed. Water content decreased in flies at both desiccation and starvation (‘no food or water’ and ‘water only’) compared to initial amount (Table 3, Figure 6 and 7). Overall flies at ‘water only’ condition lost least amount of water among all stresses, desiccated flies lost water significantly in control males and RK-fed females (Table 6). Dose and sex interaction had significant effect on the water loss in both desiccated and starved (‘no food or water’ and ‘water only’) flies (Table 3 and 6). Body size did not affect water loss in desiccation and ‘no food or water’ condition, however, in ‘water only’ condition water content was strongly affected by body size (Table 6). Low dose RK-fed flies lost more lipids significantly in ‘no food or water’ and ‘water only’ condition, on the other hand, lipids did not change significantly in desiccated flies (Table 3 and 7). Body size did not affect the changes of flies’ lipids neither in desiccation nor starvation (‘no food or water’ and ‘water only’) stress (Table 7).

Table 1: Effect of RK dose (0% control, 1.25% low, 5% high), sex (male, female) and stress treatment ('full diet', 'no food or water', 'water only') on survival of Qfly at nutritional stress (N=3276, R^2 = 0.966), and effect of sex, dose and body size on survival at desiccated (N=360, R^2 = 0.053) condition.

Source	**Nutritional stress**			**Source**	**Desiccation stress**		
	df	**F**	***P***		**df**	**F**	***P***
Dose	2	15.263	**<0.001**	**Dose**	2	9.380	**<0.001**
Sex	1	19.599	**<0.001**	**Sex**	1	7.117	**0.008**
Dose*Sex	2	1.486	0.226	**Dose*Sex**	2	0.143	0.867
Stress treatment	2	46561.037	**<0.001**	**Body size (Wing length)**	1	.006	.940
Dose * Stress treatment	4	4.093	**0.003**				
Sex * Stress treatment	2	5.836	**0.003**				
Sex* Dose * Stress treatment	4	0.304	0.876				

Table 2: Initial body size (N=356, R^2 = 0.391) and body weight (N=357, R^2 = 0.162) of *B. tryoni* at different RK dose (0% control, 1.25% low, 5% high), sex (male, female)

Source	**Body size**			**Body weight**		
	df	**F**	***P***	**df**	**F**	***P***
Dose	2	4.804	**0.009**	2	1.654	0.193
Sex	1	215.198	**<0.001**	1	59.35	**<0.001**
Dose * Sex	2	3.153	**0.044**	2	5.633	**0.004**

Table 3: Lipid and water reserves in Qfly at Initial (i.e., immediately after RK treatment), desiccation, 'no food or water' and 'water only' condition at different RK dose (0% control, 1.25% low, 5% high) and sex (male, female).

		Lipid (mg) / fly (mean+SD)				**Water content (mg in mass) / fly (mean+SD)**			
Dose	Sex	Initial	Desiccation	No food or water	Water only	Initial	Desiccation	No food or water	Water only
Control	Female	0.27±0.18	0.46±0.34	0.10±0.04	0.13±0.05	7.71±1.72	4.36±0.67	3.86±1.02	5.48±1.74
	Male	0.24±0.13	0.30±0.21	0.12±0.05	0.11±0.06	7.38±1.56	3.64±0.65	3.09±0.97	4.58±1.38
Low	Female	0.23±0.20	0.30±0.25	0.04±0.06	0.05±0.08	8.07±1.05	4.09±0.89	3.86±1.16	5.58±1.35
	Male	0.25±0.23	0.36±0.26	0.07±0.06	0.05±0.03	6.48±1.23	3.52±0.71	2.89±1.12	4.51±1.30
High	Female	0.33±0.28	0.38±0.33	0.131±0.04	0.11±0.03	7.69±1.30	4.09±0.54	3.07±1.18	5.17±1.62
	Male	0.32±0.20	0.33±0.25	0.10±0.06	0.10±0.08	6.90±1.09	3.79±0.73	3.023±1.15	4.42±1.63

Table 4. Effect of RK dose (0% control, 1.25% low, 5% high) , sex (male, female) and body size on water content in Qfly at initial (N=356, R^2 =0.216), desiccation (N=360, R^2 = 0.131), 'no food or water' (N=354, R^2 =0.116) and 'water only' (N= 354, R^2 =0.130) condition.

Source	Initial			Desiccation stress			No food or water			Water only		
	df	**F**	***P***	**df**	**F**	***P***	**df**	**F**	***P***	**df**	**F**	***P***
Dose	2	3.840	**0.022**	2	2.521	0.082	2	1.926	0.147	2	.144	0.866
Sex	1	1.560	0.213	1	49.991	**<0.001**	1	9.530	**0.002**	1	8.673	**0.003**
Sex*Dose	2	9.208	**<0.001**	2	2.421	0.090	2	6.986	**0.001**	2	0.595	0.552
Body size (wing length)	1	41.529	**<0.001**	1	0.240	0.624	1	7.962	**0.005**	1	25.614	**<0.001**

Table 5. Effect of RK dose (0% control, 1.25% low, 5% high), sex (male, female) and body size on lipid reserves in Qfly at Initial (N=356, R^2 = 0.025), desiccation (N=360, R^2 = .037 for lipid), 'no food or water' (N=354, R^2 =0.257) and 'water only' (N=354, R^2 =0.134) condition.

Source	Initial			Desiccation stress			No food or water			Water only		
	df	**F**	***P***	**df**	**F**	***P***	**df**	**F**	***P***	**df**	**F**	***P***
Dose	2	5.774	**0.003**	2	0.660	0.518	2	54.824	**<0.001**	2	10.952	**<0.001**
Sex	1	0.382	0.537	1	2.288	0.131	1	1.927	0.166	1	0.786	**0.377**
Sex*Dose	2	0.208	0.812	2	4.743	**0.009**	2	7.907	**<0.001**	2	0.481	0.619
Body size (wing length)	1	1.715	0.191	1	5.842	**0.016**	1	6.787	**0.010**	1	2.216	0.139

Table 6: Effect of RK dose (0% control, 1.25% low, 5% high), sex (male, female) and body size on changes of water level in desiccation (N=353, R^2 =0.038), 'no food or water' (N=354, R^2 =0.025), and 'water only' (N=354, R^2 =0.048) condition.

Source	Desiccation			No food or water			Water only		
	df	**F**	***P***	**df**	**F**	***P***	**df**	**F**	***P***
Dose	2	0.539	0.584	2	0.379	0.685	2	0.300	0.741
Sex	1	3.590	0.059	1	5.265	**0.022**	1	3.506	0.062
Dose*sex	2	5.885	**0.003**	2	4.043	**0.018**	2	3.766	**0.024**
Body size (wing length)	1	2.608	0.107	1	2.110	0.147	1	17.111	**<0.001**

Table 7: Effect of RK dose (0% control, 1.25% low, 5% high), sex (male, female) and body size on changes of lipid reserves in desiccation (N=353, R^2 =0.021), 'no food or water' (N=354, R^2 =0.010), and 'water only' (N=354, R^2 =0.016) condition.

Source	Desiccation			No food or water			Water only		
	df	**F**	***P***	**df**	**F**	***P***	**df**	**F**	***P***
Dose	2	2.628	0.074	2	3.266	**0.039**	2	5.126	**0.006**
Sex	1	1.895	0.170	1	0.468	0.494	1	0.011	0.918
Dose*sex	2	1.615	0.201	2	0.378	0.685	2	0.007	0.993
Body size (wing length)	1	1.972	0.161	1	0.640	0.424	1	0.760	0.384

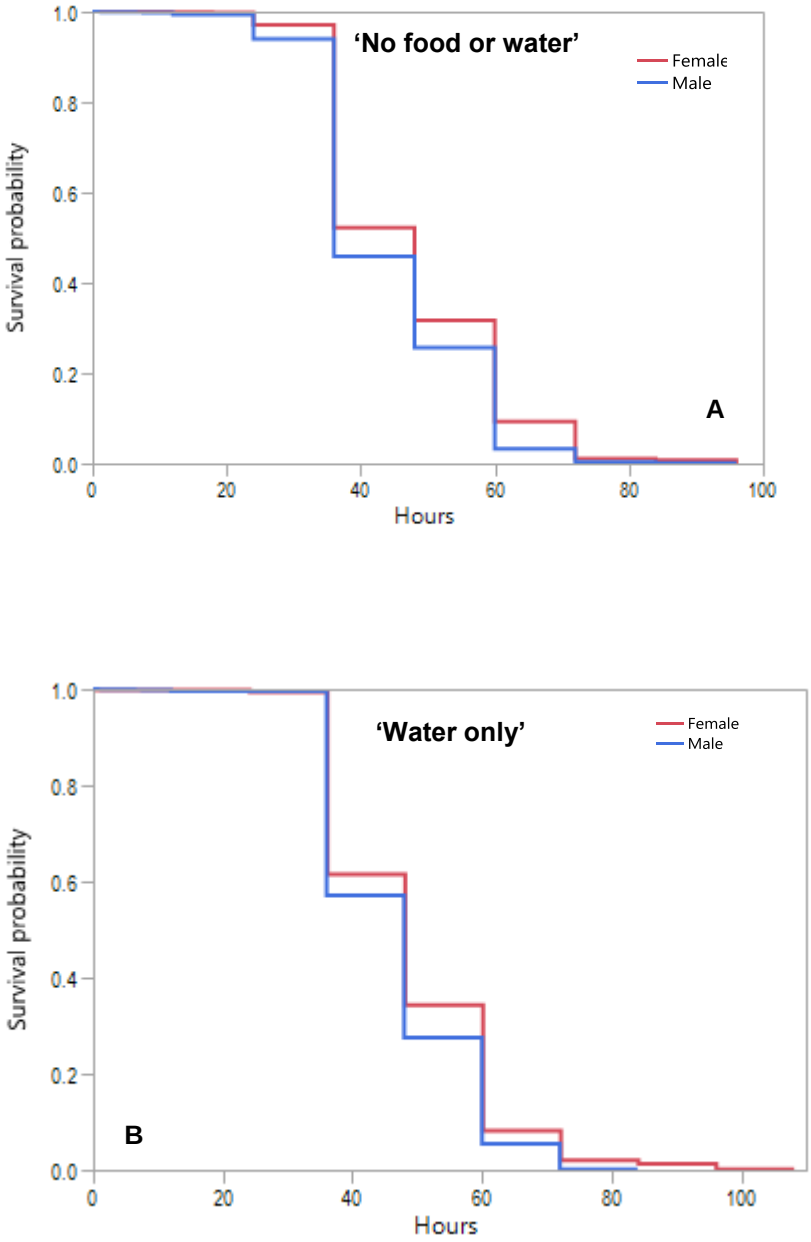
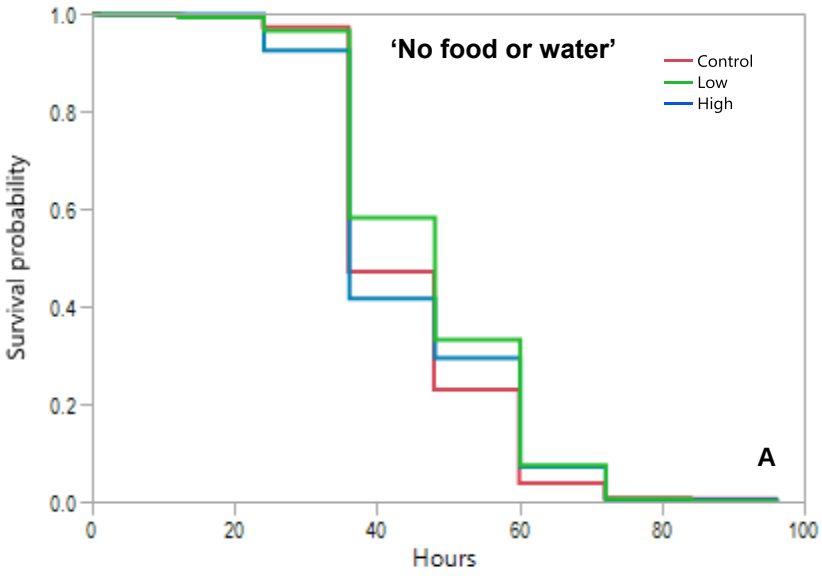


Figure 1: Survival probability of nutritional stressed male and female Qflies; A. ‘no food or water’ B. ‘water only’. Female survived longer in both stress condition compare to males.



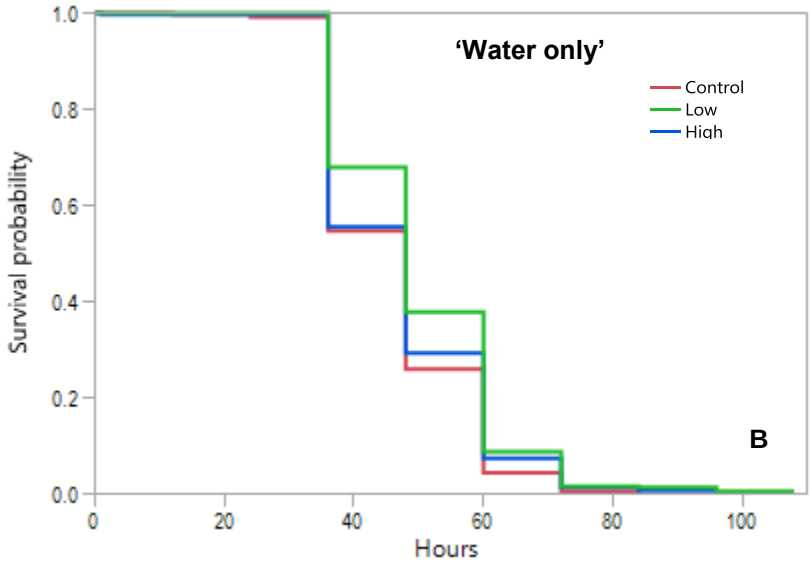


Figure 2: Survival probability of nutritional stressed Qflies receiving different RK doses; A. 'no food or water' B. 'water only' condition. In both conditions low dose treated flies survived longer than other two doses.

