Foraging Response of Female *Bactrocera dorsalis* (Hendel) to a Fruit Fly Protein Food Attractant

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Abstract

Oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) causes serious economic loss for papaya (*Carica papaya* L.) production in Hawai‘i through direct fruit damage and restriction of export commodities. Suppression of female flies was a challenge until a protein-based bait contains reduced risk insecticide spinosad became available, GF-120 NF Fruit Fly Bait (GF-120; Dow AgroScience, Indianapolis, IN). This bait provides an environmentally sound alternative to conventional cover sprays of organophosphate insecticides. Factors that influence the attractiveness of protein bait include chemical composition, visual stimuli, and competing volatiles from host fruits. This dissertation focuses on biological factors that affect the foraging response of female *B. dorsalis* to volatiles emitted by protein bait. Female reproductive state and dietary experience are two biological variables that shape the manner in which a fly searches for and responds to essential resources such as food and egg-laying sites. The experiments reported in this dissertation were conducted in order to assess the influence of physiological states on the response of female *B. dorsalis* to protein bait in papaya orchards.

In Chapter 1, baseline information on key morphological characters in *B. dorsalis* ovarian development and the associated morphometric parameter of each oogenesis stage is collected. Four oogenesis stages include previtellogenesis, vitellogenesis, gravid and parous. In Chapter 2, field observations were conducted to determine reproductive states of *B. dorsalis* females (using ovarian developmental stage as an indicator) that respond to

two protein bait trapping devices: visually enhanced attract-and-kill bait stations termed papaya leaf mimics (PLMs) treated with GF-120 and dome traps containing torula yeast solution. Females with ovaries at previtellogenesis stage and egg laying females are the two main classes that responded to protein bait. Visual stimuli from the bait stations enhanced the response of immature females to protein bait but this effect was not found in egg-laying females. Yellow color also increased the capture of females with greater egg loads compared to those captured by green bait stations. This is an important finding for improved fruit fly management because reducing numbers of egg-laying females within an area results in lower fruit infestation.

The physiological state of foraging flies determines the level of food searching behavior. In Chapter 3, the effects of female age and dietary history on the propensity of *B. dorsalis* to alight on protein bait were quantified. One week old females exposed to papaya as a diet source for 4 d in the first week of adult life showed a significantly greater propensity of alighting on GF-120 protein bait than females fed on a protein or sugar diet. Delay of ovarian development from feeding on a sugar only diet resulted in significantly higher response of 4 week old females to protein bait than females fed on papaya or protein. On the contrary, ovarian development in papaya fed females was not significantly different than that of protein fed females. Feeding on papaya during weeks 2 to 4 of adult life increased the response of female flies to protein bait at a lesser level than for 1 week old females. These results are evidence of the possible physiological profile of females attracted to protein bait in the natural environments. In addition, previous

exposure to papaya fruit enhanced the response of females to papaya compared to females without the experience. This is a finding that suggests sanitation practice of removing culled fruit not only removes a breeding source but may also reduce the number of females re-entering orchards.

Studies conducted in this dissertation are the first documentation of the response of egg-laying female *B. dorsalis* to protein bait under natural and semi-natural conditions. Results suggest that protein baits such as GF-120 attract females with developing ovaries as well as egg-laying females. In addition, fruit-based diet enhances the response of female *B. dorsalis* to protein bait and host fruit stimuli.

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Aloha

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Introduction

The impacts of oriental fruit fly (*Bactrocera dorsalis* Hendel) to Hawaiian agriculture include direct economic loss from infested fruit and early fruit abscission of over 100 species cultivated fruits. The consequent quarantine restriction obstructs the expansion and development of the export market for various Hawai‘i fruit crops (Vargas et al. 1990; Jang 1996). Among fruit fly susceptible commodities, papaya (*Carica papaya* Linn) produces fruit year round and hence, is the most vulnerable to *B. dorsalis*. Papaya becomes attractive to fruit fly females at color break stage and the susceptibility increases during fruit ripening process. Fruit fly management for papaya production in Hawai‘i is based on a multi-tactics integrated pest management program developed through the Hawai‘i Area Wide Fruit Fly Pest Management Program (HAW-FLYPM). The farm based integrated pest management (IPM) program includes field sanitation, male annihilation and the use of an environmental friendly protein bait spray (Mau et al. 2007). The goals for a successful fruit fly management include reducing local breeding population and prevent egg-laying females from reaching host fruit. Effective reduction of fruit infestation can be achieved by the combination of field sanitation and foliar protein bait spray (Piñero et al. 2009a). This “attract and kill” approach manipulates the behaviors of flies with the combination of removing stimuli (field sanitation) and providing stimuli (male lure and protein bait) in order to protect the fruit source (Foster and Harris 1997). However, the physiological profile of the *B. dorsalis* female which responds to protein bait treatment is largely unknown in the orchard agro-ecosystem. In this review, recent advances in IPM technology, fruit fly biology and behavioral ecology

are three topics of focus. Understanding the factors involved in a female's decision on alighting on a protein bait source such as the physiological states of the female, cues from visual stimuli, olfactory stimuli, and the interaction between individual factors are essential to improve fruit fly IPM.

Oriental fruit fly management in Hawai'i

Soon after its first detection in Honolulu by O. C. McBride in 1945, *B. dorsalis* became a dominant pest, which devastated local fruit production and limited tropical fruit export markets (Fullaway 1947; Bess and Haramoto 1961). The rapid establishment of *B. dorsalis* called for the largest biological control efforts at the time to introduce over 30 species of opiine parasitoids (Hymenptera: Braconidae) (Clausen et al. 1965). Successful establishment of biological control agents made it possible to manage fruit flies in agricultural areas using chemical treatments via cover spray or spot treatments of protein bait. Research and control efforts during the last half century led to the development of additional tools, including male annihilation using parapheromone attractants, sterile insect release and bait spray with toxic protein food attractants (Mau et al. 2007).

Field sanitation. Removing infested, fallen, damaged, and over-ripe fruit are the foundation for reducing the fruit fly population. Local fruit fly breeding can be greatly reduced by field sanitation on individual farms by burying or drowning culled fruits, feeding animals infested fruits, or using augmentoria developed by the HAW-FLYPM (Klungness et al. 2005; Vargas et al. 2008a; Piñero et al. 2009a). Field sanitation is

important to eliminate the breeding population and reducing emission of volatiles from ripe fruits that attract gravid females into the orchards. Liquido (1993) demonstrated that sanitation alone reduces fruit fly infestation from 20-60 % to below 20% in half to fully ripe papaya. Bagging is another sanitation practice that some papaya growers in the Puna area on the Island of Hawai‘i adopted by keeping the discarded fruit in sealed plastic bags under the sun.

Male annihilation. In addition to field sanitation, IPM suppression is effective in reducing fruit fly infestation in papaya with weekly bait and male annihilation treatments (Vargas et al. 2008a; Vargas et al. 2008b). The development of long-lasting polymer matrix cones, panels and SPLAT (specialized pheromone and lure application technology) as lure carriers has greatly improved the efficiency of male annihilation in recent years (Vargas et al. 2009; Vargas et al. 2010). Methyl eugenol lures are combined or embedded with an insecticide such as dichlorvos, spinosad, or fipronil. High density trapping reduces not only male populations but also subsequently reduces female fecundity and fruit damage. Methyl eugenol mass trapping requires minimal labor and maintenance (3-4 applications per year). In addition, the attraction of fruit flies to lures from outside the application site makes it a favorable tool for area-wide fruit fly suppression.

Protein bait. Synovigenic insects such as *B. dorsalis* require constant energy input for egg development and are highly responsive to proteinaceous volatiles during the

initial ovarian developmental stage (Miller et al. 2004; Barry et al. 2006; Piñero et al. 2011). Weekly protein bait application on roosting hosts has been adopted by cucurbit growers in Hawai‘i and Taiwan as a standard practice for fruit fly management (Mau et al. 2007; Huang personnel communication). Protein bait treatment against *B. dorsalis* in papaya orchards was achieved through the development of a rain-fast bait station; papaya leaf mimics (PLMs) (Piñero et al. 2009b). Applying protein bait on the visually enhanced bait station significantly reduced the overall female population in the orchards. However, the effect of this tactic on reducing the density of egg-laying females is still unclear.

Papaya fruit biology associated with fruit fly susceptibility.

Continuous fruit production of papaya provides an excellent environment to study *B. dorsalis* behaviors. Fruit harvest in commercial papaya orchards begins at 9-12 months after planting for up to 3 years in Hawai‘i. Weekly harvest of color break fruit is the standard practice to minimize the risk of fruit fly infestation, postharvest diseases and fruit damage during transportation (Tan and Seriti 1994). Most papaya in Hawai‘i is grown on approximately 1,200 hectares of lava rock land in Puna area at the southeastern corner of the Island of Hawai‘i. In addition to fruit flies, several arthropods and diseases require pesticide applications in order to produce blemish-free fruits for fresh consumption and prevent quarantine issues (Nishina et al. 2000). As pest management is moving away from a reliance on broad-spectrum insecticides as a single pest management strategy, a clear understanding of the pest biology and behavior associated with fruit biology is the foundation to develop sustainable IPM strategies. The

horticulture background of papaya and fruit physiology that associated with fruit fly host finding, are the focuses in this review section.

The ripening process of mature papaya fruit takes approximately 1 week from color break to fully ripe (Table A.1). Ripe fruit develops a light or deep yellow-orange skin color and aromatic volatiles that are highly attractive to tephritid females include linalool, ethyl acetate and carbon dioxide (Paull and Chen 1983; Quintana and Paull 1993; Stange 1999; Kendra et al. 2005). Egg-laying female *B. dorsalis* are most responsive to fruits that are at least ¼ ripe (Liquido and Cunningham 1990; Jang and Light 1991). Fruit fly infestation increases according to the ripeness stage. For example, field surveys found the infestation rate in 'Sunrise' variety increases from 1.5 % at half ripe to 45% at fully ripe (Liquido 1991). Development of slow ripening varieties may have the potential to increase fruit fly tolerance with less physical change in fruit color and texture during the ripening process (Chen and Paull 2003; Yeh et al. 2007; Sivakumar and Wall 2013).

From a quarantine point of view, *B. dorsalis* is the most important pest in papaya production. Mediterranean fruit fly (*Ceratitis capitata* (Wiedemann)) was the primary fruit fly species found in papaya prior to the introduction of *B. dorsalis* (Liquido et al. 1990). In addition to *B. dorsalis*, melon fly (*Bactrocera cucurbitae* (Coquillett)) is also common in papaya orchards. Surface pests such as papaya mealybug (*Paracoccus marginatus* Williams and Granara de Willink) and white peach scale (*Pseudaulacaspis pentagona* (Targioni)) are the top priorities for growers' pest management efforts

(Nishina et al. 2000). In addition, *Thrips parvispinus* Karny is the most recent established papaya pest in the Puna district. Thrips feeding causes foliar and fruit injury as well as flower drop and scarred fruits. Stevens leafhopper (*Empoasca stevensi* (Young)) is a phloem-feeder that causes hopper burn, which can result in severe stunting of young plants (Ebesu 2004). A number of predatory coccinellidid and parasitoids contribute the suppression of these pests. Three species of pest mites are common on papaya in Hawai‘i: the broad mite (*Polyphagotarsonemus latus*), flat mite (*Brevipalpus phoenicis*), and a key pest, spider mite (*Tetranychus cinnabarinus*). In addition, leaf edge roller mite (*Calacarus flagelliseta*) is the latest introduced pest mite established in Hawai‘i in 1990 (Fournier et al. 2004). An IPM program incorporating selective insecticides to target specific pests (Table A.2) is essential for sustainable fruit production to reduce the use of broad-spectrum insecticides (Hardy 2012).

Papaya orchards in Puna are ranged from 4-8 hectares with a density of 1,500-2,000 plants per ha in low elevation (< 152 m) areas to ensure production of desirable hermaphrodite fruits (Nakasone 1976; Nishina et al. 2000). High density planting reduces fruit production, in addition; fruit flies tend to spend more time resting or foraging in shaded areas (Nishida 1980; Stark 1995). Thus, adjusting planting distance to provide open space may assist fruit fly management by reducing resting area in the environment. This approach successfully reduced fruit damage by papaya fruit fly (*Toxotrypana curvicauda* Gerstaecker) through manipulating plant spacing for applying peripheral row trapping (Aluja and Rull 2009).

For high quality fruit, the shallow rooted plants require protection against wind damage. Strawberry guava (*Psidium cattleianum* Sabin) is the most common windbreaks grown naturally in Puna area which unfortunately, is also a major population reservoir for *B. dorsalis* (Vargas et al. 1990). In addition, the expansion of this invasive shrub throughout the lowland rain forest on the Island of Hawai‘i (Uowolo and Denslow 2008) is yet another hurdle for fruit fly management with increasing breeding ground in natural environment. Mass trapping and bait treatment on orchard borders provides the first line of defense against *B. dorsalis* migrating from the dense strawberry guava stands (Piñero et al. 2009a).

