THE AUTECOLOGY OF

BACTROCERA CACUMINATA (HERING)

(DIPTERA: TEPHRITIDAE: DACINAE):

FUNCTIONAL SIGNIFICANCE OF RESOURCES

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Abstract

This thesis investigated the autecology of the dacine species, *Bactrocera cacuminata* (Hering) (Diptera: Tephritidae: Dacinae). I specifically focused on the adult phase of the life cycle and resources believed to be significant to this life stage.

The prevailing paradigm in dacine ecology predicts that the larval host plant serves as the centre of dacine activity, a state mediated by mutualistic associations with fruit fly-type bacteria. Contrary to predictions, an explicit test of this hypothesis found that the host plant of *B. cacuminata*, *Solanum mauritianum* Scopoli, acted almost exclusively as a site for oviposition and larval development. Other key adult behaviours, most notably feeding and mating, were rare at the host plant. Even in disturbed habitats, the paucity of key adult behaviours such as mating was striking. Adult flies of this species were therefore hypothesized to be utilizing other components of their habitat, i.e. resources vital to their life history requirements. Some of the resources that *B. cacuminata* are known to respond to include sugar, protein, methyl eugenol and the host plant. The latter three resources are believed to be critical in the reproductive success of dacine flies in general. I assessed the physiological status of flies arriving at these resources to determine if flies of different status foraged for resources differently.

In dacines, the internal reproductive structures of the male and female flies have been used as predictors of physiological status. I quantified expansion of the male ejaculatory apodeme in *B. cacuminata* with age of fly and found that there is a threshold apodeme size that is strongly correlated

with sexual maturity. Maturity of female flies could be accurately predicted by ovarian development. Using these methods to assess the physiological and nutritional status of flies arriving at resources (larval host plant, protein and methyl eugenol) in the field, I discovered that only sexually mature and mated females were responding to the host plant, while the males at the host plant were sexually immature. This confirmed the hypothesis that the host plant primarily served as an oviposition site. Additionally, this study revealed that sexually mature males with high nutritional reserves were most commonly collected at methyl eugenol (a plant-derived chemical that elicits a strong response in males of many dacine species) at dusk, the time of peak sexual activity in this species. This indicated that methyl eugenol was perhaps a significant resource in the context of the reproductive behaviour of this species.

Methyl eugenol (ME) is one of group of phenyl propanoids to which males of certain species of Dacinae respond. The current hypothesis of the role of these phenyl propanoids is that they function as pheromone precursor chemicals. Response to these chemicals is hypothesized to be a trait under sexual selection. In *Bactrocera dorsalis* (Hendel), this effect is so strong that a single feeding on ME results in a strong mating advantage up to a month after males feed on the chemical.

Bactrocera cacuminata fed on multiple occasions on ME in a laboratory bioassay. After a single 24-hour exposure to ME, investigations of mating competitiveness did not reveal any obvious advantage for ME-fed males over unfed males. However, ME-fed males did enjoy a higher mating success 16 and 32 days after exposure to the chemical, suggesting that some physiological benefits unrelated to the pheromone synthesis was driving this delayed advantage. Investigation of the physiological consequences of feeding on ME revealed no enhancement of nutritional or energetic reserves,

suggesting that the delayed mating advantage observed was more likely a chance event.

An alternate hypothesis about the proximate function of ME, proposed by Robert Metcalf, is that it serves as a mate rendezvous site. As mating behaviour was notably absent at the host plant, I tested Metcalf's hypothesis. A field-cage experiment, spatially separating adult resources (host plant, methyl eugenol, sugar and protein) clearly demonstrated that methyl eugenol was functioning as a mate rendezvous stimulus for *B. cacuminata*. This is the first direct support for Metcalf's hypothesis.

A synthesis of the literature revealed that significantly greater ecological and evolutionary information was required to understand the basis of dacine response to phenyl propanoids. Different dacine species may be utilizing these chemicals differently, even if their evolutionary origin may have been as a plant based kairomone.

My studies show that generalizations on the ecology and behaviour of Dacinae, often extrapolated from research on a few pest species, do not hold up in the case of *B. cacuminata*. This suggests that a more autecological, species-specific approach is required in dacine research, before any predictive generalizations can be made.

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An academic endeavour such as a Ph.D., though independent, is seldom solitary. My experience is no exception. Specific help provided by various people is acknowledged at the end of each of the Chapters. Several people have collectively made my Ph.D. an enjoyable, memorable and a productively intense period of my life and I acknowledge them here.

Firstly, I thank my mother who has always encouraged me to think and work independently, a skill that was vital to the conduct and completion of this thesis. I dedicate this thesis to my grandmother and mother whose lives have been inspirations to me.

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From debating biological questions and experimental design to commenting on chapters and manuscript drafts (often within the same week as receiving them from me), Dr. Tony Clarke has guided me in this study as my principal supervisor. Tony's incisive and constructive criticism on every aspect of my research has greatly advanced my development as an ecologist. His role as a friend, colleague and mentor has been invaluable. The debt of gratitude I owe him is hard to express in words. Suffice it to say that interacting with him has been the single most profound influence on my development as a scientist and as an individual. That I feel a tremendous sense of achievement to have begun to think along the same intellectual wavelength as him at the end of this journey is a testament to his abilities as an academic.

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STATEMENT OF ROLE OF CO-AUTHORS

This thesis greatly benefited from the collaboration and help of several people. In recognition of help that was critical to this study, I have included them as co-authors in publications resulting from this study. The specific contributions are outlined below in alphabetical order.

Tony Clarke was involved in the refinement of many of the questions addressed in this thesis and in the experimental designs used in this thesis: he is recognized as a co-author on manuscripts from Chapters 2, 3, 5, 7, 8, 9. Richard Drew financially supported all the work and was actively involved in discussions related to Chapter 3 and guided me in all the morphological and anatomical aspects of the study on apodeme and ovarian development (Chapter 4). Jill Bradley taught me all the microbiological techniques vital for Chapter 2 and assisted me in several of the assays. Peter Halcoop aided me in the dissections and slide-mounted the apodemes for further measurement and analysis (Chapter 4). Amy Lawson's help was vital in simultaneously monitoring the feeding behaviour of fifty individual flies (Chapter 6). Boaz Yuval was a visiting scientist at Griffith University during 2000-2001 and his laboratory at the Hebrew University of Jerusalem helped analyse all the samples for energetic reserves (Chapter 5, 8). Each of the authors commented on the studies they were involved in.

PHOTOGRAPHS USED IN THE THESIS

Steve Wilson (Queensland Museum) took the photograph on the title page. Figure 1.1 is a composite of Steve's, Richard Drew's, Bob Cochran's and Tony Clarke's photographs. All other photographs are by the author.

STATEMENT OF ORIGINALITY

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

S. Raghu

October 2002

Chapter One

GENERAL INTRODUCTION

1.1 GENERAL INTRODUCTION

Tephritid flies (Diptera: Tephritidae) are one of the most diverse groups of insects, comprising over 4000 species in 481 genera (Thompson 1998). They have a global distribution, covering tropical, subtropical and temperate regions and occupy habitats ranging from rainforests to open savanna (Drew 1989a,b, Norrbom et al. 1998, Michaux and White 1999). Nearly all tephritid larvae are herbivorous, but have diverse feeding habits including flower feeding, galling, stem boring, bamboo feeding and fruit feeding (Hardy and Foote 1989, Headrick and Goeden 1998, Norrbom et al. 1998). The adults in contrast are free-living in the environment.

Because the larval host plant offers a known locality where flies will be present, and since larvae are the economically significant life stage of the fruit fly, the best studied aspects of fruit fly ecology and behaviour are those that concern the host plant. Thus oviposition behaviour, mating behaviour (for species that mate on the larval host plant) and within tree adult foraging have received significant attention. This is particularly evident from research on the fruit-infesting pest species of the genera *Rhagoletis* and *Anastrepha*, *Ceratitis capitata* Weidemann (Mediterranean fruit fly), *Bactrocera tryoni* Froggatt (Queensland fruit fly) and *Bactrocera dorsalis* Hendel (Oriental fruit fly). Besides this, little detailed information exists on the ecology and behaviour of adult phase of tephritids, particularly on those aspects of the adult life history that may occur away from the larval host. Notable exceptions to this are work undertaken on some non-frugivorous tephritids (Headrick and Goeden 1994, 1998) and on certain galling species of the genus *Eurosta* (Abrahamson and Weis 1996).

Generalizations for frugivorous tephritids are often extrapolated from research done at the larval host plant on the few economically significant species mentioned above. From such studies inferences on ecology and

evolution of frugivorous tephritids as a whole are made (Christenson and Foote 1960, Bateman 1968, 1972, Fletcher 1987).

1.2 ECOLOGY AND BEHAVIOUR OF DACINE FRUIT FLIES

Dacine fruit flies (Tephritidae: Dacinae[1]) have a wide zoogeographic distribution covering the Afrotropical, Oriental and Australian regions. Species occupy diverse habitats, from rainforests through to open sclerophyll and dry savanna and also highly modified habitats such as orchards and suburbia (Drew 1975, 1989a, b, Agarwal 1986, Norrbom et al. 1998). Over 750 species of dacine fruit flies are recognized, principally in two genera *viz*. *Bactrocera* (>500 species) and *Dacus* (>200 species) (Thompson 1998). Despite the variety of habitats occupied, most dacine fruit flies are exclusively frugivorous in the larval stage. Where the larval host plants include commercial species, dacines "compete" with humans and thus are classified into minor or major horticultural pests. Given this economic significance, dacines have attracted attention from both theoretical and applied research. A significant amount of information on the population or demographic ecology is available on dacine fruit flies (Bateman 1967, 1968, Bateman and Sonleitner 1967, Pritchard 1969, 1970, Fletcher 1973, 1974a, b, Fitt 1981a, 1989, O'Loughlin et al. 1984, Debouzie 1989, Meats 1989a, b), and has been summarized by Bateman (1972) and Fletcher (1987) (also see Robinson and Hooper 1989a, b and articles therein).

[1] There is considerable debate over the classification of dacine flies at the subfamily level. Some authors place the genera *Bactrocera* and *Dacus* within the Tribe Dacini, within the subfamily Dacinae, along with the genus *Ceratitis* (Tribe Ceratidini) (Kornyev 2000). Others taxonomists (Drew 1989b, Drew and Hancock 2000) prefer the placement of *Bactrocera* and *Dacus* in a different subfamily to *Ceratitis*. Norrbom et al. (1998) place *Bactrocera* and *Dacus* in one subtribe (Dacina) and *Ceratitis* in another (Ceratitidina) within the Tribe Dacini, Subfamily Trypetinae. Within the context of this thesis, I use the Subfamily Dacinae (*sensu* Drew 1989b), more for consistency with the previous ecological literature, rather than as a result of personal taxonomic/ conceptual orientation.

1.2.1. Life history of dacine fruit flies

Many aspects of the life cycle of dacine fruit flies are considered common across species, although few cross-species comparative studies have been done. Key ecological characteristics of the group are thought to include high mobility, high fecundity and prolonged life span of adults (Fletcher 1989). A standardized version of the "typical" dacine life cycle (Figure 1.1) is as follows.

Gravid female fruit flies lay their eggs in the flesh of fruit. Larvae hatch from the eggs after about 42 hours (at 25^{o}C) and feed on the fleshy fruit, reaching the prepupal stage in approximately 9 days (Bateman 1967). Bacterial decay associated with larval utilization of the fruit results in fruit fall. The prepupae emerge from the fruit and "hop", burrow and pupate into the top 2-3cm of the soil (Christenson and Foote 1960, Prokopy and Roitberg 1984, Fletcher 1987). Pupal development occurs in the soil underneath the host tree and is completed in approximately 12 days (at 25^{o}C) (Bateman 1967, Gibbs 1967). Rapid larval growth is hypothesized to be an evolved life-history strategy to minimize chances of encounter with potential predators and or dispersal agents of fruit (e.g. frugivorous birds and vertebrates), while a short pupal period is hypothesized to reduce exposure to soil based insect predators and specialized parasitoids (Zwolfer 1983, Fletcher 1989).

The teneral adults emerge from the puparia and tunnel their way out of the soil and fly into the foliage. Teneral adults are hypothesized to be governed by a strong endogenous rhythm for dispersal away from the emergence site as mechanism to overcome the density-dependent consequences of intraspecific competition (Fletcher 1974a, b). The prolonged adult phase places adult fruit flies at the mercy of variable abiotic factors such as moisture, temperature and light (Andrewartha and Birch 1984, Bateman 1968, 1972, Fletcher 1987). Adult flies are believed to compensate for

the variability in abiotic factors by seeking out microhabitats with relatively stable climatic conditions (Meats 1981, 1989a, b).

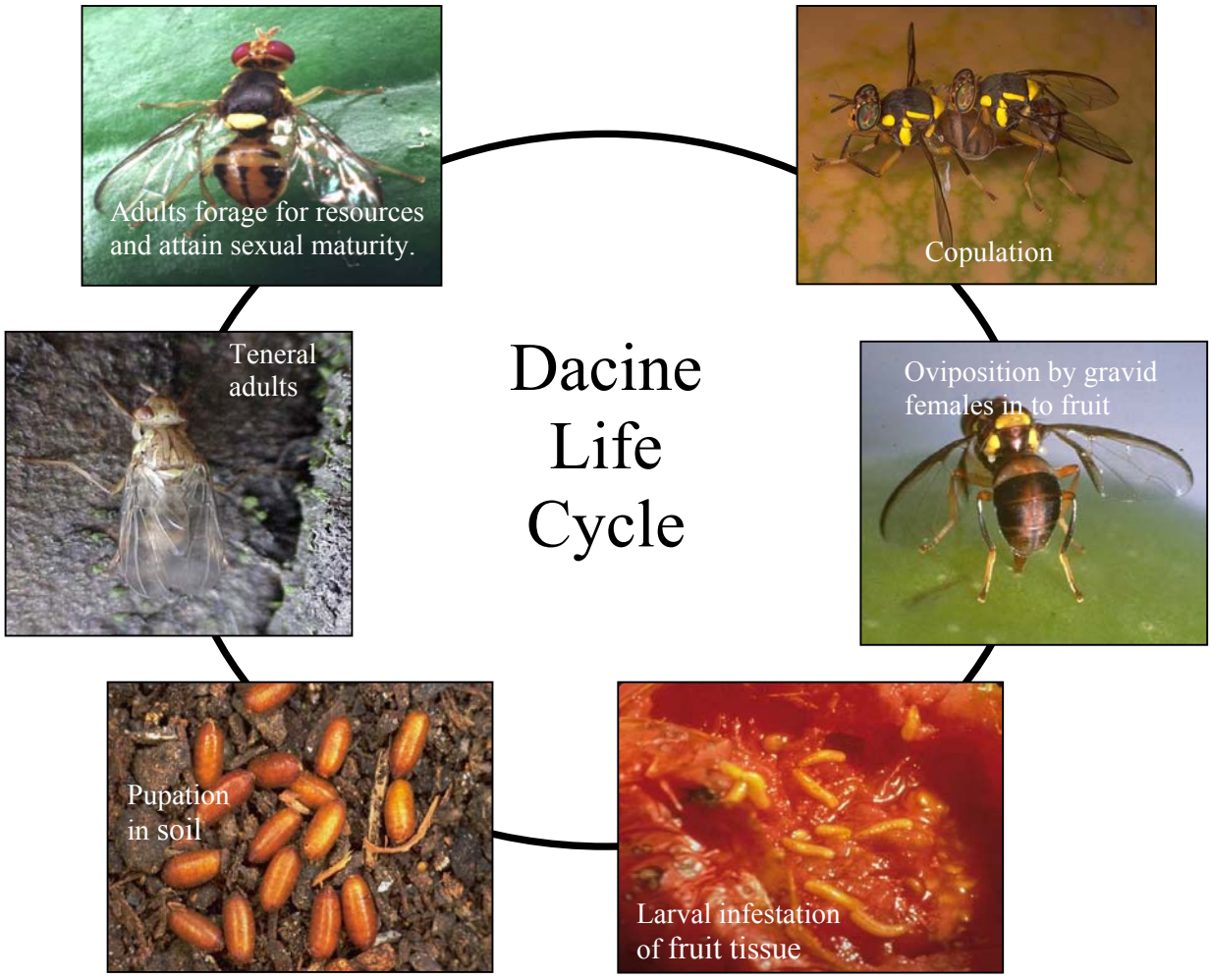

Figure 1.1. Generalized life cycle of dacine fruit flies.

Teneral adults require various resources to facilitate survival and reproduction. Key resources include moisture for metabolism, sugars for energy to sustain their highly mobile habit, protein to attain sexual maturity and, in conjunction with lipids, egg production (Fletcher 1987). Sugar sources include honeydew and other plant exudates. Protein is derived from sources such as phylloplane bacteria (Drew et al. 1983, Courtice and Drew 1984) and bird faeces (Bateman 1972, Fletcher 1987), while moisture is derived from dew and rain (Meats 1981). Adult flies forage for these resources in their environment, although lipids are possibly synthesized *de novo* (Warburg and Yuval 1996). In addition, adults may also actively seek out certain plant derived chemicals (e.g. methyl eugenol and raspberry ketone) (Metcalf 1990), that are hypothesized to pay a role in the mating behaviour of dacine species (Fitt 1981b, c, Nishida et al. 1988, 1993, 1997, Shelly and Dewire 1994, Tan and Nishida 1996, Shelly 2000)[2]. Once sexual maturity is attained, adult flies forage for a mate and copulation ensues. The resultant gravid female then re-initiates this cycle (Figure 1.1). Females generally amte only once in their life while males mate repeatedly (Barton-Browne 1957, Fay and Meats 1983, Mazomenos 1989).

Host use in frugivorous Dacinae is defined on the basis of the host plants female flies lay eggs in and that support larval development. In this respect most dacines are monophagous (predominantly utilizing a single host plant) or oligophagous (utilizing a group of closely related host plants) and the remaining (<1%) are truly polyphagous (Drew 1989b, Clarke et al. 2001).

[2] Whether flies actively forage for such plant derived chemicals, or are simply attracted to them if they chance upon them in the environment in unclear from the current literature. Results from Chapter 9 suggest that they may actively forage for them.

1.2.2. Resources for adult Dacinae

All biota require key resources to facilitate survival and reproduction (Begon et al. 1990). As mentioned above, adult fruit flies need to forage for sugars, protein, moisture and specific phytochemicals in their habitat. Resources for adult flies therefore have direct fitness consequences (Prokopy 1983, Zwolfer 1983) and in Andrewartha and Birch's (1984) *theory of environment* would be placed in the *centrum* of the environment of fruit flies. Therefore understanding "what constitutes these resources to adult fruit flies in nature?" and "what specific function these resources play in the ecology, physiology and behaviour of adults?" is critical to our understanding of fruit fly ecology.

The prevailing, but largely untested paradigm in dacine ecology, is that the host plant is the focal point of the entire life history of fruit flies, playing a role in larval and adult feeding, mating and oviposition (Prokopy 1983, Drew and Lloyd 1987, 1989, Metcalf 1990). With the exception of certain resources (e.g. methyl eugenol, raspberry ketone) fruit flies are believed to acquire all resources from the host plant. The host plant role is thought to be so critical that Drew and Lloyd (1987) labelled it as the "centre of activity". The predictability of finding the host plant (site of adult resources) in space and time and the quantity and quality of fruit at the host plant (larval resources), in association with abiotic factors, are therefore seen as key factors determining the life history strategies and ecology of dacine fruit flies (Fletcher 1989, Meats 1989a).

Given this view, the treatment of resources within dacine ecology has been principally in terms of their role in influencing population abundance through density dependent mechanisms (inter- and intra-specific competition, predation, parasitism) at the host plant (Carey 1989, Debouzie 1989, Fitt 1989, Fletcher 1989, Drew and Yuval 2000), an approach consistent

with population and community (=demographic) ecology (Tilman 1982). An alternative approach to studying resources is to understand how they are used and sought by individual flies and how these resources impact on the fitness of those individuals, i.e. an autecological approach. This is the approach that is followed in this thesis.

1.2.3. An autecological approach to the ecology of fruit flies

…an ecological study should begin with a general appreciation of the natural history, environmental physiology and behaviour of the animal so far as it is known or can be observed.

Andrewartha and Birch (1954, 1984)

Understanding of the ecology of a species is contingent upon knowledge of the effects of various biotic and abiotic factors on individuals (Andrewartha and Birch 1954, 1984). Demographic approaches in ecology deal with the implications of such factors on abundance (*sensu lato* population dynamics), often at an abstract, theoretical level, principally to explain *patterns* observed in nature (Tilman 1982, Begon et al. 1990, Peters 1991). As a result the functional/ mechanistic significance of species-specific interactions with components of their environment (i.e. *process*) remains largely unexplored or insufficiently integrated into their ecology (Hengeveld and Walter 1999, Walter and Hengeveld 2000). This is, in part, due to the complexity of the functional relationships between organisms and their environment. This situation is further complicated in fields such as entomology where numbers are often easier to record than species-specific processes and where the motivation for much research has been the development of strategies for suppression of populations (i.e. abundance) of pest species (Price 1997, Huffaker and Gutierrez 1999).

Explorations of species-specific physiological and behavioural processes have often been "relegated" to the discipline of natural history (Caughley 1994, Kareiva 1994, Shine 1994). Despite several critiques (Andrewartha 1984, Andrewartha and Birch 1954, 1984, Hengeveld 1989, Peters 1991, Hengeveld and Walter 1999, Walter and Hengeveld 2002) highlighting the significance of such process-centered autecological research to ecological theory and knowledge, it continues to remain a relatively insignificant component of contemporary ecology (Caughley 1994, Aarssen 1997, Lawton 1999, Murray 2000, Turchin 2001).

In this context, dacine ecology is no exception. As in other groups containing economic insects, the motivation for much dacine research has been the development of strategies for suppression of populations of pest species in their native and introduced zoogeographic ranges (e.g. Bateman et al. 1966a, b, Bateman 1968, 1972, Meats and Fay 1977, Fletcher 1987, 1989, Robinson and Hooper 1989a, b, Maelzer 1990a, b, Vijaysegaran and Ibrahim 1991). However, the basic understanding of key functional processes, vital to interpreting demographic patterns, remain enigmatic (Drew and Romig 2000).

After preliminary comparative studies on the population dynamics of dacine fruit flies in modified and natural habitats (Raghu et al. 2000, Raghu and Clarke 2001), my initial objective was to undertake a metapopulation approach to the ecology of fruit flies. However, this task proved impossible given the paucity of information on resource-use and functional significance of such resources in nature to fruit flies. This was compounded by a lack of resolution as to what aspects of the fly's environment constitute "resources". Delineation of "patch" and "habitat", critical to developing and (more significantly) understanding metapopulation models (Hanski and Gilpin, 1991, 1997), was unfeasible. As a result, any interpretation from such a

demographic study would essentially be speculative in the context of dacine ecology (Raghu 1997, Raghu and Clarke 2001).

Furthermore, much of the published research has been confined to the economically significant pest species in orchard environments, often outside their natural/ endemic distribution range (Raghu 1997, Drew and Romig 2000). Studies in natural systems in which the Dacinae are hypothesized to have evolved (e.g. rainforests) are rare (Drew et al. 1984, Zalucki et al. 1984). Therefore inferences about dacines in general are frequently speculative extrapolations, with untested generality.

In order to move away from a speculative/ descriptive to an explanatory/ interpretive understanding of dacine ecology, I revised the objective of my thesis to investigate the functional significance of resources identified in nature to individuals (i.e. *autecology sensu* Hengeveld and Walter 1999, Walter and Hengeveld 2000) of the fruit fly species *Bactrocera cacuminata* (Hering). Specifically, I focused on the adult phase of the life history, a phase that can be difficult to study in nature because of the mobility of individuals.

1.3 THE STUDY ORGANISM – *BACTROCERA CACUMINATA* (HERING)

Bactrocera cacuminata (Hering) (Diptera: Tephritidae: Dacinae) is native to Australia and is almost exclusively monophagous[3] on *Solanum mauritianum* Scopoli (wild tobacco) (Drew, 1989b). It is a non-pest species of the *B. dorsalis* complex, a group that includes pests of worldwide economic significance (e.g. *B. dorsalis* (Hendel) [Oriental fruit fly] and *B. papayae* Drew & Hancock [Asian papaya fruit fly]) (Drew and Hancock 1994). *Solanum mauritianum* is naturalized and widespread in eastern Australia, having been introduced

[3] Two other hosts (*Elaeocarpus* sp. and *Disoxylum* sp.) of *B. cacuminata* have been recorded (Drew 1989b), but these have a restricted distribution in northern Queensland. Outside this area, the fly is considered truly monophagous on *Solanum mauritianum*.

from South America, via the Portugese trade routes in Asia, sometime during 16th or 17th centuries, i.e. before European colonization (Roe, 1972). A medium to large shrub, *S. mauritianum* is an early succession plant that typically grows as a part of riparian vegetation along rainforest edges (Symon, 1981).

The following three key features dictated the choice of this study organism. Firstly, *B. cacuminata* is a non-pest species and therefore offers a system by which the generality of hypotheses/ theories extrapolated from pest species can be explicitly examined. Secondly, for nearly all of its geographic range, it is a monophagous species with females ovipositing only in the fruit of *Solanum mauritianum.* This makes the fly relatively immune to the complexities posed by studies on polyphagous species with their associated spatial and temporal patterns of host use. Finally, both *B. cacuminata* and *S. mauriritianum* are highly abundant in the area of study, a feature that facilitates field experimentation.

1.4 STRUCTURE OF THE THESIS/ THESIS OUTLINE

This thesis explicitly examines specific hypotheses of dacine ecology and behaviour to test their generality and, more specifically, their validity in the context of the dacine species, *Bactrocera cacuminata*. As mentioned above, these hypotheses have been generated from studies on very few polyphagous pest species in agricultural or semi-natural systems, with those results extended across the entire Dacinae (e.g. Bateman 1972, Nishida 1980, Fletcher 1987, Drew and Romig 2000). The thesis chapters, while structurally independent, have as a common thread the objective of resolving the ecology of *B. cacuminata* through an autecological approach. This is achieved by focussing on components of the fly's environment that could, or have been, regarded as resources vital to the survival and reproduction of this species.

Each chapter's introduction contains a brief review of the relevant literature, placing it within the respective conceptual framework. Contents of the research chapters are in various stages of consideration in scientific journals, with some already published or accepted for publication. All research chapters, regardless of their stage in the publication process, have been written as if for journal publication. As a result I do not present a General Materials and Methods chapter as the materials and methods section in each chapter explains the methodology in sufficient detail. The only general method common to all chapters is the rearing of flies. I followed the general procedures outlined in Heather and Cochran (1985) to rear flies for experimental work in this thesis.

A logical starting point to an autecological approach to dacine ecology would be to test the prevailing paradigm directing research i.e. the notion that the host plant (defined as the plant in which female fruit flies lay eggs) is the "centre of activity" of fruit flies (Drew and Lloyd 1987). The host plant is thought to be a critical resource in the dacine biology (see Section 1.2.2) and dacine – host plant interactions are hypothesized to be mediated through specific bacteria, referred to as "fruit fly type bacteria" (Drew and Lloyd 1987, 1989, 1991). This hypothesis was developed based on detailed studies of the polyphagous pest species *B. tryoni* (Lloyd 1991). In Chapter 2, I examine the validity of the "host plant as the centre of activity" hypothesis for *B. cacuminata* and its host plant *S. mauritianum* in a natural system, the riparian edge of a lowland rainforest. I further investigated the role of other biotic (host plant architecture) and abiotic characterisitics (microclimatic variables) of the host plant on the abundance and behaviour of *B. cacuminata* (Chapter 3). Results from Chapters 2 and 3 indicated that the host plant is primarily a larval resource and female oviposition site for *B. cacuminata*, but appears to play little further role in the ecology of the fly.

A paucity of most adult behaviours on the host plant raised the question: What is the physiological status of those adults that were on the host plant and those at other environmental resources? I address these questions in Chapters 4 and 5. Chapter 4 is a methodological chapter in which I use the rate of growth of the male ejaculatory apodeme and female ovarian development to develop predictive methods by which the age of flies trapped in the field could be assessed. Using these methods, I examined the differences in physiological status of flies coming to different resources in the field (e.g. host plant, methyl eugenol [a plant-derived lure to which male *B. cacuminata* respond] and protein [a limiting resource]) in Chapter 5. In addition to assessing the age structure of the flies at the host plant, the nutrient reserves of flies at different resources were also assessed to determine the energetic status of individuals at the host plant.

The paucity of adult behaviours, other than oviposition, at the host plant forced a reconsideration of the "nature" of adult resources in the context of this species. Given that *B. cacumintata* is an abundant, multivoltine species, the absence of mating behaviour on the host plant was particularly striking (see Chapters 2, 3). I therefore examined the role of resources important for reproduction. Chapters 6, 7 and 8 examine the role of one such resource considered in the literature to be significant, methyl eugenol, a chemical that is botanical in origin. It has been hypothesized to play a role in the pheromone system of dacine fruit flies, with sexual selection via the Fisherian runaway selection model invoked to explain the strong attractancy of male dacine flies to lures (Shelly 2000). This hypothesis and associated expectations of dacine behaviour were tested by investigating the feeding behaviour (Chapter 6) and associated mating consequences of feeding on methyl eugenol in *B. cacuminata* (Chapter 7). Since attraction to methyl eugenol has been classed as *pharmacophagous* (Tan and Nishida 1998), I examined the physiological and survival consequences of feeding on methyl eugenol, to *B. cacuminata* (Chapter 8).

Given that variation in physiological status of flies at different resources was found (Chapter 5) and that the purported role of methyl eugenol in the mating behaviour of dacines did not appear to hold true for *B. cacuminata*, I hypothesized that adult flies may partition their behaviour spatially and temporally between different resources as a function of their physiological status (as has been reported for other insect species; e.g. Wiklund, 1977). In particular, Metcalf (1990) hypothesized that phytochemicals such as methyl eugenol function as a mate aggregation stimulus. I ran a field-cage experiment to explicitly test this hypothesis in Chapter 9.

The results of Chapters 6-9 indicated a strong need to re-think the ecological and evolutionary basis for attractancy of dacine fruit flies to lures. Therefore, in Chapter 10, I present a synthetic review exploring the ecological and evolutionary basis for the attraction of dacine fruit flies to lures, integrating information from biochemistry with ecological and evolutionary knowledge. This chapter highlights the inter-disciplinary approach needed to resolve the enigma of the role of the plant-derived lures in dacine ecology and evolution.

In the final chapter (Chapter 11) I highlight the key conclusions from this study and discuss the implications of my findings to prevailing approaches in dacine and tephritid ecology and explore avenues for further research.

Chapter Two

Microbial mediation of dacine – host plant interaction: Is the host plant the "centre of activity"?

This chapter has been published in a slightly modified form:

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2.1 INTRODUCTION

Plants are heterogeneous resources to which insects have adapted to satisfy the requirements of their survival and reproduction (Wratten et al. 1988, Maschinski and Whitham 1989, Mayhew 1997). Such adaptation may involve the exploitation of microorganisms. Due to their rapid reproduction and unique metabolic capabilities, microorganisms can mediate insect – plant interactions by their capacity for rapid adaptation to plant resource heterogeneity (Jones 1984, Barbosa et al. 1991).

Such symbiotic interactions are particularly well developed and studied in social insects, such as termites and ants. Most termites rely on endosymbionts to aid cellulose digestion, while many ants rely of fungal symbionts for nutrition (Waller and LaFage, 1985, Holldobler and Wilson, 1990). In the termite species *Reticulitermes speratus,* association with its bacterial symbionts is so highly specialized that in addition to facilitating cellulose digestion, colony specific associations with bacteria are formed and are often the cue by which nestmates are recognized (Matsuura 2001). In certain ant species such symbiotic associations attain a new level of complexity with the ants not only utilizing fungal gardens, but also antibiotic producing bacteria to deal with parasites of the fungal gardens (Currie et al. 1999a,b, Wilkinson 1999). However, not all hypothesized mutualistic/ symbiotic interactions conform to theoretical expectations of mutualism (Wilkinson and Sherratt, 2001). The specific ecological role of symbionts in non-social insects is often difficult to resolve. In this study I examine one such system, where the relationship between the insect and plant host is reputed to be mediated by a specific bacterial interaction.

The family Tephritidae (Diptera), or true fruit flies, comprises some 4500 species world-wide in tropical, subtropical and temperate regions. One

subfamily, the Dacinae, consists of over 700 species that are believed to be endemic to tropical and subtropical rainforests (Drew 1989a, b, Thompson 1998). Larval dacines utilize fleshy fruits as their food resource and several species (e.g. *Bactrocera dorsalis* [Hendel], *B. papayae* Drew and Hancock, *B. cucurbitae* [Coquillett]) are major pests. With respect to the larval host plant, most species are monophagous, a few are oligophagous, while the remaining <1% are polyphagous (Drew 1989b).

The larval host plant is believed to be the focal point in dacine biology, ecology and behaviour, playing a part in adult and larval feeding, mating and oviposition (Prokopy 1983, Drew and Lloyd 1987, 1989, Metcalf 1990). So important is the host plant thought to be, that Drew and Lloyd (1987) coined the phrase "centre of activity" to describe the role of host plant to dacine fruit flies. While the larval host plant as a site for oviposition and larval feeding is self-obvious, the role of the host plant in other activities is more complex and is dependent on a hypothesis that bacteria play a major role in the biology and ecology of adult fruit flies.

Whilst there is considerable inconsistency in the literature on the precise role of bacteria, specifically Enterobacteriaceae, in dacine ecology (Fitt and O'Brien 1985, Lloyd et al. 1986, Drew and Lloyd 1987, 1989, 1991, Prokopy et al. 1991), the most parsimonious mechanism of bacterial mediation is hypothesized to occur in the following sequence.

(a) Gravid female flies are attracted to fruit bearing host plants by visual and olfactory cues for the purpose of oviposition.
(b) Pre-ovipositional foraging (which included regurgitation and "bubbling behaviour") by gravid female flies on the fruit and leaf surfaces results in the inoculation of "fruit fly- type" bacteria (viz. *Klebsiella oxytoca*, *Erwinia herbicola*, *Enterobacter cloacae*) on those surfaces.
(c) Fruit fly-type bacteria spread on the plant surface utilizing nutrients from plant leachates.

(d) Bacterial metabolites associated with such phylloplane colonies attract immature/ teneral adult flies.
(e) Attracted immature flies utilize bacteria as food (protein source) to attain sexual maturity. And
(f) Mating ensues amongst such bacteria-fed mature flies.

The cycle thus continues with the resultant mated females searching and finding fruiting hosts for oviposition (Prokopy et al. 1991). A point of critical importance is that the host plant supposedly serves as the hub for every step in the process.

Predictions that result from this hypothesized mechanism include:

(i) Until gravid females are attracted to fruit for the purpose of oviposition, there are few fruit-fly type bacteria present on the host plant and hence immature flies are not attracted to it.
(ii) Host plants that have never borne fruit will not be attractive to fruit flies, but plants that have fruited and are now without fruit should still carry fruit fly – type bacteria and so be attractive to (at least) immature flies.
(iii) All behaviours exhibited by fruit flies (resting, feeding, oviposition, courtship and mating) should predominantly be restricted to host plants bearing fruit.
(iv) The sex ratio of flies on the fruiting host plant should approximately be 1:1 as this is believed to be the primary sex ratio (Drew and Hooper 1983).

The objective of the current study was to test the above expectations.

Commonly, ecological generalizations made about dacine fruit flies are based on orchard studies of polyphagous, pestiferous species on exotic host plants, outside the endemic range of the fly (see for example Bateman

1972, Nishida 1980, Fletcher 1987). Seldom have such generalizations been tested using fruit fly species within their natural range of distribution, on natural/wild hosts. The utilization of cultivated, exotic hosts may be quite different to what occurs on natural or "primary hosts" of insect species (Fitt 1986, Walter and Benfield 1994). Therefore, understanding the behaviour of fruit flies in relation to their natural hosts is critical to our full understanding of their ecology, including interactions with possible mediating influences such as bacteria. Hence I investigated the above issues using a non-pest, monophagous fruit fly species and its natural host in a natural habitat.

2.2 MATERIALS AND METHODS

2.2.1. Natural history

Bactrocera cacuminata (Hering) is a dacine fruit fly (Diptera: Tephritidae: Dacinae) native to Australia, that is almost exclusively monophagous on *Solanum mauritianum* Scopoli (wild tobacco) (Drew 1989b). *B. cacuminata* is s multivoltine species with overlapping generations. It is a non-pest species of the *B. dorsalis* complex, a group that includes pests of worldwide economic significance (e.g. *B. dorsalis*, *B. papayae*) (Drew 1989b). *Solanum mauritianum* is naturalized and widespread in eastern Australia, having been introduced from South America, via the Portugese trade routes in Asia, sometime during 16th or 17th centuries, i.e. before European colonization (Roe 1972). A medium to large shrub, *S. mauritianum* is an early succession plant that typically grows as a part of riparian vegetation along rainforest edges (Symon 1981). It fruits year round in south eastern Queensland. The aforementioned fruit fly-type bacteria (see Introduction) have been purported to mediate the interaction between *B. cacuminata* and *S. mauritianum* (Lloyd et al. 1986, Drew and Lloyd 1989).