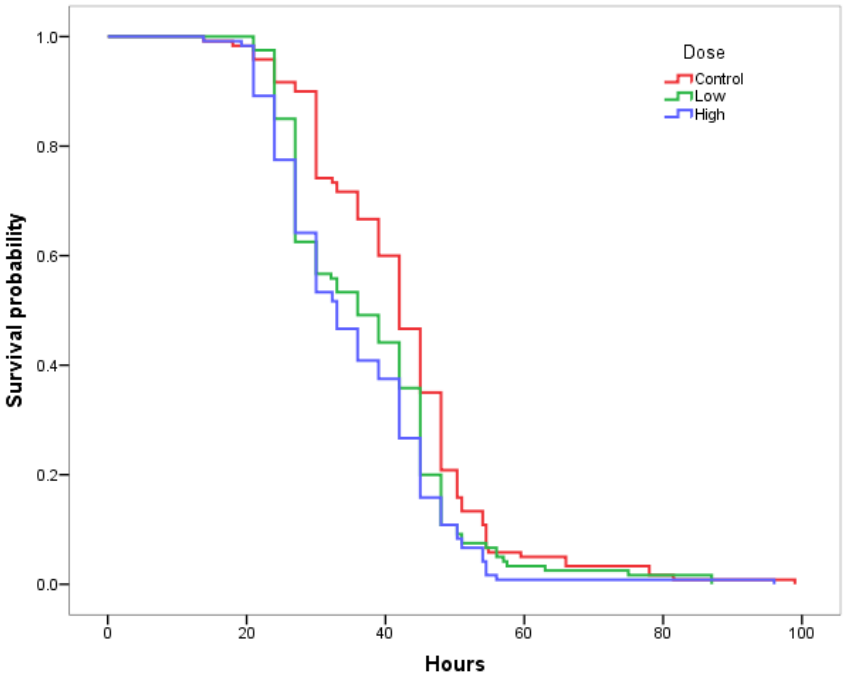


Figure 3: Survival probability of desiccated flies; control flies survived longer than RK-treated flies in desiccation condition.

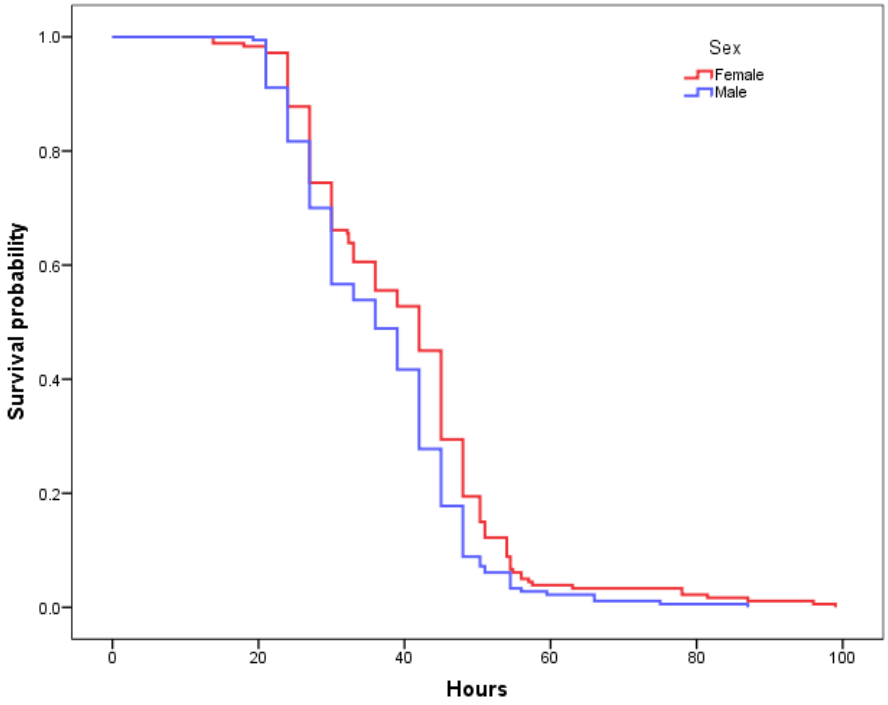


Figure 4: Survival probability of desiccated flies; overall females live longer than males regardless of RK-fed or RK-unfed.

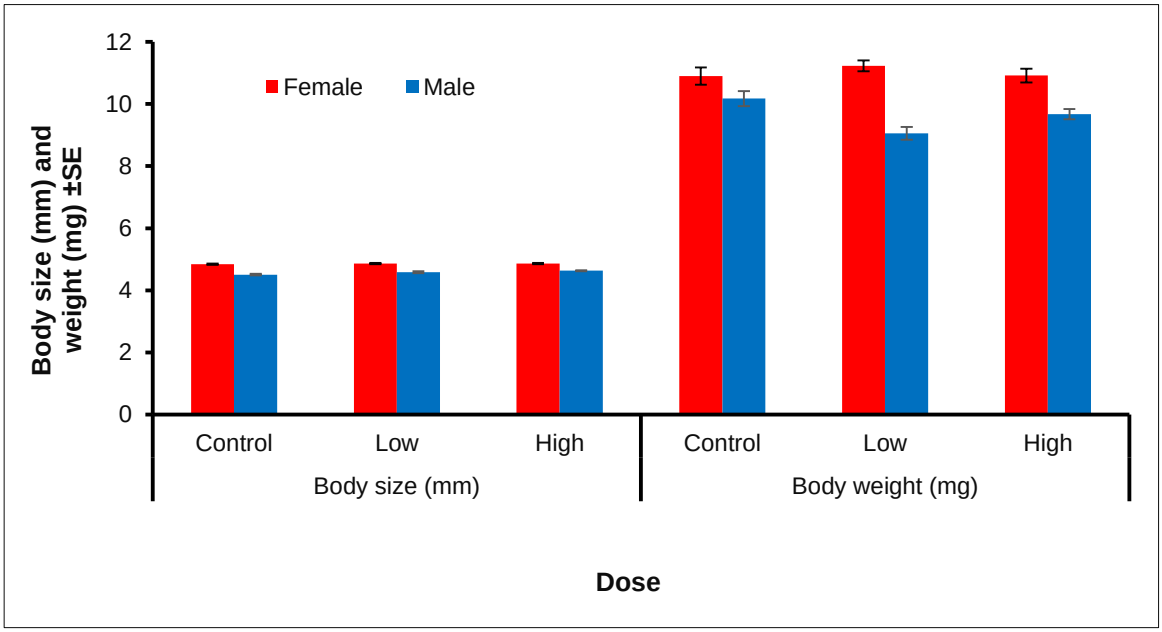
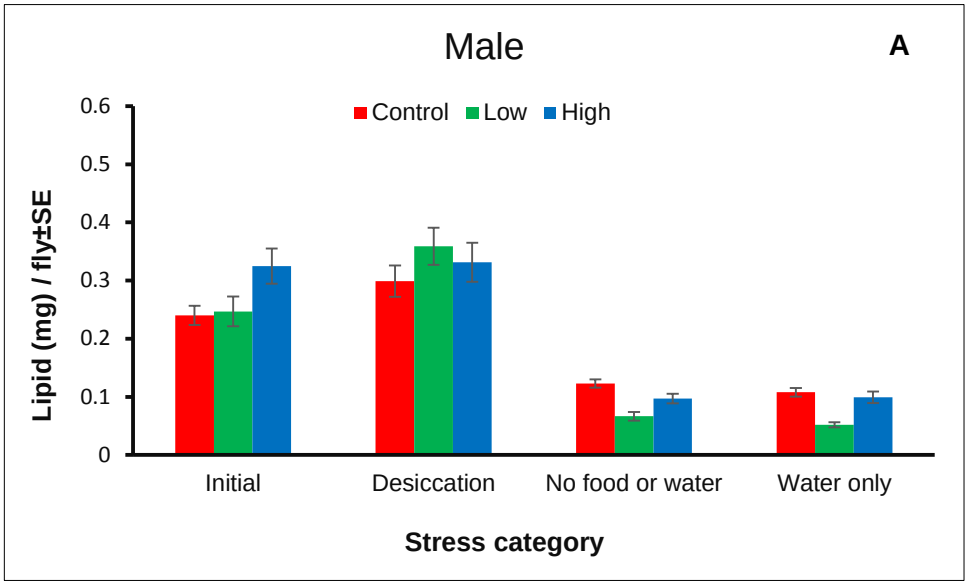


Figure 5: Initial body size and body weight of *B. tryoni*; females were bigger and heavier than males in both RK-fed and RK-unfed flies. Vertical bars represent standard errors (SE).



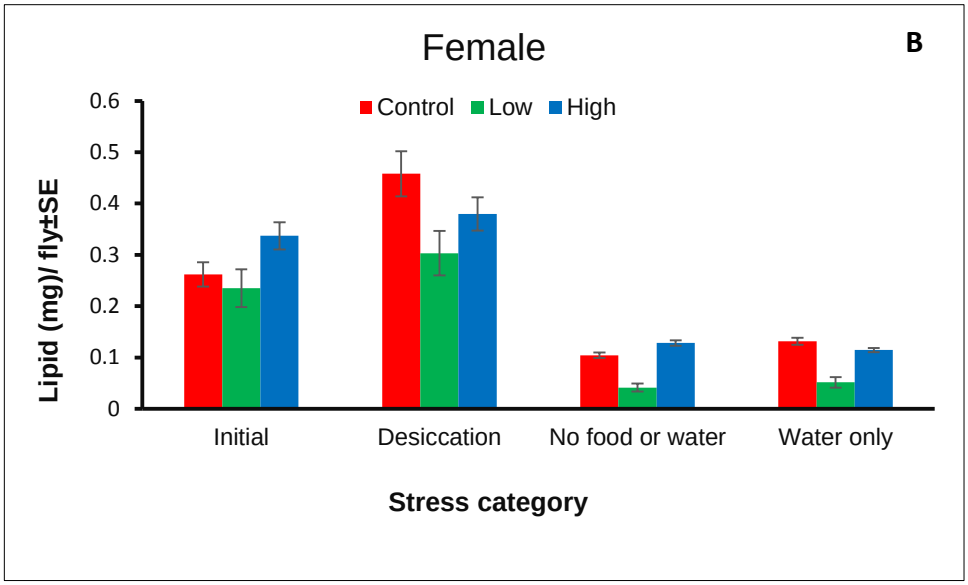
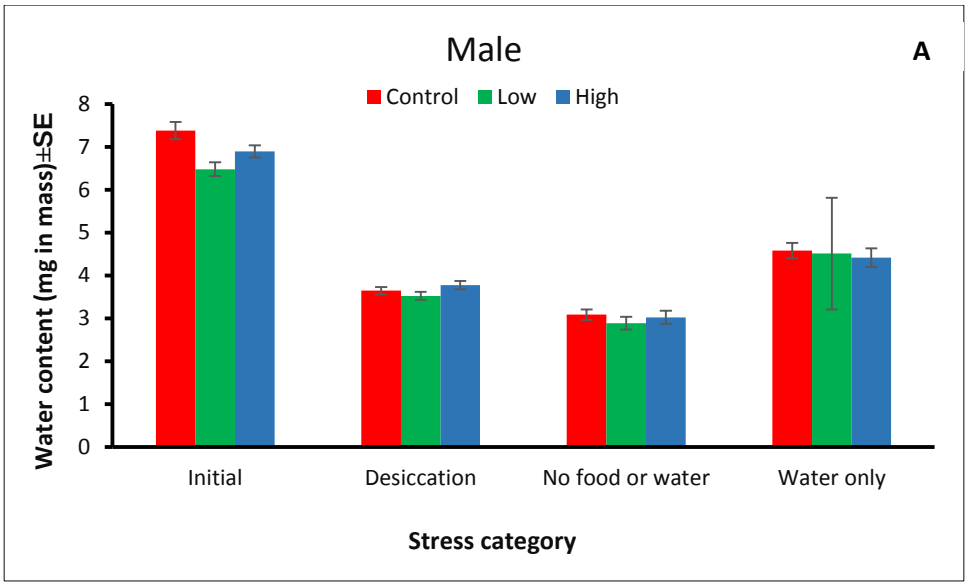


Figure 6: Initial lipid reserves and lipid reserves at death of Qfly after suffering under desiccation and starvation ('no food or water' and 'water only') stress, at different doses, control (0% RK), low dose (1.25% RK) and high dose (5% RK). Lipid level of both males (A) and females (B) decreased significantly in 'no food or water' and 'water only' condition compared to initial amount and low dose treated flies lost more lipid than other two treatment in both condition. In desiccation lipid did not change significantly compared to initial lipid level. Vertical bars represent standard errors.