Hawai‘i-grown papaya fruits are subject to postharvest treatment to disinfest fruit fly eggs and larvae due to quarantine restrictions. Two-stage, hot water treatment was the standard treatment, but other phytosanitary treatments were developed to reduce fruit damage and survival *B. dorsalis* larvae in blossom-end of some fruits (Armstrong et al. 1989; Zee et al. 1989). High temperature forced air, vapor heat, cold temperature storage and irradiation technologies are the present phytosanitary treatments used by the Hawai‘i papaya export industry (Seo et al. 1982; Lee 1986; Jang 1996). Fruits treated by the hot air treatment protocol are sometimes associated with uneven ripening (i.e. hardened lumps of flesh in ripe fruit) due to selection of immature fruit prior to mature green stage (Nishijima et al. 1992; Sangwanangkul and Paull 2005). The establishment of the generic irradiation dose (150 Gy) as a phytosanitary treatment allows papaya fruit to be harvested

at greater than 1/4 ripeness stage provides desirable qualities for shipping to U.S. mainland and international markets (Follett and Armstrong 2004). One of the limitations of marketing papaya at ¼ ripe or a greater is the potential occurrence of fruit fly eggs or larvae (Chen et al. 2007; Liquido 1991). Nonetheless, the advances made in area-wide fruit fly management during the past decade suggests that increased understanding of behavior by egg-laying females might lead to more effective field management of fruit fly populations. Specifically, detailed biological information regarding egg laying females and elements involved in their food foraging behaviors are key knowledge for improving fruit fly management.

Fruit fly reproductive biology

The reproductive state of a female fly plays a significant role in modulating her behaviors such as searching for food, mate, egg laying sites and the visual stimuli that she is attracted to, as reviewed in the previous section. Most of the frugivorous tephritids that have been studied, including *B. dorsalis,* are anautogenous insects, which need to consume protein as adults for ovary redevelopment. Based on this need for protein in female flies, a number of protein baits and trapping devices were developed (reviewed in Heath et al. 2007). The effectiveness of these attractants depends upon the ovarian developmental stage of the foraging females. A number of factors that affect ovarian development in tephritid females including nutrition, hormones and environmental conditions, are discussed in this section.

Oogenesis of *B. dorsalis* requires proteins in the form of free amino acids, in addition, carbohydrates and water are essential for survival and reproduction. (Hagen and Finney 1950). Females require a substantial and varied diet to achieve maximum fecundity; most of it is acquired from the non-host foliage as well as fruits (include host and non-host fruits). Extra-floral exudates, plant surface leachate, fruit juice and bird droppings are the most commonly observed food sources of tephritids (Hendrich and Prokopy 1994). *B. dorsalis* adults are opportunistic foragers feed on the juice in their habitat (Chang et al. 1977). The behavior of *Bactrocera* flies dabbing on leaf surfaces leads to the discovery of bacteria of the family Enterobacteriaceae as a source of protein in the adult diet (Drew and Lloyd 1987; Manrakhan and Lux 2008).

Female reproductive system. Female *Bactrocera* of all studied species has two polytrophic ovaries, each with 20~50 ovarioles; each ovariole has a long distal germarium and a proximal enlarged egg chamber (vitellarium) containing mature oocyte (Fletcher 1975). The ovarioles connect at the base of the egg chambers where they form a membranous calyx. Nurse cells are attached to the anterior of immature oocyte, which expand toward the germarium during the vitellogenesis process. *B. dorsalis* females become sexually receptive after the first egg batch complete development (Fletcher 1987). Reproduction strategies of three tephritids in Hawai‘i illustrate the variation in ovarian development. *B. dorsalis* has short egg-laying intervals and small egg clutches whereas *B. cucurbitae* deposits larger egg clutches with long inter-ovipositional intervals and the egg productions in *C. capitata* declines quickly as female age (Carey 1984).

Duration of egg-laying intervals, egg loads and longevity of the female flies are useful ecological parameters for managing tephritid pests. In many families of Diptera, parity status of wild caught females may be assessed by morphological characteristics of the ovaries (Table A. 3). The most recent advances in tephritid biology includes the discovery of genes involved in reproductive behaviors and chemosensory perception in *B. cucurbitae* and *C. capitata* (Miyatake et al. 2002; Gomulski 2012). In *B. dorsalis*, gene function for yolk protein synthesis and genes related to reproductive behaviors have been identified; however, the mechanisms and associations of these genes to behaviors have yet to be identified (Chen et al. 2008; Zheng et al. 2012).

Nutrient regulation of ovary development. Oogenesis in *B. dorsalis* requires feeding on protein sources: the role of nourishment in oogenesis is reviewed in detail by Fletcher (1987). The amount of protein required to initiate oogenesis varies drastically between species, and a lack of protein prevents egg development past the pre-vitellogenesis stage in many higher dipteran insects. Diets rich in carbohydrate and protein are essential for survival and reproduction. Studies examined the effect of protein on fecundity in tephritids show a significant decrease in the protein requirements in ovipositing females.(Jácome et al. 1995; Jácome et al. 1999; Mangan 2003; Robacker and Thomas 2007). Females fed on only protein are unable to survive (Hendrich et al. 1991; Jácome et al. 1995). In contrast, diets that consist of carbohydrate rich foods such as fruits and sugar are less detrimental than a protein alone diet. Egg production and fertility

in female *Anastrepha* were greatly reduced when fed on carbohydrate diets alone than fed with the combinations of protein and carbohydrate (Jácome et al. 1999).

Hormone regulation of ovarian development. Egg production is a cyclical process regulated by a hormonal control system which is actuated by suitable environmental stimuli. The previtellogentic period and the mechanism of yolk development coincide with an intense protein feeding phase. Many insects produce yolk protein precursors in the fat body (reviewed by Raikhel and Dhadialla 1992). The production of yolk protein precursors in tephritids and other higher Diptera insects, by contrast, is in the ovary, with very low levels derived from the adult fat body. (Rina and Mintzas 1987; Handler and Shirk 1988). Vitellogenesis is often completed within 1-2 d (Papaj 2000).

Environmental factors regulate ovary development. In addition to food, female density, presence of semiochemicals such as male pheromones and fruit volatiles also have positive effects on egg load (Pritchard 1970; Aluja et al. 2001). The variability of the pre-ovipositional period and inter-ovipositional interval is determined by the host fruit stimuli. For example, the presence of host fruit is the essential cue for to initiate oogenesis in monophagous tephritids such as olive fruit fly, *Bactrocera oleae* Gmelin (Fletcher et al. 1978; Lachmen and Papaj 2001). Female *B. oleae* enters a reproductive resting phase when the preferred host is absent (Fletcher et al. 1978; Fitt 1986). In addition, visual stimuli resembling host fruits increased egg production in *Rhagoletis*

species (Alonso-Pimentel et al. 1998; Papaj 2005; Senger et al. 2008). By contrast, there is no regulatory feedback from the terminal follicle in polyphagous *B. tryoni* in which eggs mature continuously in follicles of subsequent gonotrophic cycles even when deprived of host plant (Fitt 1986).

Temperature is the primary environmental factor controlling the speed of ovarian development, with a certain moderate temperature (varies between species) leading to maximum fecundity. The impacts of low temperature on oogenesis in *B. dorsalis* include prolonging of the preovipositional period and smaller egg clutches (Vargas et al. 1997). High temperature (> 30 ^{o}C or > 85 ^{o}F) also affects oogenesis in *B. dorsalis*, causing smaller egg clutches, and significantly reduces female lifespan (Yang et al. 1994). The number of ovarioles in the adult is determined by environmental factors associated with the pre-adult stage include density, temperature and food quality (Fitt 1990; Hodin 2009).

Adult fly foraging behavior – olfactory, gustatory and visual stimuli associated with finding proteinaceous food.

Behavioral and perceptual responses to food depend on a convergence between gustatory, olfactory, and visual information that allows insects to go from detecting stimuli in the environment to actually taking action based on that information (Belanger and Willis 1996). How insects recognize and discriminate the numerous odorants in their environment and respond to these stimuli with appropriate behaviors provides the baseline that is essential for using attractants as a pest management tactic. A series of

progressive behaviors in tephritids – arousal, orientation, taxis and alightment – followed by tasting, feeding, ovipositing, courting and mating are the outputs arising from stimulation by the outside world (Light and Jang 1996). This section discusses how a female's reproductive development and its nutritional state affect behavior towards the olfactory, gustatory and visual stimuli while acquiring nutrients – proteinaceous food in particular.

Olfaction. Olfaction is the major sensory modality mediating host-plant selection in tephritid females (Light and Jang 1987; Aluja and Prokopy 1993). Plant-derived **s**emiochemicals play a key role in host location by phytophagous insects. The detection of these plant volatiles is largely mediated by olfactory cells located on the antennae and information from these peripheral receptors is then conveyed as nerve impulses to the central nervous system, where it is processed, and further transmitted to motor neurons, resulting in behavioral responses.(Cornelius et al. 2000; Kendra et al. 2005; Hull and Cribb 1997). Host seeking females rely on the fluctuations of carbon dioxide around the ripening fruit to locate surface lesions (Stange 1999).

Most foraging behaviors of frugivorous tephritids take place on non-host plants. Food foraging by both sexes of *C. capitata* is primarily confined to leaves (Hendrichs and Prokopy 1994). Bess and Haramoto (1961) found that flies of both male and female *B. dorsalis* rest or feed on non-host plants whereas *B. cucurbitae* mate on non-host plants (Nishida and Bess 1957). A group of 6-carbon alcohols and aldehydes known as "green

leaf volatiles" and ripe fruit esters stimulate the fruit fly antennal response of *Bactrocera* flies but not *Rhagoletis* flies (Fletcher and Prokopy 1991; Jang et al. 1997).

Gustation. Contact chemoreceptor neurons perceive primary and secondary plant metabolites mediating stimulation or inhibition of feeding or oviposition. Egg laying and food sampling are closely linked and flies have been shown to exhibit various feeding related sampling behaviors on encountering a potential egg-laying site. *B. dorsalis* females exhibit several different behaviors in order to assess suitability for oviposition including antennal and tarsal palpations, proboscis extension, and ovipositor dragging and probing (Jang and Light 1991). The choice of an egg-laying site often reflects an adult's choice of food. The success of egg laying depends upon the water content, the physical and the chemical properties of the substrate sense by the taste receptors on the ovipositor (Eisemann and Rice 1989; Tousson and Hustert 2000).

Vision. Adult fruit flies follow attractive odors associated with food and oviposition sites through widely varied visual landscapes. Visual stimuli such as the yellow color elicit strong behavioral responses in many species of tephritid (reviewed by Katsoyannos 1989). Yellow with peak reflectance close to that of green leaves (550 nm) enhanced alighting of *B. dorsalis* to colored spheres and papaya leaf mimics bait stations (Vargas et al. 1991; Wu et al. 2007; Piñero et al. 2009b). In apple maggot fruit fly, *Rhagoletis pomonella* (Walsh), flies are able to detect the odor of host fruit at distances of at least 20 m, and detect visual stimuli of host trees at distances of at least 3 m (Green et al. 1994). Flat yellow boards are highly attractive to *Bactrocera* spp. (reviewed by

Fletcher 1987). At close distances, visual characteristics are the principal cues to determine food or egg laying sites. *C. capitata* immature female flies are more inclined to alight on flat-panel type traps (Heath et al. 1995). Prokopy (1972) proposed that yellow color represents a supernormal foliage type stimulus eliciting food-seeking and/or plant-seeking behavior in tephritids. In addition to the visual stimulation of color, it is well established that object size, shape and background contrast play major roles in enhancing or diminishing an insect's response to visual stimuli (reviewed by Prokopy and Owens 1983).

Table A.1. Papaya ripening time

Color	Stage of Ripeness	Ripening Time at Room Temperature
Green, with slight yellow tinge at blossom end.	¼ ripe	5 – 7 days at room temperature.
1/3 yellow, 2/3 green.	½ ripe	2 – 4 days at room temperature.
1/2 yellow, 1/2 green.	¾ ripe	1 –2 days at room temperature.
Mostly yellow or yellow-orange.	100% ripe	Ready to eat.

Table A. 2. Pesticide available in Hawai‘i for papaya pests (updated from CTAHR database, 2012)

Target pest	Common names	Active ingredient
Insects and mites	White peach scale	azadirachtin, buprofezin, imidacloprid, malathion, pyriproxyfen, petroleum oil, spirotetramat
	Steven leaf hopper	azadirachtin, imidacloprid, malathion, pyrethrin
	Papaya mealybug	azadirachtin, imidacloprid, malathion, mineral oil
	Aphids	
	Thrips	azadirachtin, imidacloprid, malathion, spinosad
	Oriental fruit fly	spinosad, malathion,
	Melon fly	male attractants with spinosad or fipronil
	Mites	abamectin, azadirachtin, bifenazate, febutatin-oxide, fenpropathrin, mineral oil, pyridaben, spirodiclofen, sulfur

Table A. 3 Morphological characters of parity in dipteran insects

Family	Species	Type of character	Reference
Muscidae	*Musca sorbens*	Follicular relics	(Mau et al. 1981)
Glossinidae	*Glossina palpalis*	developing oocyte Nulli-parous Young-parous Old-parous	(Jarry et al. 1999)
Culicidae	*Anopheles multicolor* *Anopheles pharoensis* *Culiseta maelanur*	Follicular dilatations	(Shalaby 1971) (Grodowitz et al. 1997)
	Anopheles gambiae *Aedes aegypti*	Basal bodies	(Ferro et al. 1995) (Hoc and Schaub 1995)
Calliphoridae	*Chrysomya bezziana* Villeneuve	Follicular remains	(Spradbery and Sands 1976)
Simuliidae	*Simulium woodi*	Degenerating follicles Granulation in the basal body	(Craddock and Boake 1992)
Psychodidae	*Lutzomyia migonei* *Lutzomyia youngi* *Lutzomyia spinicrassa* *Lutzomyia ovallesi*	Genital atrium (parous-nulliparous)	(Hoc 1996)
Ephydridae	*Hydrellia* pakistanae	Follicle relics	(Lenz et al. 2007)
Tabanidae	*Chrysops atlanticus*	Follicular relics (parous –nulliparous)	(Magnarelli and Anderson 1979)
Tephritidae	*Anastrepha suspense*	Residual follicular bodies (corpora lutea)	(Kendra et al. 2006)
	Bactrocera oleae *Bactrocera cacuminata*	Corpus luteum	(Fletcher et al. 1978) (Raghu et al. 2003)

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Chapter 1.