2.2.2. Study site

South-east Queensland is sub-tropical in climate and originally contained large tracts of coastal and subcoastal rainforests (Beard 2001). The study site was located within a large training area maintained by the Australian Army at Canungra (28°01'S 152°09'E), in the Gold Coast hinterland. The vegetation is largely undisturbed and is representative of rainforest/edge systems in which *S. mauritianum* typically occurs. Along one creek line, where *S. mauritianum* was growing naturally intermixed with other riparian vegetation, twelve groups of three neighbouring plants (2-3 metres apart) were chosen. Each group of three plants (a replicate) consisted of two fruiting and one non-fruiting plant. Frequent visits to the field site were made to strip floral structures to ensure that the plants designated as "non-fruiting" did not bear fruit. Twenty-four hours prior to behavioural observations, one of the fruiting plants was completely stripped of all its fruit. Therefore, for each of the twelve replicates, there was one host plant of each of three different states viz. host that had never borne fruit, host with fruit, and host with fruit removed. The logic of the three treatments was that a never fruited host should offer neither bacterial nor oviposition resources to flies, a fruiting host should offer both bacterial and oviposition resources, while the fruit-removed plant should have bacterial resources, but not oviposition resources.

2.2.3. Behavioural studies

Predictions from the hypothesized mechanism of bacterial mediation suggest that the entire suite of fruit fly behaviours would be observed on fruiting host plants. With the exception of oviposition, all other behaviours should be equally frequent in host plants with fruit removed, while non-fruiting host plants should be unattractive to fruit flies and hence no adults will be seen visiting them (other than those expected by chance).

The specific behaviours I observed are standard behaviours previously defined in the tephritid literature (Malavasi et al. 1983, Hendrichs et al. 1991). They include;

(a) Resting = a stationary fly with minimal movement with the exception of occasional cleaning.

(b) Feeding = arrestment with repetitive lowering of the proboscis to touch the surface on which the fly was standing.

(c) Ovipositing = insertion of ovipositor into a fruit.

(d) Calling = conspicuous presence of a clear droplet of pheromone everted from the anal gland of a male fly (Nation 1981).

(e) Male aggregation = an aggregation of at least three males calling simultaneously on adjacent leaves with an estimated distance of no greater than 10-15 cm between neighbouring males. This behaviour has been referred to as a lekking in behavioural studies of other fruit flies (Hendrichs et al. 1991). Since there is no prior evidence of a lek-based mating system in *B. cacuminata* this behaviour is referred to as male aggregation in the present study.

(f) Mating = a male and female fly in copulation. Mating is restricted to dusk in this species (Myers 1952).

Examination of diurnal patterns in these behaviours were made with observations commencing at 0600 (dawn) and ending at 1900 hours (full night). During a focussed observation period of five minutes per plant per hour, the entire host plant was scanned and the number of individuals engaging in the different behaviours recorded. Each day, one replicate was observed, with all observations made during December 1999. This period was selected as previous studies (Drew and Hooper 1983, Drew et al. 1984) had shown it to be one of high abundance of *B. cacuminata*.

2.2.4. Trapping and fruit dissection

Trapping using male-specific parapheromones is an established method of assessing populations of fruit flies (Cunningham 1989a, b). In order to help establish the size of the background fly population at the study site, 20 Steiner traps, baited with 4 ml of methyl eugenol (ME) and 1 ml of malathion, were distributed around the area where behavioural observations were made. Ten of the traps were suspended from host vegetation and the other ten were suspended from non-host plants that were located at least 200 metres from wild tobacco host plants (approximate attractancy radius of Steiner traps = 50-100 metres; Cunningham 1989b). Traps were serviced daily, for a fortnight, and numbers of *B. cacuminata* recorded. Trapping was done after the completion of all behavioural observations to prevent any interference between the traps and the host plant stimuli during behavioural observations.

Mature fruits were collected during the period of trapping. One-hundred and nine fruits, all greater than 1cm diameter, were dissected. This size was selected because this appears to be the threshold diameter above which maggots are found in wild tobacco fruit (S. Raghu - unpublished data). Fruit were dissected to ascertain natural levels of fruit fly infestation and to corroborate trapping data in establishment of the presence and size of the background population of *B. cacuminata*.

2.2.5. Microbiological assays

Predictions from the microbial mediation hypothesis (see Introduction) suggest that fruit fly-type bacteria should only be present on fruiting host plants. Hence, of the three host plant treatments used for behavioural observations, one would expect only the fruiting and fruit-removed treatments to have fruit fly – type Enterobacteriaceae. Microbiological assays were done to test the validity of this hypothesis. For each plant in each of the

12 replicates, ten leaf discs of 3 cm diameter were singly collected in sterile tubes from the cluster of leaves immediately surrounding the fruit, or where the fruit had been in the case of the fruit-removed treatment. No more than one disc was collected from each leaf. In the case of the never-fruited treatment, the ten leaf discs were sampled from various parts of the plant. In addition, ten fruit (of varying stages of ripening) from each plant in the "with-fruit" treatments were collected into sterile tubes. All these samples were collected within one week after behavioural observations.

All ten leaf discs from each plant were transferred into a sterile container in the laboratory. Ten ml of peptone water was added to this container and the container was agitated using a mechanical stirrer to wash the phylloplane bacteria off into the nutrient peptone water (Oxoid Peptone H_20 CM9, Oxoid Ltd., Basingstoke, Hampshire, England). This resultant solution was taken to be the stock solution. This was diluted by 10^{-1} and 10^{-2} in sterile water. A similar procedure was adopted for the fruit.

One hundred micro litres (0.1 ml) of each of the three dilutions (stock, 10^{-1}, 10^{-2}) were plated onto MacConkey Agar (CM109, Oxoid Ltd., Basingstoke, Hampshire, England) plates that permit growth of gram negative bacteria. All plating was done in duplicate. All plates were incubated aerobically at 30^oC for 36 hours. Following a qualitative assessment, only the plates from the 10^{-2} dilutions were used for subsequent sub-culturing. Numbers of the each of the different colony types on plates were counted using a colony counter (Applethorn Pty. Ltd., Australia). If the number of colonies was <300 then entire plate counts were made. If >300 colonies were present on the plate, based on a visual estimate, then four randomly allocated squares of 1 cm^2 were counted and used to estimate number of colonies over the entire plate.

Each of the colony types were sub-cultured to obtain pure stock cultures. All bacterial isolates were initially Gram-stained and oxidase/ catalase activity and oxidative fermentation (O/F) tests were performed. The Enterobacteriaceae can be separated from other common environmental microbes (e.g. *Pseudomonas*) as they are oxidase negative, catalase positive and fermentative (Brenner 1992). Subsequently, their specific status was determined using the API-20E system for Enterobacteriaceae (Cat#20 100, bioMerieux sa 69280, Marcy-l' Etoile, France). The organisms isolated that did not belong to this family were not further characterized. Motility was determined by growing organisms in a motility medium (peptone 10g, distilled water 1 litre; incubation duration 30°C for 12 hours) followed by microscopic examination of hanging-drop preparations. The APILAB Plus (v3.3.3) identification database was used to identify the bacterial species based on the biochemical profile. The microbiological assay methods used in the current study are similar to prior pathological (Dillon and Charney 1995, 1996), ecological (Fitt and O' Brien 1985, Lloyd et al. 1986, Drew and Lloyd 1987, Lauzon et al. 1998) and evolutionary (Howard et al. 1985) studies.

Based on previous work (Lloyd et al. 1986, Drew and Lloyd 1987), the "fruit fly type" bacteria that I focussed on was *Enterobacter cloacae*, *Klebsiella oxytoca* and *Erwinia herbicola*. Since earlier studies, *E. herbicola* has been synonymized into the Genus *Pantoea* (Ewing and Fife 1972, Brenner et al. 1984, Beji et al. 1988, Gavini et al. 1989). Hence I also included *Pantoea* species in the analysis.

2.2.6. Data analysis

Prior to the analysis, all data were tested for heteroscedasticity using Levene's test. A one-way analysis of variance (ANOVA) was used to analyse the differences in daily numbers of flies of each sex present on the different host states. A similar analysis was done on different behaviours observed

with respect to host plant status. Where the assumptions of the ANOVA were violated (i.e. variance was heterogeneous in spite of standard transformations), the non-parametric equivalent, Kruskal-Wallis test used instead. The trapping data was also analysed using an ANOVA with vegetation in which the trap was suspended as the factor. Data (number of fruit fly-type bacterial colonies) from the microbiological assays were analysed using a one-way ANOVA with host status as the factor. In the analysis of the microbial colony counts, the fruiting bodies were included as a treatment as the females are hypothesised to be attracted to the fruit prior to their inoculation of the phylloplane with bacteria (see Introduction). When the analyses revealed significant differences, specific differences between pairs of means were tested with least significant differences (LSD) tests for homoscedastic data and with Games-Howell tests for heteroscedastic data (Zar 1999).

2.3 RESULTS

2.3.1. Number of flies vs. Host status

The number of male flies on the host plant were far fewer than the number of female flies (♂:♀ = 75:423; summed over all treatments and replicates). The daily number of male flies present on the host differed significantly in relation to the host status ($F_{2,33}$ = 4.071, P = 0.026, $\log_{10}(x+1)$ transformed data). Host plants with fruit had a higher number of males than those that had never fruited (Figure 2.1a; P = 0.008), while there was no significant difference between hosts with fruit removed and hosts with fruit (Figure 2.1a; P = 0.074) or hosts that had never fruited (Figure 2.1a; P = 0.343). The daily number of female flies present on the host also differed with respect to host status ($F_{2,33}$ = 71.889, P < 0.001, $\log_{10}(x+1)$ transformed data). Post hoc LSD tests revealed that all three host states differed significantly from each other in terms of the number of female flies present on them (Figure 2.1b).

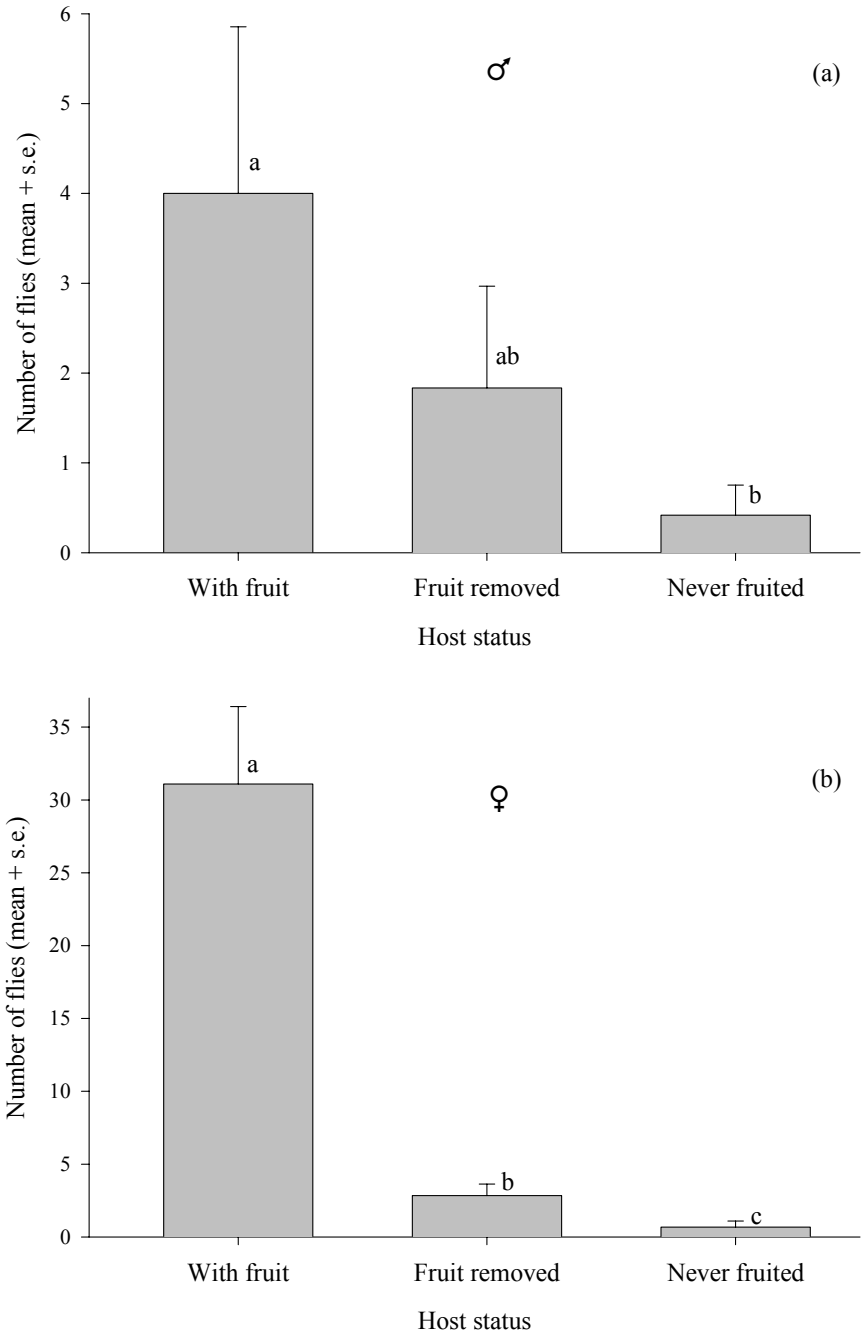


Figure 2.1. Distribution of *B. cacuminata* in relation to the fruiting status of its larval host plant, *S. mauritianum* (a) male flies (b) female flies. (*N*=12 for both graphs; Bars with same letters adjacent to them are not significantly different as indicated by post hoc pairwise comparisons).

2.3.2. Behaviours vs. Host status

Only feeding, resting and oviposition behaviours were observed during the course of the experiment. Male aggregation, courtship and mating behaviour were not observed. In addition these behaviours were also not seen during fortnightly casual observations at dusk (1700 - 1900 h), over an eight-month period (August 1999 to April 2000), across five different sites (S. Raghu - unpublished data).

Of the three observed behaviours, feeding was the least common. The number of males and females engaged in this behaviour differed significantly in relation to host status (Figure 2.2a, b; Male – Kruskal-Wallis H = 8.368, df = 2, P = 0.015; Females – Kruskal-Wallis H = 14.692, df =2, P = 0.001). Post hoc comparisons revealed that the number of females feeding on host plants with fruit differed significantly from the fruit-removed and never-fruited host states (Figure 2.2b; Games-Howell test, P = 0.008 and P = 0.008 respectively). There was no difference in the number of females feeding between the fruit-removed and never-fruited host states (Figure 2.2b). Similar post hoc comparisons for males did not reveal any significant differences in spite of such differences indicated by the Kruskal-Wallis test. This is possibly due to the weakness of the non-parametric post hoc test rather than any lack of difference between treatments. Graphical interpretation (Figure 2.2a) reveals that host plants with fruit have a higher number of feeding males than the other two states.

Resting was most common on host plants with fruit, and there was a greater number of females engaged in this behaviour than males (Figures 2.2c, d). The number of resting males did not differ significantly between host states (Figure 2.2c; $F_{2,33}$ = 2.383, P = 0.108, , $\log_{10}(x+1)$ transformed data), while the number of resting females did differ significantly (Figure 2.2d; $F_{2,33}$ = 32.732, P < 0.001, $\log_{10}(x+1)$ transformed data). Post hoc LSD tests revealed

that all three host states differed significantly in terms of number of resting females (Figure 2.2d).

A total of 216 oviposition events were recorded during the course of the study, with flies ovipositing more frequently into unripe, than ripe fruit.

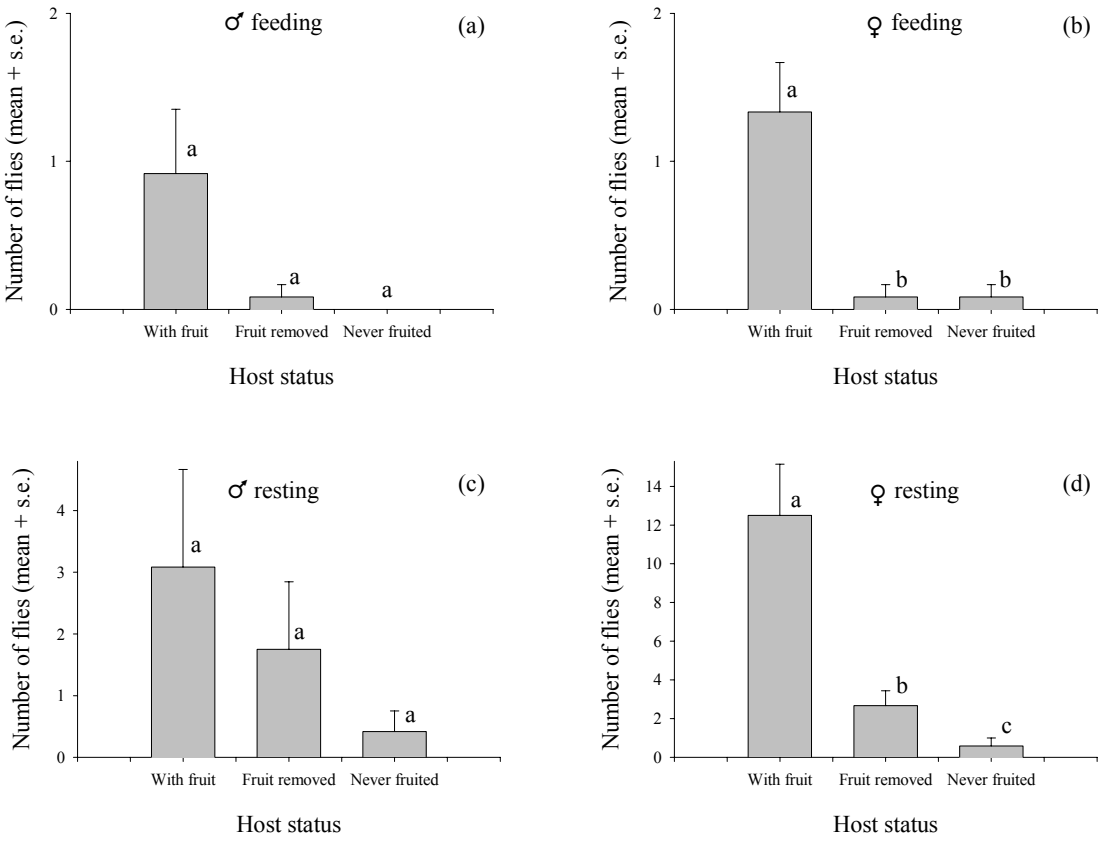


Figure 2.2. Distribution of behaviours of *B. cacuminata* in relation to fruiting status of its larval host plant, *S. mauritianum* (a) male- feeding behaviour (b) female- feeding behaviour (c) male- resting behaviour (d) female- resting behaviour (*N*=12 for all graphs; Bars with same letters adjacent to them are not significantly different as indicated by post hoc pairwise comparisons).

2.3.3. Diurnal patterns in behaviour

The presence of male flies on the host plant was both erratic and relatively uncommon compared to the presence of female flies (Figures 2.3a, b). At no time of day, on any treatment, was there an average of more than one male per plant. The greatest number of males were found between 0900-1000h and 1300-1400h in the with-fruit treatment (Figure 2.3a). In the fruit-removed treatment, the abundance of males was lower and the peak periods were between 1100-1200h and 1400-1500h. The never-fruited state had very few male flies (Figure 2.3a). No male flies were observed to be present on the host plant at dusk (1700-1900h). Female flies were largely restricted to the host plants with fruit (Figure 2.3b) and peaked between 1100-1200h and 1600-1700h.

There was no evident diurnal pattern in the feeding behaviour of either male or female flies (Figure 2.4a). Very few flies were observed to be feeding on the host plant during the study. Resting was the most common of behaviours. The number of resting males peaked between 0900-1000h and then gradually declined over the day. The number of resting females on the other hand was bimodal (Figure 2.4b) with a major peak between 1100-1200h and one to a lesser degree between 1400-1500h and then declining gradually. Oviposition behaviour by females increased sharply from about midday and peaked between 1600-1700h, gradually declining at dusk (Figure 2.4c).

2.3.4. Trapping and fruit dissection

In all 23,078 male flies were trapped in the 16 day trapping period. There was no significant difference between the number of flies trapped in non-host vegetation (11,825) and those trapped in host vegetation (11,253) (Figure 2.5; $F_{1,18} = 0.090$, $P = 0.768$). The fruit dissection revealed that the natural level of infestation in the vicinity of the study site was 75% (N=109 fruits).

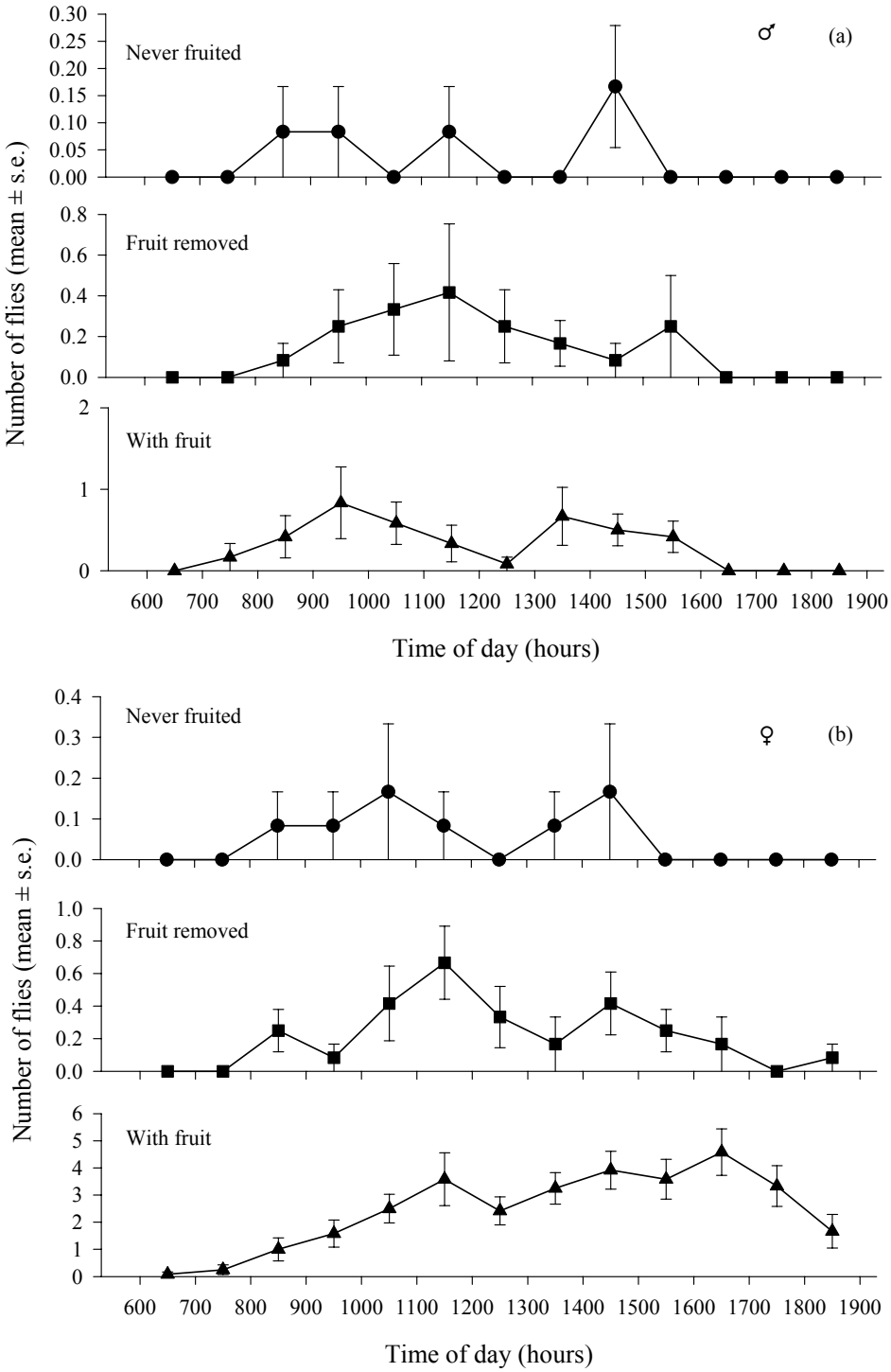


Figure 2.3. Diurnal patterns in distribution of *B. cacuminata* in relation to the fruiting status of its larval host plant, *S. mauritianum* (a) male (b) female (*N*=12 for both graphs).

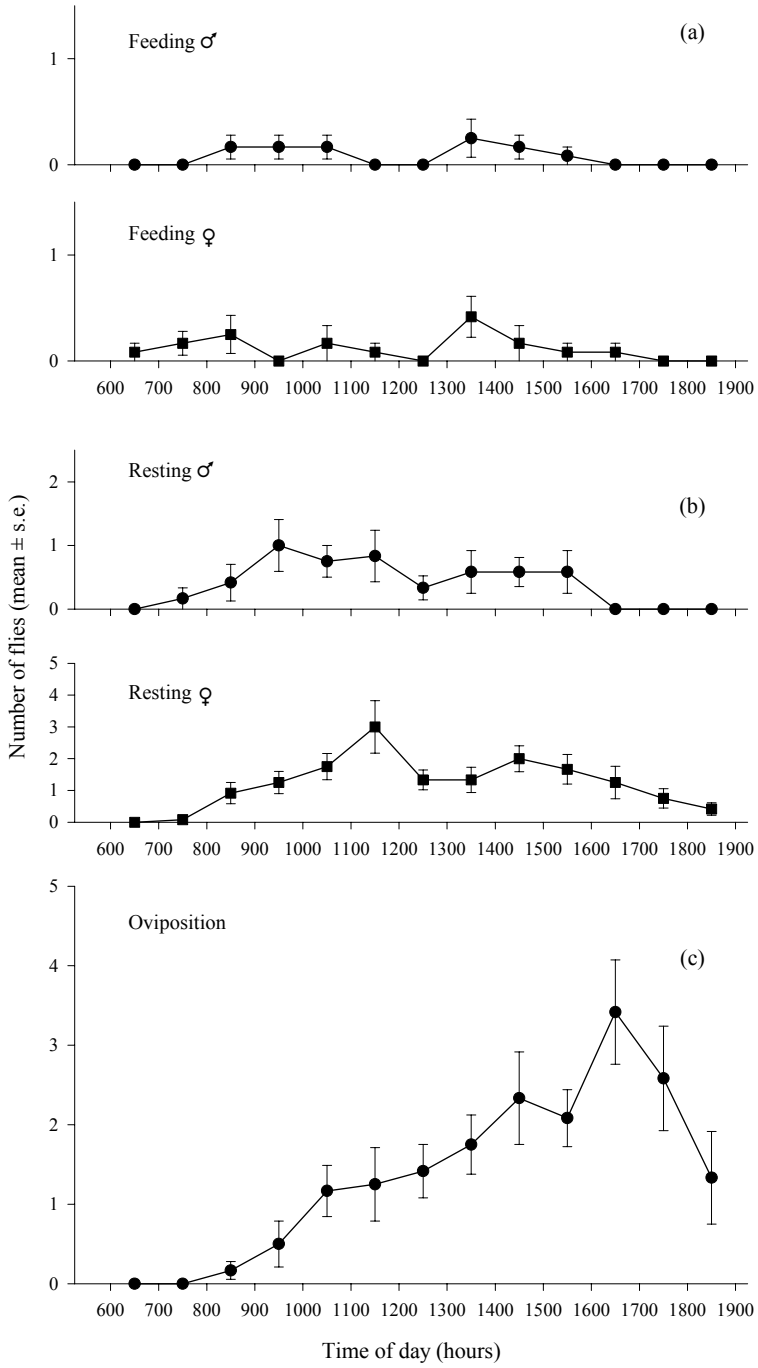


Figure 2.4. Diurnal patterns in behaviour *B. cacuminata* on fruiting larval host plants, *S. mauritianum* (a) feeding (b) resting (c) oviposition (*N*=12 for all graphs).

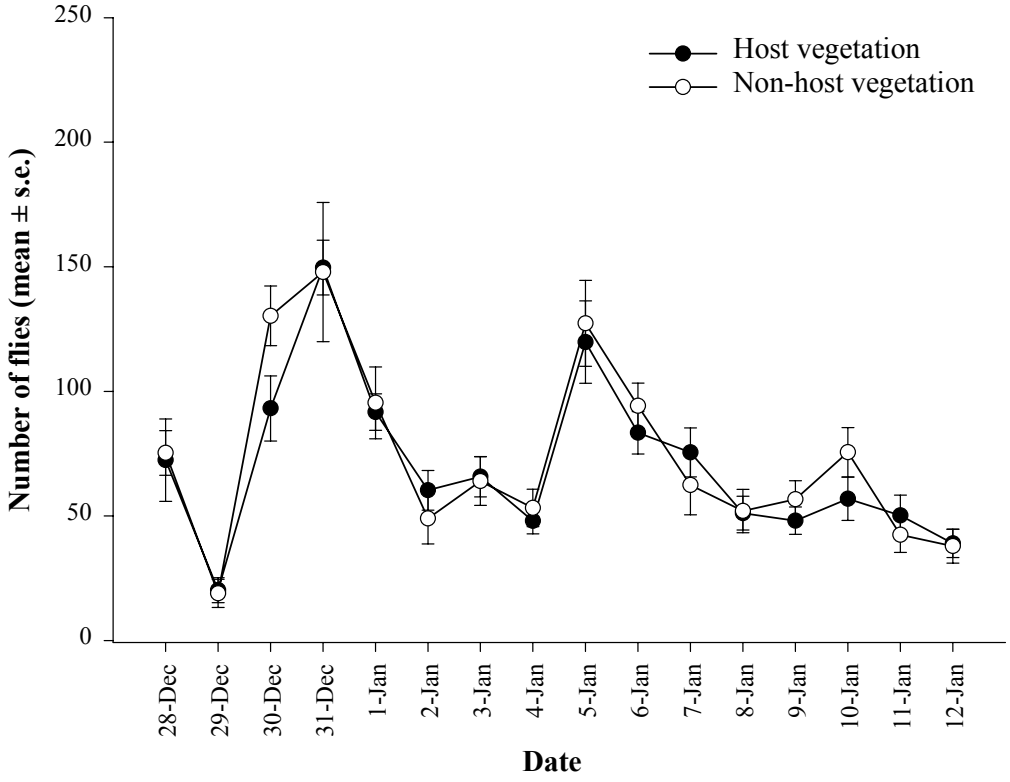


Figure 2.5. Number of male *B. cacuminata* trapped at host and non-host vegetation (*N*=10 traps in each vegetation type).

2.3.5. Microbiological assays

Klebsiella oxytoca and *E. cloacae* were rare on all three host states and on fruit. Hence no statistical analysis were done on their presence or abundance. *Pantoea* spp. 2 and 3 were the most abundant fruit fly-type bacteria and were present on all three host states, but were largely restricted to host plants in the with-fruit and fruit-removed treatments. Pantoea spp. 3. was more abundant on host plants that had never fruited in comparison to those with fruit removed. Both species were either rare or absent on fruit (Figure 2.6a, b). Both *Pantoea* spp. differed significantly between the host states/ structures (*Pantoea* spp. 2 – Kruskal-Wallis H = 10.650, df = 3, P = 0.014; *Pantoea* spp. 3 – Kruskal-Wallis H = 9.059, df = 3, P = 0.029).

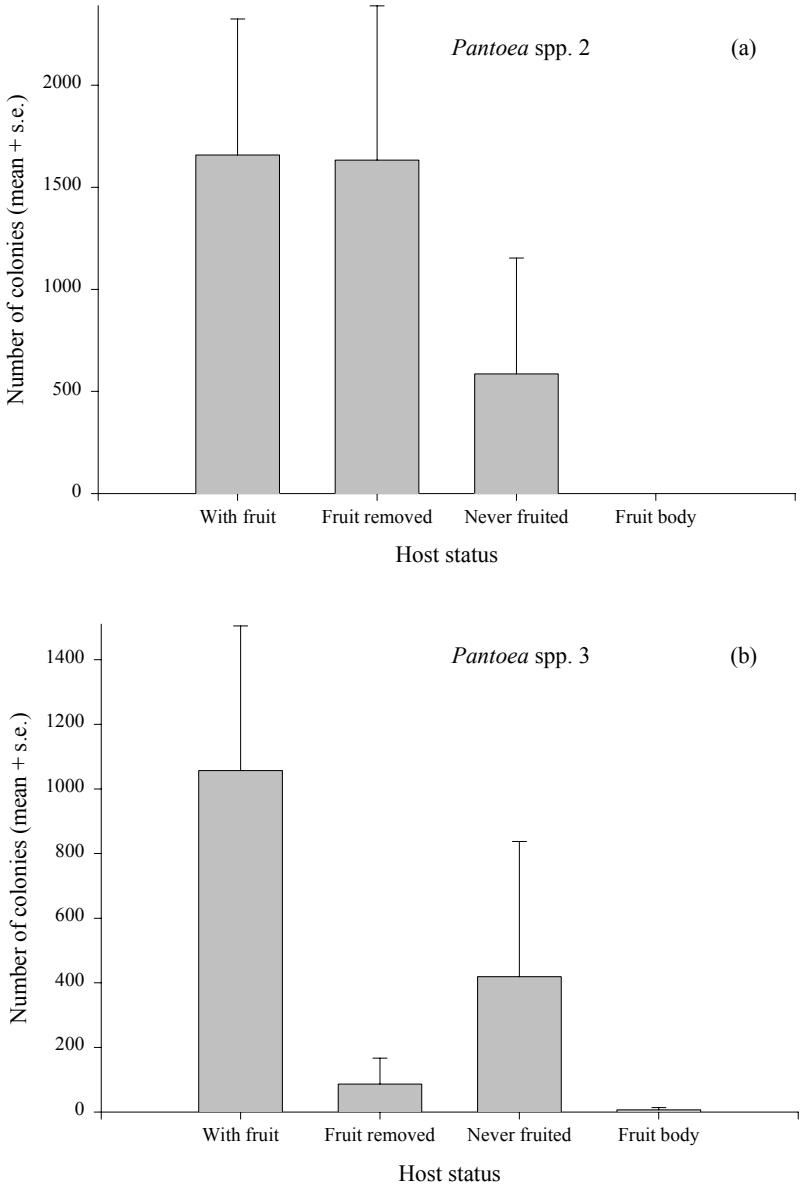


Figure 2.6. Distribution of fruit fly – type bacteria in relation to the fruiting status of *S. mauritianum* (a) *Pantoea* spp. 2, (b) *Pantoea* spp. 3 (*N*=12 for both graphs).

2.4 DISCUSSION

The results of this study present both positive and negative evidence for Drew and Lloyd's (1987, 1989, 1991) hypothesis on fruit fly - host plant interactions. The phylloplane microflora data offer evidence that substantiates the hypothesis partly (supports parts a-c of the hypothesis outlined in the Introduction), whilst the insect behaviour data constitute contrary evidence (does not support parts d-f). I deal with these issues individually in the following sections.

The microbiological assays of the Enterobacteriaceae associated with the phylloplane of *S. mauritianum* reveal that two of the fruit fly-type bacteria viz. *K. oxytoca*, *E. cloacae* were either rare or absent. The data for *Pantoea* spp. 2 showed patterns that are expected if Drew and Lloyd's hypothesis is true (Figure 2.6a) while *Pantoea* spp. 3 (formerly *E. herbicola*) did not. *Pantoea* spp. 2 was relatively abundant on with-fruit or fruit-removed treatments, but rare or absent on never-fruited plants. However, an interesting finding is that the fruit itself, to which bacteria-spreading gravid females are supposedly attracted, had only a very small complement of only one of these bacteria species (*Pantoea* spp. 3, Figure 2.6b). The general trend in these data is consistent with the view that gravid females may be moving bacteria onto plants to which they come to oviposit.

While the microbiological assay results are consistent with the hypothesis that ovipositing females are responsible for the spread of *Pantoea* spp. on fruiting host plants, the behavioral observations are not. Bacteria are believed to serve as food for sexually immature flies (Drew et al. 1983, Courtice and Drew 1984, Lloyd et al. 1986, Drew and Lloyd 1987, 1989, 1991) and if this were the case one would expect a significant number of flies to be feeding on the fruiting, or fruit-removed host plants. Contrary to this expectation, observations of flies feeding were rare (Figure 2.2a, b). Whilst

the numbers of flies feeding differed significantly between the host plants with fruit and the other two host states, the paucity of this behaviour is striking. Also, I suspect that more flies were feeding on the with-fruit plants simply because there were more flies on those plants (primarily ovipositing females), rather than because of any microbial mediation.