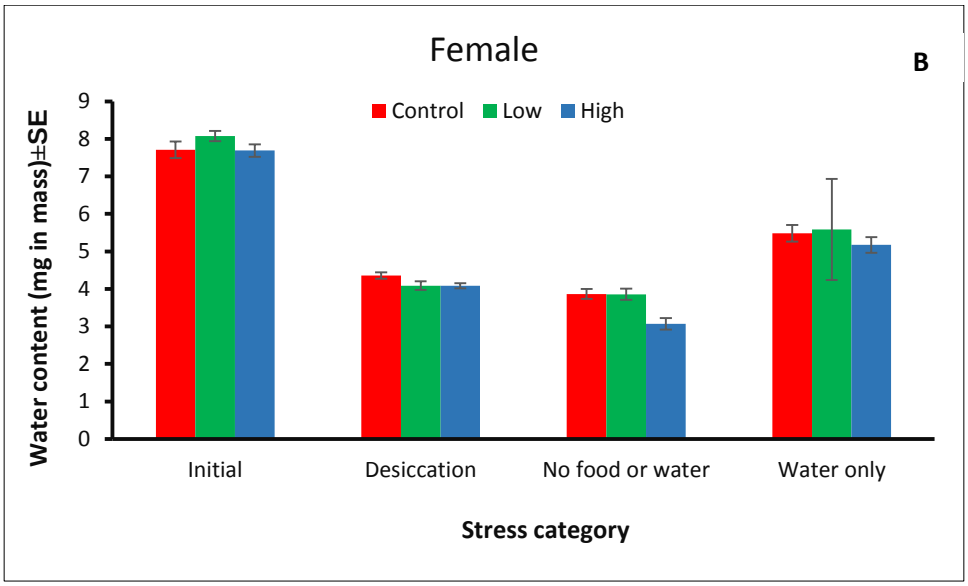


Figure 7: Initial water contents and water contents at death of Qfly after suffering under desiccation and starvation ('no food or water' and 'water only') stress, at different doses, control (0% RK), low dose (1.25% RK) and high dose (5% RK). Water level of both males (A) and females (B) decreased in both desiccated and starved ('no food or water' and 'water only') flies compared to initial amount; flies at 'water only' condition lost least amount of water among all stresses. Vertical bars represent standard errors.

4. Discussion

4.1. General discussion

Mass reared sterile flies may need to survive in a period, immediately following release, when nutrition might be scarce as well as dry condition may appear. Under such conditions longevity of mass-reared *B. tryoni* is significantly less than that of their wild counterparts (Weldon et al., 2013). Pre-release supplements may appear an obvious way to prepare flies for hardship following release, but this is not always straightforward. While it seems sensible to maintain sterile flies in ideal conditions in terms of temperature/humidity and food availability, this can prove detrimental. For instance, pre-release yeast hydrolysate supplements make Qfly more vulnerable to starvation (Taylor et al., 2013). Therefore, nutritional and desiccation stress cannot be disregarded when new supplements are considered to exploit in SIT application. As RK could be considered as a promising supplement as it accelerated and enhanced sexual (Akter et al, 2017) and reproductive maturation (chapter 3 of this thesis), its effect on survival in stress was assessed. Unlike yeast hydrolysate (Taylor et al., 2013) RK has no detrimental effects, rather RK supplementation to YH+sugar diet extended the survived of Qfly in starvation ('no food or water' and 'water only'). Similarly, exposure to ginger root oil (GRO) had a significant positive effect on survival in starvation when combined with the sugar + protein diet in wild males of medfly (Levy et al. 2005). Similarly, application of methoprene with protein diet had no adverse effect on starvation survival of melon fly (Haq et al., 2013). Along with the beneficial effect of RK, there is also disadvantage of RK supplementation as RK-fed flies are less resistant to desiccation. However, this issues could be minimized by careful release planning such as releasing sterile flies during summer rainfall when Qfly usually survive well (Bateman, 1972) or arranging irrigation to increase humidity that is beneficial for flies' survival.

Females of Qfly survived longer in both desiccation and starvation condition than males in the present study, however, in other experiment with same species it was observed that desiccation resistance of adult female Qflies is generally lower than that of adult males (Weldon and Taylor 2010, Weldon et al., 2013) which contradict present findings. Seemingly contradictory results for desiccation study

were also found in *Drosophila*; when body size was not considered females showed more resistant to desiccation, however, when size was taken into account males were as or more resistant than females (Matzkin et al., 2007, 2009). Consistent with present findings there are several examples where female resistance to desiccation stress exceeded that of males. For instance, in *Drosophilla* species (Gibbs and Markow, 2001; Matzkin et al., 2009), *D. mimica* females also showed survival for longer than males under desiccating conditions (Steiner, 1974), however, another study of *D. mimica* found no sex difference in water loss rate (Eckstrand and Richardson, 1980). Sex difference in survival at starvation is also common in fruit flies, *D. busckii*, showed extremely difference in survival at starvation where females survived 104 hour and males survived for 58 hour (Matzkin et al., 2009). There are also other such examples where females outperformed in starvation than males such as *D. anceps*, *D. mojavensis*, *D. paulistorum* and *D. simulans* (Matzkin et al., 2009).However, *D. melanogaster* is exception in this instance as males significantly resisted starvation better than females (Matzkin et al., 2009). Extended survival of Qfly females are possibly related to the body size, as females are larger than males they might have advantageous position to survive in desiccation and starvation as bigger flies are more stress-resistant than smaller flies (Tejeda et al., 2014). However, Weldon and Taylor (2010) showed that survival under starvation was not related to body size in Qfly, on the other hand, other studies showed very weak, but significant, positive relationship between body size and survival of adult Qfly under nutritional stress (Collins et al., 2009). In the present study relationship between body size and survival at starvation was not studied directly, but considering female's size it could be interpreted that there is positive relationship between body size and survival in starvation. Like Qfly such differences in the relationship between body size and stresses were also observed in *Drosophila*. Some studies of *Drosophila* showed a positive relationship between size and starvation stress resistance (Parsons, 1970, Matzkin et al., 2009, Chippindale et al., 1996; Gibbs and Matzkin, 2001), while other studies have not found such a relationship (Gibbs et al., 1997; Hoffmann and Harshman, 1999; Hoffmann and Parsons, 1989a). Survival of Qfly in desiccation was not affected by body size in the present study, however, the strong relationship was found in both females and males in *Drosophila* (Matzkin et al., 2009).

Resistance to starvation largely depends on changes in lipid content in *D. melanogaster* (Hoffmann and Harshman, 1999) which is also evident in Qfly in the present study where lipid content decreased significantly along with water in low dose RK-fed flies (that survived longer) in starvation ('no food or water' and 'water only') compare to initial lipid. Similar to Qfly in A. *serpentine* lipid reserves in starved flies dropped drastically during the first days of adult life (Jacome et al., 1995). Also, in *A. ludens* flies that died under the starvation treatment used high amount of teneral lipid (Tejeda et al., 2014) and in Mediterranean fruit fly, the level of lipids decreased significantly with days in both protein-fed and protein-deprived released sterile males (Maor et. al. 2004) detected. This depletion of lipid indicates a role of energy reserves for survival (Yuval et al., 1998). As there was no food availability for starved flies ('no food or water' and 'water only'), initial lipid presumably was metabolised to provide energy during extended starvation periods (Arrese and Soulages, 2010; Park, 2010). Consistent with the present findings, males of *A. ludens* used greater amounts of lipids to survive longer during stress (Tejeda et al., 2014) and *D. melanogaster* used lipids to release more energy in starvation (Djawdan et al., 1997). However, in starvation selected *Drosophila* populations showed increased accumulation of lipid than controls under starved condition (Chippindale et al., 1996, 1998) which is different from the present study. In another study with *Drosophila* also showed that starvation resistance is positively correlated with lipid levels (van Herrewege and David, 1997). While starvation resistance is linked to lipid level, desiccation resistance, on the other hand, very much related to the water level (Gibbs et al., 1997; Hoffmann and Harshman, 1999; Chown and Nicolson, 2004). Reduced rates of water loss without changes in the minimum water content facilitated increased survival of *D. melanogaster* in desiccation (Hoffmann and Harshman, 1999). Desiccated Qfly lost significant amount of water without changing lipid level in the present study whereas in *Drosophila* desiccation resistance is positively correlated with lipid levels (van Herrewege and David, 1997). Similarly *A. ludens* in the desiccation treatment showed the highest use of water reserves and the lowest use of lipid reserves (Tejeda et al., 2014). Which energetic substrates flies will metabolise depends on the type of stress imposed, and the energy and water contents of different stored compounds (Marron et al., 2003). For instance, in one experiment

it showed that metabolic rates of lipid and protein were similar during starvation and desiccation, but carbohydrate metabolic rate was higher during desiccation in *Drosophila* (Marron et al., 2003). The reason behind this differential metabolic rate could be because lipids provide over twice as much energy per gram as carbohydrates (Withers, 1992) which is an appropriate fuel to store for starvation resistance and the total amount of water available after metabolism is much lower for lipids than carbohydrates (Gibbs et al., 1997) thus carbohydrates would be more appropriate fuel under desiccation.

4.2. *Implications for SIT*

Raspberry ketone treatments accelerate maturation in Qfly (Akter et al., 2017) and show promise as pre-release supplements for SIT. However, potential negative effects also need to be considered, especially effects in adverse environments. In this study it was revealed that RK makes flies more vulnerable to desiccation but not starvation. Flies are usually released in towns where some sources of food and water might usually be available. Overall, in most conditions it is expected that the shortcoming of increased desiccation vulnerability would be outweighed by the benefits of accelerated mating and reproductive organ development in RK-fed young adults.

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Chapter Seven

Suppression of cuelure attraction in male Queensland fruit fly *Bactrocera tryoni* (Froggatt) provided raspberry ketone supplements as immature adults

Photo: Humayra Akter

Abstract

Tephritid fruit flies are amongst the most damaging insect pests of horticulture globally. Some of the worst fruit fly species are managed using the sterile insect technique (SIT) whereby millions of sterile males are released to impede reproduction of pest populations. Male annihilation technique (MAT), whereby lures are used to attract and kill males, is often used to reduce wild male numbers before SIT programs commence, providing released sterile males an increased numerical advantage. Overall program efficacy would be improved if MAT and SIT were deployed simultaneously, but this requires that attraction of released sterile males to MAT devices be inhibited. Previous studies have found that exposure of fruit flies to lure compounds as mature adults can suppress subsequent response to those lures, raising the possibility of pre-release treatment. However, this approach requires holding flies until after maturation. The present study takes a novel approach of exposing immature adult male Queensland fruit flies (*Bactrocera tryoni*, or 'Qfly') to raspberry ketone (RK) mixed in food, forcing these flies to ingest RK at ages far younger than they would naturally. After feeding on RK-supplemented food for two days after emergence, male Qflies exhibited a reduction in attraction to cuelure traps that lasted more than 20 days. This approach to RK exposure is compatible with current practises, in which Qflies are released as immature adults, and also yields advantages of accelerated reproductive development and increased mating propensity at young ages.

Introduction

Tephritid fruit flies are significant pests of horticulture in most regions of the world, causing direct damage to crops and restricting trade. The sterile insect technique (SIT) is used to manage some of the most serious fruit fly pests (Orozco-Dávila et al. 2007; Hendrichs et al. 2002; Enkerlin et al. 2015). In SIT, millions of flies are reared in factories, sterilised, and then released into the field. Sterile males mate with females of pest populations, curtailing their reproduction, such that over generations the pest population is reduced (Knipling 1955; Krafsur 1998).

For species that respond to strong male attractants such as methyl eugenol or cuelure (Beroza et al. 1960; Bateman 1972; Cunningham and Suda 1986; Seewooruthun et al. 2000), the male annihilation technique (MAT) is often used before the deployment of SIT (Bateman et al. 1966; Barclay and Hendrichs 2014). MAT reduces the number of males in the pest population, and this increases the effective overflooding ratio when sterile males are released.

Because released sterile males are also attracted to male attractants, MAT and SIT have traditionally been used sequentially. However, massive advantages in overall control levels and efficiency would be achieved if MAT and SIT could be used simultaneously through the release of male flies that are unresponsive to the male lures used in MAT (Barclay et al. 2014). In some species there is compelling evidence that pre-prelease treatment with male lures can have a lasting effect of reduced responsiveness to those lures and might provide a basis for development of simultaneous deployment of MAT and SIT. Chambers et al. (1972) exposed mature males of Mediterranean fruit fly (*Ceratitis capitata*, or ‘medfly’) to trimedlure and mature males of Oriental fruit fly (*Bactrocera dorsalis*) to methyl eugenol, and in both cases found that the lure exposure resulted in persistent reduction is responsiveness to traps containing these lures. Shelly et al. (2007) exposed medfly males to ginger root oil, which contains α-copaene, and found reduced attraction to trimedlure traps. Although Shelly (2000) found no evidence that feeding on natural sources of methyl eugenol by mature male oriental fruit flies reduces subsequent trap capture, in an earlier study Shelly (1994) found that more

controlled pre-release exposure of oriental fruit flies to synthesised methyl eugenol resulted in significantly reduced responsiveness to traps containing this lure. Traps were checked only at five days after release, and so it is not clear whether the effect lasted this full period, or lasted longer. Similarly, Shelly and Villalobos (1995) found that mature male melon flies (*Zeugodacus cucurbitae*) provided a wick containing cuelure, a synthetic and more volatile analogue of naturally occurring raspberry ketone (RK) (Park et al. 2016 a,b), for two hours one day prior to release showed reduced responsiveness to cuelure traps during the two days following release. Longer periods were not investigated, and so the persistence of this effect is unknown.