Morphological features of the ovaries during oogenesis of the oriental fruit fly, *Bactrocera dorsalis*, in relation to the physiological state

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Abstract

Determination of physiological state in insects is useful in furthering the understanding of how insect behavior changes with age. Central to this determination is the identification of characters that allow assessment of physiological age. While non-destructive measures produce the most desired outcomes, internal markers may be more diagnostic and reliable. In this study, key morphological characters during previtellogenesis through vitellogenesis and ovulation were assessed as markers to determine physiological states of the oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae). Ovary length and width, ovarian index (length x width), and egg load of laboratory-reared *B. dorsalis* females recorded daily from eclosion up to 80 days old suggested significant differences in the ovarian index and egg load between females from each oogenesis stage. Parity status determined by the presence of follicular relics was found to provide high-accuracy classifications for *B. dorsalis* females. The presence of follicular relics with distinct morphological features provides a reliable identification tool to determine the physiological state of wild female oriental fruit fly. The potential applications of this technique to identify the physiological age of female fruit flies to study behavioral attributes in their natural habitat, and also the potential applications in relation to field control, are discussed.

Introduction

In female oriental fruit flies, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), ovarian development represents a time line from previtellogenesis to complete egg development for the purpose of male fertilization. Changes of the ovary's morphological characters from oogenesis and ovulation are key signifiers of physiological age for long-lived female tephritids (Carey 2001; Kendra et al. 2006). Changes in the length and width of the ovaries as a result of yolk protein accumulation in the follicles are followed by a concomitant expansion of egg chambers during vitellogenesis. The end of the egg production process is marked by the formation of follicular relics (corpora lutea) at the basal part of the ovarioles after ovulation (Fletcher et al. 1978). Nonrecurring changes of ovary morphology from continuous egg production in conjunction with accumulations of follicular relics are two main indicators of physiological age for dipteran pest species of medical and economic importance (Nation 1972; Kapatos and Fletcher 1984; Magnarelli et al. 1984; Gryaznov 1995).

The association between the age of females and their physiological status is the foundation of many control strategies developed for tephritid fruit fly pest management (Aluja 1994; Prokopy 2003; Vargas et al. 2010). Age-related demographic parameters have been used for predicting field establishment of pest populations and for timing augmentative biological control programs (Vargas and Ramadan 2000). The physiological age of an individual modulates the behavioral ontogeny of a female tephritid in response to inherited genetic traits, food quality and quantity, temperature,

and other environmental factors (Prokopy et al. 1994; Vargas et al. 1997; Wang et al. 2009). In this context, physiological age involves behavioral modification of response threshold to stimuli with time. Females switch from food-foraging to host-searching oriented behaviors as they approach sexual maturity; this switch is a result of modifying threshold to odors in the environment (Siderhurst and Jang 2006; Siderhurst and Jang 2010). Monitoring programs with food or host attractants may thus provide quantitative information relevant to the age structure of a population.

Various methods have been evaluated for age determination in insects, including approaches based on adult morphology, physiology, and biochemistry (Hayes and Wall 1999; Hugo et al. 2008). Accumulation of pterin fluorescent pigment compounds over time in the head capsule of *Anastrepha ludens* (Loew), *Bactrocera cucurbitae* (Coquillett), and *Ceratitis capitata* (Wiedemann) was measured to determine age dependent linear regressions (Hayes and Wall 1999). This method needs to factor in increasing pterin level due to ambient temperature, light level, and protein feeding (Hugo et al. 2008). For sterile insects released into the environment, the degree of wing abrasion gives a useful estimate of survival age (Dyck et al. 2005). Cuticle deterioration in age determination has the advantage of being independent of factors affecting the reproductive system, such as protein availability, although other factors, for example temperature or habitat, may influence fly activity and hence the rate of degradation. Physiological age-grading systems are useful, particularly for female insects, where physiological (rather than chronological) age determines key life history traits such as

lifespan, fecundity, and foraging behaviors (Tyndale-Biscoe 1984). The study of physiological age distribution gives insights for understanding population biology and describing behaviors of tephritid populations. Knowledge of the physiological age distribution of a particular tephritid population holds a crucial key to successful pest management. Tephritid pests, such as olive fly (*Bactrocera oleae* Gmelin), walnut fly (*Rhagoletis juglandis* Cresson), cherry fruit fly (*R. indifferens* Curran), and Chinese citrus fruit fly (*B. minax* Enderlein), have been reported to synchronize their ovarian development to coincide with host-fruit ripening (Delrio and Cavalloro 1977; Kapatos and Fletcher 1983; Messina et al. 1991; Carsten and Papaj 2005; Dorji et al. 2006). In orchards, these monophagous tephritids were effectively controlled when management efforts began after the capture of the first egg-bearing female.

Many tropical tephritid pests are polyphagous fruit flies, which seek oviposition sites according to available host fruits in the environment. Effective pest management depends on determining the movement of mature females. Studies have found that the response of *Anastrepha suspensa* (Loew) to proteinaceous odors and distance of attraction varied with sexual maturity of the females (Kendra 2009; Kendra et al. 2010).

Physiological age and parity have not yet been investigated in the oriental fruit fly, a major economic pest in tropical and subtropical fruit-producing areas in Asia and Hawai‘i (Seo et al. 1982; Vargas et al. 1983; Mau et al. 2007). Suitable tools are needed in order to assess the physiological age of a targeted population for optimal timing of

application of food-based attractants. The objectives of this study were (1) to describe in detail the basic reproductive morphology as well as the overall development of the reproductive system of female *B. dorsalis* through time, (2) to describe the morphological features of oogenesis stages based on ovary length and width, and (3) to assess the characters of the parous females by examining the presence of retained mature eggs, corpora lutea, and other morphometric features of the ovaries.

Materials and methods

Flies. *B. dorsalis* were obtained from cultures maintained under laboratory conditions for > 300 generations at the Pacific Basin Agricultural Research Center, Honolulu, Hawai‘i, and maintained in a room at 24 ± 2° C and 50 ± 5% RH, with a photoperiod of about 16:8 L:D (Vargas et al 1984), to document the morphological changes during ovarian development. Newly emerged adults were supplied with sugar and enzymatic yeast hydrolysate protein (United States Biochemical Corp., Cleveland, Ohio) mixed in water in a 3:1 ratio *ad libitum*. Four cages with 100 pairs of females and males each were sample cohorts. Ten female individuals were dissected daily from emergence to day seven to record the morphometric changes during the first gonotrophic cycle, and then every 10 days for 80 days after the sampled cohort grew fully developed ovaries, which occurred at day seven. Fresh papaya was provided in the cage for oviposition from day seven onwards, when all sampled flies were bearing fully developed eggs.

As oogenesis is asynchronous in dacine fruit flies, the classification of ovarian development was based on the condition of the most advanced follicles when assigning individuals to a particular oogenesis stage (Fletcher et al. 1978; Klowden 2007). Stage 1 and 2 were part of the previtellogenic phase. Ovaries with no visible follicle cells presented were categorized as Stage 1. Stage 2 began when the developing oocytes entered the vitellarium region once it had been completely surrounded by the follicular epithelium. The nurse cells were located at the anterior end of the follicle, and the oocyte at its posterior end (Stage 3). The size of the terminal follicle increased rapidly during the vitellogenesis phase (Stage 4) as yolk protein was being transported into the developing oocyte. The follicular epithelium and nurse cells degenerated at the end of vitellogenesis after the secretion of chorion was completed (Stage 5). As ovulation proceeded, the egg was ejected from the ovariole while the empty follicle shrunk up and collapsed, forming a spherical body known as corpus luteum. Stretched, empty ovarioles and the presence of follicle relics at the calyx and lateral oviduct areas marked the parous phase (Stage 6).

Flies were placed in a 0° C freezer for 30 minutes prior to examination. The reproductive system was extracted from the abdomen under a stereomicroscope equipped with an ocular micrometer at 10–20× magnification. Dissection was conducted by grasping the abdominal cuticle along the mid-dorsal line with a fine forceps and tearing open the cuticle, exposing the ovaries in the abdominal cavity. Ovaries were removed and excised from the ovipositor and adhering tissues and rinsed with phosphate buffered saline (pH = 7 PBS). Ovary samples were stained with aqueous neutral red (dimethyl

diaminophenazine chloride; toluylene red) saline solution (0.001%) for 30–40 seconds (Gryaznov 1995). The biometric parameters recorded included the length of the ovaries from the anterior end of the germarium to the calyx area and the width of the ovaries taken from the anterior end of the vitellarium. The Ovarian index, as one of the parameters to determine the stage of oogenesis, was obtained by multiplying ovary length by ovary width. Parous females were identified by the presence of follicular relics, often a light yellow color after the neutral red stain. The egg load was determined by counting the number of chorionated fully-developed oocytes in the egg chambers.

Data analysis. One-way analysis of variance (ANOVA) was performed to compare changes in key ovarian characters and egg loads (SAS Institute 1998). The morphometric data of ovary length and width were square-root (x + 0.5) transformed prior to analysis. Data of ovarian index were log (x + 1) transformed prior to analysis. Tukey's HSD was performed to determine the difference of morphometric data between each oogenesis stage. Student's *t*-test was performed for two samples to determine the differences between the two measurements. The correlation between the morphometric parameters, ovary length, ovary width, and egg load were tested with Pearson correlation at $p = 0.05$.

Results

Table 1 presents the morphometrics of ovarian development in *B. dorsalis*. The first oogenesis process was completed by seven days after adult eclosion. Stages 1 and 2 were the pre-vitellogenesis phase of the ovarian development. Each oocyte at the pre-

vitellogenesis phase lacked a visible egg chamber. Stage 1 lasted for the first three days after emergence, with the ovaries approximately equal in length and width (Figure1.1A). The vitellarium was not distinguishable from the lateral oviduct in Stage 1. As the developing oocytes moved down the ovariole and entered the vitellarium region between day three and four, the vitellarium area gradually became visible (Figure 1.1B). The length of the ovarioles averaged 0.4 ± 0.01 mm for Stage 1 and 0.6 ± 0.03 mm for Stage 2. Developing oocytes increased the ovary length significantly from Stage 1 to Stage 2 with no significant change in the width.

Stage 3 (Figure 1.1C) was characterized by the onset of vitellogenesis, with nurse cells occupying the anterior end of the terminal follicle. There was a rapid transition between Stage 3 and 4 in four to six day old females once yolk began accumulating. The length and width of Stage 4 ovaries were significantly greater than both the pre-vitellogenesis phase and Stage 3. Stages 1–4 comprised the classes of nulliparous females (Table1.1). The size of ovaries increased significantly from the growth of egg chambers during vitellogenesis. The duration of the vitellogenesis phase was on average 3 days from Stage 3 to the first batch of fully developed eggs. Nurse cells degenerated once the vitellogenesis was completed, and the process of forming chorion eggshells began.

The mature oocytes with chorion shells first appeared in the sampled females (Stage 5) as early as day five (Figure 1.1E). The egg load increased significantly between day five and day seven during the development of the first egg batch (Figure1.2A), and fully

developed ovaries were recorded in all samples by day seven. Egg load was correlated to ovary width (Pearson correlation = 0.40; $p < 0.001$) and length (Pearson correlation = 0.61; $p < 0.001$). The first oviposition marked the transition to Stage 6 (Figure 1.1F), as determined by the presence of follicular relics (corpora lutea) formed after the release of the terminal follicles. The follicular relics accumulated and the calyx became swollen with increasing chronological age. One possible source of error in the determining the stage of an ovary was that a mature female might be recorded as a Stage 3 if she happened to be sampled in the interval between the laying of a complete batch of eggs and the maturation of the next. Empty ovaries with stretched and straightened vitellarium section were found in 20% (n = 22) of the dissected parous females aged between eight and 80 days old. The index, length, and width of empty ovaries from parous females were significantly different compared to females at Stage 3. Once ovulation was initiated, the ovary width varied according to the egg load, but the length did not change significantly. Stage 6 was characterized by an overall decline in egg load (Figure1. 2A) and ovary width (Figure 2B) with increasing age. The average egg load in females older than eight days (Stage 6) was significantly lower ($t = 4.14$, $p < 0.0001$, df =32) than Stage 5 females (Figure1. 2A). In Stage 6, the egg load of parous females was an average of 20 (± 5.6 S.E.) eggs per female throughout the observation, regardless of age. Developmental asynchrony increased in secondary oogenesis with aging. Despite this increase, ovary width and ovarian index (Figure 1.2C) were significantly greater in Stage 6 than in Stage 4. Follicular relics were found in 98% of the ovipositing females. At the beginning of

Stage 6, follicular relics were difficult to see because the ovarioles have to be well spread to allow a clear view of pedicel.