Another key result that is counter to Drew and Lloyd's hypothesis is the absence of male aggregation, courtship and mating behaviours on the host plants. This absence of mating is not unique to this study site. Preliminary observations prior to these experiments hinted at the absence of mating on host plants. Therefore, observations for mating behaviour of *B. cacuminata* were made on a regularly two-weekly basis between August 1999 and March 2000 at patches of *S. mauritianum*. Sites for these observations were selected haphazardly and observations were made at dusk (1600 to 1900h). In spite of this effort, no mating behaviour was observed. Hence the validity of the claim that mating occurs on the host plant is in doubt for this fly species. Fletcher (1987) indicated that this could be the case in some dacine species.

One explanation for the rarity or absence of feeding and mating behaviours on the host plant could be that our background population at the study site was very low and I missed these behaviours by chance. However, the trapping data (23,078 *B. cacuminata* trapped over the fortnight following the behaviour observations; Figure 2.5) and the natural fruit infestation levels (75%) indicate the presence of a large population.

These findings are anomalous in light of expectations from currently hypothesized microbial mediation of dacine – host plant interactions (see Introduction). In light of the current results the physiological status of adult flies on the larval host plants needs to be ascertained to establish both their age and mating status of females (i.e. mated or unmated). An alternate

explanation for the rarity of feeding behaviour on the host plant could be that females arriving at the host plant have already fed elsewhere in the environment and subsequently arrive at the host plant for oviposition only. I examine this possibility both by physiological analyses and dissections of individuals at the host plant (Chapter 5). If only mated female flies are coming to host plants for the purpose of oviposition, as the current study indicates, the site of mating must be elsewhere in the habitat. Spatial partitioning of feeding, oviposition and mating sites in not uncommon in insects (Wiklund 1977) and this may explain the paucity of behaviours, other than oviposition, observed for this fruit fly. Should mating sites be identified away from the larval host plant, the results will not only contradict the "host plant as centre of activity" model, but also the Prokopy-Burk model of fruit fly mating strategies (Prokopy 1980, Burk 1981), which suggests that monophagous fruit fly species mate on the larval host plant.

While I observed no fly behaviors consistent with the hypothesis of bacteria mediated host-plant interactions, it remains that fruit fly type bacteria were found on fruiting hosts and rarely on never-fruited hosts. The most likely hypothesis that may explain this pattern, without invoking any underlying mutualism, is the incidental spread of bacteria by ovipositing flies (Fitt and O'Brien 1985). While fruit flies use certain species of bacteria as food (Drew et al. 1983), the three species of Enterobacteriaceae that are believed to be associated with fruit flies are common in the gut of animals (Brenner 1992, Grimont and Grimont 1992, Grimont et al. 1992). The fruits of *S. mauritianum* are dispersed by birds (Crome 1975, Symon 1979) and bird faeces are common on the leaves of fruiting wild tobacco plants (personal observation). Fruit flies, including *B. cacuminata,* feed on bird faeces (Hendrichs et al. 1991, personal observation), or the bacteria it contains (Lauzon et al. 1998), and through such feeding flies would imbibe and subsequently spread, through defecation and/or crop regurgitation, those gut bacteria. Such an explanation is consistent with the occasional presence

of large concentrations of these bacteria on host plants that had never borne fruit (Figure 2.6). Bacterial establishment on larval host plants then becomes a purely incidental aspect of female flies coming to oviposit on the plant, rather than a specific mechanism of resource enhancement.

Acknowledgments – I thank D. Lynch and Colonel W.T. Bowen for enabling the fieldwork to be conducted on the premises of the Land Warfare Centre, Canungra, Queensland. I also thank Dr. D. Teakle, Dr. C. Hayward and Dr. H. Stratton for interesting discussions during the course of this research.

Chapter Three

Effect of host plant structure and microclimate on the abundance and behaviour of *Bactrocera cacuminata*

This chapter has been submitted for review in a slightly modified form:

Raghu, S., Clarke, A.R. and Drew, R.A.I. Influence of host plant structure and microclimate on the abundance and behaviour of a tephritid fly. *Journal of Insect Behaviour* (in review).

3.1 INTRODUCTION

Various factors influence the interactions of insects with their host plants. These include biotic factors such as structural and physiological attributes of the host plant (Lawton 1983, Juniper and Southwood 1986, Steinbauer et al. 1998) and the presence of conspecifics, predators and parasitoids (Janssen et al. 1997, Pallini et al. 1997). In addition, abiotic factors such as temperature, relative humidity and light intensity (Willmer 1982, Kaspi and Yuval 1999) influence insect plant interactions. The relative importance of these biotic and abiotic variables differs with respect to the insect-host plant system under scrutiny.

The influence of host plant structural traits on insects has predominantly focused on questions of abundance and/or diversity of species assemblages within a plant species (Haysom and Coulson 1998), or across plant species (Lawton 1978, 1983, Neuvonen and Niemalä 1981, Peeters et al. 2001). The variation in abundance and behaviour of a single insect species in relation to the architecture and associated microclimate of a single host plant species has been less frequently examined (Willmer 1982, Juniper and Southwood 1986, Steinbauer et al. 1998).

Tephritid fruit flies (Diptera: Tephritidae) are considered to have close evolutionary and ecological associations with their larval host plants (Prokopy 1983, Drew 1989). Host plant attributes, and the associated microclimate, are therefore expected to have a significant influence on the abundance and behaviour of fruit flies (Prokopy and Hendrichs 1979, Kaspi and Yuval 1999). For the tephritid subfamily Dacinae, the fly/larval host plant relationship is thought to be particularly strong as the host plant is considered central to larval and adult feeding, mating and oviposition (Prokopy 1983, Drew and Lloyd 1987, 1991, Metcalf 1990). However, what if any role, plant structure and microclimate play, in the host plant interactions

of the Dacinae have never been explored. In this study I investigated the influence of microclimate and aspects of host plant architecture on the abundance and behaviour of a dacine fruit fly. The wild tobacco plant *Solanum mauritianum* Scopoli and its associated dacine species *Bactrocera cacuminata* (Hering) (Diptera: Tephritidae) was the system I examined. This fly species is almost exclusively monophagous on *S. mauritianum* (Drew 1989b) and hence serves as an ideal system to investigate the influence of host plant characteristics and associated abiotic factors on the behaviour of a fly species.

3.2 MATERIALS AND METHODS

The first of two studies was carried out along a rainforest edge in Canungra (28°01'S 152°09'E), Queensland (described in Chapter 2). *Solanum mauritianum* occurs naturally at this site as a part of the riparian vegetation. Observations of the presence of this fruit fly species and associated behaviours were made commencing at 0600 (dawn) and ending at 1900 (full night) (= 13 observations/day). Twelve days of observations were made, each day on a different mature, fruiting host plant.

The specific behaviours I scanned for were resting, feeding, ovipositing, calling, male aggregation and mating. These are standard behaviours defined in the fruit fly literature (Malavasi et al. 1983, Hendrichs et al. 1991, Chapter 2). During a focussed observation period of five minutes per plant per hour, the entire host plant was scanned and the number of individuals engaged in the different behaviours recorded. During each observation period microclimate variables, including temperature (°C), relative humidity (%) and light intensity (lux) were recorded adjacent to the canopy of the plant. Temperature was recorded using a temperature sensor (AIRFLOW Instrumentation, DVA6000T) and light intensity with a digital light meter (Lutron, LX-101). Relative humidity was estimated using a wet

and dry bulb thermometer and standard tables. In addition, the host plant structural characters height, number of branches and number of leaves were recorded.

The data were analysed using multiple regression. Multicollinearity (correlation between independent variables) in multiple regression analyses results in unstable and unreliable partial regression coefficients of the correlated variables (Sen and Srivastava 1990, Chatterjee and Price 1991, Draper and Smith 1998). In this study the number of branches and number of leaves were significantly correlated (r=0.903, P<0.001) and hence could not be used as reliable predictors in the regression model. Therefore a foliage density index was calculated (foliage density = number of leaves/ number of branches) and used as a surrogate measure of these host plant characters. Therefore the independent variables used in the multiple regression models and path analyses were foliage density and height of host plant (i.e. host plant attributes) and temperature, relative humidity and light intensity (i.e. microclimate variables). Examination of tolerance and variance inflation factors of these independent variables revealed that the multicollinearity was sufficiently below acceptable norms (Chatterjee and Price 1991, Draper and Smith 1998) so as to allow their use in multiple regression analyses effectively. Dependent variables in the path analysis included number of males and females and the number of individuals of each sex engaged in the different behaviours as a proportion of total number of individuals observed on the host plants.

The significance level was preset at 0.05. The results are presented in the form of path diagrams. In path diagrams single headed arrows link independent variables (tail) to dependent variables (head). The number adjacent to the arrow represents the standardised partial regression coefficient. Double headed arrows represent correlations between independent variables and the number adjacent to these arrows represent

correlation coefficients. The error term R_e is estimated as $R_e = \sqrt{(1-R^2)}$ (Li 1975, Matsuki and MacLean 1994). For a detailed discussion on the method of path analysis the reader is referred to Li (1975), Matsuki and MacLean (1994), Shipley (1997) and Ozaki (2000).

The second study was undertaken in a disturbed habitat along a creek in Brisbane (27°28' S, 153°2'E). In the first study of this pair, no matings were observed (Chapter 2). However, preliminary observations at the second site had revealed some on-plant matings, albeit rare and restricted to certain host plants. To ascertain if plants where mating was occuring showed any particular traits, I measured abiotic and biotic variables (as above) at 12 *S. mauritianum* plants and noted the number of different flies and the different behaviours they were engaged in during the dusk photophase (1730-1930h), the mating time of *B. cacuminata* (Myers 1952). Principal component analyses were undertaken to determine if plants where flies mated were different in any way from those where mating behaviour was not observed. The principal components were rotated (orthogonal varimax) to simplify the structure and maintain independence of the components (Quinn and Keough 2002).

3.3 RESULTS

In the first study only resting and oviposition behaviours were observed (Chapter 2) and there was a significantly greater total number of female flies on the host plant than male flies (♂:♀ = 75:423). The overall regression model(s) testing the effects of microclimate and host characteristics on the abundance and behaviours of *B. cacuminata* were significant in all cases except the feeding behaviour of female flies (Table 3.1). The multiple regression models explain 16.7% and 35.2% in the variation in abundance of male and female *B. cacuminata* at the host plant. The independent variables explain 8.3% and 4% of the variability in feeding behaviour of males and

females respectively and 7.3% of the variability in the total number of flies exhibiting this behaviour (Table 3.1). This relatively small amount of variation in feeding behaviour, explained by the measured biotic and abiotic variables, is possibly a result of the rarity of this behaviour at the host plant. Therefore further interpretation of the regression model for this behaviour is tenuous and I do not discuss this further.

The explanatory variables account for 23.8% and 35.1% in the variability in resting behaviour of male and female *B. cacuminata* respectively and 39.8% of the variability in the total number of flies resting on the host plant. Twenty three percent of the variance in female oviposition behaviour is explained by the independent variables (Table 3.1).

Table 3.1. Summary of regression analyses of the effect of microclimate variables (temperature, relative humidity, light intensity) and host plant structural characteristics (host plant height, foliage density) on different dependent variables (n = 156 for all analyses).

Dependent Variable	R^2	F	Probability
No. of male flies present	0.167	6.032	<0.001
No. of female flies present	0.352	16.325	<0.001
No. of male flies resting	0.238	9.372	<0.001
No. of female flies resting	0.351	16.260	<0.001
No. of flies resting	0.398	19.861	<0.001
No. of male flies feeding	0.083	2.699	0.023
No. of female flies feeding	0.040	1.244	0.291
No. of flies feeding	0.073	2.356	0.043
No. of female flies ovipositing	0.230	8.969	<0.001

3.3.1. Effects of host plant attributes on *Bactrocera cacuminata* behaviour and abundance

The number of resting males and females was positively influenced by foliage density (Figures 3.1a, b) and this trend is reflected in the influence of the plant characteristics on total number of flies resting on the host plant (Figure 3.1c).

The density of the foliage on the host plant had a significant positive effect on the number of ovipositing female *B. cacuminata* (Figure 3.2a). The density of the foliage was the only significant positive predictor among the host plant attributes on the overall abundance of males and females on the host plant (Figures 3.2b, c).

3.3.2. Effects of microclimate on *Bactrocera cacuminata* behaviour and abundance

The number of resting male and female *B. cacuminata* was positively influenced by light intensity (Figures 3.1a, b). In addition, temperature positively affects the numbers of resting females at the host plant (Figure 3.1b). The effect of microclimate variables on the abundance of total number of resting individuals was identical to their effects on number of female flies (Figure 3.1c). This may be an artefact of the fact that there were many more female flies at the host plant than male flies.

Temperature had a significant positive effect on the abundance of ovipositing female flies, while the number of ovipositing females increased with declining light intensity and relative humidity (Figure 3.2a). The abundance of male *B. cacuminata* at the host plant was positively influenced by light intensity while the abundance of females was positively affected by

temperature and negatively influenced by relative humidity (Figures 3.2b and 3.2c).

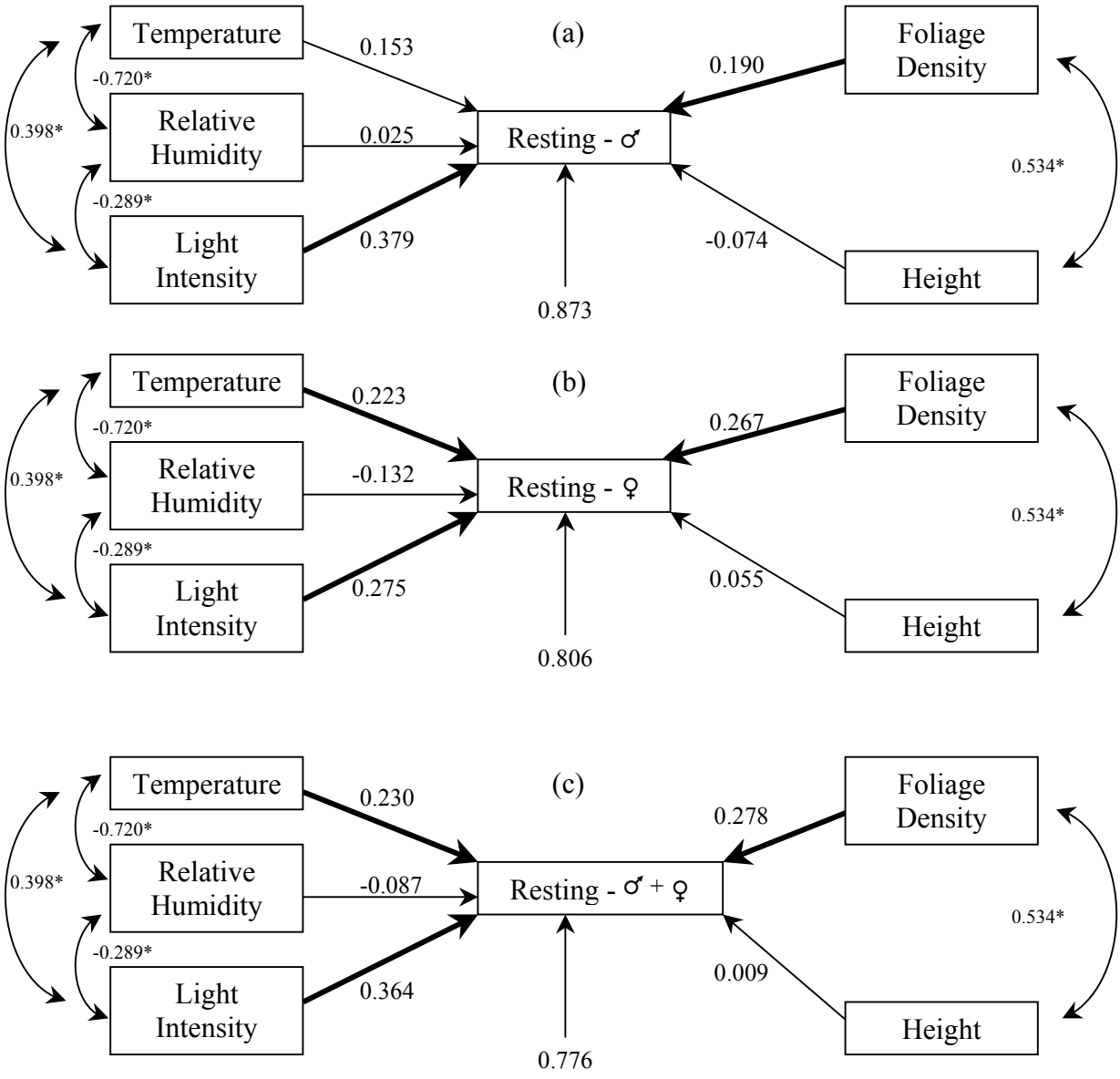


Figure 3.1. Path diagrams showing effects of host plant attributes and microclimate on the numbers of *Bactrocera cacuminata* "resting" on the host plant, *Solanum mauritianum* (a) Male flies, (b) Female flies and (c) Total number of resting flies (males + females). Bold arrows represent statistically significant (P<0.05) path coefficients. * Indicates statistically significant (P<0.05) correlation coefficients. The single headed arrow directed at the dependent variable from below represents the error term (R_e).

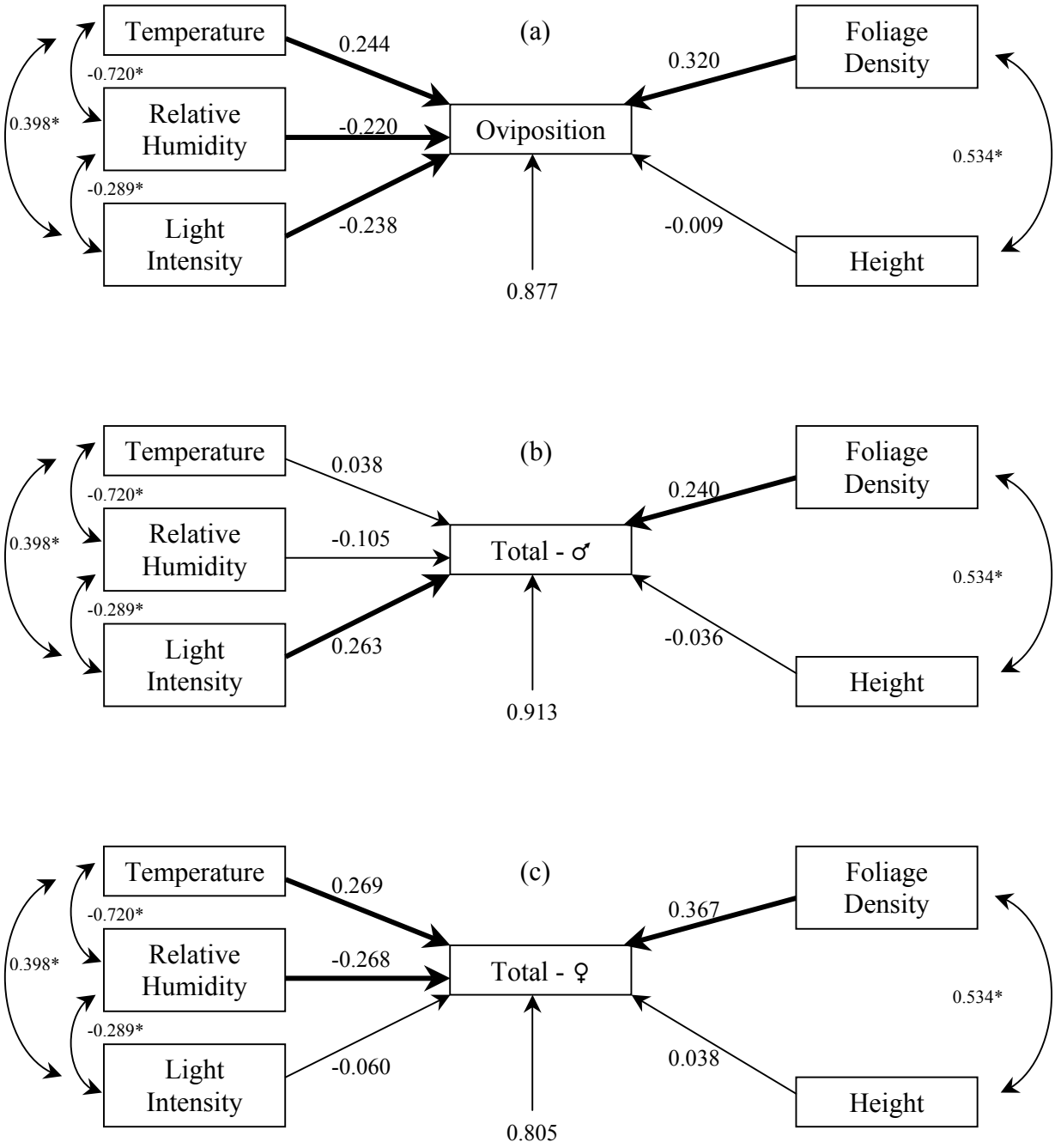


Figure 3.2. Path diagrams showing effects of host plant attributes and microclimate on the oviposition behaviour and abundance of *Bactrocera cacuminata* on the host plant *Solanum mauritianum* (a) Oviposition behaviour, (b) Total number of male flies and (c) Total number of female flies. Bold arrows represent statistically significant (P<0.05) path coefficients. * Indicates statistically significant (P<0.05) correlation coefficients. The single headed arrow directed at the dependent variable from below represents the error term (R_e).

3.3.3. Microclimate and structural attributes determining selection of mating site

In the second study, a total of five mating pairs were recorded over the entire observation period and all of these were restricted to one of the host plants (Plant number 7, Figure 3.3). Ordination analyses revealed that the first three principal components explained 82.834% of the variation in microclimate and plant structure variables (Figure 3.3, Table 3.2). Each of the variables loads strongly on only one of the principal components (Table 3.2). Foliage density, temperature and relative humidity were positively correlated with Principal Component (PC) 1, while fruit was positively correlated with PC3. Height of the plant was positively correlated with PC2, while light intensity was negatively correlated with the same principal component (Table 3.2). The only site where mating was observed differed from the other sites in the plant being taller, bearing more fruit and having an intermediate light intensity at dusk in comparison to the other plants surveyed (Figure 3.3).

Table 3.2. Rotated (Varimax) factor loadings of the microclimate and plant structure variables. Number is brackets represents proportion of variation explained by the principal component. Strong correlations of variable with principal components are highlighted in bold.

Variable	Principal Components		
	PC1 (35.428%)	PC2 (24.980%)	PC3 (22.425%)
Fruit	0.0386	0.0854	**0.937**
Foliage Density	**0.755**	-0.0587	0.422
Height	0.121	**0.808**	0.374
Light Intensity	0.0669	**-0.907**	0.141
Temperature	**0.818**	-0.0306	-0.335
Relative Humidity	**0.931**	0.110	0.135

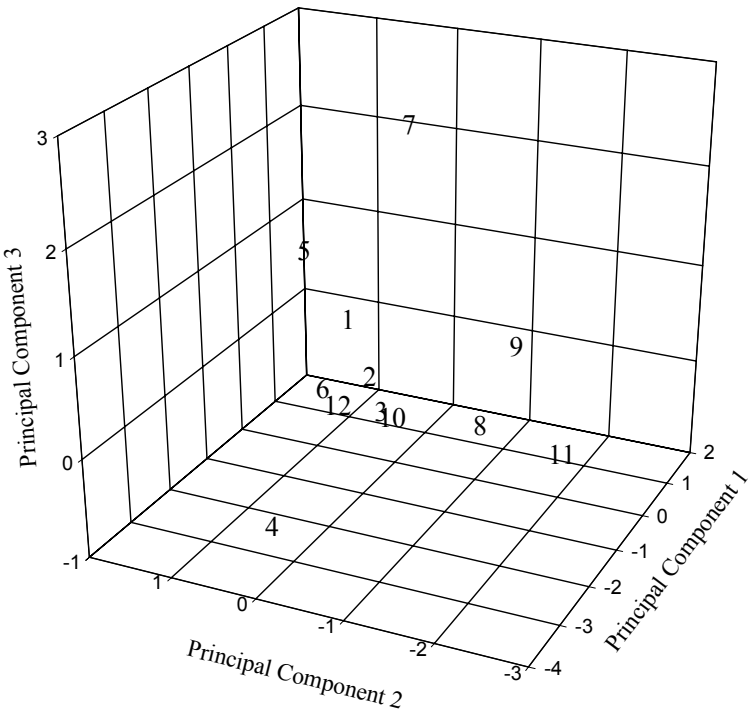


Figure 3.3. Plot of host plants (*Solanum mauritianum*) in the space defined by the first three principal components (see Table 3.2 for factor loadings of different variables). Numbers represent host plant identification numbers (see Results).

3.4 DISCUSSION

Abundance and behaviour of *B. cacuminata* on *S. mauritianum* are positively influenced by the density of foliage on the host plant (Figures 3.1, 3.2). The selection of host plants with dense foliage may shield flies from predation by airborne predators such as dragonflies (Fletcher and Prokopy 1991, Hendrichs et al. 1991). This may particularly be true in the case of females engaged in oviposition. In addition, dense foliage would also provide shelter from the elements. Tephritid flies are known to seek shaded and moist regions of the host plant with increasing temperature and declining relative humidity (Gibbs 1967, Meats 1981, Kaspi and Yuval 1999), a phenomenon common in insects (Willmer 1982).

Dacine fruit flies have a flight threshold temperature of approximately 20^{o}C, but once this threshold is reached the flies actively forage for resources and potential mates (Drew and Hooper 1983). This is consistent with the observations that numbers of flies at the host plant increases with an increase in temperature and light intensity and a decrease in relative humidity towards the middle of the day (Figures 3.1, 3.2). There was a significantly greater number of female *B. cacuminata* on the host plant than males. The diurnal pattern of oviposition peaks between midday and dusk (Chapter 2, Figure 2.4) and this is reflected in the significant effect of declining light intensity on the number of ovipositing females (Figure 3.2a). The rarity of feeding and the absence of mating behaviours on the host plant indicate that adults may encounter these resources (i.e. food and mates) away from the larval host plant. While this contrasts with some generalisations of dacine ecology (Prokopy 1980, Burk 1981, Prokopy et al. 1991), feeding and mating behaviours are reported to principally occur away from the host plant in other dacine species (Fletcher 1987, Fletcher and Prokopy 1991).

Microclimate and host plant characteristics are believed to be significant in the reproductive behaviour of polyphagous fruit flies mating at the host plant (Kaspi and Yuval 1999). The site where mating was observed (plant 7, Figure 3.3), differed from the other sights in the plant being taller, bearing more fruit and having an intermediate light intensity at dusk in comparison to the other plants. These characteristics may be what mature flies respond to in selecting a mating site. However, the paucity of mating (only five mating pairs, also see Chapter 2) observed over the course of the study warrants caution of inferring too much from the data. I also advocate caution in inferring this spatial restriction of mating behaviour as evidence for "lekking" (*sensu* Shelly and Whittier 1997) in this species for the following reasons. Firstly the rarity of mating behaviour associated with the host plant does not allow sufficiently strong inference on the nature of mating systems in this fly. Secondly, several aspects of the mating behaviour (Hoglund and Alatalo 1995) need to be explicitly examined prior to attributing a lek-based mating system to *B. cacuminata*.

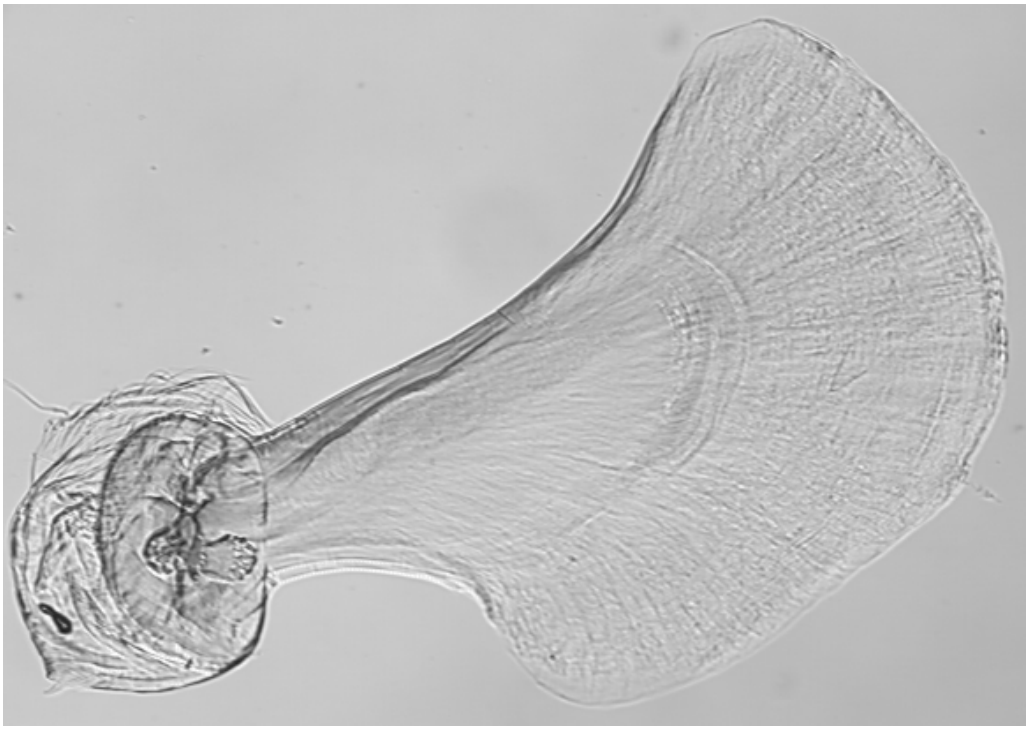
The present study shows that microhabitat variables may also influence behaviours other than reproduction, in natural systems. However, as indicated by the large R_e value in all path analyses, factors other than those measured may influence the abundance of *B. cacuminata* on *S. mauritianum* (Juniper and Southwood 1986). These could include olfactory (fruit odours, lures) and visual (foliage colour, reflectance of leaves and fruit) cues, that have been suggested as being important in host detection and host selection (Fletcher and Prokopy 1991, Dalby-Ball and Meats 2000a, b). Many of these studies, however, have investigated the role of such cues on adult behaviour in laboratory or glasshouse environments, with numbers of male and female flies. The differences between the numbers of male and female *B. cacuminata* and the paucity of mating behaviour on the host plant suggests that, at least in this species, resource use and associated behaviours may

differ between the sexes. Future experimentation needs to take such natural densities into account when investigating dacine – host plant relationships.

Acknowledgments – I thank Don Lynch and Colonel William T. Bowen for enabling the fieldwork to be conducted on the premises of the Land Warfare Centre Canungra, Queensland. I also thank Mamoru Matsuki for valuable discussions and suggestions on the data analysis used in this study.

Chapter Four

Apodeme and ovarian development as predictors of physiological status in *Bactrocera cacuminata*



This chapter has been accepted for publication in a slightly modified form:

Raghu, S., Halcoop, P. and Drew, R.A.I. 2003. Apodeme and ovarian development as predictors of physiological status in *Bactrocera cacuminata* (Hering) (Diptera: Tephritidae). *Australian Journal of Entomology* (in press).

4.1 INTRODUCTION

Resource use in insects is determined by intrinsic factors such as age and physiological status (Chapman 1998). Field based ecological research often requires tools so that such intrinsic factors can be identified. In fruit flies (Diptera: Tephritidae: Dacinae) the host plant has been hypothesized to be the centre of activity (Prokopy 1983, Drew and Lloyd 1987, 1989, Metcalf 1990), playing a pivotal role in all larval and adult life stages. However, a recent study of the wild tobacco fly, *Bactrocera cacuminata* (Hering), has revealed that in this species not all key adult behaviours (e.g. mating, feeding) occur on the host plant (Raghu et al. 2002, Chapter 2). This study raised the question of the physiological status of flies that were observed at the larval host plant, but suitable methodological tools to address the question were not available for this species. Developing a method by which flies of different physiological ages can be identified is therefore critical to the further development of this research. This was the motivation behind the present study.

The reproductive endoskeleton of the Dacinae is suspended internally from the anterior wall of abdominal segment 9 as an ectodermal cuticular invagination of this segment (Drew 1969, Bitsch and Bitsch 2002). The ejaculatory apodeme is a part of the erecting and pumping organ and serves as a point of attachment for the musculature of the pumping organ, facilitating the transfer of the seminal fluid down the ejaculatory duct during copulation (Figure 4.1). Drew (1969) showed that the ejaculatory apodeme of *Bactrocera* (= *Strumeta*) *tryoni* (Froggatt) grew with age and argued that the use of male internal genitalia in taxonomic studies of the group would render species definitions imprecise. However, it was also evident from that study that such measurements of growth stages can be used as surrogate measures for physiological status for male *B. tryoni*. For female flies, Fletcher et al. (1978) demonstrated that ovarian development (Figure 4.2) could be

used as a reliable predictor of the physiological status of female olive fruit flies, *Bactrocera oleae* (Gmelin). Using these two studies as guides I develop similar methods for determining the physiological age of *B. cacuminata*.