In the present study, we consider the potential of pre-release RK supplements as a means of reducing response of male Queensland fruit fly *Bactrocera tryoni* (Qfly) to cuelure, the attractant used in MAT for this species, to potentially enable to simultaneous deployment of MAT and SIT for this species. As a significant departure from previous studies of lure response, we adopt a novel approach to deployment of RK that has been recently developed by Akter et al. (2017). Rather than waiting for flies to respond naturally to RK once mature, this approach entails mixing RK in food provided to immature flies, enabling exposure to RK over a time frame that is compatible with standard pre-release holding periods.

Qfly is Australia's most damaging fruit fly pest and in some non-endemic regions outbreaks have been managed by the sequential combination of MAT followed by SIT (Monro and Osborn 1967; Horwood and Keenan 1994; Dominiak et al., 2003; Meats et al. 2003). With increased restrictions on the use of insecticides, SIT and MAT are set to become more routine management tools (Dominiak and Ekman 2013) and significant research programs are currently under way to maximise the efficacy of these tools.

Qfly is usually released two to three days following emergence, and this holding period provides an opportunity for the implementation of pre-release

treatments that enhance the performance of flies following release. Previous studies have highlighted the potential benefits of access to yeast hydrolysate in addition to sugar as pre-release diet, as this treatment promotes reproductive development and mating performance (Pérez-Staples et al. 2008, 2009, 2011; Weldon et al. 2008) and results in increased prevalence of sexually mature (i.e., cuelure responsive) males in the field (Reynolds et al. 2014). In addition to benefits of yeast hydrolysate feeding, treatment of pupae or recently emerged adults with methoprene, a juvenile hormone analogue, has been found to accelerate development (Collins et al. 2014).

In addition to yeast hydrolysate and methoprene treatment of immature Qflies, phytochemicals such as cuelure and zingerone have also shown promise but until recently these had only been considered in terms of mating performance of males that feed on these compounds as mature adults (Tan & Nishida 1995; Kumaran et al. 2013, 2014ab). Immature males show little or no attraction to RK analogues, and previous studies have consequently focused on the responses of mature males. The need to wait for flies to mature before providing phytochemical supplements constrains the operational viability of such supplements, as in the case of Qfly this would require holding the flies for more than a week before release. However, Akter et al. (2017) took a novel approach of mixing RK supplements in the diet of immature Qfly for two days following emergence, forcing them to ingest RK at much younger ages than they would naturally be attracted and feed on RK. This RK supplementation of diets of immature Qflies substantially increased the mating propensity of young males, this being the first demonstration that RK might also show potential as a supplement for immature fruit flies. This is important because the approach of Akter et al. (2017) enables deployment of RK supplements within the usual short pre-release holding period.

Given that inclusion of RK in the diet of immature adult Qflies yields sexual benefits leads naturally to consideration of what other aspects of physiology and behaviour might be affected. Modified responses to male lures is an interesting prospect. As was until recently also the case for studies of mating performance, the effects of access to RK, cuelure, methyl eugenol and related compounds on

subsequent responses of fruit flies to lures has only been considered following exposure of mature males. In the present study we consider whether the RK treatment of immature males using the approach of Akter et al. (2017) reduces subsequent responses to cuelure, the standard lure used in MAT for this species. If the promising effects reported previously for mature male medfly, oriental fruit fly and melonfly (Chambers et al. 1972; Shelly 1994) are also evident when RK is provided to immature Qflies, this would potential enable simultaneous deployment of MAT and SIT within the current standard pre-release holding period.

Materials and Methods

Source and maintenance of flies

Q-fly pupae were supplied by the New South Wales Department of Primary Industries Fruit Fly Production Facility at the Elizabeth Macarthur Agricultural Institute at Camden, New South Wales, Australia (for production details, see Dominiak et al. 2008). Pupae were kept under controlled temperature (25 ± 1°C) and relative humidity (65 ± 5 %) on a 13:11 h light: dark cycle in which the first and last 30 min of the light phase were simulated dawn and dusk in which light level gradually ramped up to full output, and down to darkness, respectively.

Raspberry ketone treatment

Approximately 2000 pupae (estimated by weight) were placed in 47.5×47.5×47.5 cm fine mesh cages (Bugdorm 44545, Megaview, Taichung, Taiwan) for adult emergence with only water provided for sustenance. Because only a few flies usually emerge on the first day, the first day of emergence was discarded and only adults that emerged over the second day were used in the experiment.

After fly emergence, the cages were provided with water and diet (1:3 yeast hydrolysate:sugar) containing raspberry ketone, 4-(4-Hydroxyphenyl)-2-butanone (≥98%, Sigma–Aldrich®) at high dose (5% RK), low dose (1.25% RK), or no RK (control) for 48 hours. RK was ground using a blender before being mixed in the diet and to differentiate among treatments, both the diet and water was mixed with

food colour (blue, red and yellow) that were clearly visible in the gut of the flies for few days. Three millilitres of dye was added to 30 g of diet and the powdered diet became a paste that was spread over a filter paper and dried for 48 hours. Two millilitres of dye was added into 50 mL of water in a 70 mL plastic container. The dye was rotated among treatments between replicates. After providing food and water cages were placed in sheltered outdoor conditions where the flies were kept until being used in the small field cage experiments. After 48 hours of feeding, the diet was removed from the cages. Males were removed from mixed-sex cages when 3 days old, to ensure they were virgin (Meats et al. 2004) when used in the experiments, and placed in clean 47.5×47.55×47.5 cm fine mesh cages (Megaview Bugdorm 44545) (700 - 1,000 male flies per cage) provided with sugar and water that were also coloured with corresponding food colour used for water and RK-treated food.

For large field cage study water and RK-treated diet were not coloured as mentioned above. In this case pupae were coloured with fluorescent dye (strong magenta 21, lunar yellow 27, stellar green 8) (Swada, UK), as is standard in SIT programs, to identify treatment groups and dye was rotated among treatments between replicates. After emergence, the cages were provided with water and diet (1:3 yeast hydrolysate:sugar) containing a high dose (5% RK), low dose (1.25% RK), or no RK (control). After 48 hours feeding treated food was removed and flies were ready to release in large field cage.

Treated flies were kept separately from the control flies to avoid exposure to RK odour released by treated flies (Tan and Nishida 1995).

Small field cage experiments

Four small field cages (3 m diameter, 2.2 m high) were used (Figure 1A-B). An artificial tree (Ikea Fejka) was placed in the middle of each field cage. Male Q-flies were tested for attraction to cuelure at 5, 7, 9, 11, 13, 15, 20 and 25 days after emergence. Fifty male Q-flies from each treatment group were released into each field cage 30 minutes before sunrise (i.e., 150 flies per cage on each test day).

Thirty minutes later, one Lynfield trap baited with 200 µL of cuelure on a cotton wick was suspended from the ceiling of each cage. Instead of using insecticide, each trap contained a white plastic sheet that was covered with adhesive (Tanglefoot) to capture attracted flies. Traps were collected after sunset. Captured flies were sorted by treatment and counted. The experiment was repeated 10 times, using flies from three production batches obtained at least four weeks apart.

Figure 1. Field cages where experiments were run; (A-B) small field cage (3 m diameter, 2.2 m high) with 1 artificial plant and 1 lure trap and (C-D) large field cage (8 x 24 m) where citrus plants and both lure and control traps were suspended.

Large field cage experiments

In this experiment, flies were released in large field cages (8 m wide and 24 m long metal frame covered with white mesh, 5 m at the highest point) that contained 6 lemon trees (*Citrus limon*) each (Figure 1C-D). To simulate an SIT release under contained conditions, rather than releasing and testing flies at specific ages as in small field cage experiments, in this experiment flies were released immediately after the 48-hour treatment period was complete and were then sampled over days. This experiment was repeated twelve times, with four large field cages each being used three times. Four Lynfield traps were suspended in each large field cage, two containing cuelure as an attractant ('cuelure traps') plus malathion as killing agent and two containing malathion only without attractant ('control traps'). To remove effects of incidental captures unrelated to cuelure attraction, the number of flies captured in the control traps was subtracted from the number captured in the cuelure traps within each field cage on each day of trap clearance.

Statistical analysis

The number of flies from each treatment captured in traps was analysed by a mixed model. RK treatment was treated as nominal and Age was treated as ordinal. Batch number and cage number were included as random effects. All statistical analysis were carried out using JMP version 10.0.0 (SAS Institute, Cary, NC, USA).

Results

Small field cages

The effect of RK supplementation on responses to cuelure traps varied across the days of testing (RK Treatment $F_{2,241.8}$= 2.510, P=0.083, Age $F_{7,242.2}$=13.874, P<0.001, RK Treatment x Age $F_{14,241.8}$=5.544, P<0.001), with significantly fewer

RK-supplemented flies captured at some ages but not at others (Figure 2, 3). When the flies were 5, 7, 9 and 25 days of age there was no evidence of differences in capture rates of RK-supplemented and control flies, but at 11, 13, 15 and 20 days of age significantly fewer RK-supplemented flies were captured compared with the controls. At each of these ages the effects were significant for both RK doses, and there were no significant differences between the two doses. At the peak of differences in daily captures when flies were twenty days of age, approximately five and a half times as many control flies were captured compared with those that received RK.

Large field cages

For males there was significant variation across the tested days in the number captured in cuelure traps ($F_{9,224}$=12.409, P<0.001) and significant variation amongst RK treatment groups ($F_{2,224}$=7.273, P<0.001), with significantly more control flies being captured than either of the RK treated groups and no difference between the RK treatments (Figure 4, 5). There was no evidence of variation across the tested days in the effect of RK treatment (Age*Treatment interaction $F_{18,206}$=0.489, P=0.961). At the peak of differences in daily captures when flies were eight days of age, approximately fourfold more control flies were captured compared with those that received the high RK dose and eight-fold more control flies were captured compared with those that received the low RK dose.

For females there was significant variation across the tested days in the number captured in cuelure traps ($F_{9,224}$=2.815, P=0.004) but no significant variation amongst RK treatment groups ($F_{2,224}$=0.861, P=0.424).

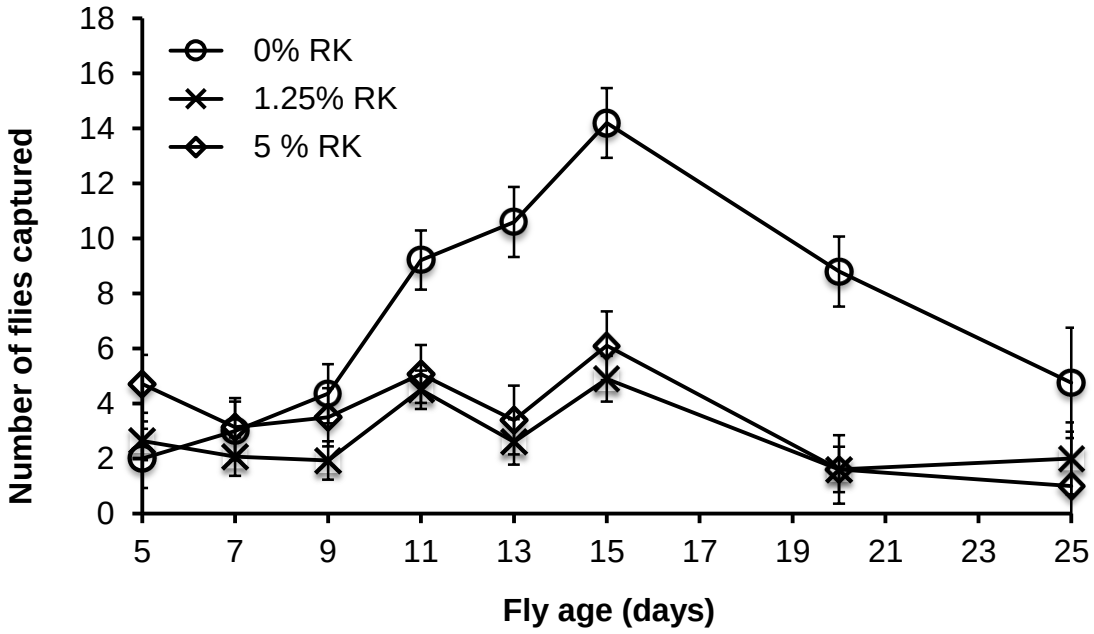

Figure 2. Mean number of male Qflies captured in cuelure traps in small field cage trials. Vertical bars represent standard errors.