Discussion

The overall objectives of this study were to identify the morphometric parameters for each oogenesis stage and to identify characters to identify parous female oriental fruit flies. The six stages of ovarian development proposed for *B. cacuminata* (Raghu et al. 2003) and *B. oleae* (Fletcher et al. 1978) fit the stages we reported here for *B. dorsalis*. The morphometry of the ovary increased significantly between each oogenesis stage during the first gonotrophic cycle whereas few changes were found in the parous females. The presence of corpora lutea and the ovary length were two key characters to identify parous females. The morphometric index was found significantly different between parous and nulliparous females.

The ovaries of the first oogenesis phase in *B. dorsalis* initially increased in length and subsequently in width during the first seven days after eclosion. Our results support the seven to ten day (at 24 ^{o}C) preoviposition period previously recorded in the laboratory strain of *B. dorsalis* (Foote and Carey 1987; Vargas et al. 1997). Of the four characters examined, ovary length was the most reliable indicator for pre-vitellogenesis and vitellogenesis during the developmental stage. The vitellogenesis phase was marked by the appearance of nurse cells at the anterior end of the developing oocyte, and lasted until the complete development of chorion eggshell. A large amount of yolk protein was

deposited into the developing oocyte during this stage, which caused a rapid change in the ovarian structure. Characters of the terminal follicles, in combination with the ovarian index, provided accurate identification for the stages of oogenesis and were consistent with the findings of Kendra et al. (2006).

Once a mature egg ovulates, the next distal follicle begins to mature, regardless of the stage of the proximate follicles in the other ovarioles. Laboratory and wild *B. dorsalis* have similar reproductive cycles once the female reaches maturity (Vargas et al. 1984; Vargas and Carey 1990). The rapid development in the following oocytes therefore resulted in an increasingly asynchronous egg development in each ovariole. The follicular relics of *B. dorsalis* observed in this study appeared as clumps of cells in the calyx (Figure 1.1F). Individual follicular relics were visible by stretching the tissue with a fine needle. However, the heterogenic number of follicular relics between the ovarioles, due to developmental asynchrony, resulted in the increased difficulty to determine the exact number of gonotrophic cycles. The two-class age-grading system widely used in field studies to determine the age structure of hematophagous dipterans, such as *Anopheles* and *Culex* mosquitoes, and the screw worm (*Cochliomyia hominivorax* (Coquerel), are also applicable to *B. dorsalis* (Hayes and Wall 1999). The pre-oviposition development time in *B. dorsalis* colonies derived from wild flies was longer, with a larger range in variation, than the laboratory strain (Arakaki et al. 1984; Foote and Carey 1987), suggesting that morphological characters are suitable candidates in determining the physiological age of feral populations. This age-grading technique was applied to

determine the parity status of field captured *B.oleae*, *B. cacuminata*, and *B. dorsalis* (Fletcher et al. 1978; Raghu 2003). Based on comparisons of the morphological characters examined in this study, follicular relics and ovary length are reliable indicators to determine the age structure of *B. dorsalis*.

Physiological states (i.e., nutritional state, mating status, etc.) coordinated with oogenesis influence females' foraging behaviors (Jang and Light 1991; Prokopy et al. 1991; Prokopy et al. 1995; Jang et al. 1998). One of the main tasks for a newly eclosed female is to forage for the nutrients required for egg production. A surge in protein feeding was recorded in *B. tryoni* at this stage (Meats et al. 2004). Behavior studies confirmed that female tephritids with developing ovaries have a stronger response to proteinaceous odors compared to mature females, which respond more strongly to host-fruit odors (Prokopy et al. 1991; Nigg et al. 1995; Cornelius et al. 2000; Rull and Prokopy 2000). Physiological changes triggered by mating consequentially alter female behaviors from food- and mate-oriented olfactory behaviors to a strong preference for host-fruit stimuli. Fluctuations of the egg load with age suggest alternating between food seeking and oviposition-site seeking behaviors in order to obtain the protein for egg development (Kendra et al. 2006). The physiological age of flies collected from food-based traps may provide more complete information for monitoring purposes. From an applied perspective, the classification of ovarian developmental stages in conjunction with assessment of egg load and parity status will facilitate the evaluation of the age structure of a fly population responding to specific lures in field trapping studies.

Detailed behavioral studies are required to determine the effects of egg load on food-foraging behavior of female *B. dorsalis*, including its relationships with host-seeking and proteinaceous food-seeking decisions. The proposed classification system based on *B. dorsalis* in this study has applications for both laboratory and field research based on the observed characters of ovarian development as indicators for the female physiological age. The method described to characterize the parity status of *B. dorsalis* is the main key to determine the target physiological age group for control using food-based attractants in natural environments.

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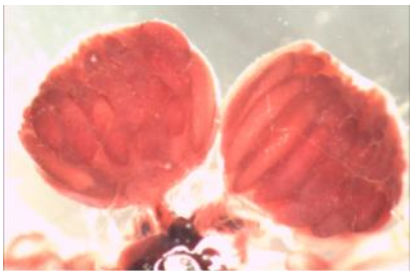
Table 1.1. Ovarian characters, chronological age range and egg load at each developmental stage of *Bactrocera dorsalis*.

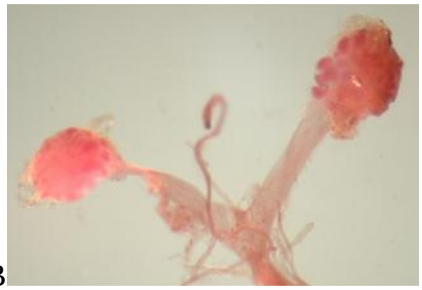
Means within a column followed by the same letter are not significantly different (Tukey's HSD test (p = 0.05)). [1]Data were square-root (x + 0.5) transformed prior to analysis, non-transformed means are shown. [2]Data were log (x + 1) transformed prior to analysis, non-transformed means are shown.

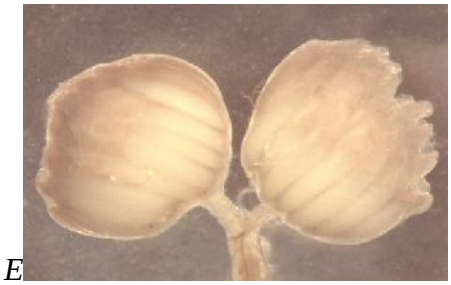
					Morphological parameters (Mean ± SEM)		
Ovarian stages		n	Age (d)	Egg load	Length (mm)[1]	Width (mm)[1]	Index (mm^2)[2]
Previtellogenesis	1	34	1-4	0	0.4 ± 0.01a	0.4 ± 0.01a	0.2 ± 0.01a
	2	17	2-4	0	0.6 ± 0.03b	0.6 ± 0.03ab	0.3 ± 0.03b
Vitellogenesis	3	10	4-6	0	0.7 ± 0.05b	0.8 ± 0.05b	0.6 ± 0.08b
	4	12	4-7	0	1.0 ± 0.05c	1.2 ± 0.07c	1.3 ± 0.11c
Gravid	5	25	5-7	59.9 ± 7.6	1.8 ± 0.07d	1.8 ± 0.07d	3.2 ± 0.22d
Parous	6	112	7-80	20.7 ± 2.7	1.7 ± 0.03d	1.4 ± 0.05c	2.4 ± 0.10d

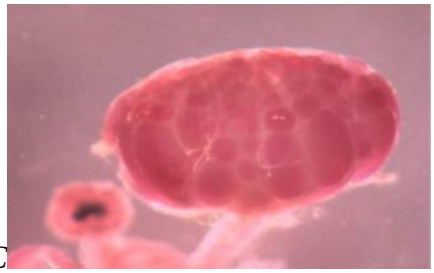
Figure 1.1. Stages of ovarian development in *Bactrocera dorsalis*. Stage 1 (A, black circle) and 2 (B) represent previtellogenesis development. Stage 3 (C) marks the initiation of vitellogenesis and 4 (D) indicates late vitellogenesis, at which point the yolk occupies more than half the follicle. The presence of first mature oocyte, characterized by an intact chorion with a reflective surface, indicating the beginning of stage 5 (E). Stage 6 (F) denotes parous females at the onset of oviposition, with the presence of follicular relics (F.R.) at base of the ovary (circle).

A 

D 

B 

E 

C 

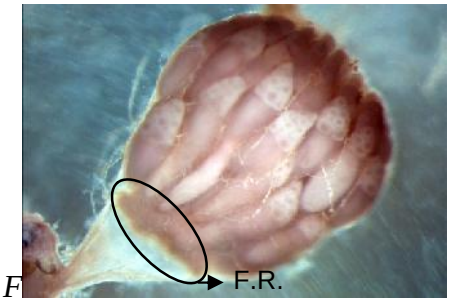
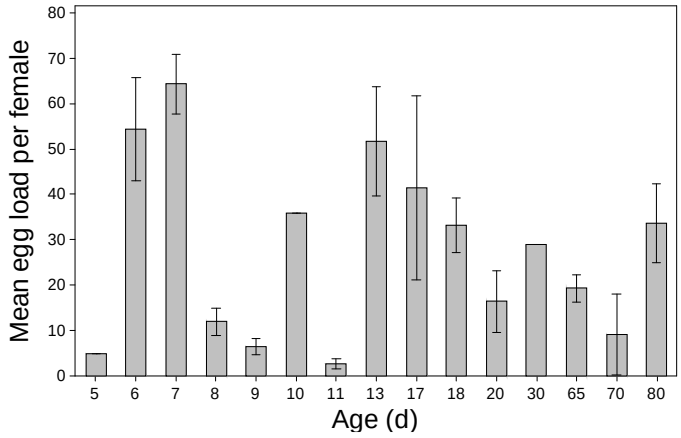
F 

Figure1.2. Egg load and ovary measurements (means and standard errors) of *B. dorsalis* age between 5 to 80 d.

A



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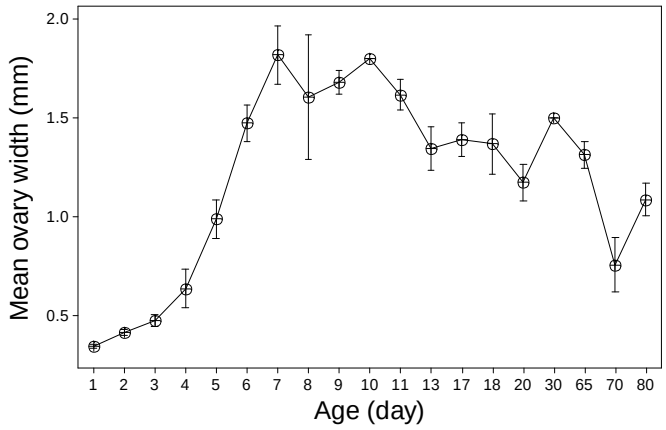

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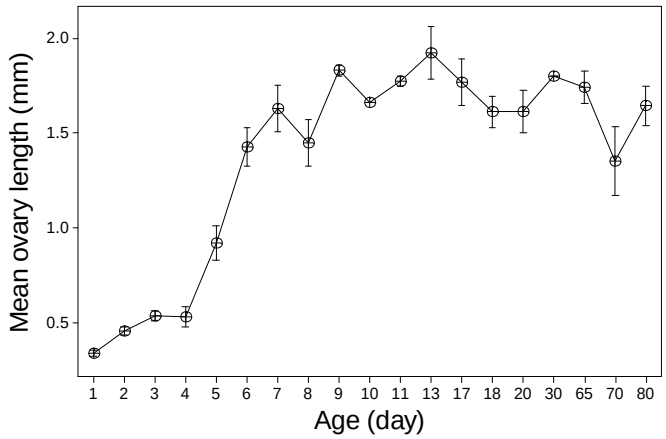

Chapter 2

Effects of visual and olfactory stimuli on the response of oriental fruit flies (Diptera: Tephritidae) to bait stations with protein or methyl eugenol in Hawai‘i

Abstract

Protein-based baits and methyl eugenol (a parapheromone) are attractants widely used for tephritid fruit fly management. Protein bait spot treatments and mass trapping with aqueous protein bait in McPhail-type traps (i.e. dome traps) are tactics available for suppression of feral female oriental fruit fly, *Bactrocera dorsalis* (Hendel) attacking economically important fruit crops in many regions around the world. In this study, the influence of color of dome traps and bait stations termed Papaya Leaf Mimics (PLMs) on the response of male and female *B. dorsalis* was evaluated with methyl eugenol (PLMs) and GF-120 NF Naturalyte Fruit Fly Bait (Dow AgroScience, Indianapolis, IN) (PLMs and dome traps) in commercial papaya orchards in Hawai‘i. The response of male *B. dorsalis* to methyl eugenol was significantly enhanced by the yellow color of the PLMs compared to similarly-baited green-painted bait stations. Trap capture data revealed that for PLMs baited with GF-120 (a highly attractive bait) a non-significant effect of color was recorded, whereas for dome traps baited with torula yeast (a comparatively less attractive bait) the effect of color become apparent, with significantly more males and females being attracted to yellow traps than to green-painted traps. Results from this study indicate that the visual stimulus from yellow- PLMs significantly increased the response of male *B. dorsalis* to methyl eugenol whereas in the case of protein baits the effect of color was dependent upon the type of bait used.