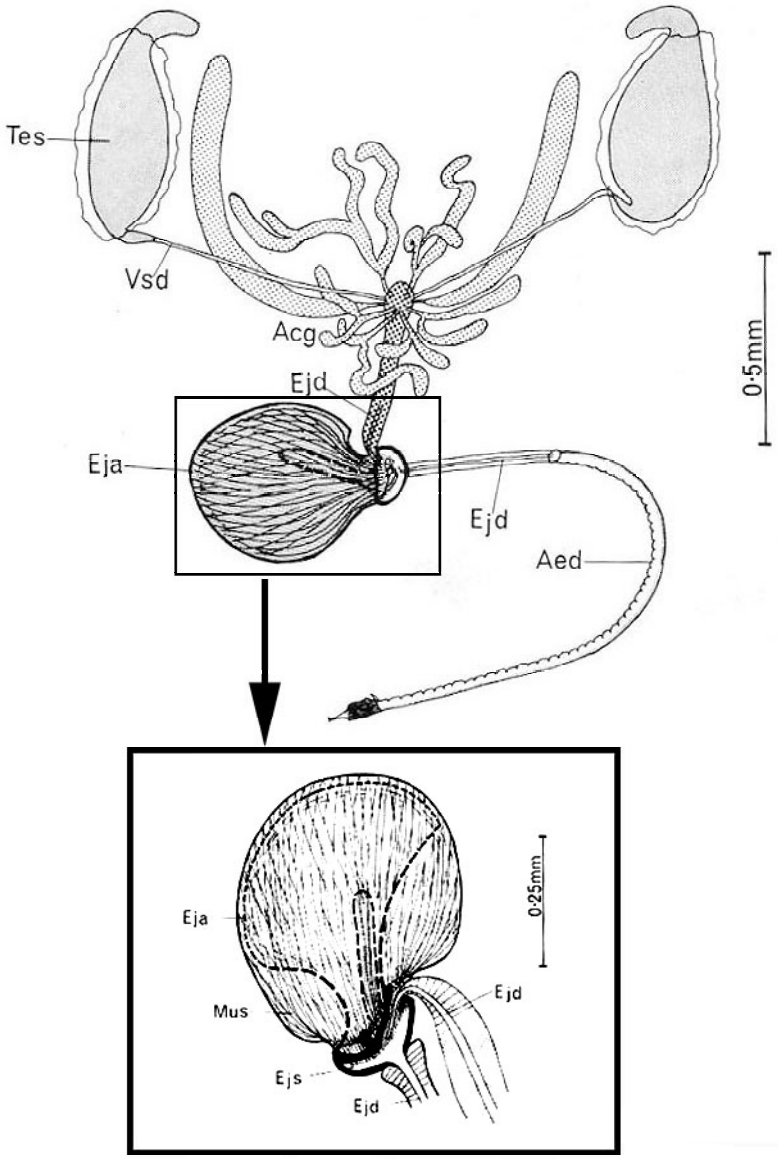
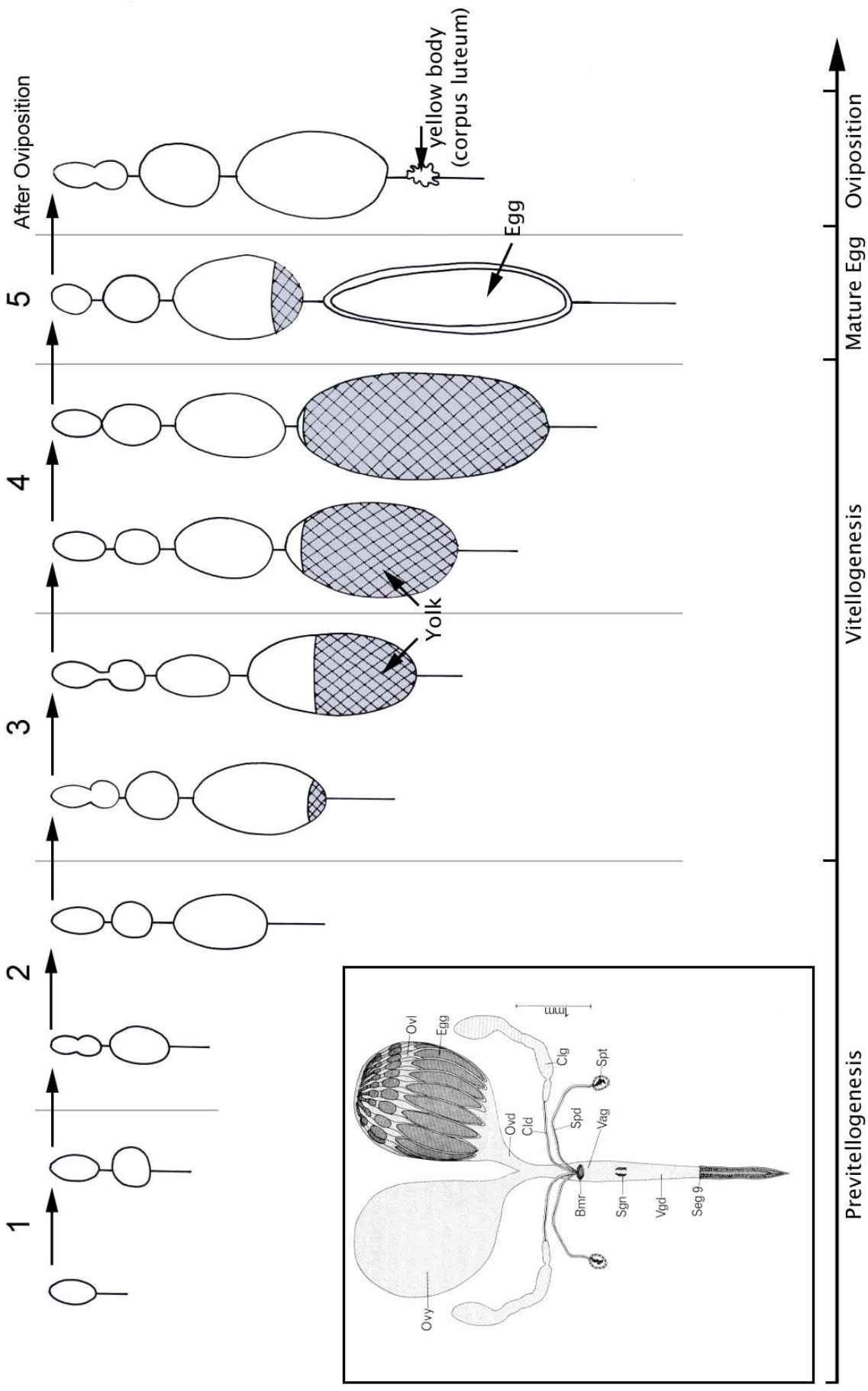


Figure 4.1. Typical male reproductive system of genus *Bactrocera* showing location of ejaculatory apodeme – Acg., accessory glands; Aed., aedeagus; Eja., ejaculatory apodeme; Tes., testis; Vsd., vas deferens. ***Inset*** – Erecting and pumping organ – Eja., ejaculatory apodeme; Ejd., ejaculatory duct; Ejs., ejaculatory sac; Mus., musculature. (Adapted from Drew [1969] with permission)

Figure 4.2. Schematic representation of ovarian development in *Bactrocera cacuminata* (Hering) (adapted from Fletcher et al. 1978): Stages 1 & 2 = Previtellogenesis; Stages 3 & 4 = Vitellogenesis, accumulation of yolk in terminal follicles prior to egg formation; Stage 5 = Egg formation; Stage 6 = Yellow body (corpus luteum) left behind after oviposition. ***Inset*** – Typical female reproductive system of genus *Bactrocera* – Bmr. = Base of morula gland; Cld. = Duct of Collaterial gland; Clg. = Collaterial gland; Egg = Egg; Ovd. = Lateral Oviduct; Ovl. = Ovariole; Ovy = Ovary; Seg. 9 = Abdominal segment 9; Sgn. = Signum; Spd. = Spermathecal duct; Spt. = Spermatheca; Vag. = Vagina; Vgd. = Vaginal duct. (Inset reprinted from Drew [1969] with permission)


1
2
3
4
5
After Oviposition
yellow body
(corpus luteum)
Egg
Yolk
Previtellogenesis
Vitellogenesis
Mature Egg
Oviposition
1mm
Ovl
Egg
Clg
Spt
Ovd
Cld
Spd
Vag
Bmr
Sgn
Vgd
Seg 9
Ovy

4.2 MATERIALS AND METHODS

4.2.1. Cultures

Bactrocera cacuminata is a non-pest species that is almost exclusively monophagous on the wild tobacco plant, *Solanum mauritianum* Scopoli, in southeastern Queensland. Adult flies for the study were sampled from a colony being maintained at Griffith University. Pupae of *B. cacuminata* were originally obtained from the University of Sydney. These were approximately 8 generations removed from the wild (Dr. A. W. Meats – pers. comm.). Wild flies (collected from rearing larvae through from field sampled fruit) were introduced into the colony in a 1:1 ratio every 3-4 generations to minimize the effects of any laboratory induced selection pressures.

Flies were maintained in 30 Δ 30 Δ 30cm sleeve cages and fed water, sugar and protein (in the form of yeast autolysate) *ad libitum*. The ambient conditions during the course of the entire experiment were 23Γ2^{o}C and 60-65% relative humidity. A minimum of 12 flies of each sex were sampled daily from the day of emergence (Day 0) till 17 days (Day 17) after emergence. Flies were transferred into 100% alcohol and stored in a freezer till dissection. All flies used in the experiment were from the same cohort.

4.2.2. Morphological Studies

Male flies

The reproductive cuticular endoskeleton was immersed in 10% cold potassium hydroxide (KOH) for approximately 5 hours to dissolve attached musculature and other soft tissue. The ejaculatory apodeme was excised under water from other tissues and washed sequentially in water, 70% alcohol and water again. It was then mounted in polyvinyl alcohol (PVA) on a flat microscope slide and sealed with a cover slip.

All apodemes were examined under a Zeiss Axioskop FS microscope (10X CP-ACHROMAT objective). Images were captured using a Cohu gray-scale charge-coupled device camera (Cohu, Inc., San Diego, CA), digitized and analyzed with a Scion framegrabber under control of Scion Image 1.62 (Scion Corporation, MD). Apodeme perimeters were delineated using the threshold command, apodeme areas measured in pixels and converted to square microns after calibration against a stage micrometer.

In addition, morphological measurements were made to standardize for variations in apodeme size as a function of size of fly. Length of wing (from base to tip), length of wing vein (CuA_1), length of thorax (from base of neck to the apex of scutellum) and the length of the aedeagus were measured for this purpose.

Female flies

The ovaries of teneral females are small because of immature ovarioles, while at sexual maturity the egg is formed at the basal section of the ovariole, expanding the ovaries to occupy almost the entire body cavity (Drew, 1969; Figure 4.2 – Inset). Female flies were dissected under water similar to male flies. Female flies were classified based on ovarian development using modified categories (Figure 4.2) based on Fletcher et al's (1978) classification of ovarian development stages. As gonadotrophic cycles are asynchronous (Fletcher et al. 1978; R.A.I. Drew – personal communication), the condition of the most advanced follicles was used in assigning individuals to a particular class. Stages 1 and 2 are part of the pre-vitellogenic phase, while stages 3 to 5 represent the vitellogenic phase. Flies were assigned to stage 5 if the most advanced ovarian follicles had mature eggs. Oviposition results in a yellow residual follicular relic, the *corpus luteum* (Figure 4.2). This stage was not observed in this study.

Daily observations were made at dusk to note the number of pairs in copulation.

4.2.3. Data analysis

Within day correlation of measures of fly size and apodeme size revealed that apodeme area was not consistently significantly (P>0.05) correlated with fly size. Hence absolute measures of area were used in ascertaining the rate of apodeme growth. Observations of exploratory scatterplots of the data revealed that a logistic regression model (SPSS 1998) would be the most appropriate way to estimate the growth rate of apodemes and ovaries in the present study (Daniel and Wood 1971, Chatterjee and Price 1977). Spearman's rank-order correlation (Zar 1999) was used to analyze the relationship between apodeme and ovarian development.

4.3 RESULTS

Male endoskeletal structures increased in size from day of emergence till approximately 9 days after emergence. The ejaculatory apodeme increased in area with a widening of the vanes. Growth in this structure is clearly expressed in the form of growth lines and formation of sutures (Figure 4.3).

The growth of the ejaculatory apodeme was sigmoid (Figure 4.4a), with a slow development phase from the day of emergence for the first 4 days. After that a rapid growth phase was observed from Day 5 until the apodeme reached a maximum size between Days 9 and 10, with little growth occurring subsequently (Figure 4.4a). The regression model explained 99.5% of the variation in the development of the ejaculatory apodeme. I estimated the threshold apodeme area to be 151752.32 $\Leftarrow m^2$ by solving the regression equation for $x \rightarrow \infty$. Ovarian development was similarly sigmoidal (Figure 4.4b), with a slow development phase between Day 0 and Day 4 and full maturity reached by Day 9. The regression model explained 96% of the

variation in the ovarian development. The development of the sexes were synchronous, as indicated by a significant correlation between the mean apodeme area and ovarian stage (r_S = 0.954, p <0.0001, df = 17).

Forty mating pairs were observed in the cages over the duration of the study. Mating was first observed 7 days after emergence and copulating pairs were observed till the end of the study (Figure 4.4c). The number of copulating pairs peaked 9 days after emergence, with the frequency of mating remaining consistent between Days 9 and 13, before gradually declining (Figure 4.4c).

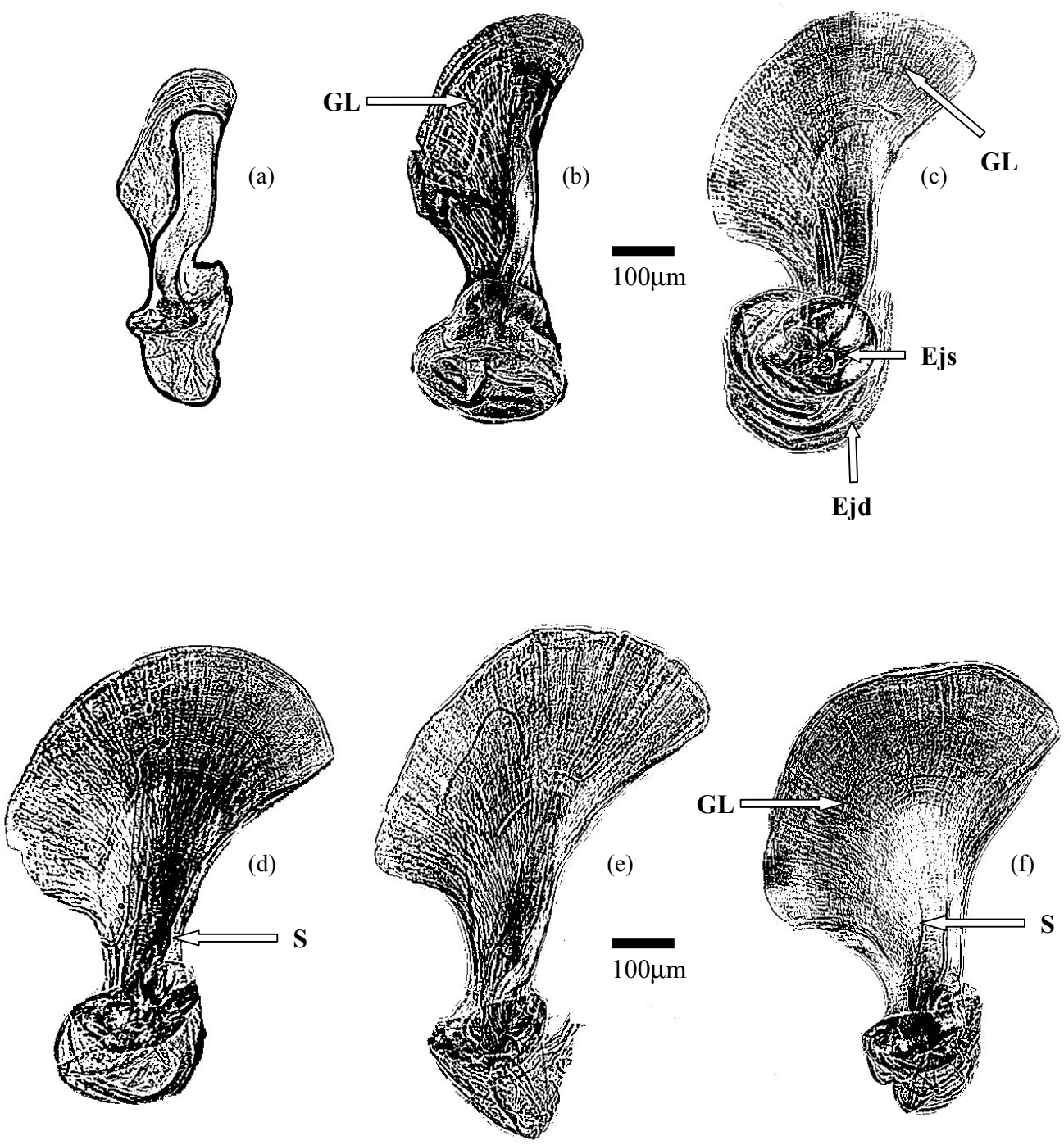
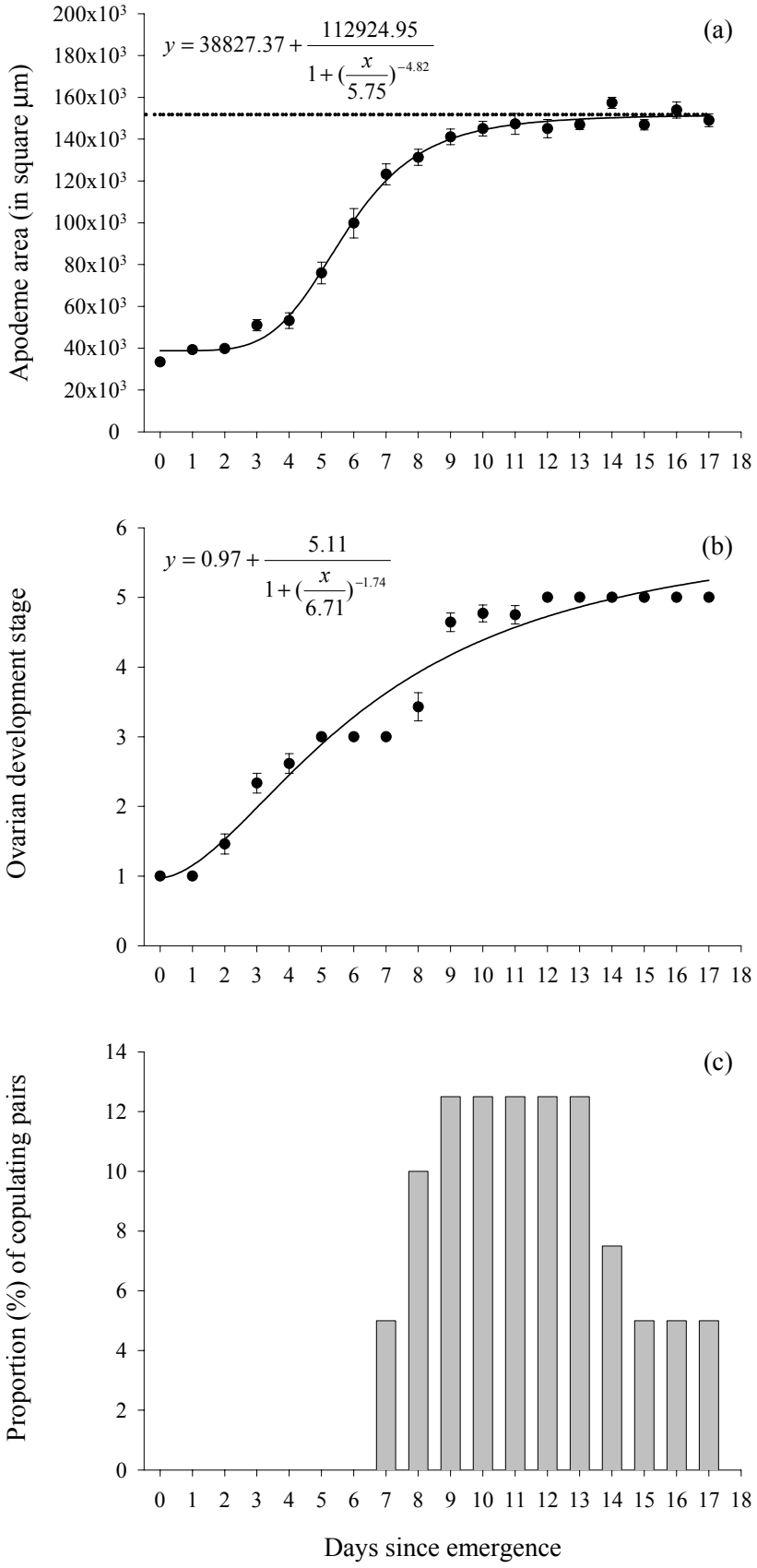


Figure 4.3. Development of ejaculatory apodeme over time in male *Bactrocera cacuminata* (Hering). Thresholded images of apodeme at (a) Day 0, (b) Day 4, (c) Day 8, (d) Day 10, (e) Day 12 and (f) Day 16. Arrows depict ejaculatory duct (Ejd.), ejaculatory sac (Ejs.), growth lines (GL) and sutures (S).

Figure 4.4. Development of the reproductive system in *Bactrocera cacuminata* (Hering) in relation to age and mating status (a) Growth of ejaculatory apodeme (mean ± st. err.) in μm^2 over time in male flies. Dotted line represents estimated apodeme growth threshold (= 151752.32 μm^2) (b) Ovarian development (mean ± st. err.) over time in female flies. (Stages refer to modifications of the system developed by Fletcher et al. 1978) (c) Number of pairs in copulation over time as a proportion (%) of total number of copulating pairs observed over the entire experiment.


(a)
$y = 38827.37 + \frac{112924.95}{1 + (\frac{x}{5.75})^{-4.82}}$
Apodeme area (in square µm)
200x10³
180x10³
160x10³
140x10³
120x10³
100x10³
80x10³
60x10³
40x10³
20x10³
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
(b)
$y = 0.97 + \frac{5.11}{1 + (\frac{x}{6.71})^{-1.74}}$
Ovarian development stage
6
5
4
3
2
1
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
(c)
Proportion (%) of copulating pairs
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Days since emergence

4.4 DISCUSSION

The specific objective of this study was to provide a reliable method by which physiological status (i.e. sexual maturity) of flies in the field can be ascertained based on structural evidence from the reproductive systems of males and females. The data show that there is a very good correlation between size of male ejaculatory apodemes and stage of female ovarian development with age at which flies attain sexual maturity and mate (Figure 4.4). However, caution needs to be exercised given that the data is representative of development rates at a particular temperature-humidity regime. Under fluctuating regimes of abiotic factors, as would occur in natural environments, the rate of sexual development is likely to vary from those observed in the current study (Drew 1969, Fletcher et al. 1978, Chapman 1998). Furthermore, flies may exercise physiological control over their reproductive development in the presence of unfavourable ambient biotic and abiotic conditions. In *B. oleae*, presence of fruit significantly enhanced the rate of ovarian development under constant temperature regimes (Fletcher et al. 1978). Female flies also resorb ovarian follicles at the end of previtellogenesis when exposed to unfavourable conditions such as high temperature and low humidity (Fletcher et al. 1978).

A high level of synchrony between male and female reproductive development was observed in our study (Figure 4.4a, b). This is unusual as in many insect species protandry (males attaining sexual maturity prior to females) is a common strategy, maintained by direct and indirect fitness consequences for males or females (Thornhill and Alcock 1983, Morbey and Ydenberg 2001). However, studies dealing with life-history timing have dealt principally with reproductively autogenous species (species in which adults emerge sexually mature from the pupal stage) (e.g. butterflies – Wiklund and Fagerström 1977, Fagerström and Wiklund 1982). An explanation for the synchronization of development between the sexes in our study could be

that it is a characteristic of reproductively anautogenous insects (species that spend a proportion of their adult life as sexually immature adults and need to forage for resources to attain sexual/ reproductive maturity; e.g. dacine fruit flies, some species of mosquitoes, blowflies and mayflies). However, previous laboratory studies on feeding behaviour in dacine and related tephritid species have noted that males required little or no protein to achieve fertilization while female flies required at least one protein feed to ensure egg production (Drew 1987, Webster et al. 1979). This suggests that the synchrony in our study may just be an artefact of our provision of protein *ad libitum*.

The present study indicates that there is a growth threshold for the ejaculatory apodeme (also found in *B. tryoni* by Drew [1969]) and ovarian stage. This threshold appears to be a good predictor for time of first mating in *B. cacuminata*. Dissections of field sampled flies would therefore enable clear distinctions between sexually mature and immature males and females. Secondly, assuming that wild flies have a similar growth threshold (found to hold true in *B. tryoni* by Drew [1969]), the size of the apodeme of immature wild caught flies could be used as a proportional measure of development through comparison to the maximum apodeme size (Figure 4.4a). For female flies, ovarian dissections coupled with spermathecal squashes would enable identification of maturity and mating status. The results from this study are used as a tool in assessing the physiological status and sexual maturity of *B. cacuminata* sampled at different resources (Chapter 5).

Acknowledgments – I am grateful to Dr. David Merritt, Department of Zoology and Entomology, The University of Queensland for access to microscopy and digital imaging facilities and for discussions on the apodeme mensuration techniques.

Chapter Five

Physiological and nutritional status of *Bactrocera cacuminata* at different resources

This chapter has been submitted for review in a slightly modified form:

Raghu, S., Yuval, B. and Clarke, A.R. Physiological and nutritional status of *Bactrocera cacuminata* (Hering) at different resources: Evidence for spatial and temporal partitioning of behaviour by adult flies. *Physiological Entomology* (in review).

5.1 INTRODUCTION

The ability of individuals of a species to survive and reproduce depends largely on their ability to budget their activities between spatially and temporally variable resources in their environment (Roitberg 1985, Slansky and Scriber 1985, Bell 1990). Autogenous insects have overcome the ecological constraints posed by such variability by emerging as reproductive adults from the larval stage (Chapman 1998). Anautogenous insects, however, need to forage as adults for critical resources, principally protein to attain sexual maturity and carbohydrates to fuel foraging and courtship behaviour (Slansky and Scriber 1985, Yuval et al. 1994, Blay and Yuval 1997, Watanabe and Hirota 1999, Kaspi and Yuval 2000).

Dacine fruit flies (Diptera: Tephritidae: Dacinae) are anautogenous, requiring proteins and carbohydrates to mature sexually (Fletcher 1987). Sources of these nutrients for tephritids in nature are believed to be glandular secretions of plants, nectar, plant-wound exudates, bird faeces, decaying insects and homopteran honeydew (Bateman 1972, Drew and Yuval 2000, Fletcher 1987, Warburg and Yuval 1997a). Phylloplane bacteria have also been shown to be a significant protein source for adult fruit flies (Drew et al. 1983, Drew and Lloyd 1987, 1989, Prokopy et al. 1991).

In addition to nutritional resources, dacine fruit flies are attracted to, and ingest, certain plant-derived chemicals, such as methyl eugenol (ME) and raspberry ketone (Meats and Hartland, 1999, Meats and Osborne 2000). Strong response to pure forms of these substances (and synthetic derivatives) has enabled them to be used successfully in the pest management of these insects. Though response of dacine species to lures suggests that they actively forage for these chemicals, rather than randomly chancing upon them, this has never been explicitly tested. These chemicals are widely distributed in the plant kingdom and have been hypothesized to be

chemicals found in ancestral hosts of the Dacinae (Metcalf 1990, Metcalf and Metcalf 1992). Alternatively, a role in sexual selection has been suggested for these chemicals, as pheromone precursors (Fitt 1981b, c, Nishida et al. 1988, 1993, 1997, Shelly 2000). Their ecological and evolutionary significance remains an intriguing issue (Chapters 7, 8, 9).

With the exception of ME, dacine fruit flies were believed to acquire all required adult resources from the larval host plant and hence the host plant has been labeled as the "centre of activity" in dacine ecology (Prokopy et al. 1991, Drew and Lloyd 1987, 1989, Metcalf 1990). A recent study has shown that in certain dacine species the host plant is only visited by gravid adult females for the purpose of oviposition (Raghu et al. 2002). This suggests that not all resources vital for the survival and reproduction of dacine species are available at the host plant. Individuals may therefore partition their activities between different locations to acquire resources critical for their survival and reproduction, a pattern common in insects in general (Johnson 1969, Wiklund 1977) and other tephritids (Hendrichs et al 1991; Warburg and Yuval 1997a). Furthermore, these patterns are highly sex specific (Hendrichs et al 1991; Warburg and Yuval 1997a) and nutritional status and age may determine thresholds for specific activities. Thus, the nutritional status of male Mediterranean fruit flies regulates their participation in various discrete activities (Warburg & Yuval 1997b, Yuval et al. 1998).

Accordingly, I hypothesized that the spatial distribution of dacine flies amongst different resources (nutritional and reproductive) is non random, and that the segment of the fly population present at any of these resources will have a typical physiological profile (in terms of age and nutritional state).

To investigate whether this is the case, I asked the following specific questions:

1. Is there a sex-related difference in spatial and temporal distribution?
2. Is there a difference between the physiological status, as indicated by sexual maturation, between individuals present at different resources?
3. Is there a difference in nutritional status, as indicated by lipid, protein and carbohydrate reserves, between individuals present at different resources?
4. Does the physiological and nutritional status of individuals at any given resource vary circadianly?

5.2 MATERIAL AND METHODS

The monophagous dacine fruit fly *Bactrocera cacuminata* was my study organism. Female flies of this species oviposit almost exclusively in the fruit of *Solanum maurtianum* (Drew 1989). This species requires a protein source to attain sexual maturity (Fletcher 1987) and is attracted to and ingests ME (Meats and Osborne 2000, Raghu et al. – in press). Hence the 'resources' used in this study were the host plant, a protein source and ME.

5.2.1. Field sampling

Flies were sampled from four different locations on four different days. The host plants (*S. mauritianum*) had wild flies present on them and were selected from those growing along a creek in Brisbane (27^{o} 28' S, 153^{o} 2' E). Solitary host plants were selected. These plants were scanned to ensure that they did not contain any bird faeces or other obvious natural protein source that could affect the response to the spatially separated protein source. Two pedestals (1.40m in height) were set up approximately 10m away from the host plant and from each other. The 'resources' were thus equidistant from each other at vertices of an equilateral triangle. A Petri dish containing a cotton wick with 1ml of ME (International Pheromone Systems Ltd.) was placed on one of the pedestals and a cotton wick with 2ml of protein (yeast autolysate, ICN

Biomedicals) was placed on the other. Flies were sampled at each of these resources continuously in the morning (0700-0800h), noon (1200-1300h) and at dusk (1700-1800h). The pedestals and the resource they offered were removed between sampling events.

Individuals were then randomly separated into two groups for assessment of their physiological status by dissection, and nutritional status by biochemical analyses.

5.2.2. Dissection – Assessment of physiological status

Male flies

In male dacine flies, the ejaculatory apodeme has been shown to grow logistically with age of fly and its area asymptotes at an age that is correlated with attainment of sexual maturity. This estimated threshold apodeme area was 151752.32 μm^2 (Chapter 4). Therefore the area of the apodeme was used as a surrogate measure of physiological status.

The reproductive cuticular endoskeleton was immersed in 10% cold potassium hydroxide (KOH) for approximately 5 hours to dissolve attached musculature and other soft tissue. The ejaculatory apodeme was excised under water from other tissues and washed sequentially in water, 70% alcohol and water again. It was then mounted in polyvinyl alcohol (PVA) on a flat microscope slide and sealed with a cover slip.

All apodemes were examined under a Zeiss Axioskop FS microscope (10X CP-ACHROMAT objective). Images were captured using a Cohu gray-scale, charge-coupled device camera (Cohu, Inc., San Diego, CA), digitized and analyzed with a Scion framegrabber under control of Scion Image 1.62 (Scion Corporation, MD). Apodeme perimeters were delineated using the

threshold command, apodeme areas measured in pixels and converted to square microns after calibration against a stage micrometer.

Female flies

Female flies were dissected under water similar to male flies and the ovaries removed for examination. Females were classified based on ovarian development into one of five stages using a modified version of Fletcher et al.'s (1978) classification of ovarian development stages (Chapter 4). Stages 1 and 2 are part of the pre-vitellogenic phase, while stages 3 to 5 represent the vitellogenic phase. Flies were assigned to stage 5 if the most advanced ovarian follicles had mature eggs. As gonadotrophic cycles are asynchronous (Fletcher et al. 1978, Chapter 4), the condition of the most advanced follicles was used in assigning individuals to a particular class.

In addition, spermathecal squashes were done to assess presence of sperm and hence ascertain mating status. Spermathecae were dissected under water and crushed under a cover slip after immersing them in a drop of physiological saline.

Data from 3 males and 3 females were not included in further analyses as dissection revealed abnormalities in their reproductive system.

5.2.3. Biochemical analyses – Assessment of nutritional status

A wing of from each fly was removed and measured as an index of fly size (see Data Analyses [5.2.4.]).

Each of the flies were dessicated at 30^{o} C for 24 h and weighed on an analytical balance (± 0.01 mg). To determine the levels of protein, lipid and carbohydrates present in the flies, the biochemical techniques of Van Handel and Day (1988), as modified by Warburg and Yuval (1996, 1997b) and Yuval et al. (1998) were used as described below.

Flies were homogenized individually in 200 µl of 2% Na_2SO_4. Carbohydrates and lipids were extracted in 1300 µl of chloroform : methanol (1:2). Individual tubes were centrifuged at 8000 rev per min and 500 µl were taken from the supernatant of each sample and dried. Samples were then dissolved in 500 µl H_2SO_4 and incubated for 10 minutes at 90^{o}C. Samples of 30 µl were put into wells on ELISA plates together with 270 µl of vanillin reagent (600 mg vanillin dissolved in 100 ml of distilled water and 400 ml of 85% H_3PO_4). The plate was shaken at room temperature for 30 min and then the optical density was read at 530 nm on an EL311SX Bio-tek Spectrophotometer. Total lipids per fly were calculated from standard curves using the KCJR EIA application software (Bio-tek Instruments Inc., Winooski, Vermont).

Sugar content per fly was assessed using 300 µl from the supernantant of the chloroform : methanol extract. After adding 200 µl of water the sample was reacted with 1 ml of anthrone reagent (500 mg of anthrone dissolved in 500ml of conc. H_2SO_4) at 90^{o}C. Samples of 300 µl were then put into wells on ELISA plates and the optical density was read at 630 nm. Similar to the lipid content analysis, total carbohydrates per fly was estimated using standard curves.

Dissolved protein was extracted in 1200 µl phosphate buffer saline (PBS). Samples of 300 µl were taken and after adding 500 µl of PBS, were reacted with 200 µl of Bradford reagent (Bradford 1976). Samples of 300 µl were then put into wells on ELISA plates and optical density was read at 595 nm. Total dissolved protein per fly was calculated from standard curves.

In addition to the flies caught on the various resources in the field, a group of newly emerged (teneral) flies (15 male and 15 female) were also analyzed. This gave us a baseline against which the nutritional status of flies sampled at the different resources was compared.

5.2.4. Data analysis

Preliminary data analyses found no significant differences in the size distribution (wing length) of flies between different times of day (Males: $F_{2,151}$ = 0.818, P = 0.443; Females: $F_{2,82}$ = 2.758, P = 0.069) or between resources (Males: $F_{2,166}$ = 1.207, P = 0.302; Females: $F_{1,98}$ = 0.033, P = 0.856). Therefore, the total amount of nutrient (lipids, proteins or carbohydrates) per fly were used in further analyses. Since size distributions did not differ between the different days of sampling and tenerals sampled from the lab colony (Males: $F_{4,164}$ = 1.889, P = 0.115; Females: $F_{4,95}$ = 0.955, P = 0.436) the data were pooled from the different collection days.

Data were analyzed using one-way Analysis of Variance (ANOVA) with resource or time of day as factors. Where assumptions of the analysis were violated, the data were log-transformed prior to ANOVA. For data that still violated assumptions after transformation, the equivalent non-parametric Kruskal-Wallis test was used. Pair-wise, post-hoc, parametric (LSD) and non-parametric comparisons (Games-Howell) were used to compare means. The significance level was preset at P = 0.05.

5.3 RESULTS

Male flies responded to the host plant and ME, while female flies only responded to the host plant. Neither sex responded to protein. Reasons for this are explored in the 'Discussion'. In total, 104 males were sampled at the host plant and 110 at ME, while 123 females B. *cacuminata* were sampled from the host plant (Table 5.1).

Table 5.1. Summary of number of individuals sampled at different resources at the three different time periods.

Time of Day	Males		Females
	Host plant	Methyl eugenol	Host plant
Morning	35	56	47
Noon	20	20	23
Dusk	49	34	53

5.3.1. Abundance in relation to resources

The number of males sampled at the host plant or ME did not differ significantly between different time periods (Host plant: $F_{2,9}$ = 0.590, P = 0.574; ME: $F_{2,9}$ = 3.127, P = 0.093; Figure 5.1) nor was there a significant difference in the number of individuals between resources within time periods (Morning: $F_{1,6}$ = 0.930, P = 0.362; Noon: $F_{1,6}$ = 0.615, P = 0.463; Dusk: $F_{1,6}$ = 0.054, P = 0.824; Figure 5.1). The number of females at the host plant did not vary significantly between the different times ($F_{2,9}$ = 2.083, P = 0.181; Figure 5.1).

5.3.2. Physiological status in relation to resources

Male flies

The size of the ejaculatory apodeme did not differ significantly between males sampled at host versus those at ME in the morning (H_1 = 0.077, P = 0.782; Figure 5.2a). There was a significant difference in the size of ejaculatory apodeme between males at host versus those at ME at both noon (H_1 = 4.20, P = 0.040; Figure 5.2b) and at dusk (H_1 = 6.031, P = 0.014; Figure 5.2c) with the apodeme of males at ME being larger than those at host plant for both time periods. There was no difference in physiological status of males at the host plant or ME between the different time periods (Host: $F_{2,22}$ = 0.779, P = 0.471; ME: $F_{2, 30}$ = 1.239, P = 0.304; Figure 5.2). The apodemes of males sampled at ME were closer in size to the estimated apodeme development threshold (5.2.2.1., Chapter 4, indicated by dotted line in Figure 5.2) than those sampled at the host plant at all three time periods.