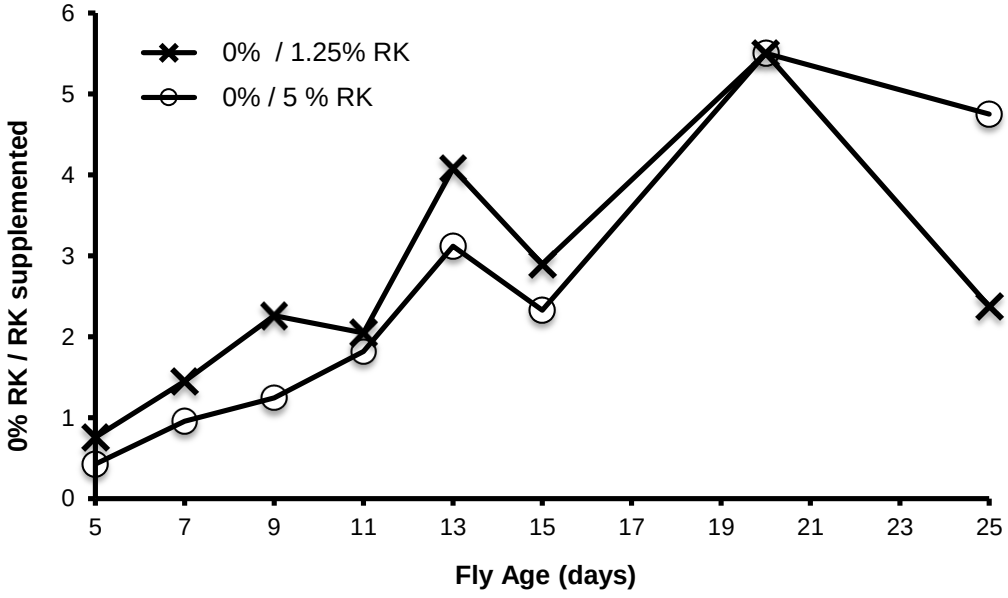

Figure 3. Relative number of control vs. RK treated male Qflies captured in cuelure traps in small field cage trials.

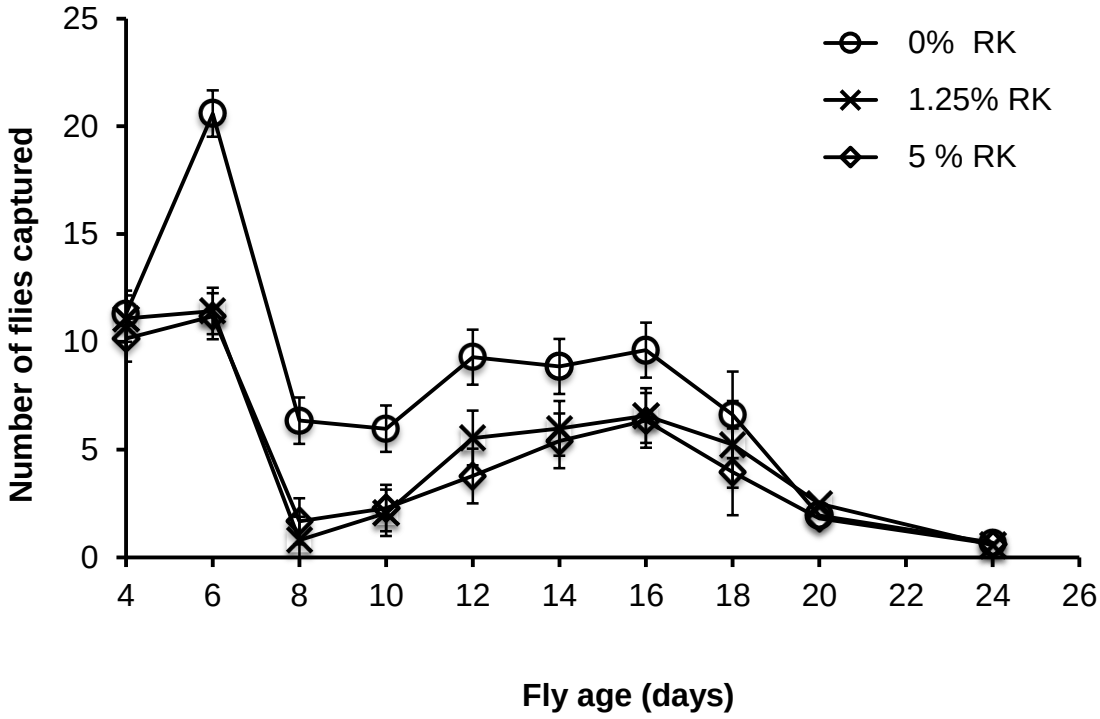

Figure 4. Number of male Qflies captured in cuelure traps minus the number captured in control traps in large field cage trials. Vertical bars represent standard errors.

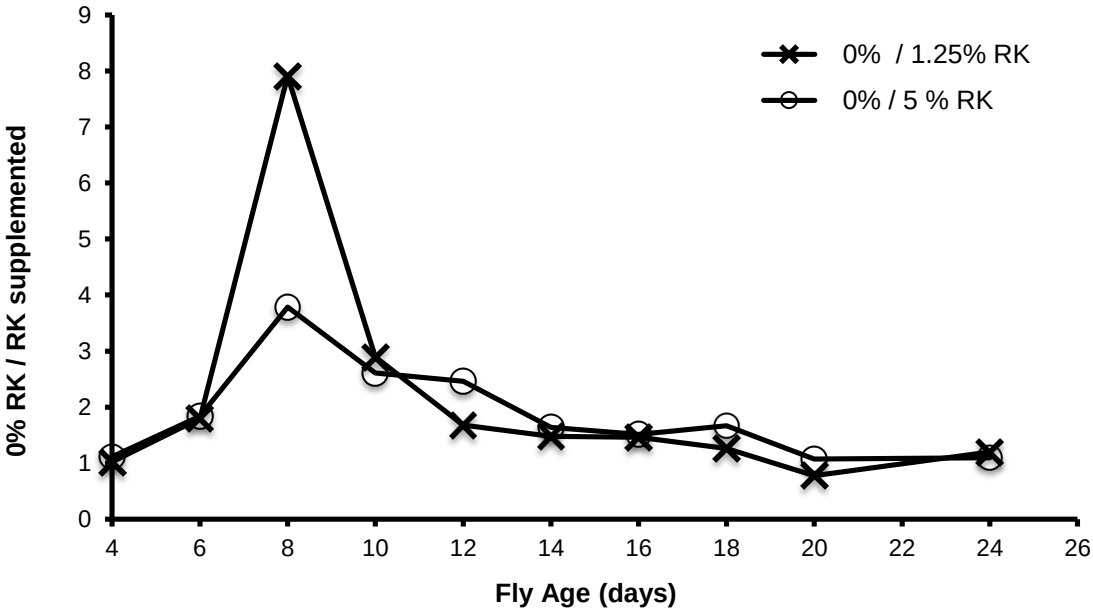

Figure 5. Relative number of control vs. RK treated male Qflies captured in cuelure traps in large field cage trials.

Discussion

In fruit fly SIT programs, MAT is commonly used to reduce wild male numbers before the release of sterile flies (Steiner et al. 1970; Koyama et al. 1984; Barclay and Hendrichs 2014). However significant improvements in overall program efficacy could theoretically be achieved if it was possible to deploy MAT and SIT simultaneously, with MAT continuously depleting wild males from the field SIT continuously replacing them with sterile males (Barclay et al. 2014). Simultaneous deployment of MAT and SIT would also enable the immediate use of SIT to combat outbreaks rather than waiting until completing an MAT program before commencing the release of sterile flies and would also negate the need to retrieve MAT devices from the field. Usually, the Qflies released in SIT programs are attracted to cuelure (Reynolds et al. 2014), the lure used for MAT against this species (Dominiak et al. 2016). For simultaneous deployment of MAT and SIT to be viable, there first needs to be an effective means of inhibiting attraction of released sterile flies to cuelure-based MAT devices that can be implemented within operational practises. The present study identifies a potential means of achieving this.

Incorporation of RK in the diet of immature male Qfly for two days following emergence induced a persistent reduction in the number of flies captured in cuelure traps. Previous studies of oriental fruit fly, medfly and melon fly have reported persistent reduction in responses to lures used in monitoring traps and MAT following previous exposure (Chambers et al. 1972; Shelly 1994; Shelly and Villalobos 1995; Shelly et al. 2007). However, each of these studies entailed exposure of adult flies once they had already matured. This approach would require holding Qflies for a week or longer after emerging (Meats et al. 2004; Pérez-Staples et al. 2007), which would be a significant departure from the current practises under which flies are typically released two to three days following emergence (Reynolds et al. 2014). The present study finds that effects paralleling those of previous studies of mature adults can be achieved by including RK in the diet of recently emerged adult Qflies.

RK and closely related compounds have diverse effects on the behaviour and physiology when ingested by mature male Qflies. For example, ingested cuelure is incorporated in pheromone as RK (Tan and Nishida 2012; Kumaran et al. 2014), and male Qflies that ingest these compounds gain significant advantages in mating performance over the following two days. Akter at al. (2017) was the first to find that RK can have significant effects even when incorporated into the diet of immature male Qflies. Male Qflies that ingest RK as immature adults exhibit greatly elevated mating propensity up to 10 days of age (Akter et al. 2017), and this tendency corresponds with significant acceleration in the development of reproductive organs over this period (Akter et al. unpublished data; Chapter 3). Usually, attraction to cuelure is associated with mature Qflies (Weldon et al. 2008) and one this basis it might be expected that the accelerated development of RK-treated males would result in increased capture rates in cuelure traps at young ages. In contrast, despite being mature at younger ages, RK-fed flies exhibited significantly reduced capture rates. Amongst the youngest ages of flies tested in small field cages, the difference between capture rates of RK treated and untreated flies was not significant. With differences only becoming evident once flies reached 10 days of age. Rather than indicating failure of RK treatment, this reflects the low responses of flies from the control group at these ages. As the control flies aged their attraction to cuelure traps increased sharply, but this was not observed in the RK treated flies.

Pre-release RK treatment shows promise as a means of accelerating development (Chapter 3), increasing mating propensity at young ages (Akter et al. 2017) and inhibiting attraction to cuelure traps, thereby potentially enabling the simultaneous deployment of MAT and SIT. This is a relatively simple approach, and while the effects found so far are promising they could likely be improved by further refinement of the RK-exposure protocol. In addition to pre-release RK supplements, there are alternative approaches that might be considered to suppress response of released Qflies to cuelure-based MAT devices. Selective breeding of non-responsive flies is one option, and there is some support for this possibility already in oriental fruit fly studies. Shelly (1997) selected oriental fruit fly males for non-response to methyl eugenol and was able to create two lines that maintained low response to this lure over many generations. Similarly, Ito and Iwahashi (1974) succeeded in selecting for lure

non-responsiveness in oriental fruit fly after just two generations. Both studies concluded that while lure non-responsiveness is a quite rare trait it is amenable to selection in oriental fruit fly colonies. In the studies of Shelly (1997) and Ito and Iwahashi (1974) the evolution of lure non-responsiveness was considered as a risk to MAT programs. On the other hand, establishment of mass-reared populations with suppressed response to lures used in MAT would be extremely beneficial for the prospective simultaneous deployment of MAT and SIT. To date there has been no investigation of within or between population variation in male Qfly response to cuelure, and such studies are now warranted to explore this possibility. However, there may also be disadvantages that should be considered in an overall program setting. Usually the abundance of mature released flies in the field is monitored using cuelure traps and these data can be used as an indirect measure of sterile fly field performance (Meats 1998). Inhibition of responses to cuelure traps would impede monitoring of released sterile flies. To counter this limitation, it might be possible to develop a calibration for the abundance of RK treated flies that would estimate an equivalency in terms of current practises. Also, under some conditions alternative monitoring systems, such as sticky traps (Weldon and Meats 2007), could be used, although this would result in significantly increased labour costs. Under many conditions, however, the constraint on ability to monitor released flies may be a minor consideration next to the substantial potential increases in overall program efficacy (Barclay et al. 2014).

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Chapter Eight

General Discussion


Photo: Humayra Akter

8.1. Introduction

Evolution has shaped plant and insect interactions in many ways, resulting in many mutually beneficial insect-plant interactions. Some of these plant-insect relationships are centred on phytochemicals. One example of this is that mature fruit fly males (of some species) seek plant secondary phytochemicals that promote mating performance (Papadopoulos et al. 2006; Shelly 2000a; Shelly & Dewire 1994; Shelly et al. 2005; Shelly et al. 2008; Shelly & Villalobos 1995; Khoo & Tan 2000; Kumaran et al. 2013; Shelly & Nishida 2004), and in return, the flies contribute to plant pollen dispersal (Clarke et al. 2002; Tan et al. 2006). In addition to their attractive properties, phytochemicals can physiologically or behaviourally affect fruit fly sexual communication systems, evoking a response similar to that of a 'true' pheromone (Renou & Guerrero 2000; Raghu 2004; Shelly 2010).

Methyl eugenol (ME) and Cuelure (CL) are most common compounds among all other phytochemocals that has been shown to affect fly behaviour and physiology, CL does not occur directly in nature, it is hydrolysed product of raspberry ketone (RK), 4-(4-hydroxyphenyl)-2-butanone, a natural plant constituent found in orchids, raspberries and cranberries (White 2000; Benelli et al. 2014). Biologically, these compounds have several effects on mature flies. Cuelure and ME have functional role in production of male sexual signals (Shelly & Dewire 1994) resulting in increased attractiveness to females (Shelly & Dewire, 1994; Khoo & Tan 2000). Because of the enhancement of mating performance, phytochemicals can have huge impacts in fruit fly sterile insect technique (SIT) programs (Benelli et al. 2014; Vargas et al. 2010; Shelly et al. 2010). The main focus of my thesis advances on previous knowledge about the effect of phytochemicals in mature flies by investigating effects of RK in immature Qflies. In this chapter, I summarise these results, considering possible underlying mechanisms, and discuss the practical implications of my findings for SIT and future directions for research on fruit fly-phytochemical interactions in the light of chemical ecology and applied entomology.