Key Words: Methyl eugenol, protein bait, reproductive state, behavior, Integrated Pest Management.

Introduction

Vision and olfaction are key sensory modalities used by tephritid fruit flies to locate hosts and food sources (Roitberg et al. 2009). Attract-and-kill methods that combine visual and/or olfactory stimuli are implemented or being developed for the control of tephritid flies (IAEA 2007). For example, in terms of visual responses, colored fruit-mimicking spheres in conjunction with semiochemicals are applied on a commercial scale to manage apple maggot fly, *Rhagoletis pomonella* (Walsh) (Prokopy 2003). Promising field trial results were also obtained with baited yellow spheres to manage female Mediterranean fruit fly, *Ceratitis capitata* (Wiedmann) (Katsoyannos and Papadopoulos 2004). One of the most successful applications of semiochemical-based fruit fly control systems includes a multi-tactic approach using highly attractive olfactory stimuli such as methyl eugenol for male annihilation and protein-based baits to target females. This approach successfully reduced the use of organophosphate pesticide for fruit fly control in Hawai‘i (Mau et al. 2007; Vargas et al. 2008a).

Protein bait spot sprays, mass trapping and bait stations are the only fruit fly control method available targeting female fruit flies. An example of a bait spray is GF-120 NF Naturalyte Fruit Fly Bait, which incorporates spinosad with a protein-based feeding attractant (Moreno and Mangan 2002). As a result of its effectiveness and environmentally friendly properties, GF-120 has become the primary tool for the area-wide control and suppression of tephritid fruit flies in the Hawaiian Islands (Mangan et al. 2006; Vargas et al. 2008b). In terms of mass-trapping, aqueous protein baits in

combination with McPhail-type traps baited such as torula yeast or Nulure (Miller Chemical & Fertilizer Corporation, Hanover, PA) were shown to be an economical and effective mass trapping tactic for *B. cucurbitae* (Chen et al. 2001) and *B. oleae* (Burrack et al. 2008). However, the trap catches can be compromised when large amounts of non-target flies are attracted to volatiles emitted from decomposed protein bait and flies (Heath et al. 2009; Leblanc et al. 2009). In addition, changes of bait consistency and pH value of the aqueous protein bait solutions over time remain as challenges to be solved for McPhail type trapping devices (Epsky et al. 1993).

A desirable characteristic of attract-and-kill systems is the presence of visual cues that are known to synergistically enhance the response of fruit flies to odor sources (Epsky et al. 1999; Mangan and Moreno 2007; Piñero et al. 2006; 2009a). Recently, a rain-fast attract-and-kill bait station termed Papaya Leaf Mimics (PLMs) was developed in Hawai‘i for suppression of pestiferous fruit flies (Piñero et al. 2009a). PLMs represent a supernormal visual stimulus of papaya foliage and serves as an attract-and-kill method to which insecticidal baits can be applied. PLM baited with protein bait sprays such as GF-120 was found to be effective at protecting GF-120 from rainfall and UV breakdown while enhancing the response of female *B. dorsalis*, melon fly, *Bactrocera cucurbitae* (Coquillett), and Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) to this bait (Piñero et al. 2009b; Piñero et al. 2010; 2011).

The physiological state of foraging fruit flies, in particular hunger, prior experience to food and reproductive state, is known to modulate the response of fruit flies to olfactory stimuli in the environment. For instance, female fruit flies are generally more responsive to protein food odors compared to males (Nigg et al. 1995; Vargas et al. 2002), in addition, age modulates the females' response to ammonium acetate, an attractive compound present in various protein-based baits (Piñero et al. 2011). It has been suggested that the effectiveness of protein-based trapping systems depend on the sex of the flies and physiological state of responding females (i.e. ovarian developmental stage, egg load, mating status) (Prokopy et al. 1995; Diaz-Fleischer et al. 2009). The main objective of this study was to examine the influence of the yellow color on the response of *B. dorsalis* males to methyl eugenol and of females to GF-120 (using PLMs) and torula yeast (using McPhail-type dome traps) in commercial papaya orchards. In addition, captured females were examined in the laboratory to determine whether the response of females to the combinations of olfactory/visual treatments may have been influenced by the reproductive state of the females.

Materials and Methods

Study Site. All experiments were conducted in an unsprayed commercial papaya orchard located near Keaau, Hawai‘i Island (19°37’15” N, 155°04’22” W, avg. elevation: 208 m). Perimeter-row papaya trees (plants 2.5- 3.0 m tall) were randomly selected as observation sites in a 1-ha orchard block.

Experiment 1. Response of male *B. dorsalis* to methyl eugenol to the color of PLMs bait stations. Field trials were conducted during March to May 2011 using bait stations (PLMs) (36 cm diameter) made from plant saucers (described in Piñero et al. 2009a). PLMs were painted either, sap green or cadmium yellow using artist's pigments (Krylon Products Group, Cleveland, OH), as described in Piñero et al. (2006).

The olfactory treatment for this test consisted of the Static- Spinosad - ME, containing 50% methyl eugenol (ME) and 0.2% spinosad (Dow AgroSciences, Indianapolis, IN). One tongue depressor with 2 g of Static- Spinosad - ME was prepared in the laboratory and then attached to the interior of each of the green and yellow PLMs with wire. Clear plant saucer liners were attached to the bait stations with zip ties (Fig 2.1) to retain responding males in the bait stations to be able to record the number of responding males at each observation interval. Yellow and green PLMs baited with the male lures were deployed in pairs spaced 15-20 m apart in each row and 25-30 m apart between blocks. Each bait station was secured on the papaya plant trunk at approximately 1.5 m above ground with a zip-tie.

On each trial day, observations started between 800 to 900 h for a 3-h period. Six replicated trials (three replicates per observation day) were conducted on sunny days to collected total of 18 samples. The number of males that entered the bait stations was recorded at 30 min intervals and the location of the bait stations was switched at each

observation to minimize the effect of location. Flies that entered the clear vinyl liner were discarded after counting.

Experiment 2. Response of female *B. dorsalis* to PLMs baited with GF-120 and to dome traps baited with torula yeast. These field trials were conducted between July and August 2009 during the peak of *B. dorsalis* adult seasonal activity. The four treatments evaluated were: (1) a yellow PLM sprayed with 10 ml of 40% (vol: vol) GF-120 solution, (2) a green PLM sprayed with 10 ml of 40% (vol: vol) GF-120 solution, (3) a yellow plastic McPhail type trap (dome trap) (Suterra LLC, Bend, OR) baited with 300 ml of torula yeast (BetterWorld Manufacturing, Fresno, CA) solution (prepared by mixing 1 torula-borax pellet with 100 ml water), and (4) a green-painted dome trap baited with 300 ml of torula yeast solution. For this study, we tested the null hypothesis that the response of female *B. dorsalis* to PLMs baited with GF-120 and to dome traps baited with torula yeast is not affected by the color of the bait station.

The experimental design was a complete randomized block with four replicates. A replicate consisted of a randomly chosen perimeter row where all four treatments were represented. Dome traps were hung from a metal elbow bracket (L×W: 12”×12” or 30 cm × 30 cm) secured to papaya plant trunk 1-2 inch away from the tree trunk at approx. 1.5 m above the ground with a zip-tie. Placement of PLMs was as described in Experiment 1. All GF-120 bait treatments were prepared on-site and applied to a PLM with a hand sprayer calibrated to apply 10 ml of the bait. Observations were conducted on sunny days

between 700 to 1100 h at 0.5 h intervals for 2 h, period during which the number of males and females captured in each bait station and dome trap was recorded. Bait stations and traps were switched one position after each observation to compensate for potential effects of trap position. Flies alighted on the PLMs or caught in the dome traps were removed prior to each rotation to ensure that each responder was counted only once. Five weekly observations were conducted to obtain a total of 20 replicates.

Physiological states of captured female *B. dorsalis*. The reproductive state of captured females was assessed via dissections of the ovaries from samples collected during 24 h to obtain the physiological profiles of females alighted to the two tested bait stations. Clear polyethylene films (12 by 22 cm) coated with Tangle-Trap (The Tanglefoot Company, Grand Rapids, MI) were placed along the edge of the PLM bait station to collect flies alighting to PLM bait stations. Samples were brought to the USDA-ARS-PBARC laboratory (Hilo, HI) for examination. Methyl soyate solution (Goof Off Stain Remover, W.M. Barr, Memphis, TN) was applied to dilute the Tangle-Trap. Flies were dissected under a dissecting microscope (at 20X magnification) to examine the morphological features of the ovaries, presence of mature eggs, presence of follicular relics, and egg load (Chou et al. 2012). The two reproductive states recorded according to ovarian development stages were: (1) immature female: with previtellogenesis or vitellogenesis stage ovaries, there was an absence of mature eggs and absence of follicular relics; or (2) egg laying female: ovaries with completely developed eggs and/or presence of follicular relics.

Data analysis. Fly capture data in both experiments were analyzed by the total number of flies pooled from each observation period. Male capture data collected in experiment 1 were compared according to the trap color with a t-test. For experiment 2, one-way ANOVA was applied to compare the captures of male and female flies according to the four trapping devices for the 2 h period. The effect of yellow color on capturing females of different ovary developmental status in PLMs and dome traps was determined with ANOVA GLM. The influence of egg load on the response of female to the four trapping devices was determined with one-way ANOVA. Data were transformed with $\sqrt{(x+0.5)}$ whenever necessary to meet the ANOVA assumptions. All data analyses were performed in Minitab 15 Statistical Software (Minitab 2004).

Results

Experiment 1. Response of male *B. dorsalis* to methyl eugenol to the color of PLMs bait stations. The visual stimuli provided by yellow PLMs increased significantly the response of male *B. dorsalis* to methyl eugenol compared to green PLMs (t =4.96; df =32 $P < 0.001$) (Table 2.1). The number of males responded to yellow PLMs in a 3-hour period was nearly twice the number of males that responded to green PLMs.

Experiment 2. Response of female *B. dorsalis* to PLMs baited with GF-120 and dome traps baited with torula yeast. Significant differences in the number of male (F = 9.6; df = 3, 76; $P< 0.001$) and female (F = 9.8; df = 3, 76; $P< 0.001$) *B. dorsalis* that

responded to the trapping devices were recorded. Overall, yellow and green PLMs captured significantly more males and females than did yellow and green dome traps (Table 2.2). Adding yellow color as a visual stimulus significantly increased the response of male and female *B. dorsalis* to dome traps, however, the effect was not statistically significant for PLMs.

Female samples collected in 24 h revealed that yellow-PLM, green-PLM, and yellow-dome traps had significantly greater captures than green-dome traps (Figure 2.2). The color of the PLM did not have significant effect on the number of females captured ($F = 0.34$; df = 1, 16; $P = 0.57$) with no significant interaction effect between the ovarian status and color ($F = 0.11$; df = 1, 16; $P = 0.74$). In contrast, dome traps had significantly higher captures of egg-laying females than immature females ($F = 5.42$; df = 1, 16; $P = 0.03$). In addition, yellow color of dome traps significantly increased female captures ($F = 7.94$; df = 1, 16; $P = 0.01$) with no significant interaction found between the ovarian status and color ($F = 0.71$; df = 1, 16; $P = 0.13$).

Overall, immature and egg laying females represented 53.9% and 46.1% of the total females collected, respectively. A previtellogenesis ovary was recorded in 96.6% of the immature females with most of the samples possessed a light body color and soft exoskeleton. The ovarian developmental stage and the physical characters indicated that these females recently emerged from puparia in the nearby area. Egg laying females with follicular relics but zero egg load (n=7) represented 2% of total female samples collected,

but the sample size was too small to compare the effects of the bait stations on capturing females from this category of ovarian status. Of the egg laying females dissected from the protein bait stations, female egg load varied significantly across trap types and baits (F = 5.76; df = 3, 173; $P < 0.001$). Irrespective of color, females captured in dome traps baited with torula bait had significantly higher egg loads than those captured in PLMs (Table 2.3).

Discussion

Here, we demonstrated that the field performance of methyl eugenol, a powerful male attractant, was significantly enhanced by the yellow color of PLMs compared to green-painted PLMs. This result is in agreement with previous findings indicating that the color yellow increased male fly captures in bucket traps baited with methyl eugenol compared to the colors green, red and black (Stark and Vargas 1992). Significantly higher captures of females in PLMs than dome traps found in this study are similar to results of the field studies of *C. capitata*, in which females were more attracted to flat surface type traps than dome traps (Heath et al. 1995). The physical structure of PLMs represents a large surface area that increases trap captures by eliciting food searching behavior in females and more volatiles are presumably emitted from the open surface of PLMs compared to the enclosed system represented by the dome trap.