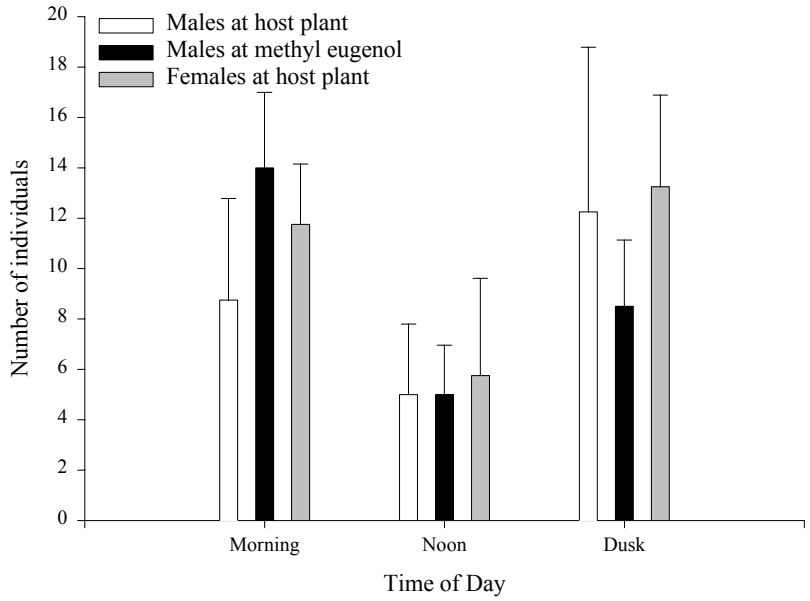
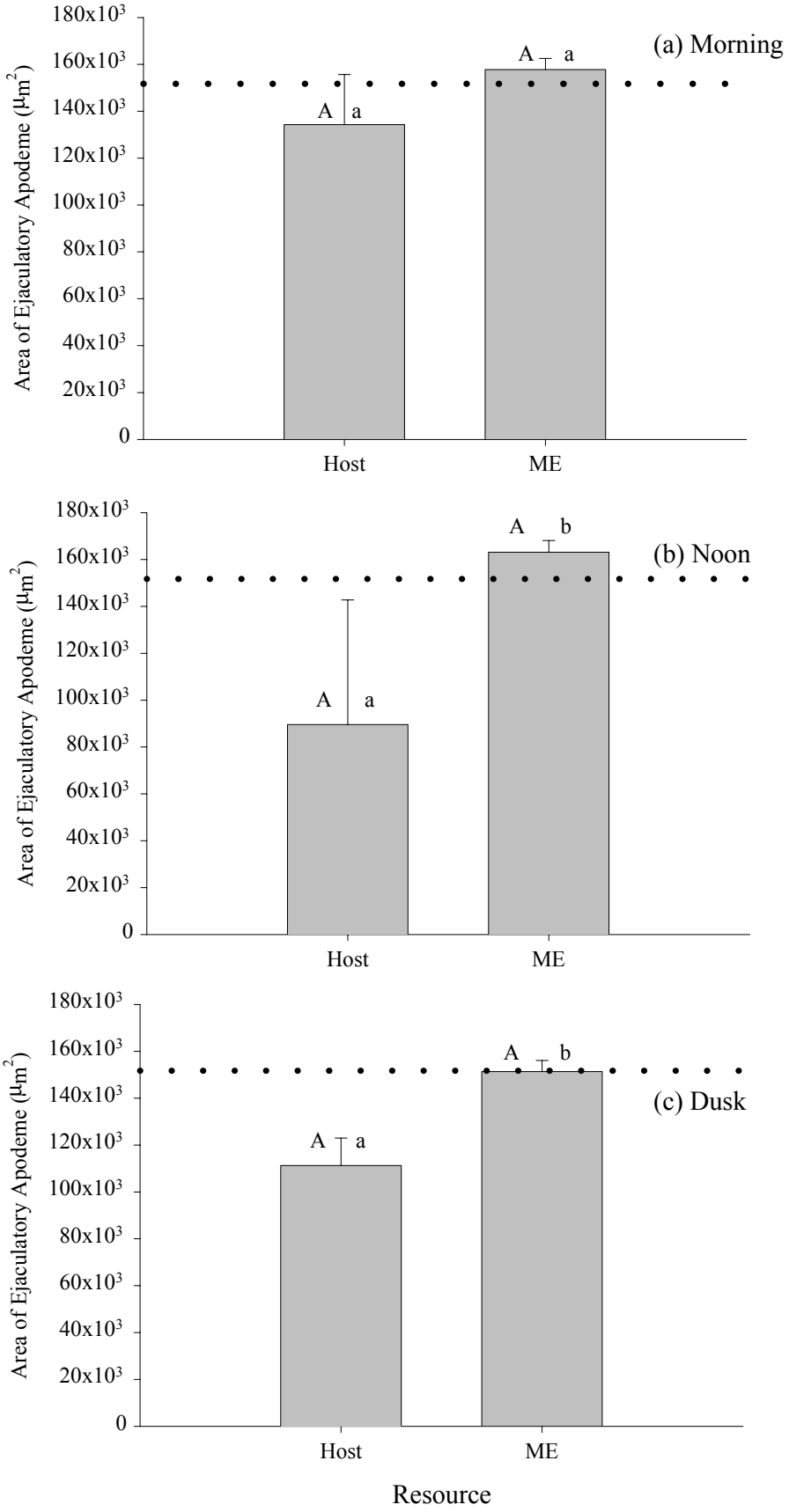


Figure 5.1. Number of individuals sampled (mean + standard error) at different resources at different times of day (N = 4 sampling days).

Figure 5.2. Physiological status of *Bactrocera cacuminata* in relation to resources. Area of ejaculatory apodeme (in square μm) of male flies at host and methyl eugenol in the (a) Morning (b) Noon and (c) Dusk. Bars represent means + standard error. Bars with same letters adjacent to them are not significantly different. Capital letters represent between time comparisons at a particular resource and lower case letters represent between resource comparisons within a particular time period. The dotted line represents the estimated apodeme growth threshold (Chapter 4).


(a) Morning
(b) Noon
(c) Dusk
Area of Ejaculatory Apodeme (μm²)
180x10³
160x10³
140x10³
120x10³
100x10³
80x10³
60x10³
40x10³
20x10³
0
A a
A b
Host
ME
Resource

Female flies

Females collected from the host tree were typically at an advanced stage of ovarian development and had previously copulated. Most of the females sampled from the host tree were in stage 4-5 of ovarian development. Very few of the females dissected had previtellogenic ovaries. There was no difference in the ovarian development stage of females at the host plant at the three different times (H_1 = 3.087, P = 0.738; Figure 5.3). At all three time periods there were more sexually mature females (as indicated by ovarian development) than immature females (Figure 5.3)

A high proportion (>70%) of female flies sampled on the host plant had mated, as indicated by the presence of sperm in the spermathecae (Figure 5.4). Only female flies whose ovaries were fully developed (Stage 5) had sperm in their spermathecae. There was no significant difference between the proportion of mated females sampled at the host plant at morning, noon and dusk (H_1 = 1.566, P = 0.457; Figure 5.4). Greater than 90% of the females at the host plant at dusk already had sperm in their spermathecae (Figure 5.4).

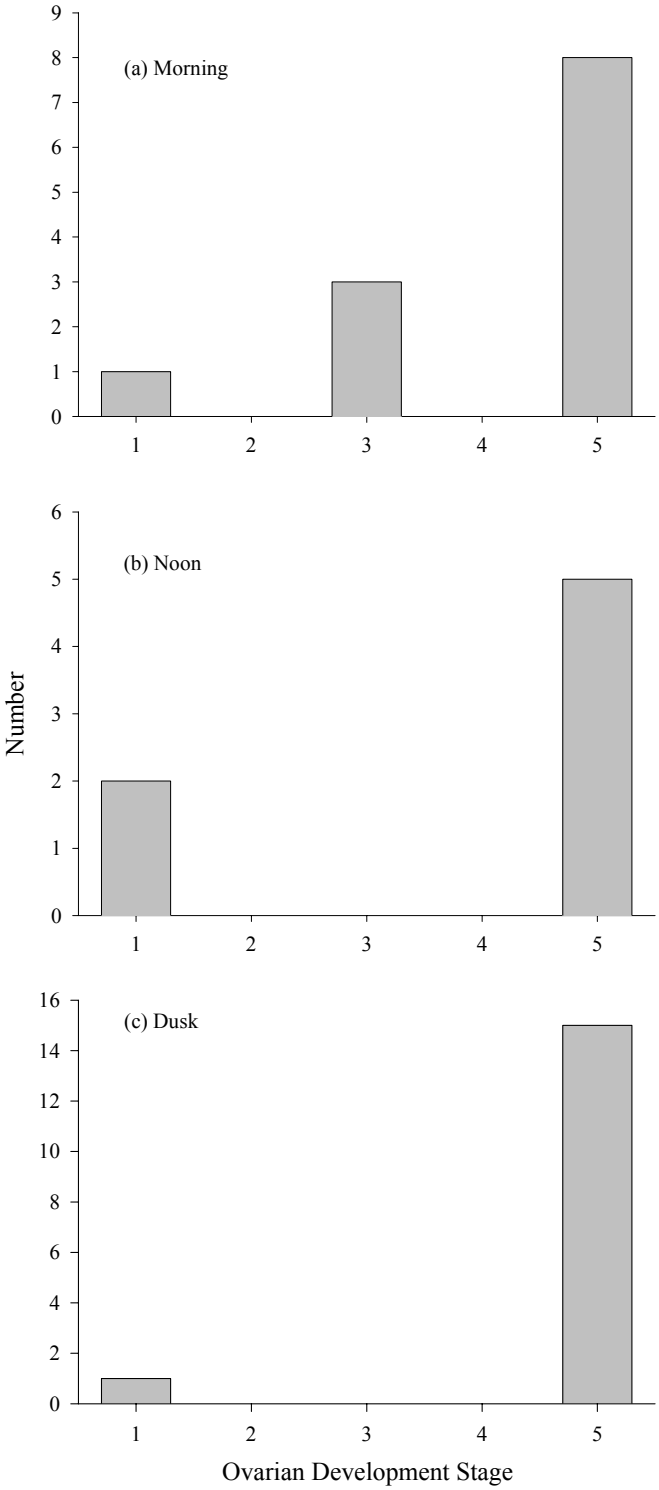


Figure 5.3. Frequency distribution of ovarian development stage of female *Bactrocera cacuminata* sampled at the host plant at (a) Morning (b) Noon and (c) Dusk.

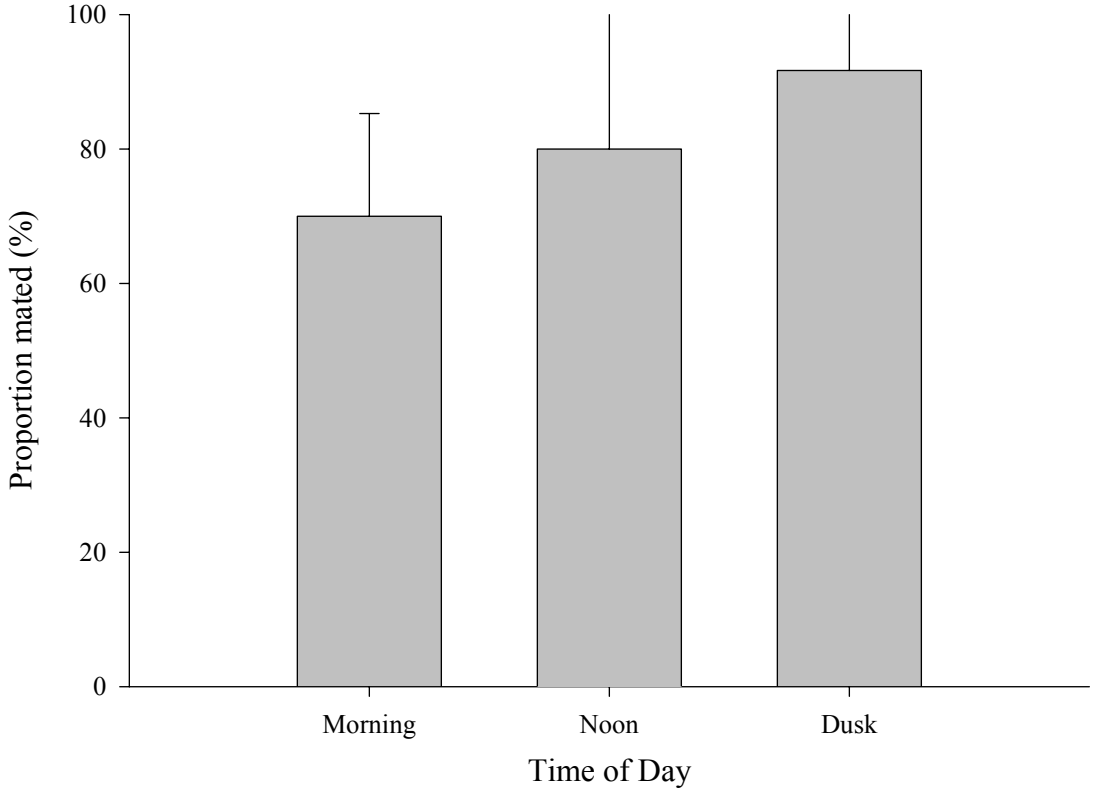


Figure 5.4. Mating status (mean proportion (%) mated + standard error) of female flies sampled at the host plant at different times of day (N = 4 sampling days)

5.3.3. Nutritional status in relation to resources

Lipids

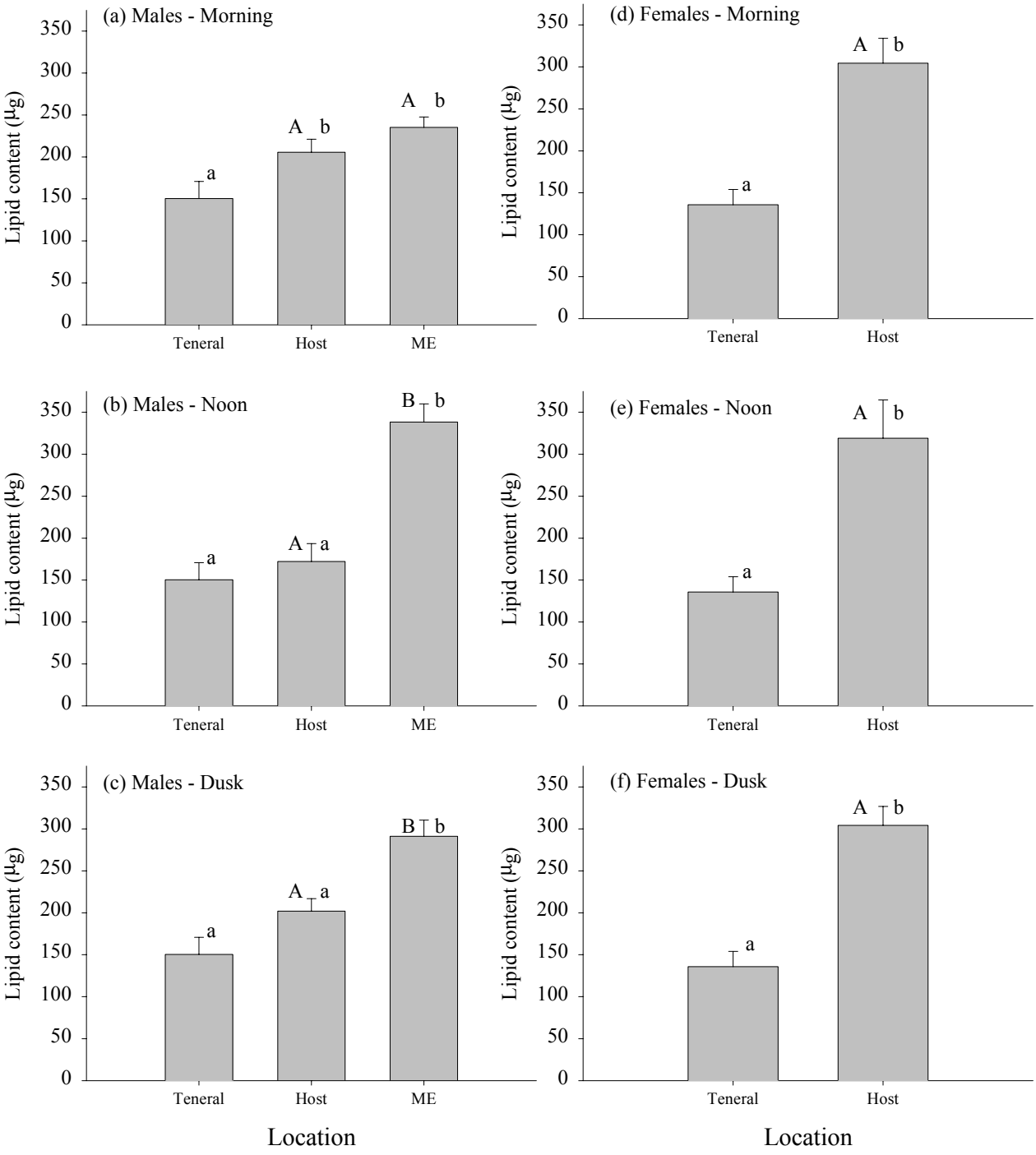
The lipid reserves in males differed significantly between resources (Table 5.2). Males sampled in the morning from host and from ME had significantly greater lipid content than that of teneral flies (Figure 5.5a), but the lipid content did not differ between males at host and ME (Figure 5.5a). At noon males at host did not differ significantly from teneral males in terms of lipid content, but both teneral and males at host had a significantly lower lipid content than males at ME (Figure 5.5b). This trend was similar at dusk (Figure 5.5c). Between time comparisons at host revealed that there was no significant difference in terms of lipid content at the plant host (Table 5.2), while there were significant differences at ME (Table 5.2). Males at ME at noon and dusk were not significantly different from each other but had significantly higher lipid content than those at ME in the morning (Figures 5.5a, b, c).

Lipid reserves in female flies sampled at host at all time periods differed significantly from those in teneral females, while the lipid content of female flies at the host plant did not differ significantly over time (Table 5.2, Figures 5.5d, e, f).

Table 5.2. Summary of analyses comparing nutritional status of flies between resources and teneral adults at a particular time of day and between time comparisons at a particular resource.

Sex	Nutritional Reserve	Time of day	Between Resource Comparisons	Resource	Between Time Comparisons
Male	Lipids	Morning	$F_{2,82} = 6.222$, $P = 0.003$	Host Plant	$F_{2,75} = 0.933$, $P = 0.398$
		Noon	$F_{2,42} = 21.667$, $P < 0.001$	ME	$F_{2,73} = 9.033$, $P < 0.001$
		Dusk	$F_{2,66} = 13.217$, $P < 0.001$		
	Proteins	Morning	$H_2 = 3.875$, $P = 0.144$	Host Plant	$F_{2,75} = 0.272$, $P = 0.763$
		Noon	$H_2 = 5.989$, $P = 0.050$	ME	$F_{2,73} = 13.754$, $P < 0.001$
		Dusk	$H_2 = 22.253$, $P < 0.001$		
	Carbohydrates	Morning	$F_{2,82} = 21.183$, $P < 0.001$	Host Plant	$H_2 = 17.762$, $P < 0.001$
		Noon	$H_2 = 7.636$, $P = 0.022$	ME	$F_{2,66} = 49.396$, $P < 0.001$
		Dusk	$F_{2,66} = 49.396$, $P < 0.001$		
Female	Lipids	Morning	$F_{1,48} = 17.461$, $P < 0.001$	Host Plant	$F_{2,82} = 0.103$, $P = 0.902$
		Noon	$F_{1,29} = 13.163$, $P = 0.001$		
		Dusk	$F_{1,47} = 27.167$, $P < 0.001$		
	Proteins	Morning	$H_1 = 15.843$, $P < 0.001$	Host Plant	$F_{2,82} = 2.072$, $P = 0.133$
		Noon	$H_1 = 7.656$, $P = 0.006$		
		Dusk	$H_1 = 11.090$, $P = 0.001$		
	Carbohydrates	Morning	$H_1 = 7.343$, $P = 0.007$	Host Plant	$F_{2,82} = 0.476$, $P = 0.623$
		Noon	$H_1 = 8.789$, $P = 0.003$		
		Dusk	$H_1 = 13.124$, $P < 0.001$		

Figure 5.5. Nutritional status of *Bactrocera cacuminata* in relation to resources. Lipid reserves (µg/ fly) in male flies at (a) Morning (b) Noon and (c) Dusk and female flies at (d) Morning (e) Noon and (f) Dusk at different resources. Bars with same letters adjacent to them are not significantly different. Capital letters represent between time comparisons at a particular resource and lower case letters represent between resource comparisons within a particular time period.

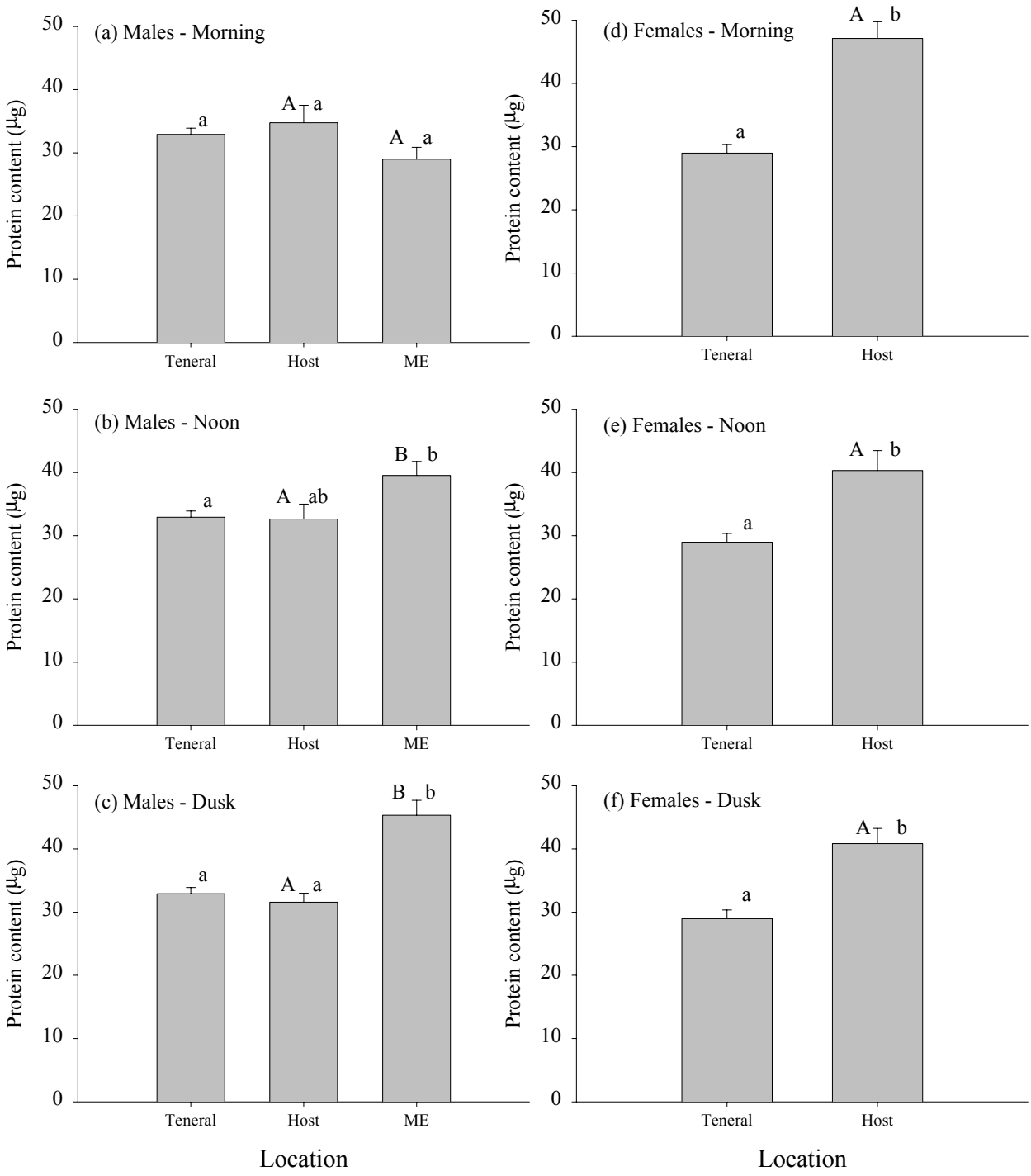

(a) Males - Morning
(b) Males - Noon
(c) Males - Dusk
(d) Females - Morning
(e) Females - Noon
(f) Females - Dusk
Lipid content (μg)
0
50
100
150
200
250
300
350
a
A b
B b
A a
Teneral
Host
ME
Location

Proteins

The protein content of males did not differ between resources in the morning (Figure 5.6a), but did so at noon and dusk (Table 5.2). At noon, the protein content of males at ME were significantly higher than teneral males, while males sampled at host did not differ from either teneral males or males sampled at ME (Figure 5.6b). Males sampled at ME at dusk had a significantly higher protein content than males at host and teneral males (Figure 5.6c). Males at host did not differ significantly from teneral males in terms of protein content (Figure 5.6c).

Female flies sampled at the host plant at different time periods did not differ from each other in terms of protein content, but had significantly higher protein reserves than teneral females at morning, noon and dusk (Table 5.2, Figures 5.6d, e, f).

Figure 5.6. Nutritional status of *Bactrocera cacuminata* in relation to resources. Protein reserves (µg/ fly) in male flies at (a) Morning (b) Noon and (c) Dusk and female flies at (d) Morning (e) Noon and (f) Dusk at different resources. Bars with same letters adjacent to them are not significantly different. Capital letters represent between time comparisons at a particular resource and lower case letters represent between resource comparisons within a particular time period.

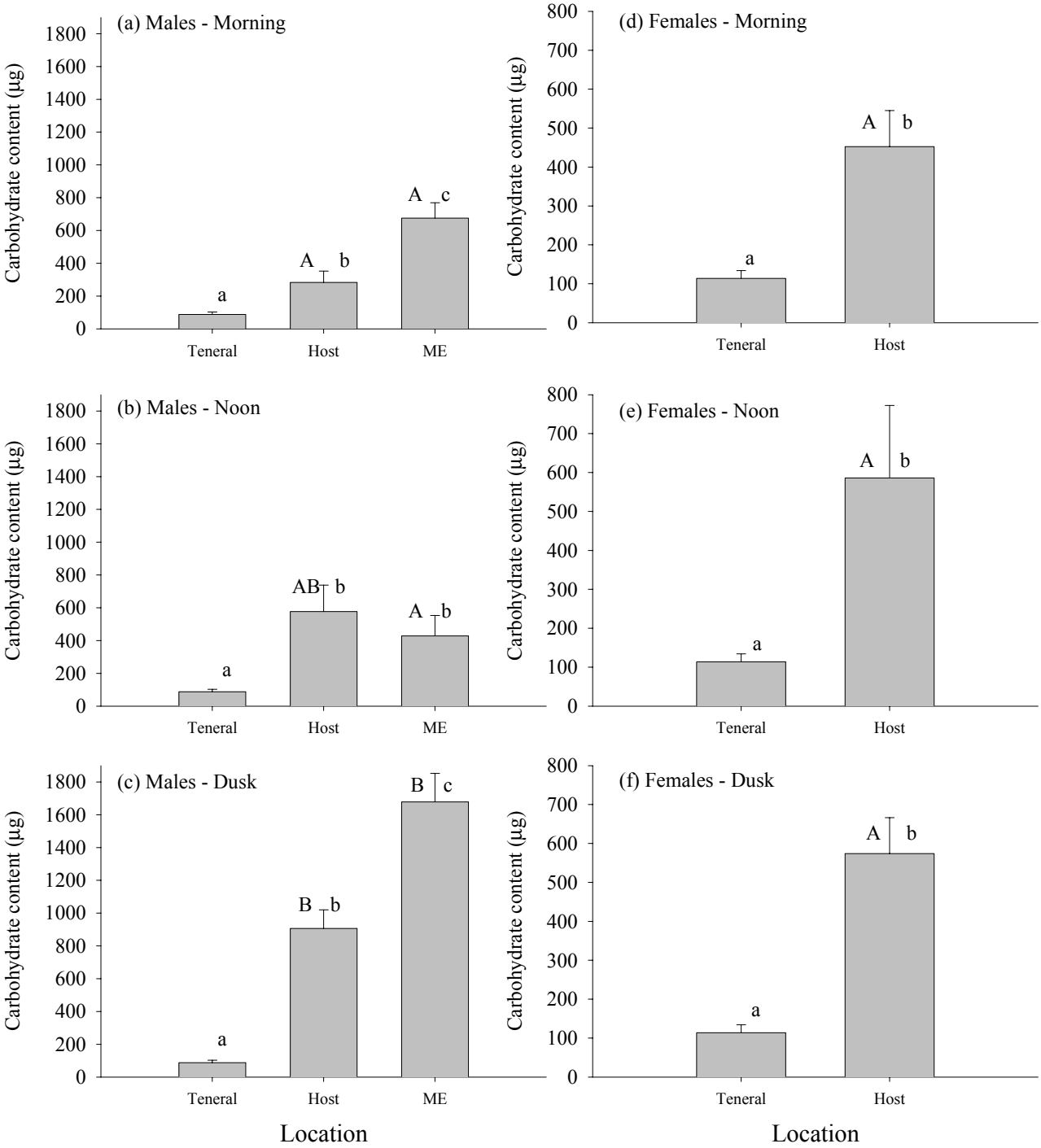

(a) Males - Morning
(b) Males - Noon
(c) Males - Dusk
(d) Females - Morning
(e) Females - Noon
(f) Females - Dusk
Protein content (μg)
0
10
20
30
40
50
a
A a
A a
A b
B b
A ab
Teneral
Host
ME
Location

Carbohydrates

The carbohydrate reserves differed significantly between the resources at morning, noon and dusk (Table 5.2). In the morning, males at ME had a significantly higher carbohydrate content than males at host and teneral males (Figure 5.7a) and males at the host plant had a significantly higher carbohydrate content than teneral males (Figure 5.7a). Males at host and ME did not differ significantly from each other at noon, but had significantly higher carbohydrate reserves than teneral males at noon (Figure 5.7b). Male flies at ME at dusk had a significantly higher carbohydrate content than teneral males and those at the host plant (Figure 5.7c). Males at the host plants had higher carbohydrate reserves than teneral males (Figure 5.7c).

Female flies at the host plant had significantly higher carbohydrate reserves than teneral females at all three time periods (Table 5.2, Figures 5.7d, e, f). Carbohydrate reserves in female flies at host did not differ between the three different time periods (Table 5.2, Figures 5.7d, e, f).

Figure 5.7. Nutritional status of *Bactrocera cacuminata* in relation to resources. Carbohydrate reserves (µg/ fly) in male flies at (a) Morning (b) Noon and (c) Dusk and female flies at (d) Morning (e) Noon and (f) Dusk at different resources. Bars with same letters adjacent to them are not significantly different. Capital letters represent between time comparisons at a particular resource and lower case letters represent between resource comparisons within a particular time period.


(a) Males - Morning
(b) Males - Noon
(c) Males - Dusk
(d) Females - Morning
(e) Females - Noon
(f) Females - Dusk
Carbohydrate content (µg)
Teneral
Host
ME
Location
a
A b
A c
AB b
A b
B b
B c

5.4 DISCUSSION

Flies responded to both the host plant and methyl eugenol, albeit the latter elicited a sex-specific response. A striking observation was the lack of response to protein in the present study. Strong response to the type of protein used has enabled it to be used in protein baiting for pest management of fruit flies in the field. I infer from this that protein was a reasonably abundant resource in the habitat where I sampled flies. Casual observations indicated that natural protein in the form of bird faeces deposited on leaves was common in the habitat, even though it was not present on the host plants used. An alternative explanation is that flies may respond to protein on foliage and so the manner in which I presented the resource may have led to a non-response.

The physiological and nutritional status of flies responding to the resources showed significant differences. Males sampled at ME were significantly more mature than the males at the host plant. Males at the host plant were, on average, immature at all three sampling times. This suggests that males partition their time between the host plant and ME as a function of their physiological status. Males at host may therefore be immature males foraging for nutrients, particularly sugars from wounds on the fruit surface and associated exudates, and proteins in the form of bird faeces and/ or bacteria (Drew and Yuval 2000). The carbohydrate reserves in males at different times are consistent with such a pattern of resource use (Figure 5.7). The protein content in males sampled at the host plant is not significantly different from that in teneral males, further suggesting that males at the host plant are sexually immature (Figure 5.6). An important finding is that sexually mature males are found at ME at dusk, the time of mating of B. *cacuminata* (Fletcher 1987), and not the host plant (Figure 5.2c). This explains the rarity of males and mating pairs at the host plant at dusk in previous studies (Raghu et al. 2002, Chapters 2, 3).

Another key finding is that teneral males appear to remain around the host plant while teneral females are quick to disperse (Figures 5.2-5.6). The final instar larvae pupate beneath the host tree whose fruit they infested. Teneral dacine adults are believed to disperse on emergence, a mechanism hypothesized to minimize competition for resources (Bateman 1972, Fletcher 1987). This phenomenon of sex-specific post-teneral dispersal in B. *cacuminata* is possibly adaptive and appears to conform to an "oogenesis-flight syndrome" postulated by Johnson (1969). However, given that *S. mauritianum* produces fruit for most of year, and since ripening of fruit within clusters is asynchronous (Symon 1979, 1981), oviposition resources are seldom in short supply at any given host plant patch (Drew and Hooper 1983). Therefore, further studies exploring the trade-offs made by individuals between energetic investment in dispersal, versus reproductive development needs to be conclusively established in order to demonstrate this to be the case (Dingle 1985).

I anticipated to sample females at both protein and the host plant. However, I only encountered female flies at the host plant. Dissections of female flies sampled at the host plant indicated that most of them had fully developed eggs in their ovaries and a significant proportion of them had already mated (Figure 5.3). This indicates that female *B. cacuminata* at the host plant were gravid females, visiting the host plant for the principal purpose of oviposition. The nutritional status of females at the host plant supports this argument. Lipids and proteins are an integral component of oogenesis (Beenakers et al. 1981). The high quantities of these nutrients in females (Figures 5.5, 5.6), in conjunction with their ovarian development and mating status, indicates that they are indeed gravid. Diurnal oviposition patterns in *B. cacuminata* peaks between late morning and dusk (Raghu et al. 2002) and the marginal decrease in average protein content between females sampled in the morning at the host plant versus those sampled at noon and dusk (Figure 5.6) could be as a result of oviposition. The marginally elevated

carbohydrate levels at noon and dusk compared to the morning (Figure 5.7) could be a result of feeding on fruit exudates during pre-ovipositional foraging.

The nutritional status of field caught flies compared with teneral flies reveals that tenerals emerge from puparia with low carbohydrate and lipids levels. While the former appears to be acquired in the field, lipids are synthesized de novo by the adult flies (Figures 5.5, 5.7, Warburg and Yuval 1996).

5.4.1. Functional Significance of resources

These findings substantiate Raghu et al.'s (2002) claims (see Chapter 2) that mating occurs elsewhere in the habitat, possibly at a natural ME source, a hypothesis suggested by Metcalf (1990). Copulation is energetically expensive (Slansky and Scriber 1985, Chapman 1998) and sugar and protein reserves are known to influence male copulatory behaviour in *Ceratitis capitata* (Mediterranean fruit fly): only flies with high levels of these reserves are found at the mating site (Blay and Yuval 1997, Warburg and Yuval 1997b, Yuval et al. 1998, Shelly et al. 2002). Lipids meanwhile serve as precursors in pheromone synthesis (Chapman 1998). Similar patterns of energetic status at the mating site have been recorded in damselflies (Marden and Waage 1990), caddis flies (Petersson and Hasselrot 1994), mosquitoes (Yuval et al. 1994) and dung flies (Otronen 1995). Therefore it can be expected that males at the mating site will be energetically superior compared to males elsewhere in the habitat. The trends in the nutritional reserves indicate this to be the case for B. *cacuminata* at ME (Figures 5.5-5.7). However, evidence contrary to this is that no female flies were sampled at ME.

Fitt (1981b) documented a response to ME by female dacine flies and similar observations have been made in recent studies in *B. cacuminata*

(Chapter 9). Both these studies presented ME amidst foliage. If female flies require visual cues in addition to olfactory cues in arriving at natural ME sources (e.g. Meats and Osbourne 2000), then the lack of response by female flies in this study may be an unintentional artefact of the sampling method, as no foliage was present at the pedestal where ME was present. The presence of foliage at natural ME sources (Nishida et al. 1993, 1997, Shelly 2000) may be a critical cue for females foraging for this resource. This may also explain the non-response of either sex to protein.

The detection of distinct physiological and developmental differences of males at ME over different times of day, in comparison to those at the host plant, strongly suggests that they actively forage for this resource. This is in contrast to the hypothesis that flies are exhibiting a positive anemotactic response to an odour they randomly encounter in their environment. While active foraging has been suspected, this may be the first field-based study of this that provides direct evidence.

Understanding resource use by individuals of a species is critical to understanding their ecology. Earlier behavioural observations (Chapter 2) and the present study indicates that though the host plant is a critical resource, it is not the hub of all adult behaviour. Flies may partition their activities between different resources in their habitat. In particular the physiological profile of flies at ME suggests that it may play a role as a key resource. The functional significance of ME in dacine ecology and behaviour is examined in the following chapters (Chapter 6-8). Behavioural partitioning is investigated in Chapter 9).

Acknowledgments – I thank Shlomit Shloush and Batya Kamenski, Hebrew University of Jerusalem and Peter Halcoop, Griffith University for their indispensable technical assistance.

Chapter Six

Feeding behaviour of *Bactrocera cacuminata* on methyl eugenol

This chapter has been accepted for publication in a slightly modified form:

Raghu, S. and Lawson, A.E. 2003. Feeding behaviour of *Bactrocera cacuminata* (Hering) on methyl eugenol: a laboratory assay. *Australian Journal of Entomology* (in press).

6.1 INTRODUCTION

Chemical lures attractive to tephritid fruit flies (Diptera: Tephritidae) have long been recognized (Howlett 1915, Steiner 1952, Beroza et al. 1960, Cunningham 1989a, b) and are a vital tool in the monitoring and management of the populations of these species (Cunningham and Steiner 1972, Cunningham et al. 1972, Sivinski and Calkins 1986). Examples of such chemicals and responding species include methyl eugenol (ME) (Oriental fruit fly, *Bactrocera dorsalis* [Hendel]), cue lure (Queensland fruit fly, *Bactrocera tryoni* [Frogg.]) and trimedlure (Mediterranean fruit fly, *Ceratitis capitata* [Weidemann]) (Fletcher 1987). Despite their widespread use in pest management and research, the biological significance of these chemicals remains enigmatic (Cunningham 1989a, b, Shelly 2000).