8.2. Summary of results

In Chapter 2, I showed that feeding of RK-rich diet only for 48 hours significantly enhanced reproductive development of male Qflies (Chapter 2). Previous studies have shown that phytochemicals have enhanced mating performance in mature males of some *Bactrocera* species, such as oriental fruit fly (methyl eugenol) (Shelly et al. 2005; Shelly 2010; Tan & Nishida 2012; Shelly & Dewire 1994), medfly (TML) (Shelly 1994), melon fly (RK/CL) (Shelly 2000; Shelly & Villalobos 1995), and *Bactrocera carambolae* (ME) (Haq et al. 2014). All of these studies involved mature males and had a goal of improving SIT through improving ability of released sterile males to mate with wild female flies (Shelly, 1995; Shelly et al. 1996, 2004, 2010; Vargas et al. 2010; McInnis et al. 2011; Barclay et al. 2014). However, the approaches used in these studies are not useful in flies that are released at younger ages such as Qflies (1-3 days old) (Dominiak et al. 2003; Reynolds et al. 2014). Considering this issue, the finding that RK supplements fed to immature flies accelerated reproductive development of Qflies is an enormously important outcome of my PhD research.

Early maturation and the willingness to mate alone are not effective for SIT if the flies' reproductive development remains insufficiently advanced of this early mating age. As previously reported protein feeding not only enhance mating in fruit fly but their reproductive organs also developed accordingly in response to protein feeding (Vijaysegaran et al. 2002; Pérez-Staples et al. 2011), RK-feeding also might have role in development of reproductive organs parallel to mating acceleration which led me to investigate the effect of RK on reproductive organ development of Qfly. I demonstrated that early mating by males is associated with accelerated development of reproductive organs (Chapter 3). That means RK feeding not only accelerates the mating activity of young flies but also enhances their reproductive organ development. Raspberry ketone feeding could elevate the metabolic rate in sexually immature Qflies in the same manner as reported for mature adult males (Kumaran et al. 2014b) to consume higher levels of protein and carbohydrates (during RK treatment for 48 hours) than normal and to convert them into lipids (chapter 6). This increased lipid storage might assist in energy for the energetically expensive activities of reproduction (Arrese & Soulages, 2010;

Warburg & Yuval 1996, 1997; Reynolds & Gross 1990). After finding positive effect of RK on male development, I examined how mating interactions differ in pairs where the male was treated with RK. I tested the remating receptivity of females that mated with RK-fed males of different ages to check the effectiveness of early mating (Chapter 4). I found no effect of RK on the remating tendency of females. In contrast, reduction of remating receptivity was observed in Qflies provided CL as mature adults (Kumaran et al. 2013) and in medflies provided GRO (Morelli et al. 2013). Consistent with the present findings in Qflies, no effect of ME feeding of male on the re-mating tendency of oriental fruit fly females was observed (Shelly & Edu 2008).

As RK feeding showed accelerated mating I investigated whether this advantage has any relation with the pheromone, whether any changes in pheromone occur as a consequence of RK feeding (Chapter 5) as found in some *Bactrocera* species in response to phytochemicals (Tan & Nishida 1995, 1996, 2000; Tan, 2000; Hee and Tan 2004; Tan et al. 2011, 2014). Indeed ingested RK was found in rectal as well as released volatiles with other endogenous compound in RK-fed males although in released volatiles RK was detected very inconsistently. Also, RK showed strong effect on the volume of endogenous pheromone in rectal gland but did not show such effect in released pheromone. Thus, present findings do not support that RK presence or the volume of endogenous pheromone in released pheromone played role in precocious mating in *B. tryoni* (Akter et al 2017) as demonstrated previously for mature *B. tryoni* (Kumaran et al., 2013, 2014a). However, RK presence in pheromone of rectal gland may affect the speed or rate of sexual signalling such as calling and wing fanning (Whittier et al., 1994, Whittier and Kaneshiro, 1995, Shelly, 2000c; Nation 1972, 1990; Teal et al., 1999) to initiate early mating as evidenced in *B. dorsalis* in response to consumption of ME or methyl eugenol-containing flowers (Shelly and Dewire 1994; Shelly 2000b).

As there might be trade-offs associated with using energy to develop and copulate at early ages through increasing metabolic rate, such as reducing body weight (Kumaran et al. 2014b), I studied survival under nutritional stress and desiccation conditions (Chapter 6). Results showed that females are more

resistant to starvation and desiccation than males and that RK-feeding did increase susceptibility to desiccation but RK-feeding showed a positive effect on survival in starvation. Similarly exposure to ginger root oil (GRO) had a significant positive effect when combined with the sugar + protein diet in wild medfly males (Levy et al. 2005). In addition, analysis of lipid levels showed that just after finishing RK treatment, lipid levels were greater in RK-fed males than RK-deprived males (Chapter 6). However, after being exposed to starvation conditions, the lipid levels of RK-fed flies were lower than control flies and in both RK-fed and control flies, lipids decreased compared to the initial lipid levels. Consistent to this study, in medfly, Maor et. al. (2004) detected the level of lipids decreased significantly from d 0 to d 6 in both protein-fed and protein-deprived released sterile males. Considering accelerated sexual and reproductive development RK has potential as pre-release supplement in SIT, thus, RK-fed male's attraction behaviour to lure in the field cage was also studied (Chapter 7). I observed significant reduced attraction of RK-fed males to cuelure traps. Similar findings have been reported following phytochemical treatment in mature oriental, melon and medflies (Shelly 1994, 2000; Shelly et al. 2007b) and in immature melon flies (Chambers et al. 1972). As sexual maturation and attraction to lure is positively correlated, consuming RK and gaining sexual maturity at younger age might reduce the necessity of lure feeding and thus RK-fed males were less attracted to the cuelure trap.

8.3. Implications in SIT

The success of sterile males is dependent on a wide range of factors, including survival until sexual maturity, successfully finding, courting and competing with wild males for mating with wild females and cost effectiveness (Calkins et al. 1994; Hendrichs et al. 2002; Lance et al. 2000). In SIT programs, released males decline rapidly within about one week in the field (Hendrichs et al. 2007) and forwarding mating age along with accelerated reproductive organ development due to RK feeding is of particular importance to get maximum mating from released males and eventually to improve SIT. While phytochemicals have used with mature flies to improve SIT through increased mating success (Shelly et al. 2004, 2007a; Shelly & Nishida 2004; Shelly 2005), similar or even better

benefits could be achieved in Qfly SIT through RK feeding because of its effective early sexual and reproductive maturation. Not only the precocious matings by RK-supplemented males, but these matings were also effective as the female remating was unrelated to whether her mate was fed RK. This is also another key point of successful SIT because if females mated with RK-fed sterile males, and then remated with males from the wild population frequently then the main aim of SIT i.e. to suppress population growth and eventually to eliminate it from target area (Hendrichs et al. 2005; Enkerlin 2005) would be compromised. Parallel to these sexual and reproductive advantages, the findings that RK-feeding had no adverse effect on the longevity and survival at starvation are also important for evaluating whether the RK fed males can survive long enough to compete with wild males in the field in SIT-relevant settings.

Successful cost effective SIT require additional suppression tactics to reduce the over flooding ratios of sterile to wild males by reducing the population of wild males before the sterile flies are released (Knipling 1979). Male annihilation technique (MAT) is such an additional technique where cue lure has been used in combination with the insecticide malathion (Jones & Skepper 1965; Dominiak et al. 2009; Bateman 1982, 1988) to reduce the Qfly population from the target area before attempting for any control approach using “lure and kill” (El-Sayed et al. 2009; Dominiak & Ekman 2013). Thus eventually decrease the cost of control operations by facilitating the release of fewer sterile flies’ release while there are reduced numbers of wild flies to compete with in the field (Koyama et al. 1984; Steiner et al. 1970). Usually, MAT and SIT are used sequentially, but because RK-fed males less attracted to cue lure than untreated males, control or eradication may be improved by using SIT and MAT simultaneously (Chambers et al. 1972). Releasing RK-fed sterile flies in the presence of MAT also is cost effective as because the MAT boxes could be left in the field for at least for 1 year as both lure and toxicant could remain active for more than a year (Dominiak & Nicol 2012; Dominiak & Ekman 2013). Thus, simultaneous use of MAT and SIT will have synergistic effects to make the integrated control strategy more powerful (Barclay et al. 2014) as well as cost effective. However, it is also possible that wild males present in the field mate with wild females before being captured at lure sources. Furthermore, the present finding also suggests that if a natural RK source is

available in release area and if wild males consume sufficient RK then they will not be attracted to cuelure baited MAT box, reducing the effectiveness of MAT (Shelly 1994).

8.4. Potential future research avenues

The mechanisms behind the behavioural changes expressed in *Bactrocera* in response to phytochemicals is little studied area even in mature adults. Here my main focus was RK's effect on the reproductive biology of immature Qflies. However, it remains unclear to what extent enhanced mating of young males is through a female preference for attractive pheromones or through development and behavioural changes in males. This area needs future detailed studies. In addition, studies on competition between RK-fed and RK-unfed males for females are needed, as are studies of mating competition with wild type males. The present study showed accelerated mating in RK-fed males, however, the amount of RK required for this effect is unknown. Although different doses of RK were tested and showed similar effects on mating the amount of RK ingested by individual flies is unknown.

Raspberry ketone treatment of flies was by ingestion ('pharmacophagy') in the present study, hence there is scope to study the possibility of aromatherapy treatment of immature male Qfly to accelerate development. Because inducing profound effects on mating performance by aromatherapy are already evident in some tephritids. For instance, exposure to odours from ginger root oil of medfly, *Ceratitis capitata*, (Andrés et al 2009; Shelly et al. 2004) and guava odours of South American fruit fly, *Anastrepha fraterculus* (Bachmann et al. 2015) increases mating performance. Elevated mating also has been observed in sexually mature carambola fruit fly, *B. carambolae*, following aromatherapy with ME, with effects of aromatherapy being similar to when these flies were fed ME(Haq et al. 2014, 2015). Therefore, RK ingestion and RK aromatherapy could also have similar results in accelerating mating in immature Qfly.

In Qfly SIT programs both sexes are released, thus, it is important to study mating behaviour and longevity of RK-fed females. Although RK did not accelerate development of female reproductive organs, it is worth studying female mating behaviour and longevity to obtain a more complete picture of how RK-feeding affects young Qflies.

I have found similar endogenous compounds in pheromone blends of RK-fed and RK-unfed males. However, pheromones in RK-fed males differ in the presence of RK in pheromone blends in rectal glands as well as in volatiles collected during the mating period. Therefore, it may be that RK makes pheromones more attractive to females, however, the behavioural response of virgin females to RK-fed males and glands of RK-fed males also needs to be studied.

Another interesting area in *Bactrocera* to explore is the biological role of reduced attraction of lure-fed males to the corresponding lure. Although several studies have found that ingestion of phytochemicals reduces the subsequent attraction to lures, there is no compelling explanation for this change in behaviour (Chambers et al. 1972; Shelly 1994; Shelly et al. 2007b). It is possible that the receptors in antenna cannot perceive the odour as they already exposed to it or RK-fed males detect but are no longer interested in cuelure as they already have all they need or can tolerate.

8.5. Conclusion

My work demonstrated that phytochemicals not only have effects on mature fruit flies but can also affect life events of immature fruit flies in ways that can be highly valuable to SIT programs. Many other fruit flies respond to phytochemicals as adults and having found such strong and consistent results for immature Qflies, it would be very interesting to test the effects of phytochemicals on immature stages of other fruit flies.

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Appendices

Published Papers

Akter, H., Mendez, V., Morelli, R., Pérez, J., & Taylor, P. W. (2017). Raspberry ketone supplement promotes early sexual maturation in male Queensland fruit fly, Bactrocera tryoni (Diptera: Tephritidae). *Pest management science*, *73*(8), 1764-1770. https://doi.org/10.1002/ps.4538

PLOS ONE

RESEARCH ARTICLE

Suppression of cuelure attraction in male Queensland fruit flies provided raspberry ketone supplements as immature adults

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Abstract

Tephritid fruit flies are amongst the most damaging insect pests of horticulture globally. Some of the key fruit fly species are managed using the sterile insect technique (SIT), whereby millions of sterile males are released to suppress reproduction of pest populations. Male annihilation technique (MAT), whereby sex specific lures are used to attract and kill males, is often used to reduce wild male numbers before SIT programs commence, providing released sterile males an increased numerical advantage. Overall program efficacy might be improved if MAT could be deployed simultaneously with SIT, continuously depleting fertile males from pest populations and replacing them with sterile males. However, such 'male replacement' requires a means of suppressing attraction of released sterile males to lures used in MAT. Previous studies have found that exposure of some fruit flies to lure compounds as mature adults can suppress subsequent response to those lures, raising the possibility of pre-release treatments. However, this approach requires holding flies until after maturation for treatment and then release. The present study takes a novel approach of exposing immature adult male Queensland fruit flies (*Bactrocera tryoni*, or 'Qfly') to raspberry ketone (RK) mixed in food, forcing these flies to ingest RK at ages far younger than they would naturally. After feeding on RK-supplemented food for two days after emergence, male Qflies exhibited a reduction in attraction to cuelure traps that lasted more than 20 days. This approach to RK exposure is compatible with current practises, in which Qflies are released as immature adults, and also yields advantages of accelerated reproductive development and increased mating propensity at young ages.