The use of visual cues for finding oviposition sites is well documented in *Rhagoletis* fruit flies, for which yellow panel type traps (Prokopy 1972) and red spheres (Prokopy 2003) are the most attractive. In this study, the synergistic effect of adding yellow color significantly increased captures of *B. dorsalis* females in dome traps baited with torula, a comparatively low attractiveness bait. The non-significant effect of the yellow color in PLMs baited with GF-120 is in agreement with findings of Piñero et al. (2009) also with *B. dorsalis*. Our data also indicate that newly eclosed *B. dorsalis* flies are attracted to by the combination of volatiles from GF-120 and the yellow color of PLMs.

In addition to trap color, it is well established that object size, shape and background contrast play major roles in enhancing or diminishing insects' responses to visual stimuli (reviewed by Prokopy and Owens 1983). Female age and egg load are two parameters that increase the probability of a female landing on red spheres hung in a host tree (Duan and Prokopy 1994; Prokopy et al. 1995). In this study, egg laying female *B. dorsalis* captured in dome traps had significantly higher egg loads than those in PLMs and the average egg load of egg laying females recorded in a previous laboratory study (Chou et al. 2012) suggest visual stimulus of dome traps is more attractive to females with high numbers of eggs.

McPhail type traps baited with aqueous protein solutions such as torula yeast are the standard trapping device for fruit fly detection and suppression programs (IAEA

2007). In this study, significantly more female flies were attracted to both yellow PLMs baited with GF-120 than to yellow dome traps baited with torula yeast solution. Yellow PLMs attracted both immature and egg laying females whereas yellow dome traps attracted more egg-laying females than immature females. This finding indicated that application of GF-120 NF Naturalyte Fruit Fly Bait in PLM bait stations may be a more efficient management tactic compared to mass trapping with plastic McPhail traps as an attract-and-kill method to manage female populations. Field studies conducted by Piñero et al. (2010) demonstrated that effective fruit fly suppression in papaya orchards can be achieved by deploying 30 PLMs with 0.12 liter of GF-120 NF Naturalyte Fruit Fly Bait per hectare per week. High density trapping with plastic McPhail traps of *B. dorsalis* and *B. cucurbitae* are recommended at 100-300 traps with 3 pellets of torula yeasts (1 pellet per 0.1 liter water) for the same treatment area in fruit fly high density areas (Chen et al. 2001). Successful suppression of *B. cucurbitae* in watermelon (*Citrullus lanatus* Thunb.), is achieved with weekly to biweekly recharge of torula baited McPhail type traps (Kao et al. 2011). In contrast to the number of bait stations and the high volumes of bait materials ($81 for 30 liter of torula yeast solution per hectare) for McPhail trap mass trapping, applying protein bait in PLMs ($3 for 0.3 liter of GF-120 solution per hectare) substantially reduces the cost of bait material up to 85-95%, with additional saving in the cost for bait stations and water.

In conclusion, the response of *B. dorsalis* to protein baits was found to be influenced by the type of bait station and this response depended upon the gender of the fly and the ovarian status of the females. Male and female flies were significantly more attracted to yellow PLMs during the initial 2 h of trap deployment. Data collected in 24 h showed yellow-PLM and yellow-dome traps are equally effective in capturing *B. dorsalis* females. According to the ovarian status of captured female flies, PLMs were not biased toward the type of female captured whereas higher capture of egg laying females was found in dome traps.

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Table 2.1. Response of male *B. dorsalis* to yellow- and green-painted Papaya Leaf Mimics baited with a mixture of Static- Spinosad - ME in a 3-h period in commercial papaya orchards in Hawai‘i Island. Values in a column followed by the same letter are not significantly different (T-test, $P = 0.05$).

Color	**N**	**Mean number of male catch ± SEM**
Yellow	18	79.78 ± 6.36**a**
Green	18	39.33 ± 5.10**b**

Table 2.2. Captures of *B. dorsalis* in PLMs baited with GF-120 NF Naturalyte Fruit Fly Bait and dome traps baited with torula yeast in yellow and green color during 2 h observation. Values in the same column followed by the same letter are not significantly different (Fisher's LSD test, $P = 0.05$).

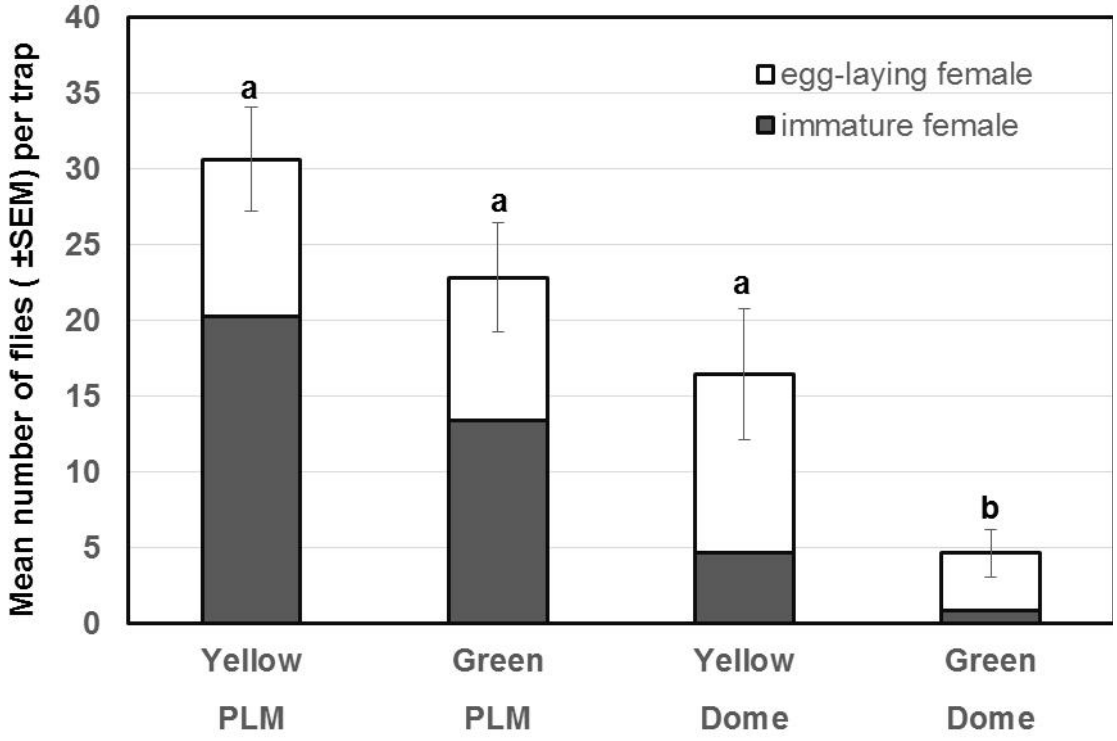
			Mean ± SEM	
Bait station	**Color**	**N**	**Male**	**Female**
PLM	Yellow	20	4.85 ± 2.13 a	6.35 ± 2.56 a
	Green	20	3.5 ± 1.19 a	5.45 ± 1.86 a
Dome trap	Yellow	20	0.3 ± 0.16 b	1.7 ± 0.55 b
	Green	20	0 ± 0.00 c	0.2 ± 0.12 c

Table 2.3. Egg load (± SEM) in ovaries of female *B. dorsalis* that responded to yellow- and green- painted Papaya Leaf Mimics (PLMs) baited with GF-120 NF Naturalyte Fruit Fly Bait and to yellow- and green- painted dome traps baited with torula yeast solution. Values in the same column followed by the same letter are not significantly different (Fisher's LSD test $P = 0.05$).

Bait station	Color	N	Mean ± SEM
PLM	Yellow	52	21.21 ± 2.31ab
	Green	47	16.85 ± 2.05b
Dome trap	Yellow	59	30.32 ± 2.65a
	Green	19	24.68 ± 4.87a

Figure 2.1. Papaya leaf mimic (PLM) bait station baited with a clear plant saucer liner to allow *B. dorsalis* males to enter the bait station for data collection during each observation interval.

Figure 2.2. 24 h trap captures of *B. dorsalis* female in green and yellow PLM stations treated with GF-120 NF Naturalyte Fruit Fly Bait and green and yellow dome traps baited with torula yeast protein solution papaya orchard, Keaau, HI. 2009. Bar values in the same category with the same letter were not significantly different means followed by Fisher LSD test (P=0.05). Statistical analysis was conducted with square-root transformed data, raw data were displayed in figure.



Chapter 3.

Fruit-based diet enhances the response of female *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) to a spinosad protein bait and host associated stimuli

Abstract

The influence of dietary history on the response of female oriental fruit fly, *Bactrocera dorsalis* (Hendel) to a protein bait contains spinosad as the active ingredient (GF-120 NF Fruit Fly Bait; Dow AgroSciences) and to host fruit stimuli represented by papaya, *Carica papaya,* was evaluated in field cages in Hawai‘i. Females were given a protein-rich diet after eclosion and followed with one of the protein-deficient diets contains papaya or sugar for half of their lifespan. Cohorts from one of the three diet regimes: papaya-fed, sugar-fed and protein-fed were tested at 1, 2 and 4-wk old. Papaya as part of fruit fly diet enhanced the response of female *B. dorsalis* to GF-120 fruit fly bait without interfering with the ovarian development when compared to protein-fed females. Response rates of female *B. dorsalis* to GF-120 were significantly higher in 1-wk, 4-wk old papaya-fed and 4-wk old sugar-fed females than protein-fed females. Significant delay of ovarian development in 4-wk sugar-fed females resulted in their high response rate to GF-120. Females with access to papaya as part of their diet exhibited a significantly higher propensity to alight on papaya than females without experience to papaya. Our findings demonstrated prior exposure to papaya increased the response of immature and egg-laying *B. dorsalis* to GF-120. Previous exposure to papaya increased the propensity of female *B. dorsalis* to alight on papaya highlights the importance of sanitation in potentially reducing the likelihood of females re-entering orchards.

Key words. Physiological state, dietary history, experience, protein bait, host stimuli, oriental fruit fly.

Introduction

Food foraging in nature is a complex and poorly understood aspect of tephritid fly biology that depends upon the interplay of the food quality, food availability, and the physiological and informational states of the insects (Papaj and Prokopy 1986; Fletcher 1987; Prokopy et al. 1994). Female reproductive status, feeding history, and the interaction of these two factors are determinants of the foraging response to hosts and food sources (Prokopy et al. 1991; Miller et al, 2004). Understanding the links between food-foraging behavior, dietary history, and ovarian status is essential for improving fruit fly management tactics based on fruit fly behaviors, including the use of food-based attractants, field sanitation and border trap crops.

Oriental fruit fly, *Bactrocera dorsalis* Hendel, is an anautogenous insect that requires continuous intake of protein to maintain 4-8-months of egg production (Vargas and Carey 1990; Chou et al. 2012). The principal food sources of this opportunistic fruit pest include fruit juice and insect honeydew (Chang et al. 1977). Additionally, bird feces and micro-organisms from rotten fruits may provide nutrients necessary for egg development (Prokopy et al. 1996). Semiochemicals from host plants and fruits are particularly important for fruit flies to locate oviposition sites and food sources (Metcalf 1990; Jang et al. 1997; Drew and Hancock 2000). The physiological states of females are known to influence the foraging response to these semiochemicals. For example, protein-deprived *B. dorsalis* females exhibited higher propensity to alight on a protein source than protein-fed females (Barry et al. 2006). Field studies showed that immature female

flies are highly attracted to proteinaceous volatiles whereas mature females are strong responders to fruit volatiles (Cornelius et al. 2000). There is limited information regarding the influence of diet on the response of mature females to protein resources. In the few comparative studies available, Piñero et al. (2011) demonstrated that continuous protein hunger (7d) increased the response of mature *B. dorsalis* female to proteinaceous bait enhanced with ammonium acetate. However, the overall response of mature females to proteinaceous volatiles was lower than immature females. In contrast to *B. dorsalis*, the response of mature female melon fly *Bactrocera cucurbitae* (Coquillett) to protein bait was higher than immature females (Vargas et al. 2009).

Papaya (*Carica papaya* L.) is a year-round fruit crop that serves as both oviposition site and food source for *B. dorsalis* in Hawai‘i (Vargas and Carey 1990). Continuous papaya fruit production increases the potential for female *B. dorsalis* to experience fruit associated stimuli during foraging between fields or between cultivated and natural environments (Prokopy et al. 1990; Vargas et al. 1990). Greater acceptance of particular fruit type in subsequent encounters through learning experience was demonstrated in *B. dorsalis*, Queensland fruit fly (*Bactrocera tryoni* (Froggatt)) and Mexican fruit fly (*Anastrepha ludens* (Loew)) (Prokopy et al. 1990; Prokopy et al. 1991; Robacker and Fraser 2005). Thus, field sanitation may reduce the risk of experienced females re-entering papaya orchards, in addition to eliminating a resource for reproduction (Klungness et al. 2005; Piñero et al. 2009a).