Some of these chemicals (e.g. ME) or their analogs (e.g. raspberry ketone, a natural analog of cue lure) are found in several plant families (Fletcher et al. 1975, Fletcher 1987, Metcalf 1990), although the natural occurrence of substances such as trimedlure are less clear (Drew 1987). Fletcher (1968), Metcalf et al. (1979) and Fitt (1981b, c) have postulated hypotheses for a role played by these substances in the pheromone systems of fruit flies and recent research has focussed on the functional significance of these substances, particularly in the context of mating behaviour (Shelly and Dewire 1994, Shelly et al. 1996a, b, Nishida et al. 1997, Shelly 2000).

Physiologically, however, some of these chemicals (e.g. ME) are principally kairomonal phagostimulants (Metcalf and Metcalf 1992) and flies in close proximity with these substances extend their proboscis in response. Response to the pure form of these chemicals can be so dramatic that "males will drink it until they fill their crops and die" (Cunningham 1989a). While mechanisms of orientation to these chemicals have been explicitly studied (Meats and Hartland 1999, Meats and Osborne 2000), the feeding behaviour

exhibited by dacine flies in relation to these chemicals have seldom been examined directly (Shelly 1994).

In this study I investigate the feeding behaviour of *Bactrocera cacuminata* (Hering) on ME in a laboratory environment. *Bactrocera cacuminata* is a non-pest member of the *B. dorsalis* complex of fruit flies (Drew 1989) and is a monophagous species that utilizes *Solanum mauritianum* Scopoli as its host plant. Males of this species respond strongly and positively to ME (Meats and Osborne 2000): in one field trial over 23,000 flies were caught in only 20 ME-baited Steiner traps over a 10 day period (Raghu et al. 2002, Chapter 2).

Specifically in this chapter I investigate the following questions.

1. Does feeding occur on ME and how often?
2. Is there a pattern in the frequency of feeding on ME?
3. Is the frequency and duration of feeding on ME related to time spent feeding on ME on a previous occasion?

These questions are critical to understanding the feeding behaviour of dacine flies on ME within an evolutionary framework (Tallamy et al. 1999), and are important in helping to understand the efficacy of ME baited lure traps. They are also significant in the context of testing generalizations that have been made from work on only one or two dacine pest species (Shelly 1994).

6.2 MATERIALS AND METHODS

All flies used in the experiment were from a colony maintained at Griffith University and were 8 generations old. Wild flies were released into the colony every 2-3 generations to minimise the effects of any laboratory-induced selection pressures.

Adult flies were separated by sex within two days of emergence, well before they attain sexual maturity at approximately 10-14 days. No more than 100 adult flies were maintained in 30 × 30 × 30cm screen cages with water, sugar and protein provided *ad libitum*. The cages were kept in a rearing room at a temperature of 25-27^{o}C and 65-70% relative humidity. The rearing room was under semi-natural light conditions, with fluorescent tubes illuminating the room between 0800 and 1600h and natural light for the remainder of the day.

Fifty sexually mature male flies (14 days old) were selected and housed individually in clear plastic containers (18 × 12 × 6cm: length × width × height) under natural light conditions. The flies had access to food (sugar + protein hydrolysate) and water continuously during the course of the experiment. Each day for 14 days, one ml of ME on a cotton wick (2cm long × 1cm diameter) was provided to each of the flies for a period of 30 min between 1100-1200h. The quantity of ME provided was similar to those in previous trials in related species (Shelly 1994). This period was selected as it has been shown to be the peak attraction period of *B. cacuminata* to ME (Brieze-Stegeman et al. 1978). Continuous observations were made over the 30 min period to document whether each individual fly was feeding, the number of feeding events/ bouts and duration of each feeding event per fly.

6.2.1. Data Analysis

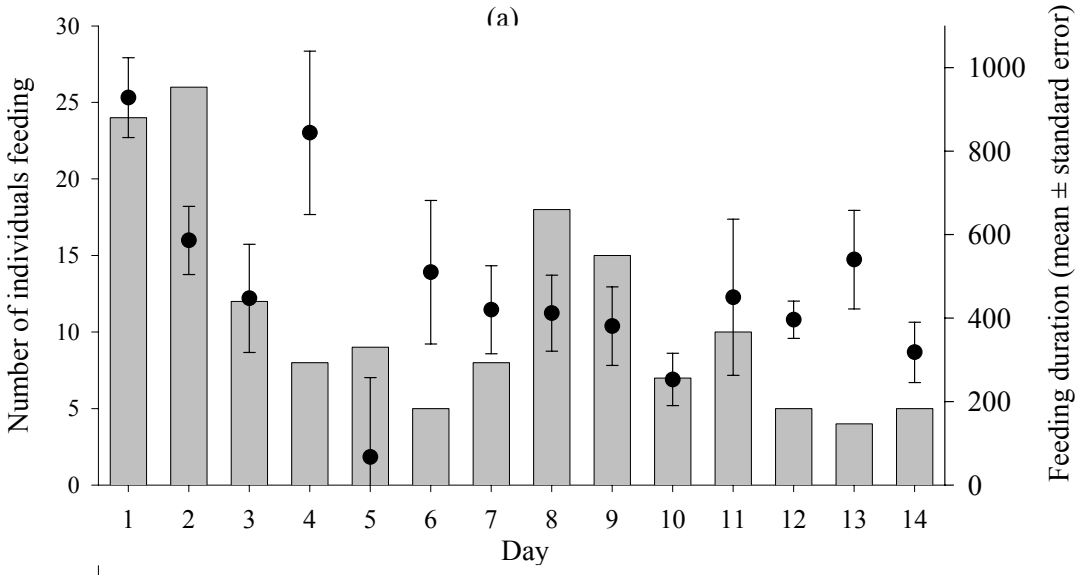
Data from these experiments were analyzed using a χ^2 Goodness of fit test (Zar 1999). As feeding on ME is expected to be a rare event (Shelly 1994), the frequency distribution of the number of times a fly fed on ME over the entire experiments and the duration since last feeding were tested against a random Poisson distribution (Zar 1999). In addition the relation between the duration of feeding event (in seconds) and the time (in days) till subsequent feeding and duration of subsequent feeding (in seconds) were examined using non-parametric correlation analyses.

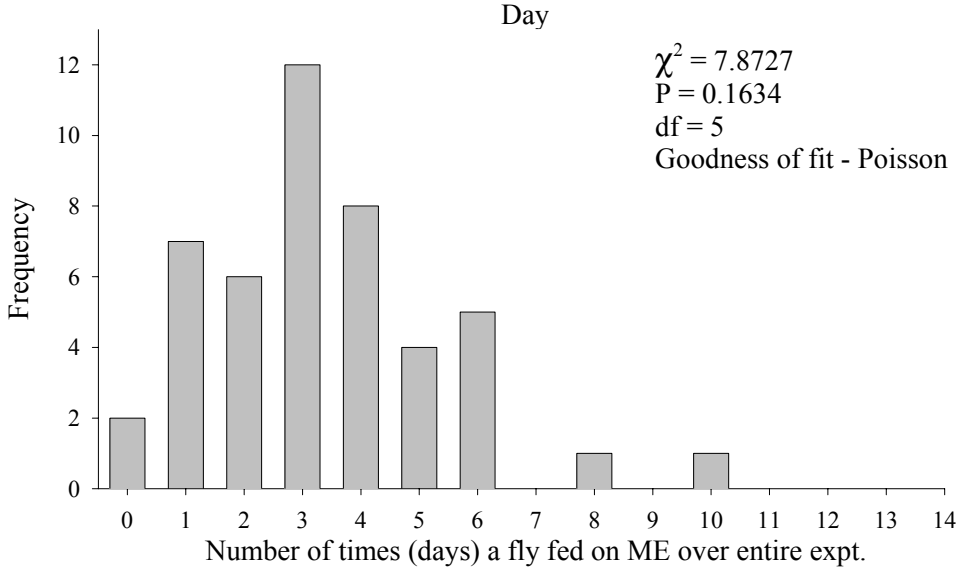
6.3 RESULTS

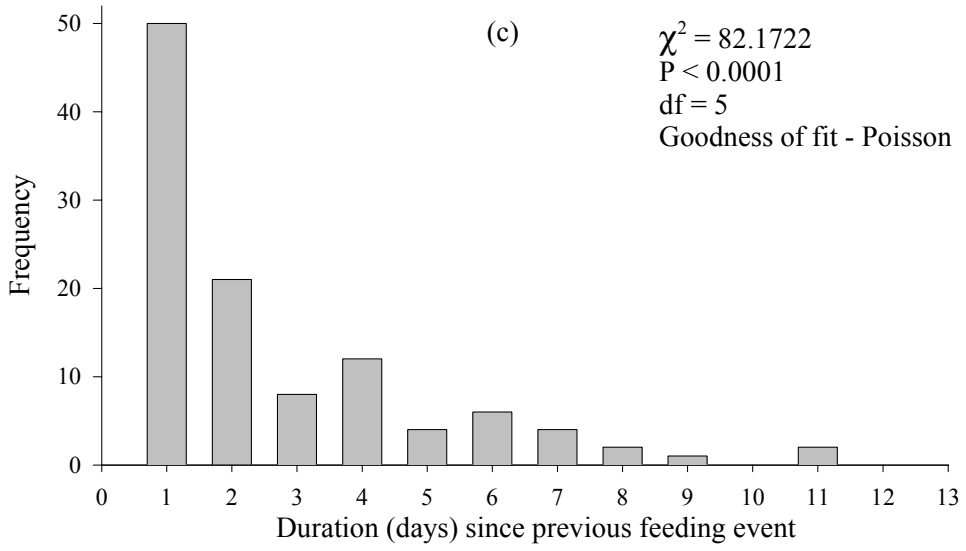
Four flies died during the course of the experiment and any data pertaining to them have been excluded from analyses. Flies were observed to feed on methyl eugenol on all days of the experiment (Figure 6.1a). The frequency distribution of the number of times a fly fed over the entire experiment was not significantly different from a random Poisson process ($\chi^2 = 7.827$, df = 5, $P = 0.1634$; Figure 6.1b). Only two flies did not feed at all throughout the experiment (Figure 6.1b). Most flies fed multiple times with a modal and median frequency of three feeding days during the entire experiment.

Where flies fed on multiple occasions, the modal duration between feeding events was one day with a median duration of two days. The frequency distribution of the duration in days since the last feeding event was significantly different from a random Poisson process ($\chi^2 = 82.1722$, df = 5, $P < 0.0001$; Figure 6.1c). Multiple bouts of feeding by an individual fly within a day were common (mean ± standard error = 2.02 ± 0.07 bouts/ fly/ day), with one fly feeding on 8 separate occasions within a single day. Duration of individual feeding bouts varied considerably (mean ± standard error = 260.63 ± 15.06 seconds/ bout; range 30-1800 seconds/ bout). The mean duration of feeding (sum of all bouts within a day) was variable (Figure 6.1a) with the longest average duration on days one and four. Flies fed for a considerable duration on each of the days they were exposed to ME (Figure 6.1a).

Figure 6.1. Feeding behaviour of male *Bactrocera cacuminata* on methyl eugenol. (a) Number of individuals feeding on methyl eugenol on each day of exposure (bar) and mean duration of feeding ± standard error (filled circle). (b) Frequency distribution of number of times an individual fly fed over the entire experiment. (c) Frequency distribution of interval between successive feeding events.


(a)
Number of individuals feeding
Feeding duration (mean ± standard error)
Day


χ2 = 7.8727
P = 0.1634
df = 5
Goodness of fit - Poisson
Frequency
Number of times (days) a fly fed on ME over entire expt.


(c)
χ2 = 82.1722
P < 0.0001
df = 5
Goodness of fit - Poisson
Frequency
Duration (days) since previous feeding event

Spearman's rank-order correlation coefficient revealed that there was no significant relationship between duration of feeding event (time of all bouts combined for a day) and time in days till next feeding event (r_S = 0.049, N = 110, P = 0.611; Figure 6.2a). There was no relationship between duration of feeding by *B. cacuminata* and duration of the subsequent feeding event (r_S = 0.074, N = 110, P = 0.440; Figure 6.2b).

6.4 DISCUSSION

The use of lures and attractants in the control of insects is quite common (Howse et al. 1998). In some cases the precise biological/ ecological reason underpinning these attractants are well understood (Kennedy 1978). However, for certain insects, such attractants have been fortuitously discovered and the biological basis for their success remains an enigma (Carde and Minks 1997, Hardie and Minks 1999). Dacine fruit flies are one such group of insects. In spite of the widespread use of lures in fruit fly management, their role in the ecology and evolution of fruit flies remain largely unresolved.

Repeat feeding on methyl eugenol has been hypothesized to be a rare occurrence (Shelly 1994). This study's results show that, in small container situations, multiple feeding on ME is a common occurrence in *B. cacuminata*, with many individuals feeding on multiple occasions within each day (Figure 6.1b) and on successive days (Figure 6.1c). One explanation for this could be that repeat feeding was only occurring in flies that were consuming small amounts of ME during first feeding. Assuming that feeding duration is a reliable indicator of ME intake, the poor correlations between duration of feeding and time to next feeding (Figure 6.2a) and duration of subsequent feeding (Figure 6.2b) suggests that this is an unlikely explanation.

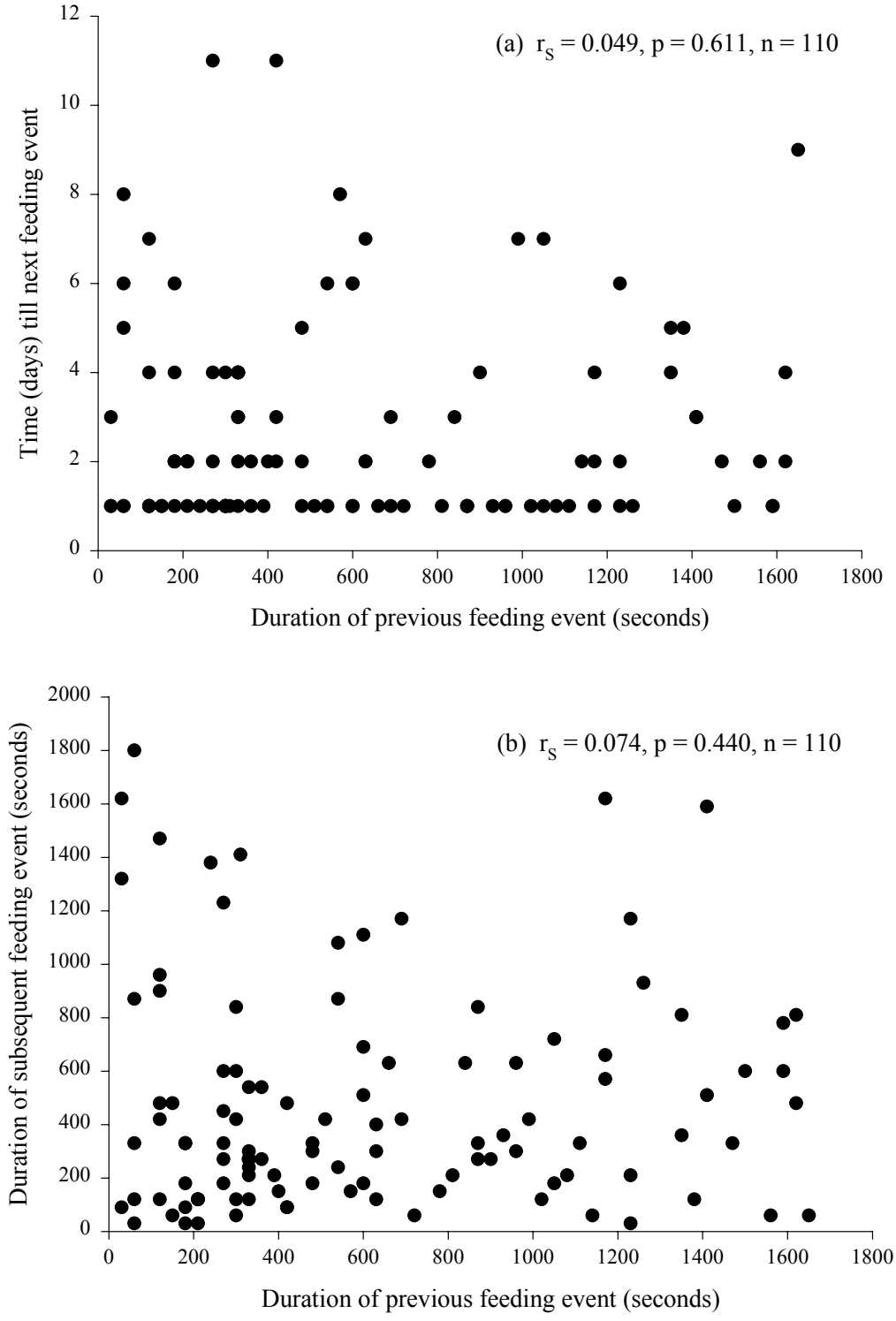


Figure 6.2. The duration of feeding on methyl eugenol (in seconds) by male *Bactrocera cacuminata* correlated with (a) Time (days) till subsequent feeding event and (b) Duration of subsequent feeding event (in seconds).

A plausible alternate explanation for the patterns observed is that the use of one ml of ME was a significantly higher dose than occurs naturally and what I observed was a case of sensory overload (Barton-Browne 1975) resulting in abnormally frequent phagostimulation. However, studies on a related species (*B. dorsalis*) using a similar dose of ME, showed that while 91% of males exposed to ME fed on first exposure to the chemical, only 38% exhibited repeat feeding (Shelly 1994). However, differences in release rates of pheromones between *B. cacuminata* and *B. dorsalis* may explain the differences in the feeding frequency between the present study and that of Shelly (1994) and further studies are required to clarify this.

The evaluation of feeding in field situations is critical. While such studies have seldom been undertaken, feeding behaviour has been inferred from visitation to ME lure-baited traps by *B. cacuminata* (Brieze-Stegeman et al. 1978) and *B. dorsalis* (Shelly 1994) and to cue lure traps by *Bactrocera cucurbiatae* (Coquillett) (Chambers et al. 1972). In all these studies the authors used re-visitation of marked flies to lure traps as a measure of responsiveness after prior exposure. Caution needs to be exercised with inferring that as evidence for rarity of repeat feeding, as has been done, given the possibility that the low rate of recapture of previously lure-fed, marked individuals may be an artefact caused by the normally low recapture rates which are a component of many mark-recapture studies. Recapture rates from population dynamics research on dacine flies using lure-baited traps vary between 0.03-0.3% (MacFarlane et al. 1987) and 9.57% (Sonleitner and Bateman 1963). Hence it is highly likely that any estimation of frequency of feeding from revisitation of lure-baited traps is likely to be a gross underestimate.

The estimation of feeding behaviour on ME in natural occurring concentrations and from natural sources in a field experiment is likely to prove more insightful than either laboratory or field based studies using

artificial lure sources. If ME is a precursor to a male sex pheromone (Shelly 2000), then repeat feeding should not be unexpected as pheromones are highly volatile (Tillman et al. 1999). Multiple feeding on a precursor chemical is likely to be common so as to allow males to replenish pheromones for subsequent release.

Chapter Seven

Does methyl eugenol play a role in mate choice in the mating behaviour of *Bactrocera cacuminata*?

This chapter has been accepted for publication in a slightly modified form:

Raghu, S. and Clarke, A.R. Sexual selection in a tropical fruit fly: role of plant derived chemical in mate choice. *Entomologia Experimentalis et Applicata* (in press).

7.1 INTRODUCTION

Darwin (1871) speculated that forces other than natural selection influence the nature of species, in particular he realized that an equally powerful influence could be sexual selection. However, the underlying mechanisms of sexual selection (i.e. male-male competition and female choice) are often difficult to resolve (Searcy 1982, Andersson 1994, Eberhard, 1997, Ryan 1997) and may not be independent of each other. For example, in certain fish species (Morris et al. 1995, Candolin 1999) and barn swallows (Galeotti et al. 1997), male-male competition influences female choice. It has been noted that difficulties of resolving the mechanisms of sexual selection are particularly evident in insect species (Thornhill and Alcock 1983, Conner 1988, Pormarcom and Boake 1991).

For one group (Dacinae) within the insect family Tephritidae (true fruit flies; Insecta: Diptera), sexual selection has been hypothesised to occur through a mechanism of female choice (Shelly 2000, 2001). Female flies are believed to preferentially mate with males that have fed on a group of chemicals known to fruit fly biologists as parapheromones or male-lures. These chemicals occur naturally in plants (e.g. methyl eugenol [ME]) or are close analogues of plant-derived chemicals (e.g. cuelure) (Fletcher et al. 1975, Sivinski and Calkins 1986, Fletcher 1987). The parapheromones elicit strong anemotaxis in male flies and, at least in some species, equally strong chemotactic feeding responses (Meats and Hartland, 1999, Meats and Osborne 2000). The ingested substances are hypothesized to be integrated into the male fly's sex pheromone system (Fitt 1981b, c), subsequently making those males more attractive to females (Nishida et al. 1988, 1993, 1997, Shelly 2000). Thus, female choice is believed to be responsible for the exceptionally strong response of male fruit flies to these chemicals (Shelly 2000).

This hypothesis has been tested and validated in *Bactrocera dorsalis*, the best-studied species. ME-fed males have enhanced mating competitiveness over unfed males (Shelly and Dewire 1994, Tan and Nishida 1996). Unfortunately, a paucity of similar work on other parapheromone responding dacine species makes it difficult to judge if the results from *B. dorsalis* are widespread in dacine flies, or if it is a species-specific characteristic.

In this chapter I examine the hypothesis that feeding on methyl eugenol (ME) enhances male mating success in *Bactrocera cacuminata*. Specifically I investigate the following questions.

1. Do females preferentially mate with ME-fed males over males that have not fed on ME?
2. Is the pattern of mating in relation to prior exposure to ME consistent across different spatial scales?

7.2 MATERIALS AND METHODS

Bactrocera cacuminata is a non-pest, monophagous species that utilizes *Solanum mauritianum* Scopoli as its host plant. It is a member of the *B. dorsalis* complex of fruit flies (Drew 1989b). This complex includes *B. dorsalis*, the subject on which previous tests of Fitt's (1981b, c) hypothesis have been done (Shelly and Dewire 1994, Tan and Nishida 1996). *Bactrocera cacuminata* males also respond strongly and positively to ME: in one field trial over 23,000 flies were caught in 20 ME-baited traps over a 10 day period (Raghu et al. 2002). This fly is therefore an appropriate candidate to examine the generality of the ME's hypothesized role in mate choice.

All flies used in the experiment were from a colony maintained at Griffith University. Flies used in the glass house experiments were in culture

for 8 generations, while those used in the field-cage had been cultured for 16 generations. Wild flies were released into the colony every 2-3 generations to minimise the effects of any laboratory-induced selection pressures.

Adult flies were separated by sex within two days of emergence, well before they attain sexual maturity at approximately 10-14 days. No more than 100 adult flies were maintained in 30 × 30 × 30 cm screen cages with water, sugar and protein provided *ad libitum*. The flies were kept in a rearing room at a temperature of 25-27^{o}C and 65-70% relative humidity. The rearing room was under semi-natural light conditions, with fluorescent tubes illuminating the room between 0800 and 1600h and natural light for the remainder of the day.

Male flies used in these studies were separated into a treatment and control group. The former was exposed to 2ml of ME on a cotton wick for a continuous 24 hour period beginning at 0600 hours. Flies were observed to feed on the wick within five minutes of initial exposure to the wick. The age of flies at time of exposure was 14 days. The control group was not exposed to ME.

Mating in *B. cacuminata* has been studied in considerable detail in the laboratory (Myers 1952) and is restricted to dusk (Fletcher 1987).

7.2.1. Small cage experiments

On the day of exposure (Day 0), 5 ME-fed virgin males, 5 unexposed virgin males (hereafter referred to as unfed) and five virgin females were released in to each of ten clear perspex cages (40 × 40 × 40 cm) at 1500h. Each cage contained a terminal portion of a *S. mauritanum* branch that comprised a cluster of fruit and a whorl of leaves, with the stalk immersed in a flask of water. The cages were housed in an ambient temperature glasshouse under natural light conditions. Prior to the release of the flies, treatment and control

males were cooled (≈ 10-12°C) and marked with a different colour on the thorax. Preliminary analyses indicated that such marking had no effect on mating competitiveness (χ^2 = 0.0196, df = 1, P = 0.8885). As prolonged cooling may influence behaviour (Barron 2000), care was taken not to expose flies to low temperatures for more than ten minutes. Flies were observed to resume normal activity within 5 minutes of being released into the cage.

Continuous observations were made from 1600 (early dusk) to 1930h (full night). Details of courtship, time of initiation and duration of copulation and type of male in copula (ME-fed vs. unfed) were recorded. If copulation had not terminated by the end of the observation period, observations were made at 0600h the following morning to determine if flies remain coupled during the night or terminated copulation during the night. If copulation had terminated during the night the duration of copulation was calculated to be the time between end of the observation period (1930h) and the time of copulation initiation. This method consistently underestimated the duration of copulation (see Results section 7.3.1). The trial was repeated on days 1, 2, 4, 8, 16 and 32 after exposure to ME. These day intervals were chosen as they were similar to previous experiments (Shelly and Dewire 1994).

7.2.2. Field-cage experiments

A cylindrical field-cage (230 cm high × 250 cm diameter) was set up housing three potted *S. mauritianum* plants. At similar intervals to the small cage experiments, 10 ME-fed, 10 unfed and 10 virgin females were released into the field-cage. Observations were made from 1600 to 1930h and data similar to that in the small cage experiment were gathered.

Flies in all mating behaviour experiments were only used once.

7.2.3. Data Analysis

A logistic regresssion analysis was used to test the effect of exposure to ME on mating success of male *B. cacuminata* (Zar 1999). The effect of exposure to ME on time of copulation initiation and copulation duration (time mating pair remained coupled) was investigated using the Kruskal–Wallis test and univariate analysis of variance respectively, with status (ME-fed vs. Unfed) as the factor. Data for these analyses were pooled across days for all mating behaviours as there were no significant within-day differences, either in time of copulation initiation or copulation duration, between flies of either status. Conformation of data to assumptions of statistical analyses (e.g. normality, homoscedasticity) was verified prior to their application.

The effects of exposure to ME on mating success of males in the field-cage study was analyzed using binomial tests (Conover 1999), to test if the ratio of successful ME-fed males to successful unfed male differed significantly from 1:1, on each of the days observations were made.

7.3 RESULTS

7.3.1. Small cage experiments

Over the entire small cage experiment more ME-fed males mated (72 copulations) than did unfed males (44 copulations). Logistic regression analyses revealed that treatment had a significant influence on mating success (χ^2 = 6.64, df = 1, P = 0.01). However, there was no consistent advantage of males of either type (ME-fed vs. unfed) over time as revealed by the significant interaction effect between treatment and days since exposure (χ^2 = 21.46, df = 6, P = 0.0015; Figure 7.1). There was no difference in the number of matings achieved by ME-fed males and unfed males on days 0, 1, 2, 4, and 8 (Figure 7.1; Day 0 – $F_{1,18}$ = 0, P = 1; Day 1 – $F_{1,18}$ = 0.559, P = 0.464; Day 2 – $F_{1,18}$ = 1.670, P = 0.213; Day 4 – $F_{1,18}$ = 0.679, P = 0.421; Day 8 – $F_{1,18}$ = 0.947, P = 0.343). However there was a significant difference on days

16 and 32 with ME-fed males having a much higher mating success than unfed males (Figure 7.1; Day 16 – $F_{1,18}$ = 6.698, P = 0.019; Day 32 – $F_{1,18}$ = 57.800, P < 0.001).

There was no difference in time of copulation initiation between ME-fed males and unfed males (Kruskal–Wallis H = 0.162, df = 1, P = 0.687; Figure 7.2a). The duration of copulation also did not significantly differ between unfed males and ME-fed males ($F_{1,114}$ = 0.978, P = 0.325; Figure 7.2b). Forty-nine ME-fed and 35 unfed flies remained in copula at the end of the observation period. Therefore, the lack of treatment effect on copulation duration was not due to any bias in estimation.

7.3.2. Field-cage experiments

Binomial tests comparing the proportions of copulations achieved by ME-fed males and unfed males revealed that there was no significant difference between males of either state on all days when observations were made (Figure 7.3).

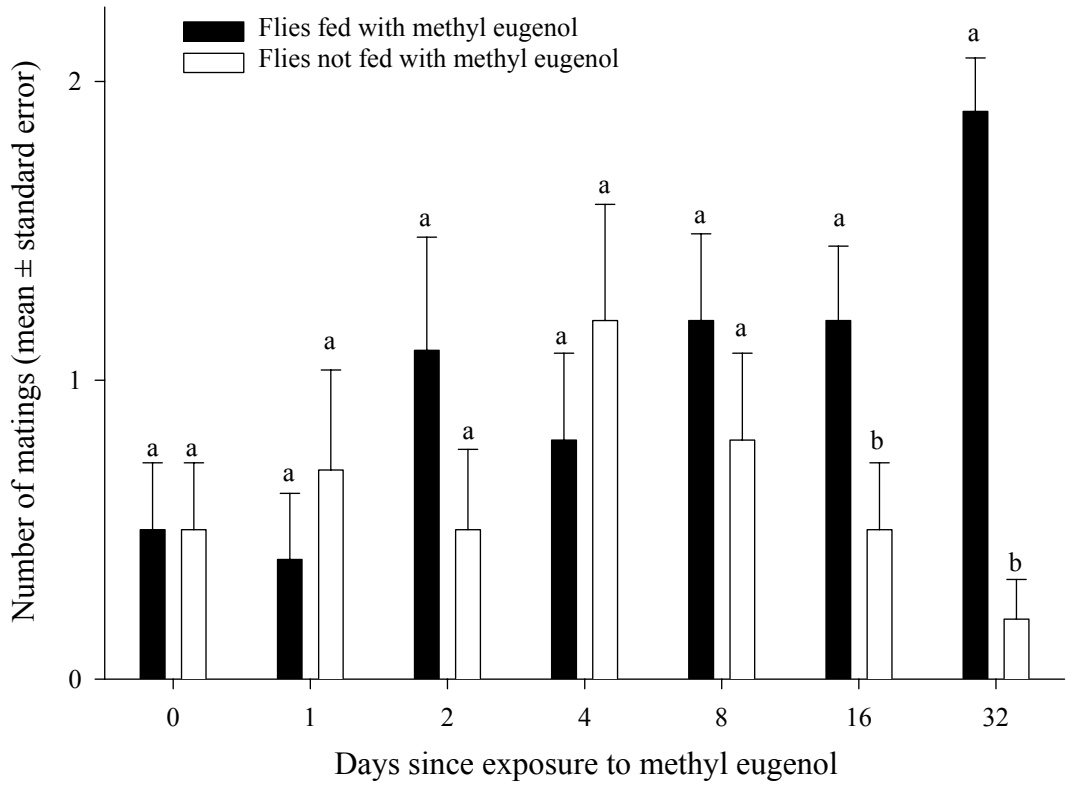
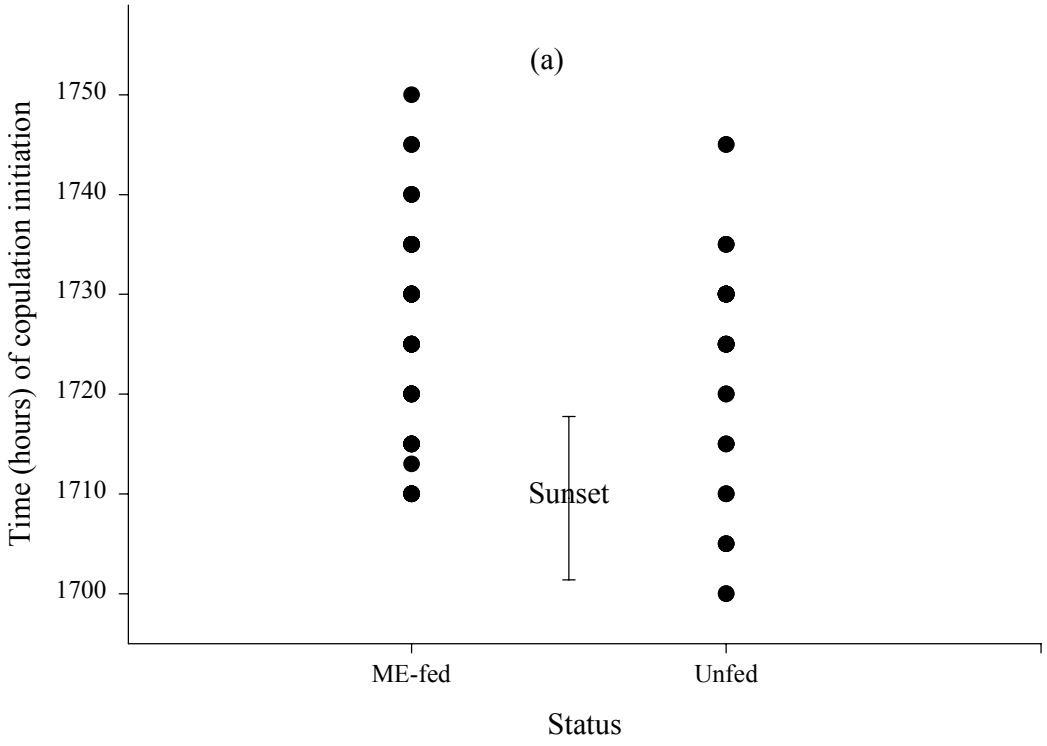
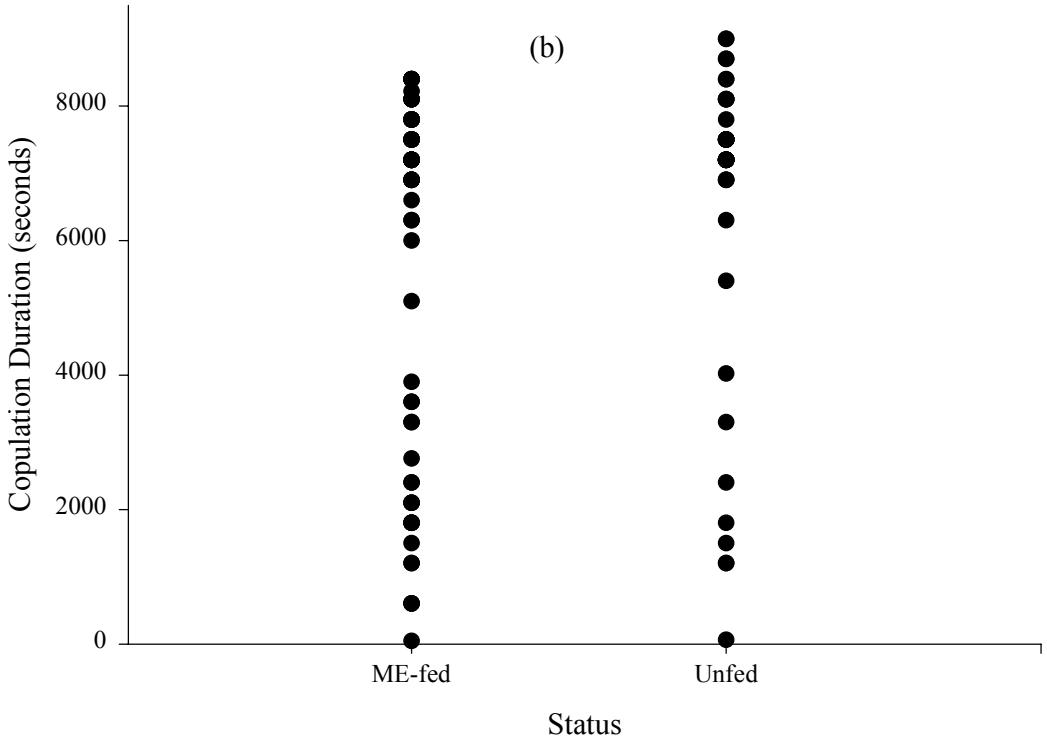


Figure 7.1. The relative mating success of methyl eugenol fed *Bactrocera cacuminata* males versus unfed males over time in small cage experiments. Shaded bars represent methyl eugenol fed males and open bars represent unfed males. The letters above the bars represent outcomes of univariate analyses of variance. Same letters on adjacent bars on any given day indicate no significant difference ($P > 0.05$) in mating success between ME-fed and unfed males.

Figure 7.2. Effect of exposure to methyl eugenol on copulation. (a) Time of copulation initiation (hours) in relation to status (methyl eugenol fed or unfed) of copulating male. (b) Copulation duration (seconds) in relation to status (ME-fed or unfed) of copulating male. *N* = 72 for methyl eugenol fed males; *N* = 44 for unfed males for both graphs.