Introduction

Tephritid fruit flies are significant pests of horticulture in most regions of the world, causing direct damage to crops and restricting trade. The sterile insect technique (SIT) is used for regional management of some of the most serious fruit fly pests [1,2,3]. In SIT, millions of flies are reared in factories, sterilised, and then released into the field. Sterile males mate with

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females of pest populations, curtailing their reproduction such that over generations the pest population is reduced [4,5].

For species that respond to strong male lures, such as methyl eugenol or cuelure [6,7,8], the male annihilation technique (MAT) is often used before the deployment of SIT [9,10]. In MAT, devices containing a male lure and an insecticide are distributed in the field to attract and kill males. MAT reduces the number of males in the pest population, skewing the sex ratio, and this increases the effective overflooding ratio when sterile males are released. A high overflooding ratio is very desirable in SIT releases, especially when released males are inferior to wild males in mating competitiveness or ecological performance [11,12].

Because released sterile males are also attracted to male lures, MAT and SIT have traditionally been used sequentially, with MAT initially reducing the abundance of wild males before commencement of SIT. This approach creates many logistical issues as the MAT devices are long lasting and are distributed through the treated region but must be retrieved prior the SIT releases. Large advantages in overall control levels and efficiency might be achieved if MAT and SIT could be used simultaneously through the release of male flies with suppressed response to the lures used in MAT [13]. In some species there is compelling evidence that pre-prelease treatment with male lures can have a lasting effect of reduced responsiveness to those lures and might provide a basis for development of simultaneous deployment of MAT and SIT. Chambers et al. [14] exposed mature males of Mediterranean fruit fly (*Ceratitis capitata*, or 'medfly') to trimedlure and mature males of Oriental fruit fly (*Bactrocera dorsalis*) to methyl eugenol and in both cases found that the lure exposure reduced responsiveness to traps containing these lures. Shelly et al. [15] exposed medfly males to ginger root oil, which contains α-copaene, and found reduced attraction to trimedlure traps. Although Shelly [16] found no evidence that feeding on natural sources of methyl eugenol by mature male oriental fruit flies reduced subsequent trap capture, an earlier study [17] found that pre-release exposure of oriental fruit flies to synthesised methyl eugenol resulted in significantly reduced responsiveness to traps containing this lure over the following five days. Similarly, Shelly and Villalobos [18] found that mature male melon flies (*Zeugodacus cucurbitae*) provided a wick containing cuelure, a synthetic and more volatile analogue of naturally occurring raspberry ketone (RK) [19,20], for two hours one day prior to release showed reduced responsiveness to cuelure traps during the two days following release. In all of these previous studies flies have been exposed to lures as mature adults, but this approach is not compatible with SIT programs in which flies are released shortly after emerging as immature adults.

In the present study, we consider the potential of pre-release RK supplements in food as a means of reducing response of male Queensland fruit fly *Bactrocera tryoni* (Qfly) to cuelure, the attractant used in MAT for this species, as a step toward enabling simultaneous deployment of MAT and SIT for this species. We here adopt a recently developed approach for exposing male flies to RK [21]. Rather than waiting for flies to respond naturally to RK once mature, this approach entails mixing doses of RK in the food provided to immature flies, enabling exposure to RK over a time frame that is compatible with pre-release holding periods of SIT programs that release these flies as immature adults [22,23].

Qfly is Australia's most damaging fruit fly pest, and in some non-endemic regions outbreaks have been managed by the sequential combination of MAT followed by SIT [22,24,25]. With increased restrictions on the use of insecticides, SIT and MAT are set to become more routine management tools [26], and significant research programs are currently under way to maximise the efficacy of these tools.

In SIT operations Qfly is usually released two to three days following emergence [22,23], and this holding period provides an opportunity for the implementation of pre-release treatments that enhance male field performance. Previous studies have highlighted the benefits of

yeast hydrolysate (in addition to sugar) in the pre-release diet, as this treatment promotes reproductive development and mating performance [27,28,29,30] and results in increased numbers of sexually mature (i.e., cuelure responsive) males in the field [23]. In addition to benefits of yeast hydrolysate feeding, treatment of pupae or recently emerged adults with methoprene, a juvenile hormone analogue, has been found to accelerate development [31].

In addition to yeast hydrolysate and methoprene treatments of immature Qflies, phytochemicals, such as zingerone, and their synthetic analogues, such as cuelure, have also shown promise, but until recently these had only been considered in terms of mating performance of males that feed on these compounds as mature adults [32,33,34,35]. Immature males show little or no attraction to RK analogues, and previous studies have consequently focused on the responses of mature males. The need to wait for flies to mature before providing supplements of phytochemicals or their analogues constrains the operational viability of such supplements, and in the case of Qfly this would require holding the flies for more than a week before release [36,37]. However, RK supplements mixed in the diet of immature Qfly for just two days following emergence, approximating the usual pre-release holding period, have been found to substantially increase the mating propensity of young males [21].

In the present study we consider whether the RK treatment of immature males [21] reduces subsequent responses to cuelure, the standard lure used in MAT for this species. If the promising effects reported previously for mature male medfly, oriental fruit fly and melon fly [14,17] are also evident when RK is provided to immature Qflies, this would be an important step toward enabling simultaneous deployment of MAT and SIT without the need to significantly modify the pre-release holding period.

Materials and methods

Source and maintenance of flies

Qfly pupae were supplied by the New South Wales Department of Primary Industries Fruit Fly Production Facility at the Elizabeth Macarthur Agricultural Institute at Camden, New South Wales, Australia (for production details, see [38]). Pupae were kept under controlled temperature (25 ± 1˚C) and relative humidity (65 ± 5%) on a 13:11 h light: dark cycle in which the first and last 30 min of the light phase were simulated dawn and dusk in which light level gradually ramped up to full output and down to darkness, respectively.

Small field cage experiment

Approximately 2000 pupae (estimated by weight) were placed in 47.5×47.5×47.5 cm fine mesh cages (Bugdorm 44545, Megaview, Taichung, Taiwan) for adult emergence with only water provided for sustenance. Because only a few flies usually emerge on the first day, the first day of emergence was discarded, and only adults that emerged over the second day were used in the experiment.

After fly emergence, the cages were provided with water and diet (1:3 yeast hydrolysate: sugar) containing high dose (5% RK) or low dose (1.25% RK) of raspberry ketone (4-(4-Hydroxyphenyl)-2-butanone, ≥98%, Sigma–Aldrich®, St. Louis, MO, USA) or no RK (control) for 48 hours. RK was ground using a blender before being mixed in the diet. To differentiate among treatments, both the diet and water were mixed with food colour (blue, red or yellow, Queen Fine Foods, Australia) that was clearly visible in the gut of the flies for 2–3 days. Three millilitres of liquid dye was added to 30 g of diet so that the powdered diet became a paste that was spread over a filter paper and dried for 48 hours before being provided to the flies. Two millilitres of dye was added to 50 mL of water in a 70 mL plastic container. A sponge extending through the lid of the container carried the dyed water to the flies in each cage. The

dye colour was rotated among treatments between replicates. Cages containing food and water were placed in sheltered outdoor conditions with ca. 5 m between cages of each treatment group where the flies were kept until being used in the small field cage experiments.

After 48 hours of feeding, the diet was removed from the cages. Males were removed from mixed-sex cages when 3 days old, to ensure they were virgin when used in the experiments [36,37], and placed in clean 47.5×47.55×47.5 cm fine mesh cages (Megaview Bugdorm 44545) at 700–1,000 male flies per cage. These cages were supplied with sugar and water containing the same dye that each cage had received previously.

Four small field cages (3 m diameter, 2.2 m high) were used for these experiments. An artificial tree (Ikea Fejka, ca. 0.5 m diameter and ca.1.5 m high) was placed in the middle of each field cage. Male Qflies were tested for attraction to cuelure at 5, 7, 9, 11, 13, 15, 20 and 25 days after emergence. Fifty male Qflies from each treatment group were released into each field cage 30 minutes before sunrise (i.e., 150 flies per cage on each test day). Thirty minutes later, one Lynfield trap baited with 200 μL of cuelure on a cotton wick was suspended from the ceiling of each cage. Instead of using insecticide, each trap contained a white plastic sheet that was covered with brush-on Tangle-Trap® sticky coating (Tanglefoot Acquisitions, Grand Rapids, MI, USA) to capture the attracted flies. Traps were collected after sunset. Captured flies were sorted by treatment and counted. This experiment was repeated 10 times, using flies from three production batches obtained at least four weeks apart.

Large field cage experiment

In this experiment, flies were released in four large field cages (8 m wide and 24 m long metal frame covered with white mesh, 5 m at the highest point) that contained 6 lemon trees each. To simulate an SIT release under those contained conditions, rather than releasing and testing flies at specific ages as in the small field cage experiment, in this experiment flies were released immediately after the 48-hour treatment period was complete and were then sampled over twenty days. To sustain the population of the flies during the experiment, four 1 L plastic containers with 15% sugar solution were suspended from the ceiling of each cage.

Rather than using food dyes, which do not persist for more than a few days, in this experiment the pupae were coloured with fluorescent dyes (Strong Magenta 21, Lunar Yellow 27, Stellar Green 8) (Swada, Stalybridge, UK) at a rate of 2 g of dye per litre of pupae to identify the different treatment groups. The dye was rotated among treatments between replicates. As in the previous experiment, after emergence, the cages were provided with water and diet (1:3 yeast hydrolysate:sugar) containing a high dose (5%) or low dose (1.25%) of RK or no RK (control). During the simulated pre-release holding period, the RK treated flies were kept in a different room from the control flies to avoid exposure to RK odour from the treated food, from treated flies, or from faeces and other residues from the flies [32]. After 48 hours of feeding the treated food was removed and the flies were released in large field cages. For this experiment, both males and females were released in the cages.

The experiment was repeated eight times, with four large field cages each being used twice. Four Lynfield traps were suspended in each large field cage, two containing cuelure as an attractant ('cuelure traps') plus malathion as killing agent and two containing malathion only without attractant ('control traps'). To remove effects of incidental captures unrelated to cuelure attraction, the number of flies captured in the control traps was subtracted from the number captured in the cuelure traps within each field cage on each day of trap clearance.

Statistical analysis

The number of flies from each treatment captured in traps was analysed by a mixed model ANOVA. RK treatment was treated as nominal, and Age was treated as ordinal. Batch number and Cage number were included as random effects. Interactions between RK Treatment and Age were considered, and significant interactions were explored using contrasts within the full model, using t-tests. Non-significant interaction terms were excluded from final models. All statistical tests were carried out using JMP version 10.0.0 (SAS Institute, Cary, NC, USA).

Results

Small field cage experiment

The effect of RK supplementation on number of flies captured in cuelure traps (square root transformed) varied across the days of testing (RK Treatment $F_{2,241.8} = 2.510$, $P = 0.083$, Age $F_{7,242.2} = 13.874$, $P<0.001$, RK Treatment x Age $F_{14,241.8} = 5.544$, $P<0.001$) (Fig 1A). Contrasts across treatments within each tested age found no evidence of differences in number of RK-supplemented and control flies captured at 5, 7, 9 and 25 days of age, the youngest and oldest ages tested, but at 11, 13, 15 and 20 days of age, the period of highest response as flies mature, significantly fewer RK-supplemented flies were captured compared with the controls. At each of these ages at which significant treatment effects were detected the contrasts between RK-supplemented treatment groups and controls were significant for both RK doses, and there were no significant differences between the two doses. At the peak of differences in daily captures when flies were twenty days of age, approximately five and a half times as many control flies were captured compared with those that received RK (Fig 1B).