The extent to which a female's previous host fruit experience influences her subsequent response to protein-based volatiles such as baits that are being used as a control measure is a question that, to our knowledge, has not yet been addressed in any species of tephritid. This type of information is required to understand food foraging behavior of tephritids in order to manipulate variables in the habitat to manage pest populations. For example, sanitation conditions affect the efficacy of protein baits such as GF-120 NF Naturalyte Fruit Fly Bait (Dow AgroSciences, Indianapolis, IN) (Piñero et al. 2009a). The spinosad-based GF-120 NF Naturalyte Fruit Fly Bait was developed as a reduced risk bait for management or eradication of fruit fly populations (Moreno and Mangan 2002). Tests in Hawai'i have demonstrated its effective field suppression of *B. dorsalis*, melon fly, *Bactrocera cucurbitae* (Coquillett) and Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) when applied on the host, border trap crop, or on visual attractant bait station (Mau et al. 2007; Piñero et al. 2009b; Vargas et al. 2010). Given that both the environmental conditions and the flora in Hawai'i provide year-round breeding grounds for these tephritid pests, a multi-tactics integrated pest management that incorporates protein baits to control female populations are essential for various species of pestiferous fruit flies (Vargas et al. 2008). The aim of this study was to assess the effects of diet on the response of female *B. dorsalis* to either, GF-120 or to host fruit stimuli, in field cages. The effect of previous exposure to papaya fruit was compared to that exerted by a protein-deficient diet and a protein-rich diet applied on females from different age groups. The ovarian developmental stage was used to determine the effect of reproductive status on the response of female *B. dorsalis* to GF-120.

Materials and Methods

Insects. All *B. dorsalis* adults used for the experiments were F2 colonies originated from infested papaya, *Carica papaya* L., collected from commercial papaya orchards on the Island of Hawai‘i. Laboratory conditions were maintained at 25 ± 1 °C, 50 ± 10% RH, and 16:8 h L: D photoperiod. All insects were kept in screened wooden cages (30 x 30 x 30 cm) with approx. 200 adults per cage (female: male ≈ 1: 1). Upon eclosion, females and males were held together in cages supplied with water and a diet comprised of a 3:1 mixture of sugar and USB enzymatic yeast hydrolysate (United States Biochemical, Cleveland, OH), referred to as protein diet hereafter, until the diet regimes were introduced prior to the experiments (see below).

We assessed the effects of age as an indicator of reproductive status by testing females that were either, 1, 2, or 4-week old. For each of these three age groups, the effects of dietary history were examined by contrasting three feeding regimes that allowed females to receive protein during the first half of their life spans. One week old females were fed: (1) continuously on the protein diet (= protein fed), (2) 3 days on the protein diet followed by 4 days of sucrose alone (= sugar fed), or (3) 3 days on the protein diet followed with 4 days exposure to a punctured papaya fruit (= papaya fed). A fresh papaya with 20-30 probe punctures (approx. 1mm diameter) was presented in the cage every other day for the flies as a food source. Two-week old females were fed: (1) continuously on the protein diet (= protein fed), (2) 7 days on the protein diet followed by

7 days on sucrose alone (= sugar fed), or (3) 7 days on the protein diet followed by 7 days on papaya fruit (= papaya fed). Four-week old females were fed: (1) continuously on the protein diet (= protein fed), (2) 14 days on the protein diet followed by 14 days on sucrose alone (= sugar fed), or (3) 14 days on protein diet followed by 14 days on papaya fruit (= papaya fed).

Experimental setup. Experimental arenas were set up according to those used in previous behavioral studies to evaluate olfactory treatments including GF-120 (Prokopy et al. 2003; Miller et al. 2004; Piñero et al. 2011). All experiments were conducted in 1 m^3 screen field cages located at University of Hawai‘i Agricultural Research Station, Panaewa. One potted coffee plant, *Coffea arabica* L. (approximately 80-90 cm in height), was placed at the center of each field cage to serve as resting site. The plants were rinsed with water before tests to remove any previous food source. Each cage was placed on a rotating platform on top of a 40 cm height platform rotation table. Field cages were set under a covered tent area. The four sides of the tent were covered with shade mesh to minimize the effects of direct sun light potentially affecting the flies' response. Temperature in the test area ranged from 22 to 30 $^{\circ}$C during the observations.

By placing GF-120 and papaya fruit simultaneously in the cage, the design of the arena simulated the display of the two resources presented spontaneously, a situation fruit flies commonly experience shortly after a bait spray application in the field. The papaya olfactory treatment consisted of two fully ripe papayas (approx. 0.5 kg) which were hung

at the diagonally opposite corners of the cage using yellow nylon mesh bags. Small probe punctures (20-30 at approx. 1mm diameter) were made on the papayas to enhance volatiles emissions. The GF-120 treatment consisted of 20 droplets (10 µl ea.) of a GF-120 solution at 40% (v/v) applied on a coffee leaf disc placed on a clear petri dish (90 mm diameter). Each Petri dish with protein bait droplets was hung in the two remaining diagonally opposite sides of the cage with a wire hanger. One water cup with cotton wick was placed at the base of the coffee plant.

Observations were conducted on clear days during the active foraging period for *B. dorsalis* between 800-1100 h. On each observation day, 10-20 females from each of the three diet regimes of a given age group were transferred to three release boxes (12 cm wide × 18 cm tall × 5 cm deep) 1-2 h prior to the observation. Sucrose and water were provided in each release box during transportation. Each of three release boxes was randomly assigned to a field cage, and females were introduced into the test arena by opening the lid of the release box. The number of flies alighting on the protein bait droplets and papaya were recorded every 5 minutes for 2 h. Field cages were rotated 90˚ counter-clockwise every 15 minutes to minimize the potential effect of sunlight and airflow. Flies that alighted on a particular source (papaya fruit or GF-120) were gently removed and placed on the coffee plant.

Ovarian developmental stage in GF-120 responded females. Females that responded to GF-120 were collected at the end of the observation to examine the ovarian

developmental stage. The oogenesis stages of protein responders were compared among diet-age treatments to determine the possible effect of physiological state to the response of GF-120. Ovarian developmental stages recorded include immature females (previtellogenesis and vitellogenesis stage) and egg laying stages from 335 females dissected (Chou et al. 2012). Immature females include females with the ovary biometric characters described in previtellogenesis and vitellogenesis ovaries with the absence of mature eggs and the absence of follicular relics. The presence of follicular relics and/ or presences of mature eggs were the characters in ovaries of egg laying females.

Statistical analysis. The proportion (percentage) of female responded to GF-120 or papaya were subject to arcsin square root transformation to meet the assumptions for analysis of variance. Data were analyzed with the average number of females that responded in the first 40 minutes to minimize the possible effect of sampling the same individual over the 2-h period. One way ANOVA was applied to determine diet effect on the response of female to protein and papaya and Fisher LSD was applied to separate the means for 5 replications of 1-wk and 2-wk old females. A total of 8 repeated trials was conducted for 4-wk old females to reduce the variability between treatments. The proportions of immature females in each diet treatment were analyzed by one-way ANOVA for each age group. The total number of eggs was subjected to one-way ANOVA to determine the effect of diet.

Results

The response of 1wk old *B. dorsalis* females to GF-120 was significantly enhanced when females had access to papaya as a food source compared to the other two diets ($F = 8.67$; df = 2, 12; $P = 0.004$). The same pattern was observed for 2-wk old females; however, the significance at $\alpha = 0.05$ was not reached ($F = 3.49$; df = 2, 12; $P = 0.06$). For 4-wk old females, feeding on sugar elicited the strongest response to GF-120 followed by the papaya and the protein diets ($F = 4.87$; df = 2, 21; $P = 0.02$). Papaya-fed females were the most active among three diet regimes tested. During observation, the average first landing on GF-120 of papaya-fed females occurred within the first minute of release, compared to 3 min for the protein-fed females. Overall, the response of papaya-fed females to GF-120 decreased with increased age whereas the response of sugar-fed females to GF-120 increased with age (Figure 3.1). In addition to the highest propensity to alight on GF-120 than sugar-fed and protein-fed females, papaya-fed females were the fastest to respond to GF-120 compared to sugar-fed and protein-fed females. Sugar-protein-fed females were the least active among females from three dietary regimes with similar response to GF-120 among three age groups (Figure 3.1). The response of sugar-protein fed females to GF-120 did not change between the three age groups. Females resting on the screen walls and coffee plant canopy were often observed among protein fed females after being released into the field cage.

For 1-wk old females that had access to papaya for 4 days, the response to papaya fruit in cages exceeded significantly ($F = 14.49$; df = 2, 12; $P < 0.001$) than that

recorded for females fed on sugar or protein diets (Figure 3.2). The effect of diet was lost for 2-wk old females (F = 0.35; df = 2, 12; P = 0.71). For 4-wk old females the response mirrored that of 1-wk old females (F = 4.18; df = 2, 21; P = 0.03), except that feeding on sugar resulted in a similar level of response compared to the papaya diet.

Ovarian developmental stage in GF-120 responded females. Immature ovaries were found in all samples collected from 1-wk old females (Figure 3.3). The ovarian developmental stage of the 1-wk old sugar-fed females was recorded at previtellogenesis stage whereas few individuals from papaya diet (n=3) and protein diet (n=2) with ovaries at vitellogenesis stage, an indication of the onset of egg development. For 2-wk old females, the proportion of immature females was not significantly different across the three diet regimes (F = 0.62; df = 2, 12; P = 0.56). The effect of diet became significant in 4-wk old females (F = 9.74; df = 2, 12; P = 0.003) with significantly more sugar-fed females recorded with immature ovaries. Immature females with ovaries at the previtellogenesis stage were recorded in 98.5% (n=101) of 2-wk and 4-wk sugar-fed females. The finding corresponded to the significantly higher response of 4-wk old sugar-fed females than papaya-fed and protein-fed females. No significant difference was found in the egg loads of 2-wk old females fed on papaya or on the protein-rich diet (F = 2.36; df = 1, 38; P = 0.13) which were13.1 ± 4.3 and 17.6 ± 4.8, respectively. Diet had significant effect for the egg loads of 4-wk old females (F = 7.93; df = 1,121; P = 0.006). Females with access to papaya had significantly smaller egg loads (18.6 ± 3.9) than protein fed females (26.4 ± 3.8).

Discussion

We demonstrated that the response of *B. dorsalis* to volatiles emitted by GF-120 and by a ripe papaya fruit was enhanced by prior experience with papaya, particularly in 1-wk old females. Previous studies have shown that the quality and availability of adult food have profound effects on egg maturation and individual fitness (Jácome et al. 1999; Aluja et al. 2001). The result in this study showed that papaya as a food source did not interrupt the ovarian development in young *B. dorsalis* females. The nutrition contents in papaya contain 0.08-0.3 % of protein and approximately 6-10 % of sugar, is a relatively poor energy source with low protein content compared to the protein diet with 25% of sugar and 75 % of protein and 100% sugar in the sugar diet (USDA 2007). Papaya-fed young females, hence, showed higher response to GF-120 than previously tested young protein-deprived *B. dorsalis* females (Barry et al. 2006). In addition, feeding on protein deficient papaya diet did not show any delay in ovarian development for 2- and 4-wk old papaya-fed *B. dorsalis*. Among papaya-fed flies that responded to GF-120, mature eggs were recorded in 16% and 86% of 2-wk and 4-wk old females, respectively. The influence of papaya feeding became not as prominent for the 2-wk and 4-wk old papaya-fed females exhibited same level of propensity to alight on GF-120 compared to protein-fed females. On the contrary, sugar diet showed significant delay in the ovarian development of 2-4 wk old females. Mature eggs were recorded in 4% and 8% of 2-wk and 4-wk old sugar-fed females, respectively. Two weeks of protein-deprived sugar-diet

resulted in delayed ovarian development and high propensity of 4-wk old sugar-fed females alighting on GF-120.

In this study, females with continuous access to papaya were constantly exposed to their egg laying site, which was served as their food source simultaneously. Interestingly, papaya-fed females displayed higher propensity to alight on papaya than other diet regime treatments without experience. Whether this is the result of associative learning from prior experience or from a potential phagostimulant effect caused by feeding on papaya is unclear and it remains to be investigated. The ovarian development in papaya-fed females were paralleled to protein-fed females, however, 1-wk and 4-wk old protein-fed females were significantly less responsive to papaya than papaya-fed females.

Results from this study are of relevance for control of *B. dorsalis*, which invade papaya orchards after breeding in wild common and strawberry guava abundant throughout the Hawaiian Islands (Vargas et al. 1990). We demonstrated that papaya as part of adult fly diet significantly enhanced the response of *B. dorsalis* females to GF-120 and stimuli associated to papaya in semi-field condition. Sanitation is an important tactic not only as removing breeding ground for *B. dorsalis*, it also minimize the risks of females repeat migration from adjacent fields (Klungness et al. 2005; Piñero et al. 2009a). As shown in this study, females have higher alighting tendency on papaya when they were previously exposed to ripe papaya. This enhanced response to papaya is important from the aspect of behavioral manipulation using trap cropping (Rull and

Prokopy 2005). Physiological state of female flies is one of the factors that influence the response of female fruit flies toward protein baits. The response of papaya-fed female to GF-120 found in this study is an indication of better results would be expected for females in natural environment with limited access to high protein food source. Exposure to fruit associated stimuli is known to increase host searching efficiency.