(a)
Time (hours) of copulation initiation
1750
1740
1730
1720
1710
1700
Sunset
ME-fed
Unfed
Status


(b)
Copulation Duration (seconds)
8000
6000
4000
2000
0
ME-fed
Unfed
Status

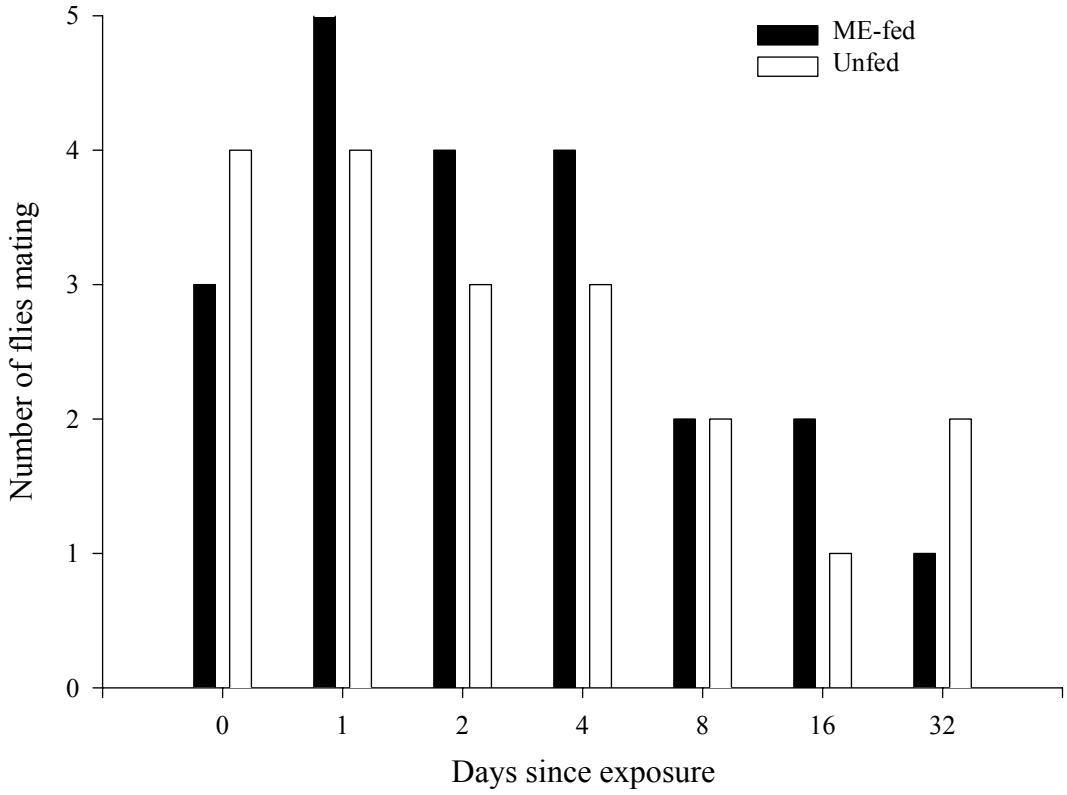


Figure 7.3. Mating success of methyl eugenol fed males versus unfed males over time in a large field-cage. Shaded bars represent methyl eugenol fed males and open bars represent unfed males.

7.4 DISCUSSION

This investigation of the influence of ME on mating success revealed that ME-fed males in the small cage were more successful in mating than unfed males only 16 and 32 days after exposure to ME (Figure 7.1). Furthermore, if females were preferentially choosing ME-fed males, these males may be expected to have an advantage in terms of copulation duration, with the selected males having prolonged copulatory or post-copulatory contact as a form of paternity insurance (Yuval et al. 1990, Alcock 1994, Andersson 1994, Radwan and Siva-Jothy 1996, Field et al. 1999). Similar benefits may be expected in terms of time of copulation initiation (i.e. "preferred" males might begin copulation earlier). However, the results presented above indicate that there is no difference between ME-fed and unfed males in copulation duration or time of copulation initiation (Figure 7.2). These results are contrary to those observed in *B. dorsalis* where ME-fed males had enhanced mating success over unfed males (Shelly and Dewire 1994, Tan and Nishida 1996, Nishida et al. 1997).

Why do ME-fed males have a mating advantage on days 16 and 32, but not on days prior to that? Could this be the physiological processing time for the transformation of ME (the precursor) into the pheromone? This appears unlikely given that transformation of precursors into pheromones is quite rapid in insects (Tillman et al. 1999, C. J. Moore – personal communication). In the case of fruit flies, Nishida et al. (1988) reported that the transformation of ME into metabolites identified from the rectal gland took between 1-3 days. Hence, if methyl eugenol is a precursor to the male sex pheromone, ME-fed males should experience higher mating success much earlier than observed in the present study. Therefore, the patterns observed on days 16 and 32 may be an artefact of spatial confinement in a small cage.

As a follow-up to the small cage and field-cage experiments I ran a field experiment, caging individuals and clusters of ME-fed and unfed flies, in cylindrical tubes that allowed air-flow, on host and non-host plants, similar to previous studies (Shelly 2000, 2001). I observed continuously at dusk for visitation of these tubes and adjacent foliage by conspecifics. Despite sufficient replication (N=10, on day intervals similar to the small age and field-cage experiments) no visitation by wild flies were recorded. This may be in part due to the fact that mating behaviour may be spatially restricted in this species (Chapter 3). However, the absence of mating advantage in the field-cage experiment (Figure 7.3) and the non-response to ME-fed individuals and clusters in the field experiment further suggests that ME (as a precursor to a long distance pheromone) may not be critical in the mating system of *B. cacuminata*. Given these findings and the results of the present study, there does not appear to be a simple pheromone based explanation, to clarify the basis for the strong attraction of this fly species to methyl eugenol.

One alternative explanation could be that feeding on ME confers some other physiological benefits to males, such as enhanced energetic reserves (referred to as "metabolic competence" by Watson and Lighton 1994) and this is the basis for female choice. This is not uncommon in insects that feed on phytochemicals believed to be pheromone precursors (Arnold and Houck 1982, Thornhill and Alcock 1983, Tillman et al. 1999). This is explored in the next chapter.

Chapter Eight

Does feeding on methyl eugenol have physiological consequences for *Bactrocera cacuminata?*

This chapter has been published in a slightly modified form:

Raghu, S., Clarke, A.R. and Yuval, B. 2002. Investigation of the physiological consequences of feeding on methyl eugenol by *Bactrocera cacuminata* (Diptera: Tephritidae) *Environmental Entomology* 31: 941–946.

8.1 INTRODUCTION

Males of many of the Tephritidae (Diptera) show strong responses to certain chemical compounds commonly referred to as male lures or parapheromones (e.g. cuelure, methyl eugenol, trimedlure). Some of these substances, such as methyl eugenol, occur naturally in plants or are analogs of substances produced by plants (Fletcher et al. 1975, Chambers 1977, Sivinski and Calkins 1986, Fletcher, 1987). The natural occurrence of others, such as cuelure and trimedlure, is less clear (Drew 1987). It is known that some of these chemicals elicit strong chemotactic feeding responses in male flies (Meats and Hartland 1999, Meats and Osborne 2000). However despite extensive studies of feeding and mating behavior in fruit flies (see reviews in Aluja and Norrbom 2000), the precise ecological role of these parapheromones remains an enigma.

Three principal hypotheses have been proposed to explain the role of parapheromones in the ecology of fruit flies. Metcalf et al. (1979) suggested that they serve as rendezvous stimuli used by males to locate mates. The second hypothesis explains attraction as a result of the parapheromones' fortuitous similarity to male aggregation pheromones (Fletcher 1968). The generality of these two hypotheses with respect to cuelure and methyl eugenol responding flies is in doubt given that males of fruit fly species are least responsive to these compounds at times of peak daily sexual activity (Brieze-Stegeman et al. 1978, Fitt 1981a). Furthermore, when populations are large, female flies are seldom attracted to these principally male lures (Fitt 1981b). The third hypothesis, first postulated by Fitt (1981b), suggests that these chemicals serve as pheromone precursors, vital to the synthesis of the male sex pheromone.

While the third hypothesis (Fitt 1981b) is a plausible explanation, there is considerable variability in the behavior of different species of flies towards

different lures. *Ceratitis capitata* (Weidemann), the Mediterranean fruit fly, does not ingest trimedlure (Shelly and Dewire 1994; Shelly et al. 1996), while species of the subfamily Dacinae do ingest cuelure or methyl eugenol (Fitt 1981b, c) and appear to integrate metabolites derived from lures into their pheromone system (Nishida et al. 1988, 1993, 1997). The strength of this hypothesis rests in the fact that females are attracted to these parapheromones when males are rare in the environment (Steiner et al. 1965, Nakagawa et al. 1970, Fletcher et al. 1975, Fitt 1981b, c), suggesting a mate seeking behavior based on a pheromone system. Female flies are believed to preferentially mate with males that have this phytochemically enhanced pheromone (Shelly and Dewire 1994, Tan and Nishida 1996, Shelly 2000).

This sequence, of attraction to and feeding on a plant derived substance, with its attendant behavioral and fitness consequences, could be described as *pharmacophagous* (sensu Boppré 1984). In order to examine whether this is the case it must be demonstrated that the benefits derived from these chemicals are ecological and *not* primarily metabolic (e.g. nutritional) or associated with host plant recognition (Boppré 1984, Nishida and Fukami 1990, Halaweish et al. 1999). In this chapter, my objective was to test the hypothesis that the attraction to and feeding on methyl eugenol by the dacine fly, *Bactrocera cacuminata* (Hering), is pharmacophagous. The behavioral consequences of feeding on methyl eugenol are being examined in a companion study (Chapters 6, 7). To test the hypothesis that *B. cacuminata* attraction to ME is pharmocophagous I examined whether exposure to methyl eugenol affects levels of fly nutritional reserves and survival. If the behavior is pharmacophagous, then there should be no physiological benefits in relation to feeding on parapheromones.

8.2 MATERIALS AND METHODS

Bactrocera cacuminata is a methyl eugenol responding, non-pest, monophagous species that utilizes *Solanum mauritianum* Scopoli as its host plant. It is a member of the *B. dorsalis* complex of fruit flies (Drew 1989b, Drew and Hancock 1994) which includes the oriental fruit fly, on which previous tests of Fitt's (1981a) hypothesis have been done (Shelly 1994, Shelly and Dewire 1994, Tan and Nishida 1996).

All flies used in the experiment were from a colony maintained at Griffith University. Wild flies were released into the colony every two to three generations to negate the effects of any laboratory induced selection pressures. Adult flies were separated by sex within 2d of emergence, well before they attain sexual maturity at approximately 10-14 d. No more than 100 adult flies were maintained in 30 × 30 × 30cm screen cages with water, sugar and protein provided *ad libitum*. The flies were maintained in a rearing room at a temperature of 25-27^{o}C and 65-70% relative humidity. The rearing room was under semi-natural light conditions, with fluorescent tubes illuminating the room between 0800 and 1600h and natural light for the remainder of the day.

For each of two experiments (see below), 400 newly emerged male flies were isolated from females within 3 days of adult emergence. Two weeks after emergence (i.e. when flies attained sexual maturity), half the flies were exposed to 4 ml methyl eugenol (ME) on a cotton wick for a period of 24h. Casual observations indicated that a majority of the flies exposed to ME began feeding on it (frequent contact of the wick by their proboscis) within the first few minutes of exposure. These flies are henceforth referred to as ME-fed flies. The remaining male flies were not provided with methyl eugenol and are hereafter referred to unfed flies.

A cylindrical field cage (230cm tall × 250cm diameter) containing three potted *S. mauritianum* plants was set up in a garden at least 3 d prior to the start of each experiment. This allowed for the colonization of the cage by potential competitors for resources (e.g. ants and bugs) and predators (e.g. ants, spiders and lizards) (pers. obs.). The potted plants had previously been grown in their natural habitat, along a rainforest edge. For each experiment, 200 ME-fed and 200 ME-unfed male flies were released into the field cage immediately after the 24h ME exposure period. Prior to release, the unfed flies were marked with a white spot on the thorax, using liquid paper. Previous observations have shown that this does not alter their mobility or behavior in any significant way.

8.2.1. Experiments

One experiment (*Experiment 1*) was run with only sugar solution (10 ml; sprayed three timers per wk on parts of the foliage) and water provided in the field cage, while in a second experiment (*Experiment 2*) sugar solution, water and a protein autolysate solution was supplied. The protein was supplied as 5ml of solution soaked into a sponge, with fresh protein/sponge provided three times per wk. The sponge was randomly re-positioned within the field cage every time the protein was provided. This was done to ensure that flies had to actively forage to find the protein source.

In *Experiment 1*, 10 flies of each status were sampled on days 0 (day of release into cage), 1, 2, 4 and 8 for biochemical analyses. In *Experiment 2*, 10 flies of each status were sampled on days 0 (day of release into cage), 1, 2, 4, 8 and 16 for biochemical analyses.

Biochemical analysis

Each of the flies were dessicated at 30^{o} C for 24 h and weighed on an analytical balance (± 0.01 mg). To determine the levels of protein, lipid and carbohydrates present in the flies, the biochemical techniques of Van Handel and Day (1988), as modified by Warburg and Yuval (1996, 1997b) and Yuval et al. (1998) were used as described below. All data were standardized based on the dry weight of the fly to correct for variations in size.

Flies were homogenized individually in 200 µl of 2% Na_2SO_4. Carbohydrates and lipids were extracted in 1300 µl of chloroform : methanol (1:2). Individual tubes were centrifuged at 8000 rev per min and 500 µl were taken from the supernatant of each sample and dried. Samples were then dissolved in 500 µl H_2SO_4 and incubated for 10 minutes at 90^{o}C. Samples of 30 µl were put into wells on ELISA plates together with 270 µl of vanillin reagent (600 mg vanillin dissolved in 100 ml of distilled water and 400 ml of 85% H_3PO_4). The plate was shaken at room temperature for 30 min and then the optical density was read at 530 nm on an EL311SX Bio-tek Spectrophotometer. Total lipids per fly were calculated from standard curves using the KCJR EIA application software (Bio-tek Instruments Inc., Winooski, Vermont).

Sugar content per fly was assessed using 300 µl from the supernantant of the chloroform : methanol extract. After adding 200 µl of water the sample was reacted with 1 ml of anthrone reagent (500 mg of anthrone dissolved in 500ml of conc. H_2SO_4) at 90^{o}C. Samples of 300 µl were then put into wells on ELISA plates and the optical density was read at 630 nm. Similar to the lipid content analysis, total carbohydrates per fly was estimated using standard curves.

Dissolved protein was extracted in 1200 µl phosphate buffer saline (PBS). Samples of 300 µl were taken and after adding 500 µl of PBS, were

reacted with 200 µl of Bradford reagent (Bradford 1976). Samples of 300 µl were then put into wells on ELISA plates and optical density was read at 595 nm. Total dissolved protein per fly was calculated from standard curves.

Survival

In each of the two experiments (mentioned above) the field cage was censused every alternate day for a period of 1 month from release of the flies. For a focussed period of 5 min, the entire cage was scanned for the two groups of flies and the number of individuals observed was noted. Since equal numbers of ME-fed and unfed flies were sampled from the field cage for biochemical analyses, the sampling protocol did not bias the survival estimates in favor of either state.

8.2.2. Data analysis

Differences in the weight, lipid, protein and carbohydrate reserves between ME-fed and unfed flies were analysed using Analyses of Variance (ANOVA). The ANOVA model used status (ME-fed vs. Unfed) as a fixed factor with days since exposure to methyl eugenol as a covariate. All data were checked for assumptions of the ANOVA and appropriately transformed (if required) prior to analysis. Specific comparisons between status within day were made using *t*-tests. In the case of carbohydrate reserves in *Experiment 2* (sugar, protein and water were provided in the field cage) heteroscedasticity could not be eliminated by standard transformations of the data for the overall analysis. Hence the data were only analyzed by ANOVAs for each of the day intervals since exposure to methyl eugenol.

Survival data was analyzed using linear regression analyses on log transformed data. The slopes were compared to examine differences between survival rates between ME-fed and unfed flies (Zar 1999).

8.3 RESULTS

8.3.1. Experiment 1 (Only sugar and water provided).

There was no significant difference in weight (F = 0.747; df = 1,97; P = 0.390; Figure 8. 1a), lipid (F = 0.100; df = 1,97; P = 0.752; Figure 8. 1b), protein (F = 0.310; df = 1,97; P = 0.579; Figure 8. 2a) or carbohydrate reserves (F = 0.882; df = 1,97; P = 0.350; Figure 8. 2b), between ME-fed and unfed flies over the course of the entire experiment. There were no differences between flies of either state in weight, lipid, protein or carbohydrate reserves within day (Figures 8.1 and 8.2).

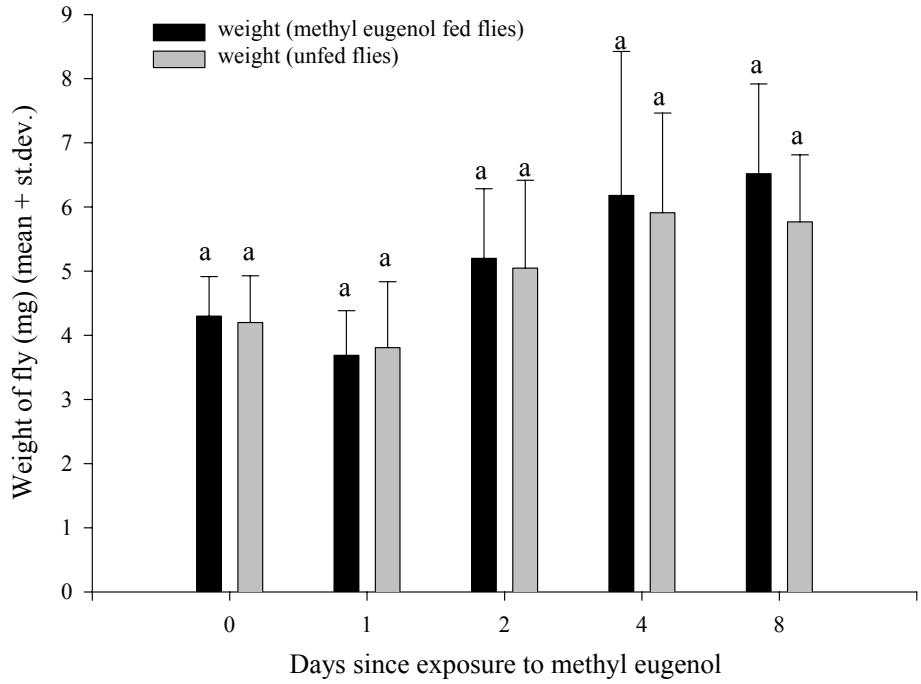
The regression models fitted to the data explained 91.9% and 95.4% of the variation in the survival of ME-fed and unfed flies respectively. Comparisons of the rate of survival of flies of the two states indicated no significant differences between them (t = 1.3059; df = 28; P = 0.2022; Figure 8.3a).

8.3.2. Experiment 2 (Sugar, water and protein provided).

There was no significant difference in weight (F = 3.094; df = 1,117; P = 0.081; Figure 8. 4a) or lipid (F = 1.668; df = 1,117; P = 0.199; Figure 8. 4b) reserves. Protein reserves did vary with status (F = 7.533; df = 1,117; P = 0.007; Figure 8. 5a). Carbohydrate reserves varied significantly on days 0, 2, 8 and 16, but not consistently (Figure 8. 5b; Day 0 – F = 25.878; df = 1,18; P < 0.001; Day 1 – F = 1.818; df = 1,18; P=0.194; Day 2 – F = 6.396; df = 1,18; P = 0.021; Day 4 – F = 1.870; df = 1,18; P = 0.188; Day 8 – F = 47.297; df = 1,18; P < 0.001 (log transformed); Day 16 – F = 27.052, df = 1,18; P < 0.001) between ME-fed and unfed flies over the course of the entire experiment. The only other difference between flies of either state within day was in protein reserves on day 4 and day 16 (Figure 8. 5a) with unfed flies having higher protein reserves than ME-fed flies on both occasions.

The regression models fitted to the data explained 94.1% and 96.3% of the variation in the survival of ME-fed and unfed flies respectively. Comparisons of the rate of survival of flies of the two states indicated no significant differences between them (t = 0.3387; df = 28; P = 0.7374; Figure 8. 3b).

Figure 8.1. Differences between ME-fed and Unfed *Bactrocera cacuminata* in (a) weight (mg) and (b) lipid reserves (µg/ mg of fly) when flies had access to sugar and water in the field cage. Bars within day that are marked with the same letter are not significantly different.


weight (methyl eugenol fed flies)
weight (unfed flies)
Weight of fly (mg) (mean + st.dev.)
0
1
2
3
4
5
6
7
8
9
a
0
1
2
4
8
Days since exposure to methyl eugenol

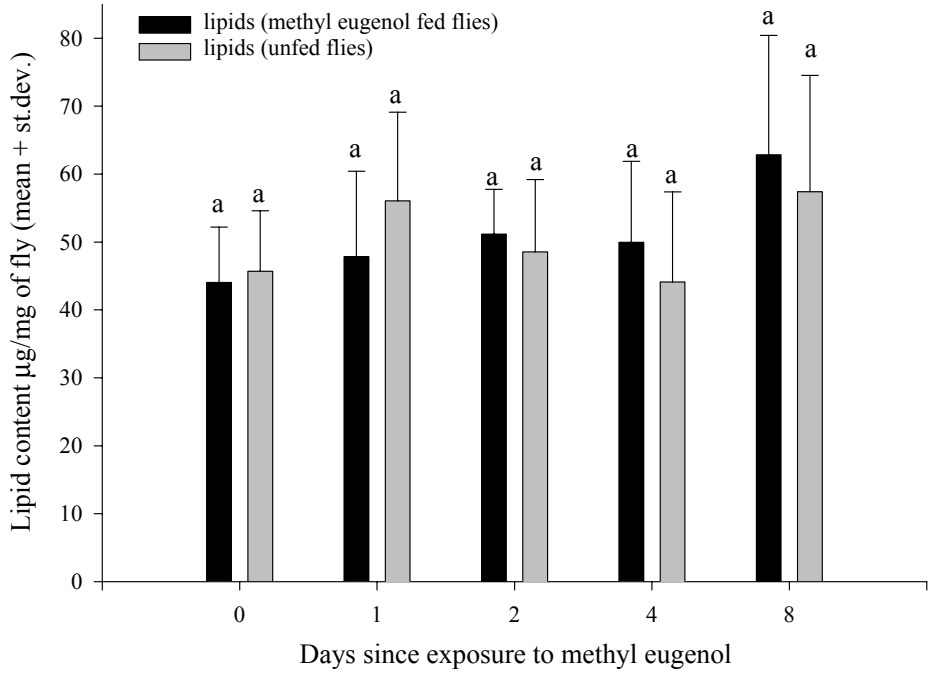
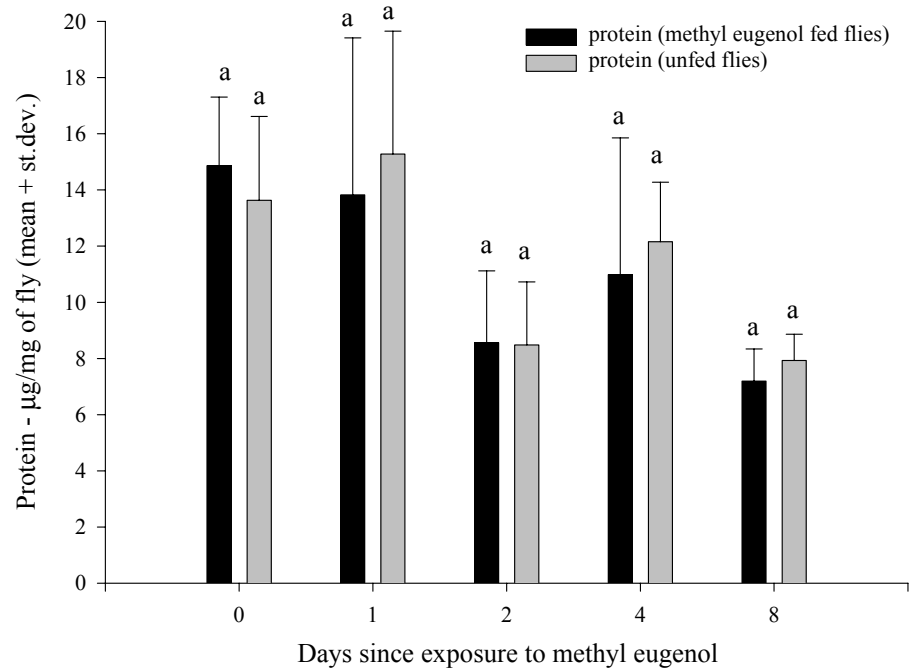

lipids (methyl eugenol fed flies)
lipids (unfed flies)
Lipid content µg/mg of fly (mean + st.dev.)
0
10
20
30
40
50
60
70
80
a
0
1
2
4
8
Days since exposure to methyl eugenol

Figure 8.2. Differences between ME-fed and Unfed *Bactrocera cacuminata* in (a) protein reserves (µg/ mg of fly) and (b) carbohydrate reserves (µg/ mg of fly) when flies had access to sugar and water in the field cage. Bars within day that are marked with the same letter are not significantly different.


protein (methyl eugenol fed flies)
protein (unfed flies)
Protein - µg/mg of fly (mean + st.dev.)
Days since exposure to methyl eugenol
0
1
2
4
8

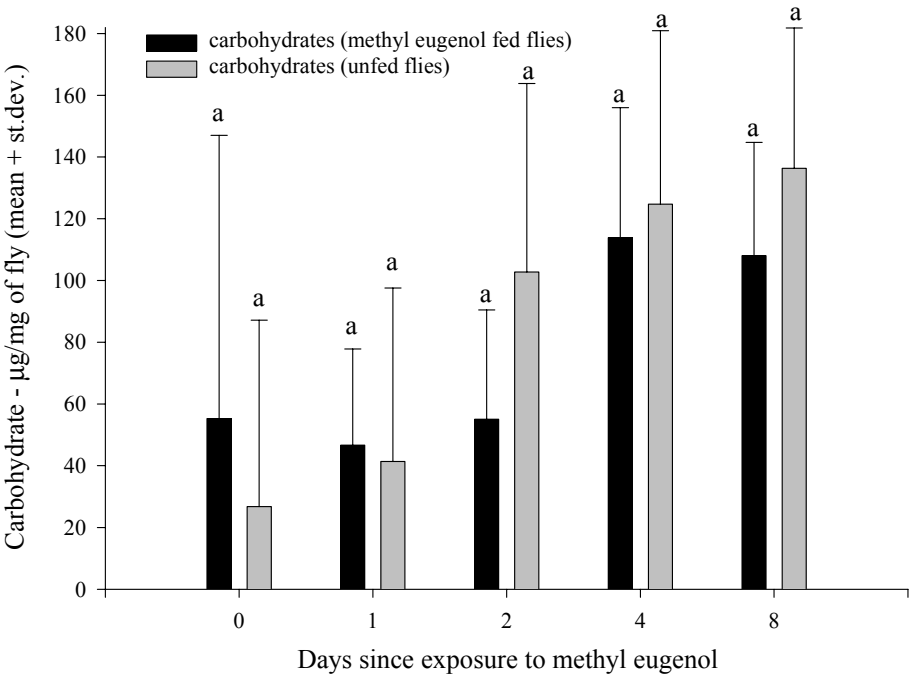
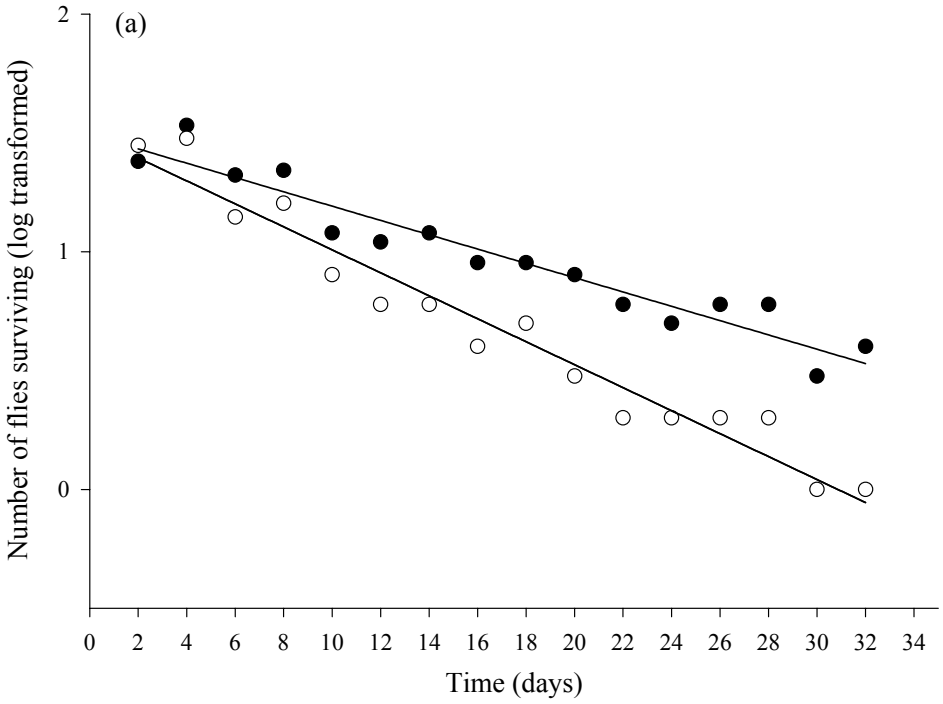

carbohydrates (methyl eugenol fed flies)
carbohydrates (unfed flies)
Carbohydrate - µg/mg of fly (mean + st.dev.)
Days since exposure to methyl eugenol
0
1
2
4
8

Figure 8.3. Difference in survival between ME-fed (filled circles) and Unfed *Bactrocera cacuminata* (open circles) (a) when provided with sugar and water and (b) when provided with sugar, protein and water.


(a)
Number of flies surviving (log transformed)
2
1
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
Time (days)

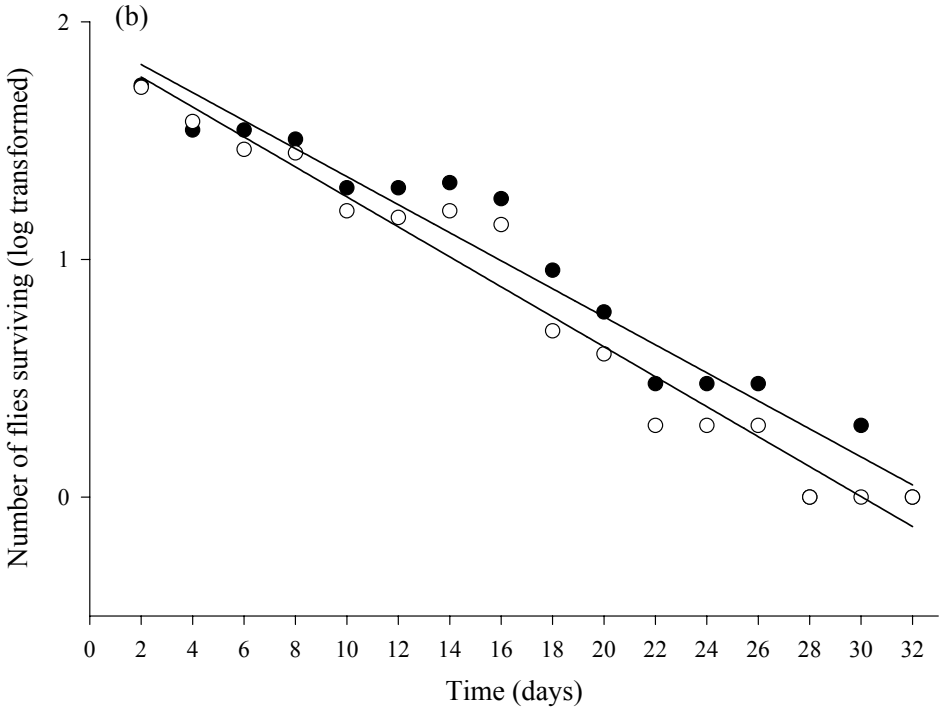
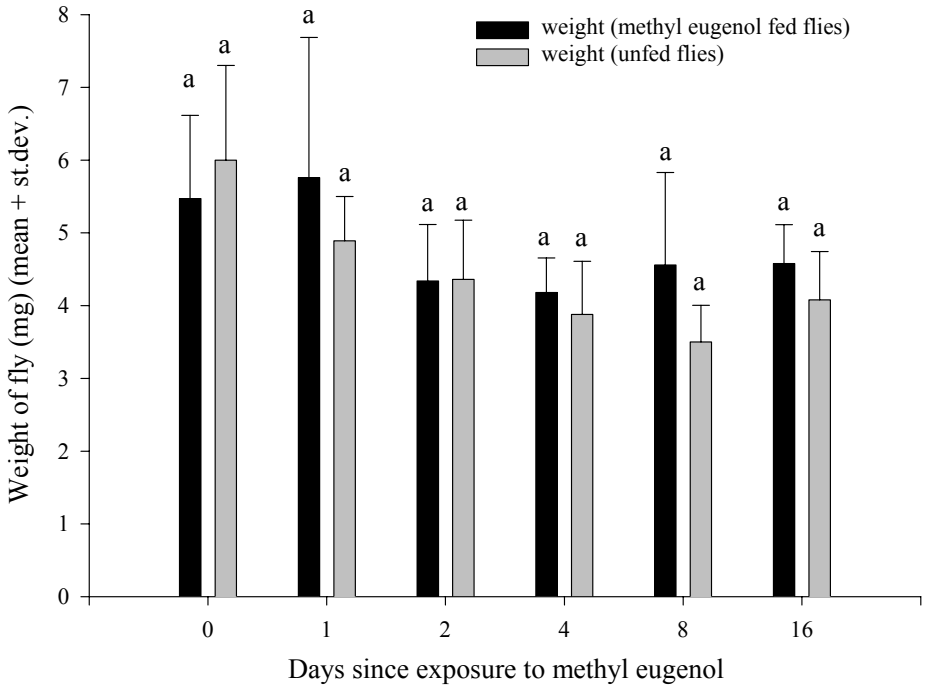

(b)
Number of flies surviving (log transformed)
2
1
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
Time (days)

Figure 8.4. Differences between ME-fed and Unfed *Bactrocera cacuminata* in (a) weight (mg) and (b) lipid reserves (µg/ mg of fly) when flies had access to sugar, protein and water in the field cage. Bars within day that are marked with the same letter are not significantly different.


weight (methyl eugenol fed flies)
weight (unfed flies)
Weight of fly (mg) (mean + st.dev.)
0
1
2
3
4
5
6
7
8
a
0
1
2
4
8
16
Days since exposure to methyl eugenol