Large field cage experiment

For males there was significant variation across the tested days in the corrected number of floes captured in cuelure traps (N cuelure traps—N control traps) ($F_{9,224} = 12.409$, $P<0.001$)

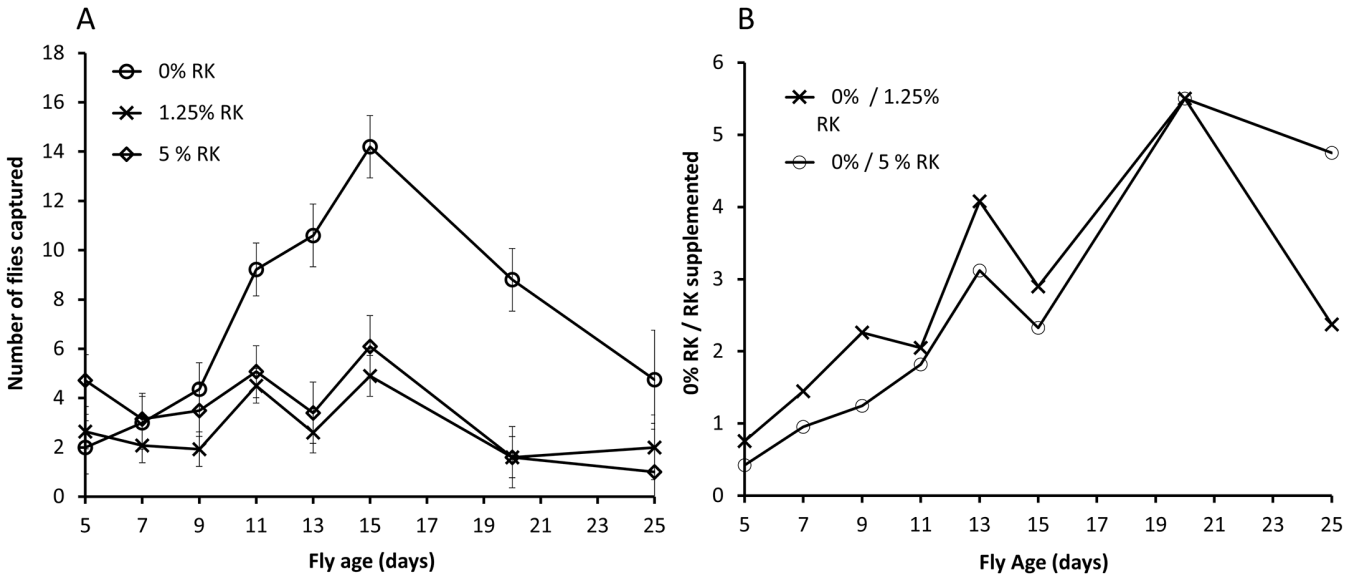

Fig 1. Mean number (± S.E.) of male Qflies captured in cuelure traps in small field cage trials (A) and relative number of control vs. RK treated male Qflies captured in cuelure traps in small field cage trials (B).

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and significant variation amongst RK treatment groups ($F_{2,224}$ = 7.273, P<0.001), with significantly more control flies being captured than either of the RK treated groups and no difference between the RK treatments (Fig 2A). There was no evidence of variation across the tested days in the effect of RK treatment (Age*Treatment interaction $F_{18,206}$ = 0.489, P = 0.961). At the peak of differences in daily captures when flies were eight days of age, approximately fourfold more control flies were captured compared with those that received the high RK dose and eight-fold more control flies were captured compared with those that received the low RK dose (Fig 2B).

Studies of lure attraction that follow a cohort in the field or in simulated field conditions in large outdoor cages are vulnerable to the possibility that rather than reflecting differences in attraction to a lure, the differences in number of flies from each treatment group captured reflect differences in survivorship To eliminate the possibility that our results reflect differential survivorship of RK-supplemented and control flies, we analysed the number of flies from each group captured in the control traps through the experiment as this value reflects abundance of flies in each cage independent of lure attraction. As expected, the number of flies captured in control traps declined through the experiment as abundance is reduced for all groups ($F_{9,224}$ = 29.828, P<0.001) but there was no significant variation amongst treatment groups in the number of flies captured in control traps ($F_{2,224}$ = 0.358 P = 0.700). There was also no evidence of differences amongst treatment groups in the rate at which number of flies captured in control traps declined through the experiment (Age*Treatment interaction $F_{18,206}$ = 0.514, P = 0.950).

For females there was significant variation across the tested days in the number captured in cuelure traps as numbers declined through the experiment ($F_{9,224}$ = 2.815, P = 0.004) but there was no significant variation amongst RK treatment groups ($F_{2,224}$ = 0.861, P = 0.424). Considering only those flies captured in the control traps as a measure of survivorship of flies from the different treatment groups, there was a significant reduction in number of flies captured each day as the experiment progressed ($F_{9,224}$ = 28.461, P<0.001) but there was no significant

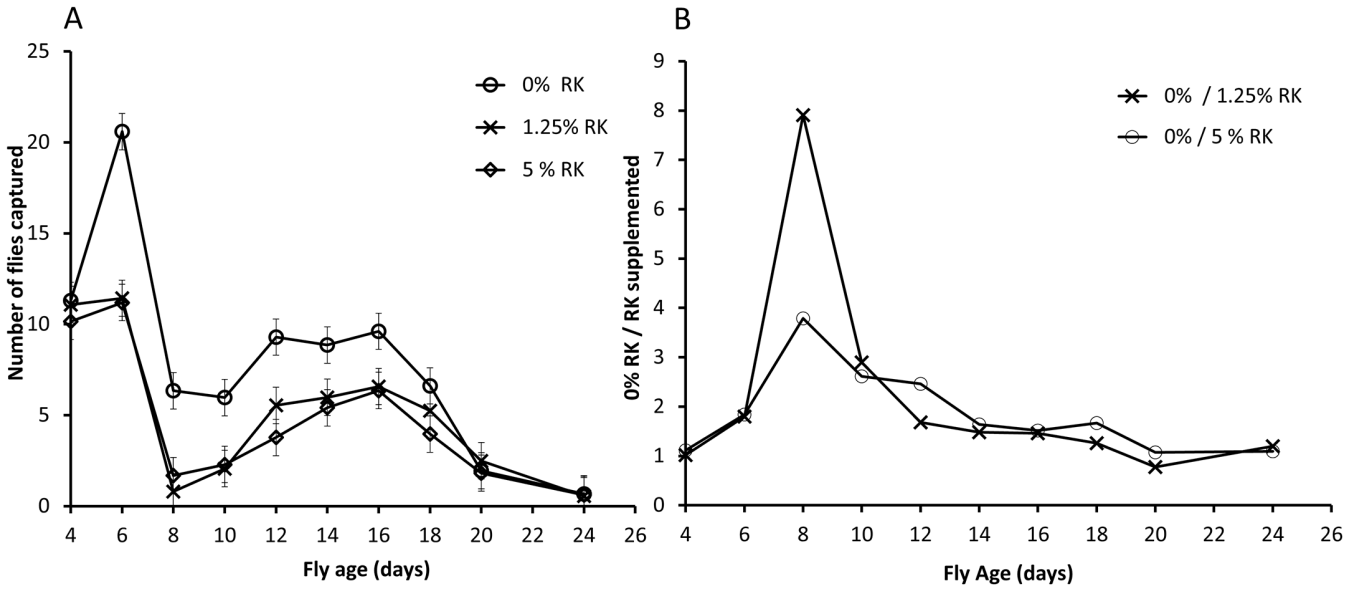

Fig 2. Mean number (± S.E.) of male Qflies captured in cuelure traps minus the number captured in control traps in large field cage trials (A) and relative number of control vs. RK treated male Qflies captured in cuelure traps in large field cage trials (B).

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variation amongst RK treatment groups in the number of flies captured ($F_{2,224} = 0.369$, $P = 0.692$) and no evidence of differences amongst treatment groups in the rate at which number of flies captured in control traps declined through the experiment (Age*Treatment interaction $F_{18,206} = 0.759$, $P = 0.746$).

Discussion

In fruit fly SIT programs, MAT is commonly used to reduce wild male numbers before the release of sterile flies [10,39,40]. However significant improvements in overall program efficacy could theoretically be achieved if it was possible to deploy MAT and SIT simultaneously, with MAT continuously depleting wild males from the field while SIT continuously replaces them with sterile males [13]. Simultaneous deployment of MAT and SIT would enable the immediate use of SIT to combat outbreaks rather than waiting until completing an MAT program before commencing the release of sterile flies and would also negate the need to retrieve MAT devices from the field before commencing SIT. Male Qflies released in SIT programs are attracted to cuelure [23], the lure used for MAT against this species [41]. For simultaneous deployment of MAT and SIT to be viable, there first needs to be an effective means of suppressing the attraction of released flies to cuelure-based MAT devices that can be implemented within operational holding and release practises. The present study identifies a potential means of achieving this.

Incorporation of RK in the diet of immature male Qfly for two days following emergence induced a persistent reduction in the number of flies captured in cuelure traps. Previous studies of oriental fruit fly, medfly and melon fly have also reported persistent reduction in responses to lures used in monitoring traps and MAT following previous exposure [14,15,17,18]. However, each of these studies entailed exposure of mature adult flies. This approach would require holding Qflies for a week or longer after emerging [37,42], which would be a significant departure from the current practises under which flies are typically released two to three days following emergence [23]. High mortality and sub-lethal stress from crowded conditions and the costs of maintaining the flies for such long periods in the rearing centres would present serious challenges to pre-release RK treatment of mature adult Qflies [25]. The present study demonstrates that effects paralleling those of previous studies of mature adults can be achieved by including RK in the diet of recently emerged immature adult male Qflies.

RK and closely related compounds have diverse effects on the behaviour and physiology when ingested by mature male Qflies. For example, ingested cuelure is incorporated in pheromone as RK [32,34], and male Qflies that ingest these compounds gain significant advantages in mating performance over the following two days. Akter et al. [21] was the first to find that RK can have significant effects even when incorporated into the diet of immature male Qflies. Male Qflies that ingest RK as immature adults exhibit greatly elevated mating propensity up to 10 days of age [21], and this tendency corresponds with significant acceleration in the development of reproductive organs over this period [43]. Usually, attraction to cuelure is associated with mature Qflies [30] and on this basis it might be expected that the accelerated development of RK-treated males would result in increased capture rates in cuelure traps at young ages. In contrast, despite being mature at younger ages, RK-fed flies exhibited significant suppression of capture rates. Amongst the youngest ages of flies tested in small field cages, the difference between capture rates of RK treated and untreated flies was not significant, with differences only becoming evident as flies matured. Rather than indicating failure of RK treatment, this reflects the low responses of flies from the control group at these ages due to them being immature. As the control flies matured their attraction to cuelure traps increased sharply, but this

tendency was suppressed in the RK treated flies. There were some differences between the small cage experiment and the large cage experiment in the timing of peak response to lures, with maximum response being at around 15 days of age in the small cage experiment and six days of age in the large cage experiment. This likely reflects differences between these experimental settings in environmental conditions and survivorship. In the small cage experiment, the flies were held in a sheltered location away from direct sunlight, whereas in the large cage experiment the flies were exposed to direct sunlight, and temperature was also elevated by a greenhouse effect in the cages. Temperature-dependent differences in development rate of the flies in these two experiments are most likely a major factor driving differences in peak lure response. In the large cage experiment it is possible that high mortality rates were also an important determinant of the identified age of peak lure response, as a peak in lure response at older ages would be increasingly difficult to detect as abundance declines. It is possible that that maximum individual responsiveness to lures in the large cage experiment occurred at an age beyond the age at which the maximum number of flies was captured but that this was not evident because of a reduced number of flies available to respond. That is, the number of flies captured in the large cage experiment reflects both levels of individual responsiveness and abundance. In the small cage experiment any effect of abundance effect was removed because a standardised number of flies were tested in each trial, thereby focusing more closely on individual responsiveness.

Pre-release RK treatment shows promise as a means of suppressing attraction to cuelure traps, thereby potentially enabling the simultaneous deployment of MAT and SIT. This is a relatively simple approach, and while the effects found so far are promising they could likely be improved by further refinement of the RK-exposure protocol. In addition to pre-release RK supplements, there are alternative approaches that might be considered to suppress response of released Qflies to cuelure-based MAT devices. Selective breeding of non-responsive flies is one option, and there is some support for this possibility already in oriental fruit fly studies. Shelly [44] selected oriental fruit fly males for non-response to methyl eugenol and was able to create two lines that maintained low response to this lure over many generations. Similarly, Ito and Iwahashi [45] succeeded in selecting for lure non-responsiveness in oriental fruit fly after just two generations. Both studies concluded that while lure non-responsiveness is a quite rare trait it is amenable to selection in oriental fruit fly colonies. In the studies of Shelly [44] and Ito and Iwahashi [45] the evolution of lure non-responsiveness was considered as a risk to MAT programs. On the other hand, establishment of mass-reared populations with suppressed response to lures used in MAT would be extremely beneficial for the prospective simultaneous deployment of MAT and SIT. To date there has been no investigation of within or between population variation in male Qfly response to cuelure, and such studies are now warranted to explore this possibility. Another alternative approach might involve treatment with RNA interference (RNAi) incorporated either in larval or adult diet as a pre-release supplement. This method could be used to deactivate genes regulating the production of odorant-binding proteins responsible for the detection of cuelure and related compounds [46]. While these alternative approaches might be more effective approaches for supressing cuelure response, they are not as easily implemented and do not include the additional benefits of accelerated sexual development that has been found when feeding RK to immature males. Even if an alternative and more effective approach to suppressing cuelure response of released flies was found, there might still be a role for pre-release RK supplements to yield addition benefits in terms of accelerated development of increased mating performance [21,43].

While there would be significant advantages to SIT efficacy if MAT could be deployed simultaneously, there may also be disadvantages that should be considered in an overall program setting. Usually the abundance of mature released flies in the field is monitored using

cuelure traps, and these data can be used as an indirect measure of sterile fly field performance [47]. Suppression of responses to cuelure traps would impede monitoring of released sterile flies. To counter this limitation, it might be possible to develop a calibration for the abundance of RK treated flies that would estimate an equivalency in terms of current practises. Also, under some conditions alternative monitoring systems, such as yellow sticky traps [48], could be used, although this would result in significantly increased labour costs. Under many conditions, however, the constraint on ability to monitor released flies may be a minor consideration next to the substantial potential increases in overall program efficacy [13].

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Writing – original draft: Phillip W. Taylor.

Writing – review & editing: Humayra Akter, Saleh Adnan, Renata Morelli, Polychronis Rempoulakis, Phillip W. Taylor.

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