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Figure 3.1. Mean (± SEM) percentage of papaya-fed, sugar-fed and protein-fed *Bactrocera dorsalis* females various ages at 1 wk, 2 wk and 4 wk old responded to GF-120 in field cages. Arcsin square-root transformation was applied for ANOVA and raw data were presented. Data are presented combining the average response at 5-min intervals in the first 40 minutes of observations.

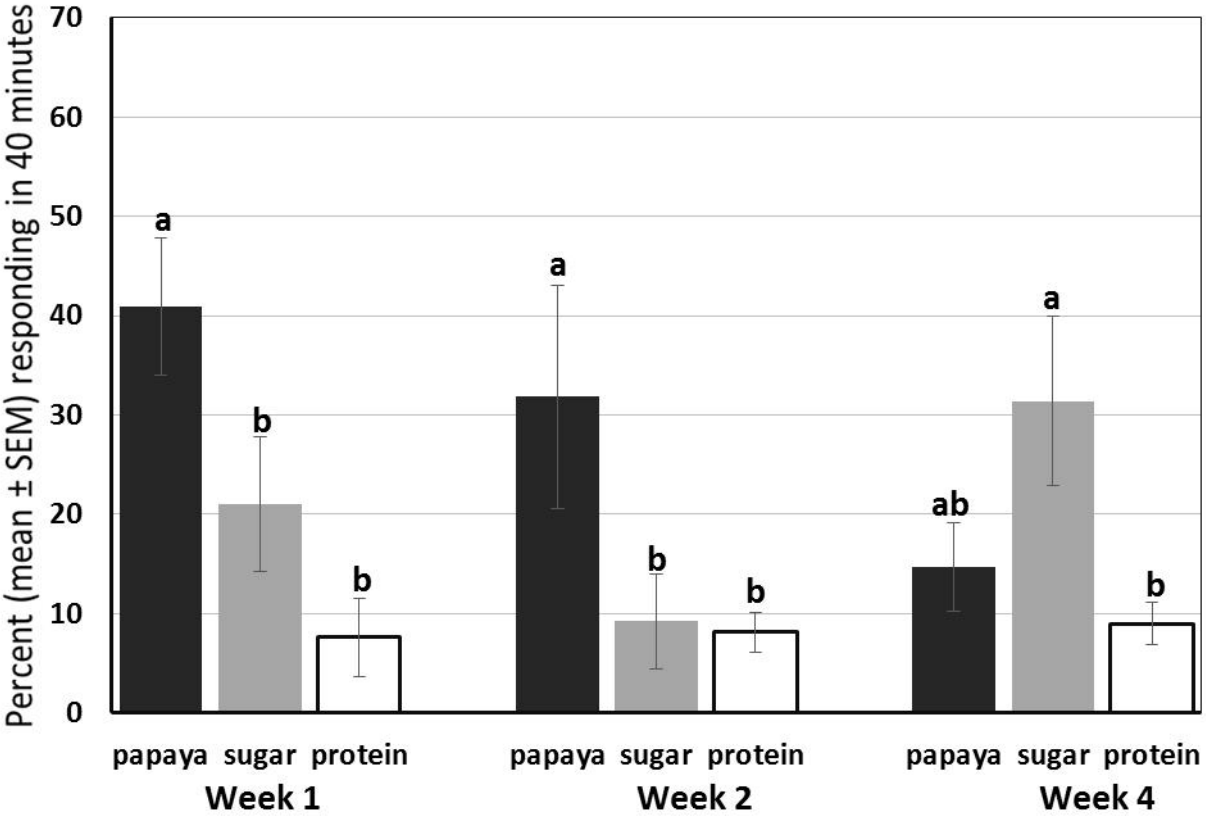


Figure 3.2. Mean (± SEM) percentage of papaya-fed, sugar-fed and protein-fed *Bactrocera dorsalis* females various ages at 1 wk, 2 wk and 4 wk old responded to papaya in field cages. Arcsin square-root transformation was applied for ANOVA and raw data were presented. Data are presented combining the average response at 5-min intervals in the first 40 minutes of observations.

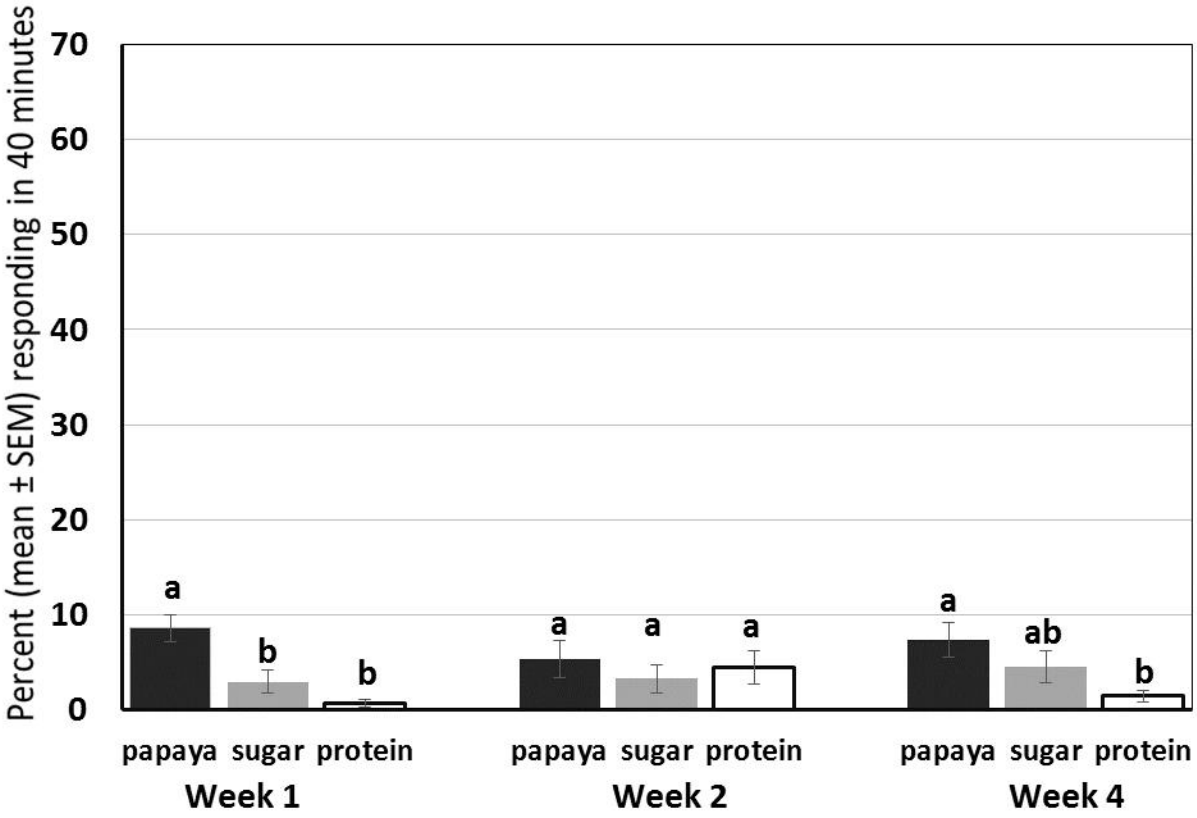
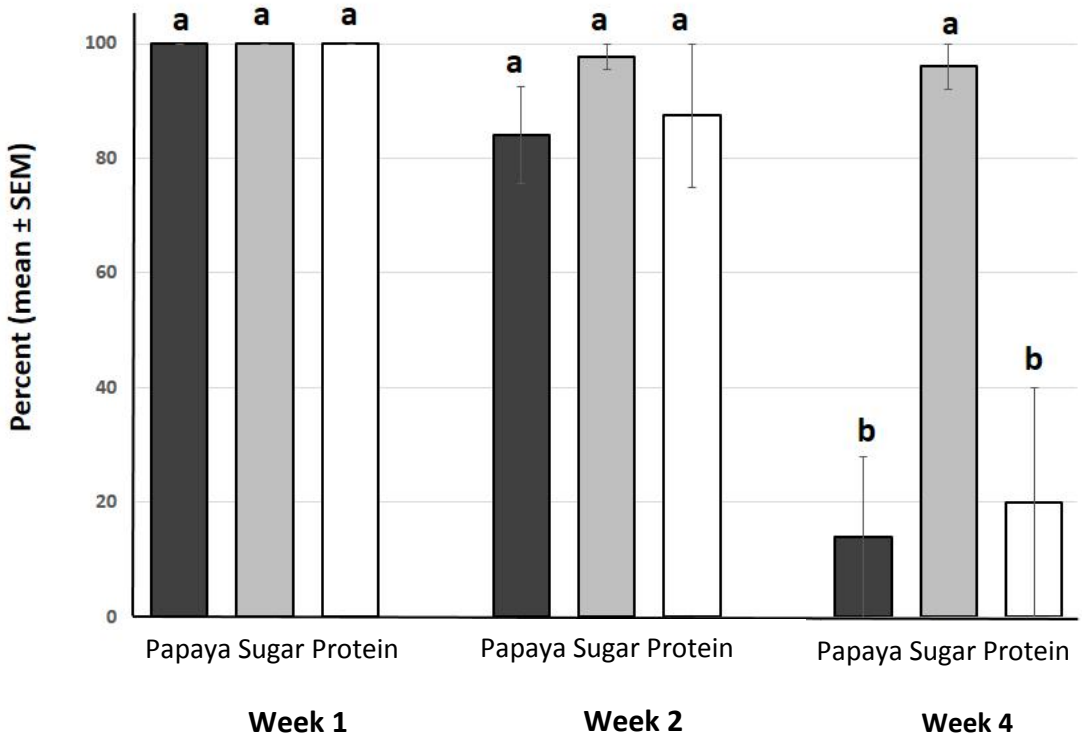


Figure 3. 3. Effect of diet on the numbers (mean percentage ± SEM) of immature female *B. dorsalis* responded to GF-120.



Conclusions.

The morphological characters and ovarian biometrics of each oogenesis stage described in Chapter 1 established a foundation to investigate foraging behaviors of *B. dorsalis* females in field environment. Neutral red staining technique makes it possible to observe the presence of nurse cells (a key oocyte character in vitellogenesis stage) and identify the presence of follicular relics (key character of parous stage) with high accuracy and efficiency. However, the heterogenic number of follicular relics between the ovarioles, due to the development asynchrony, resulted in the difficulty to determine the exact number of gonotrophic cycles. Results from chapter 1 shows that egg-laying stage is the majority part of female *B. dorsalis* life span. Immature females at the previtellogenesis stage and egg-laying females are the two most commonly found classes in field collected females. On the contrary, vitellogenesis stage females are rarely found in samples collected with protein bait. This may be the result of short period of time (2-5 d) required to complete the initial vitellogenesis process as described in Chapter 1.

Field data collected in Chapter 2 demonstrated that visual stimuli from yellow-colored bait stations enhanced the response of male flies to methyl eugenol and female flies to protein bait depending on the trap type used. PLMs baited with GF-120 captured significant more female *B. dorsalis* than did dome traps. In addition, yellow PLMs attract both immature and egg-laying females whereas yellow dome traps attract more egg-laying females than immature females. Results provide evidence that protein bait treatment of GF-120 to PLMs suppresses both immature and egg-laying female

populations in papaya orchards by applying GF-120 to PLMs. The effects of trap color became apparent when torula yeast, a bait that is comparatively less attractive than GF-120, was used.

A fruit-based diet was found to enhance the response of female flies to GF-120 protein bait, as demonstrated in Chapter 3, and is the first documentation for the influence of experiential and physiological states in terms of protein hunger, age and previous exposure to a host fruit on the foraging behaviors of *B. dorsalis*. Papaya as part of female fly diet increases the response of immature and egg-laying females to protein bait. In addition, the experience to papaya also increases the propensity of female alighting on papaya.

Implications for fruit fly management. Results from this study demonstrated that both immature and mature *B. dorsalis* females are attracted to GF-120 protein bait in yellow papaya leaf mimics (PLMs) bait stations. Higher alighting of female flies on GF-120 protein bait than papaya summarized in Chapter 3 indicated that protein bait may intercept female flies before or between foraging activities. Further work needed includes synchronizing protein bait treatment schedule with harvest schedule to target the most effective treatment window during the short papaya fruit ripening time before reaching the half ripe stage (< 1 wk).

Using PLMs as bait stations for GF-120 protect the longevity of the bait material and prevents UV break down. Results from this study further demonstrated that it attracts both immature and egg-laying females. It is essential for an effective control tactics to suppress pests with different physiological profiles and as many pest species as possible. Applying GF-120 in PLMs has been demonstrated in this study and previous studies as an effective tool to control *B. dorsalis* and *B. cucurbitae* females. Sanitation is an essential tactic to reduce fruit fly population density and infestation in agricultural area. Here, the study in Chapter 3 demonstrated that the previous experience with papaya increases the response of *B. dorsalis* female to papaya. Removing culled fruits not only reduce local breeding population, but also reduce the risk of mature females follow previous experience to re-enter the orchards.