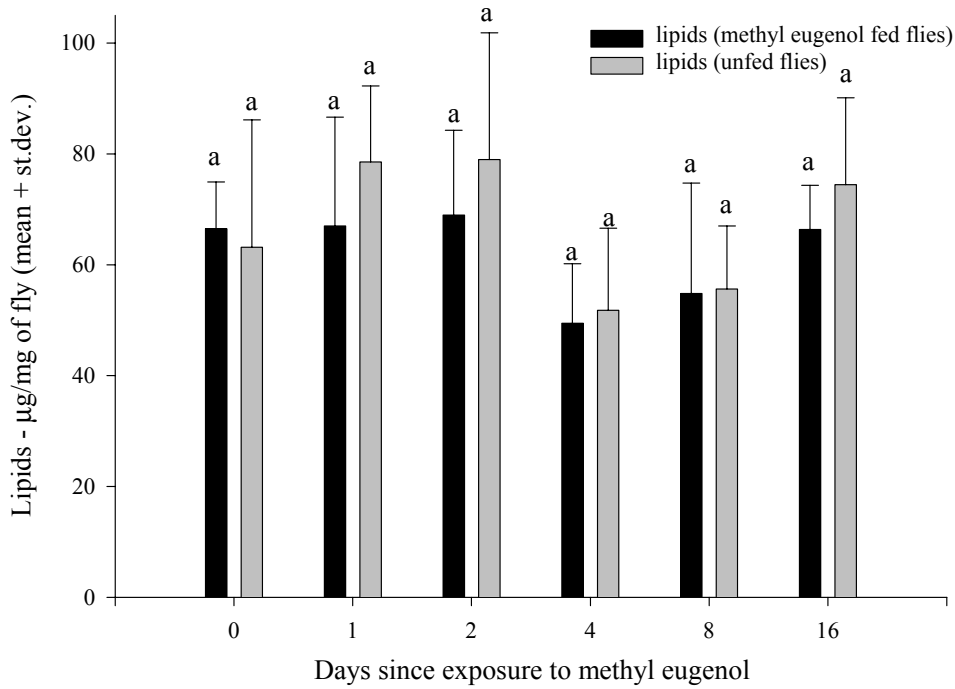
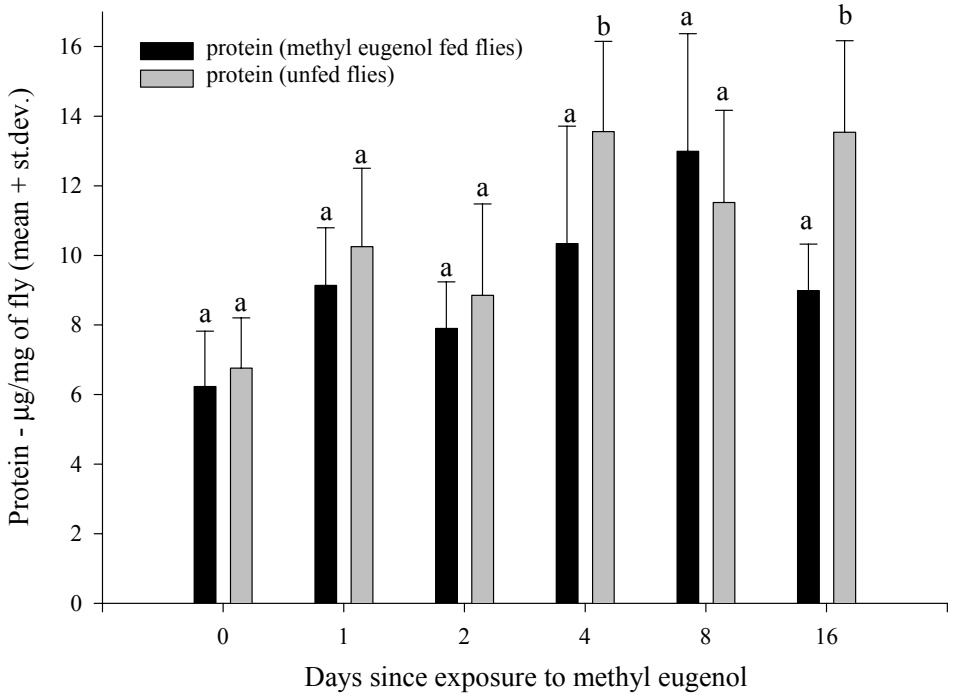
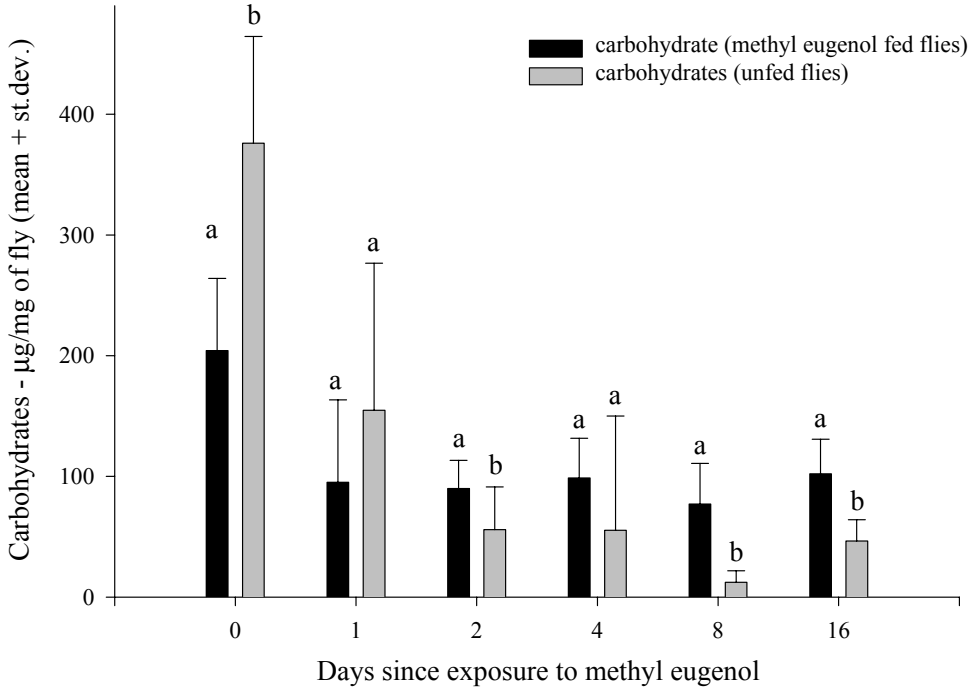

lipids (methyl eugenol fed flies)
lipids (unfed flies)
Lipids - µg/mg of fly (mean + st.dev.)
0
20
40
60
80
100
a
0
1
2
4
8
16
Days since exposure to methyl eugenol

Figure 8.5. Differences between ME-fed and Unfed *Bactrocera cacuminata* in (a) protein reserves (µg/ mg of fly) and (b) carbohydrate reserves (µg/ mg of fly) when flies had access to sugar, protein and water in the field cage. Bars within day that are marked with the same letter are not significantly different.


protein (methyl eugenol fed flies)
protein (unfed flies)
Protein - µg/mg of fly (mean + st.dev.)
Days since exposure to methyl eugenol
0
1
2
4
8
16


carbohydrate (methyl eugenol fed flies)
carbohydrates (unfed flies)
Carbohydrates - µg/mg of fly (mean + st.dev.)
Days since exposure to methyl eugenol
0
1
2
4
8
16

8.4 DISCUSSION

The functional basis of attraction of dacine fruit flies to botanically derived lures is central to our understanding of their ecology and evolution (Metcalf 1990). Male response to these lures has been inferred (Shelly and Dewire 1994, Tan and Nishida 1996, Shelly 2000), and occasionally stated to be pharmacophagy (Khoo and Tan 1998). In addition, this putative functional role of these parapheromones has been generalized to the Dacinae in general (Shelly and Dewire 1994, Tan and Nishida 1996, Shelly 2000). However, as pointed out by Boppré (1984), for a behavior to be defined as pharmacophagy, it is vital to demonstrate that the consequences of feeding on the plant-derived chemical are primarily ecological and not physiological. Hence I investigated the physiological consequences of feeding on methyl eugenol by *B. cacuminata*.

Consistent with the expectations of pharmacophagous behavior, the data show that there are no physiological benefits of feeding on methyl eugenol by *B. cacuminata*. Though the transformation of methyl eugenol directly into energetic reserves is unlikely by biochemical pathways (Fletcher and Kitching 1995), it appears to pass through the digestive tract with no obvious competitive advantage in mating (Chapter 7). Therefore, I pursued this question to assess if feeding on methyl eugenol influenced foraging for nutrients and thereby conveyed physiological benefits to males feeding on it. This does not appear to be the case for any of the measures of primary metabolism (Figures 8.1, 8.2, 8.4 and 8.5) or for survival (Figure 8. 3).

However, some differences were evident in relation to feeding on ME. When flies were not provided with a supplement of protein, no significant difference in nutrient reserves was detected on any of the days sampled (Figures 8.1b, 8.2a and 8.2b). However, when I added a source of protein to the field cage, significant differences in reserves of carbohydrate and protein

(but not lipid) were evident on some of the sampling days. Particularly striking was the trend seen in carbohydrate reserves, where after the second day in the field cage, through to day 16 (with the exception of day 4), ME fed males had significantly higher levels of carbohydrates than males who had no exposure to ME. Conversely, on day 4 and day 16 unfed males had higher protein levels than ME fed males (Figures 8.5a, b). This pattern may indicate that, when protein was available in abundance, ME fed males engaged in a different pattern of behavior than unfed flies, and (or) utilized their resources in a different manner. Whether these differences may be interpreted as a reflection of an advantage enjoyed by the ME fed flies is moot.

This result partially confirms the predictions for pharmacophagy. However, contrary to studies on other species linking feeding on methyl eugenol to mating success (Shelly and Dewire 1994, Tan and Nishida 1996, Shelly 2000), studies on *B. cacuminata* found no obvious reproductive benefits of exposure to methyl eugenol (Chapter 7).

If there are no reproductive or metabolic benefits of feeding on methyl eugenol by *B. cacuminata,* then how does one explain this strong chemotactic response of the species? Could it serve a function in defense? Though many dacine species respond to methyl eugenol, this phenyl propanoid is not commonly found in larval host plants of fruit flies. However, it occurs in numerous plant species, including some orchids (Nishida et al. 1993). This phenomenon of chemotaxy towards a chemical not currently associated with host plants is not unique to dacine fruit flies. Cucurbitacins (terpenes produced by all members of the Cucurbitaceae) elicit strong phagostimulatory response in many luperine (Chrysomelidae: Luperini) beetles that develop only on non-cucurbitaceaous host plants (Metcalf et al. 1980). This response has been hypothesised to be a relic of ancestral host associations of luperine beetles with members of the Cucurbitaceae that is

currently being maintained through secondary selection for contemporary benefits of cucurbitacin feeding such as defense (Ferguson and Metcalf 1985, Tallamy et al. 1998). This has been labelled the "ancestral host hypothesis" by Tallamy et al. (1999).

Similar benefits to defense as a result of feeding on methyl eugenol have been hypothesized for male dacine fruit flies. The Asian house gecko (*Hemidactylus frenatus* Duméril and Bibron) is deterred from feeding on methyl eugenol fed *Bactrocera papayae* Drew and Hancock males and cuelure fed *B. cucurbitae* (Coquillett) males (Tan and Nishida 1998, Tan 2000). If methyl eugenol confers similar allomonal benefits to *B. cacuminata* then feeding on this phytochemical can still be regarded as pharmacophagy. In the present study, the large field cage housed numerous predators, including ants, spiders, lizards and reduviids. Though I did not quantify predation, the survival data (Figure 8. 3) suggest that feeding on methyl eugenol did not enhance survival in the presence of potential predators in the field cage. While defensive benefits suggested in dacine species are insightful (Tan and Nishida 1998, Tan 2000), such benefits need to be determined for predators in natural systems and habitats in which these species evolved.

The fact that it is principally males that respond to ME further confounds the applicability of the ancestral host hypothesis to the Dacinae. The role these chemicals may play in female flies have seldom been explored (Fitt 1981b) and Metcalf' s hypothesis that ME may serve as a mating rendezvous stimulus has not been explicitly tested. In the following chapter (Chapter 9), I investigate the spatial and temporal partitioning of behaviour between resources by *B. cacuminata* with a view to testing Metcalf's hypothesis.

Acknowledgments – I thank Shlomit Shloush and Batya Kamenski, Hebrew University of Jerusalem for their indispensable technical assistance.

Chapter Nine

Spatial and temporal partitioning of behaviour by adult dacines: Direct evidence for methyl eugenol as a mate rendezvous site

This chapter has been accepted for publication in a slightly modified form:

Raghu, S. and Clarke A.R. 2003. Spatial and temporal partitioning of behaviour by adult dacines: Direct evidence for methyl eugenol as a mate rendezvous site. *Physiological Entomology* (in press).

9.1 INTRODUCTION

All animals require one or more vital resources to ensure their survival and reproduction and, with the exception of sedentary animals, normally actively forage to locate them (Andrewartha and Birch 1984). Within the speciose insects, these resources principally include carbohydrates and lipids to fuel short and long-distance flight activity, and protein for growth and to attain sexual maturity (Slansky and Scriber 1985). In studies of insect resource use, dacine flies (Diptera: Tephritidae) are an interesting example because resources for both of the resource dependent life-history stages (i.e. larvae and adults) are supposed to be available in one place: the plant that supplies fruit for female oviposition and larval development. This phenomenon has led to the postulation that the larval host plant is the "center of activity" of dacine populations (Drew and Lloyd 1987, 1989, Metcalf 1990, Prokopy et al. 1991). This is contrary to most anautogenouos insects (i.e. insects that emerge from puparia as sexually immature adults), which have to forage for spatially and temporally variable resources to satisfy the requirements of the different life-history stages (Johnson 1969, Wiklund 1977, Lawrence 1982, Roitberg 1985, Slansky and Scriber 1985, Bell 1990, Hendrichs et al. 1991, Warburg and Yuval 1997a).

An explicit test of the centre of activity hypothesis in the dacine species *Bactrocera cacuminata* (Hering), however, suggested that adult flies of that species do forage elsewhere, as key behaviours, particularly feeding and mating, were almost entirely absent on the larval host plant (Raghu et al. 2002). This indicates that food and mating site resources are distributed elsewhere in the habitat. Methyl eugenol (ME), considered a resource that male dacine flies need for pheromone production (Fitt 1981b, c, Shelly and Dewire 1994), is also a resource unlikely to occur at the larval host plant for most species and presumably males must forage to acquire it.

Physiological tests of nutritional status of *B. cacuminata* at different times of the day and at different resources suggest that the flies may indeed be foraging (Chapter 5), but such tests are indirect. Furthermore, given that the functional significance of ME as a pheromone precursor is still unclear, as indicated by the absence of any obvious mating advantage in *Bactrocera cacuminata* (Chapter 7), it is vital to assess if this chemical plays some other role in the ecology of fruit flies. Metcalf (1990, Metcalf and Metcalf 1992) provided an explanation, other than a pheromone precursor role, to explain the use of ME by dacines. He suggested that fly response to ME and similar botanical phenyl propanoids was a preserved, ancestral trait. Such kairomones may have facilitated host location and Metcalf (1990) hypothesized that contemporary response is maintained as ME may serve as a mating rendezvous stimulus for dacine flies.

In order to examine resource use in dacine flies, I carried out field-cage experiments to investigate if *B. cacuminata* of different physiological status (sex/ sexual maturity) partition their activities between different resources and the behaviours exhibited at each. The specific questions I asked were:

1. Do adult flies partition their behaviour between resources required for their survival and/ or reproduction?
2. Is there a difference in patterns of resource use between the sexes?
3. How do these patterns vary as a function of physiological status?
4. Are there any diurnal trends in partitioning of behaviour between resources?
5. Does methyl eugenol function as an aggregation stimulus for mating?

Based on previous studies (Raghu et al. 2002), my predictions were that adult flies would partition their behaviour between spatially separated resources, with immature flies principally foraging for resources vital for

growth and sexual development (i.e. protein and sugar). Mature-unmated flies would spend considerably lesser time in foraging for protein and at dusk, the normal mating time for this species, would spend considerably greater time foraging for mates. Mature-mated males on the other hand may be responsive to protein, given that they would have depleted their protein reserves by expenditure of sperm during mating, would also be responsive to protein and also forage for mates at dusk (as males mate repeatedly in this species). Given that mating in this species is rare at its larval host plant (Raghu et al. 2002), this study serves as a direct test of Metcalf's hypothesis that ME serves as a mate rendezvous site.

9.2 MATERIALS AND METHODS

Bactrocera cacuminata is a monophagous dacine fruit fly. Females of this species oviposit almost exclusively in the fruit of *Solanum mauritianum* (Drew 1989). Adult flies forage for food (proteins and carbohydrates), oviposition sites (fruit), mates and the plant-derived chemical, methyl eugenol. Hence the 'resources' used in this study were host fruit and foliage (referred to as host hereafter), sugar, protein and ME.

Experimental flies were from a stock laboratory colony maintained at Griffith University, which is refreshed every 3-4 generations with wild flies in approximately a 1:1 ratio. *Bactrocera cacuminata* adults take 10-12 days to attain sexual maturity and mate soon after: dacine males are polygamous, females are monandrous (Barton-Browne 1957, Fay and Meats 1983, Mazomenos 1989). Mating is restricted to the dusk photophase. Adults were separated by sex within two days of emergence from puparia and held in separate cages (30cm × 30cm × 30cm) at a density of no more than 200 individuals per cage. Flies were subsequently placed into one of three different physiological types for experiments *viz*. immature (IM) (4-5 days old), mature-unmated (MU) (13-14 days old, sexes kept separate until

released into the experimental environment) and mature-mated (MM) (20-21 days old, sexes brought together in the lab prior to the experiment, female mating rate 90% based on spermathecal squashes).

Experiments were run in field cages of dimension 4m × 4m × 3m (length × breadth × height). In each cage, four identical plastic 'plants' (150cm tall, bearing 33 identical leaves of approximately 306cm^2 area each) were placed so they were 2m apart from each other and 1m from the cage wall. The plants were used as platforms on which resources were placed. The plastic plants provided a structurally complex, shaded environment such as the flies would experience in their normal environment, but as they were identical and non-botanical, any response by the flies can be directly attributed to the resource they contained, in contrast to the situation if real plants were used. The terminal portion of a *S. mauritianum* branch, bearing leaves and fruit, was secured to one of the plants in the field cage. The other three resources (sugar, protein and ME) were provided individually on small sponges (2cm × 2cm) in Petri-dishes secured to the upper leaf surface of each of the other three plants (i.e. one resource per plant). Two ml of 20% sugar solution was used as the sugar resource, while 2ml of 10% protein (yeast autolysate, ICN Biomedicals Ltd.) solution was used as the protein source. One ml of ME (International Pheromone Systems Ltd.) was used as the ME resource.

For each fly physiological type, 200 individuals of each sex were released into a field cage at 0630h. The number of individuals of each sex at each of the resources, plus the behaviour each fly was exhibiting, was surveyed at five times of day (0800, 1100, 1300, 1500, 1800h). Behaviours recorded were resting, feeding, oviposition and mating (see Raghu et al. [2002], Chapter 2 for definitions). For feeding and oviposition only flies directly on the resource were censused, while for resting and mating all individuals on the plant holding the resource were censused. Since moisture

is vital for dacine fruit flies, an equal amount of water was sprayed on each of the plants using a hand-held atomizer after the observations at each of the time intervals. Four cages were run concurrently, repeated the following day, giving a total of 8 replicates for each physiological state.

9.2.1. Data Analysis

The data were analysed using repeated measures analysis of variance with physiological status, sex and resource as factors and the observations of abundance or behaviour at each of the five times of day as the repeated measure.

9.3 RESULTS

Flies in each of the physiological profiles responded to the resources provided in the field cage. As anticipated, there were significant diurnal patterns in abundance and behaviour (Table 9.1) and there were significant interaction effects of the factors as indicated by univariate within-subject comparisons (Table 9.1).

9.3.1. Abundance

The abundance of IM and MU males at host did not differ significantly from each other over time, while abundance of both these groups differed from MM males at 1100, 1300 and 1500h (Figure 9.1a). Abundance of females at host did not differ significantly over time as a function of their physiological profile until dusk, when the number of MM females observed were significantly greater than females of the other two physiological states (Figure 9.1b).

Table 9.1. Summary of multivariate analyses of abundance and behaviour showing the significance of the approximate *F* calculated from Pillai's Trace (PT) for each of the effects in the model and univariate tests for within-subject factors and their interaction terms based on the approximate *F* adjusted using the Greenhouse-Geisser (GG) epsilon.

Source	Abundance		Feeding		Resting	
	PT	GG	PT	GG	PT	GG
T	<0.001	<0.001	<0.001	0.001	<0.001	<0.001
T*P	<0.001	<0.001	0.001	0.003	<0.001	<0.001
T*S	0.004	<0.001	<0.001	<0.001	0.248	0.231
T*R	<0.001	<0.066	<0.001	<0.001	<0.001	<0.001
T*P*S	0.039	<0.001	0.079	0.019	0.005	0.052
T*P*R	<0.001	<0.001	<0.001	<0.001	0.025	0.016
T*S*R	<0.001	<0.001	<0.001	<0.001	0.001	<0.001
T*P*S*R	<0.001	<0.001	0.005	<0.001	<0.001	<0.001

Where T = time, P = physiological status, S = sex, R = resource

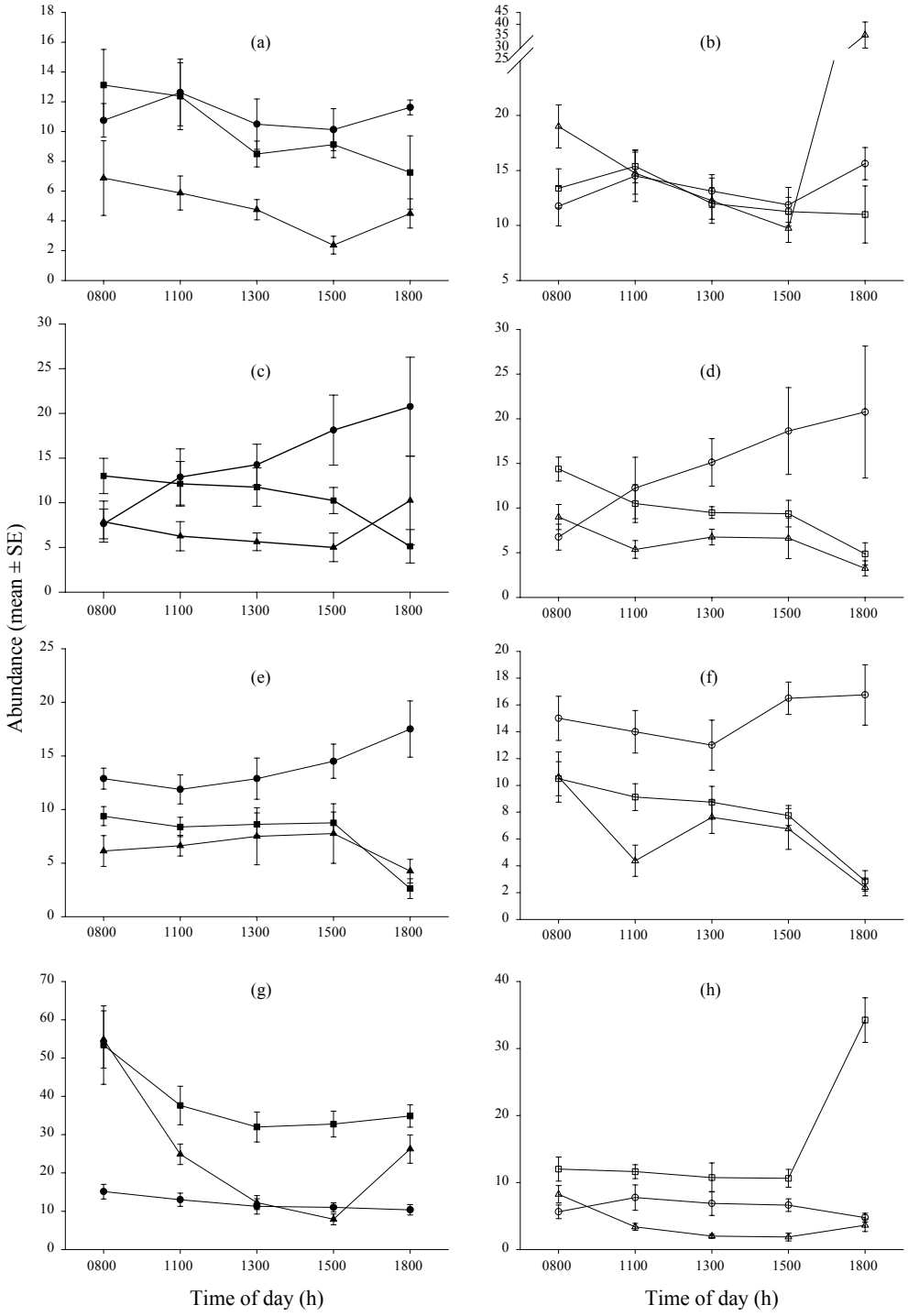
The number of IM males at sugar increased monotonically over time (Figure 9.1c). The numbers of the MU and MM males at sugar were relatively constant over time with the numbers of the latter being significantly lower than the former at 1100, 1300 and 1500h (Figure 9.1c). The trends in abundance of female flies were similar to that in the males with abundance of IM females increasing morning to dusk (Figure 9.1d). There was no difference in numbers of females of different physiological profiles at 0800 and 1100h. Numbers of IM females were significantly more abundant than the other two states at other time intervals (Figure 9.1d).

Protein was significantly more attractive to IM males than the other two states at all time intervals (Figure 9.1e). The abundance of MU and MM males were not significantly different at this resource at any time of day other than 0800h. Similar trends were observed in the abundance of females

at protein (Figure 9.1f). The only difference in abundance between MU and MM females was at 1100h with significantly fewer of the latter at protein (Figure 9.1f).

ME was significantly more attractive to MU and MM males at 0800 than IM males (Figure 9.1g) while the three states differed significantly in abundance at ME at 1100h. At all other times of day number of IM and MM males at ME did not differ significantly from each, but both were significantly lower than MU males (Figure 9.1g). Response to ME by female flies was trivial at all time intervals except dusk when the number of MU females was significantly higher than the other two physiological states (Figure 9.1h).

Figure 9.1. Diurnal patterns in abundance of *Bactrocera cacuminata* of different physiological profiles at different resources. (a) & (b) Fruit; (c) & (d) Sugar; (e) & (f) Protein; (g) & (h) Methyl Eugenol. [Circles = immature; squares = mature, unmated; and triangles = mature, mated flies. Filled symbols = males, open symbols = females]


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
Abundance (mean ± SE)
Time of day (h)
Time of day (h)
0800
1100
1300
1500
1800

9.3.2. Feeding behaviour

The number of IM males feeding on the fruit surface was significantly higher than the males of the other two states at 0800 and 1100h, while the number of MU and MM males feeding did not differ from each other (Figure 9.2a). Similar trends were seen in the number of females feeding on fruit, but there was greater variability in the number of IM females (Figure 9.2b).

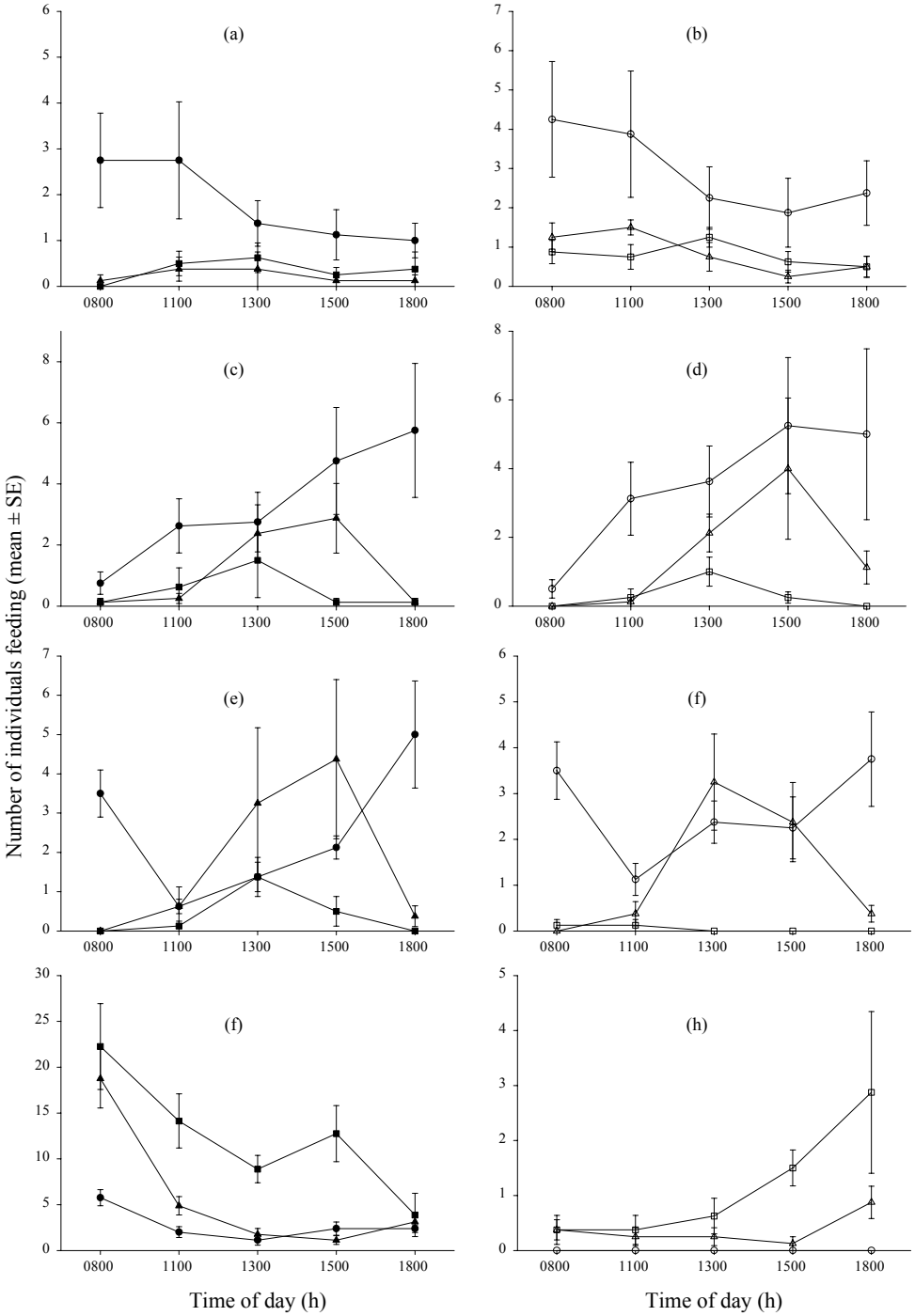
The number of IM males feeding at the sugar resource increased over time of day from a minimum at 0800 to a maximum at dusk. Their numbers differed from the other two states at 1100 and, more markedly, at dusk. While similar increases in number of individuals feeding were seen in MU and MM males, they stopped feeding on sugar by 1500h (Figure 9.2c). The pattern of feeding behaviour over time in female flies of different physiological profiles was similar to males of similar states (Figure 9.2d).

Feeding on protein by IM males was significantly higher than the MU and MM males at 0800 and 1800h (Figure 9.2e). The number of MM males feeding on protein was highly variable and increased with time of day until 1500h and declining after. The pattern in feeding behaviour in MU males was unimodal with a maximum number feeding at midday and reduced numbers at other times (Figure 9.2e). Feeding on protein by MU females was rare in comparison to IM and MM females (Figure 9.2f). While the diurnal patterns of feeding was erratic in IM and MM females, they were significantly more individuals of these states feeding on protein than MU females at most time intervals (Figure 9.2f).

The number of males feeding on ME generally declined with time of day (Figure 9.2g). At 0800h the number of IM males feeding on ME was significantly lower than the other two states. The number of MU and MM males feeding on ME did not differ at 0800 and at 1800h. However, the number of MU males feeding on ME was significantly higher than IM and

MM males at 1100, 1300 and 1500h (Figure 9.2g). Immature females did not feed on ME at any time of day (Figure 9.2h). The numbers of MU and MM females feeding on ME did not differ from each other until 1500h when the number of MU females feeding was significantly higher than MM females (Figure 9.2h). On average more MU females were feeding on ME at dusk (1800h) than the MM females (Figure 9.2h).

Figure 9.2. Diurnal patterns in feeding behaviour of *Bactrocera cacuminata* of different physiological profiles at different resources. (a) & (b) Fruit; (c) & (d) Sugar; (e) & (f) Protein; (g) & (h) Methyl Eugenol. [Circles = immature; squares = mature, unmated; and triangles = mature, mated flies. Filled symbols = males, open symbols = females]


(a)
(b)
(c)
(d)
(e)
(f)
(f)
(h)
Number of individuals feeding (mean ± SE)
Time of day (h)
Time of day (h)
0800
1100
1300
1500
1800

9.3.3. Resting behaviour

The number of IM males resting at host at dusk (1800h) was significantly higher than the number of resting MU and MM males (Figure 9.3a). The number of MU males resting at host did not vary significantly between 0800 and 1500h, but their numbers at dusk was significantly lower than at other time intervals. Between 1100 and 1500h, a lower number of MM males were resting at host than the other physiological states (Figure 9.3a). In the case of females resting at host, the number of IM females was lower than the other two states at 0800h while the number of MM females resting on fruit increased significantly at dusk (Figure 9.3b).

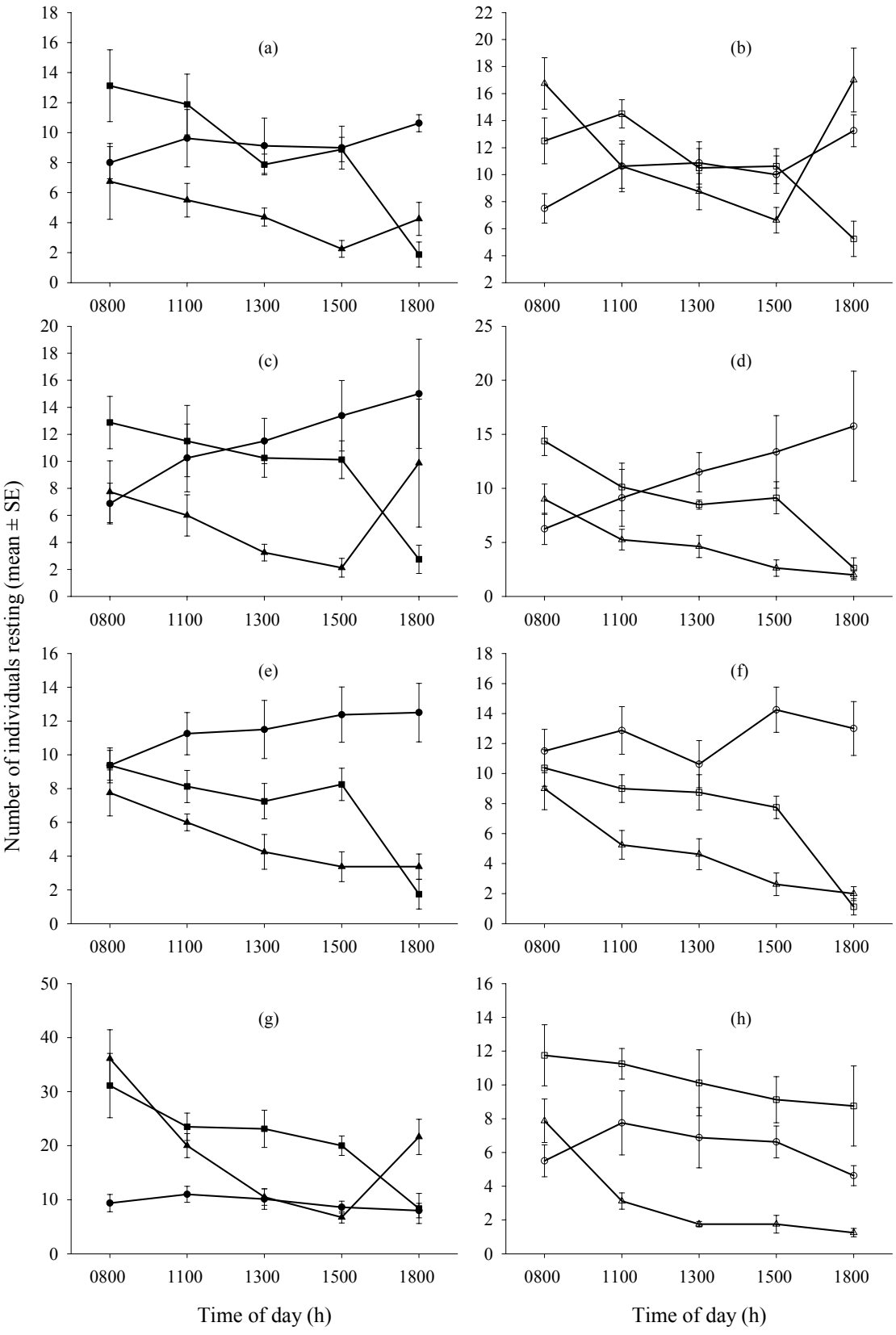
The number of IM males resting at sugar increased monotonically from morning to dusk while the number of MU males exhibiting the same behaviour declined over time, dropping sharply at dusk (Figure 9.3c). There was a decline in numbers of MU males resting at sugar between 0800 and 1500h, but their numbers increased sharply at dusk (Figure 9.3c). The trend in numbers of IM females resting at sugar was similar to IM males while the numbers of females of the other two physiological states gradually declined over time (Figure 9.3d).

The plant containing protein was equally favoured as a resting site by males of all three physiological profiles at 0800h (Figure 9.3e). While the number of IM males resting at protein increased gradually over time, the numbers of resting individuals of the other two states declined over time. This decline was more markedly so at dusk in the case of MU males (Figure 9.3e). While the number of IM females resting at protein stayed relatively constant over time, the number of MU and MM females declined from a maximum at 0800 to a minimum at dusk (Figure 9.3f).

Resting IM males were rare at ME in comparison to the other two states and their numbers stayed constant over time (Figure 9.3g). Numbers of

MU and MM males resting at ME declined from 0800 to 1500h, the rate of decline being more gradual in the former than the latter. At dusk however, there was a sharp decline in the number of resting MU males, while the number of resting MM males at ME increased sharply (Figure 9.3g). The number of IM females resting at ME stayed relatively constant in comparison to females of the other two states (Figure 9.3h). While there was a general decline in the number of resting MU and MM females from 0800 to 1800h, the numbers of the former were significantly higher than the latter at most time intervals (Figure 9.3h).

Figure 9.3. Diurnal patterns in resting behaviour of *Bactrocera cacuminata* of different physiological profiles at different resources. (a) & (b) Fruit; (c) & (d) Sugar; (e) & (f) Protein; (g) & (h) Methyl Eugenol. [Circles = immature; squares = mature, unmated; and triangles = mature, mated flies. Filled symbols = males, open symbols = females]


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
Number of individuals resting (mean ± SE)
Time of day (h)
Time of day (h)
0800
1100
1300
1500
1800

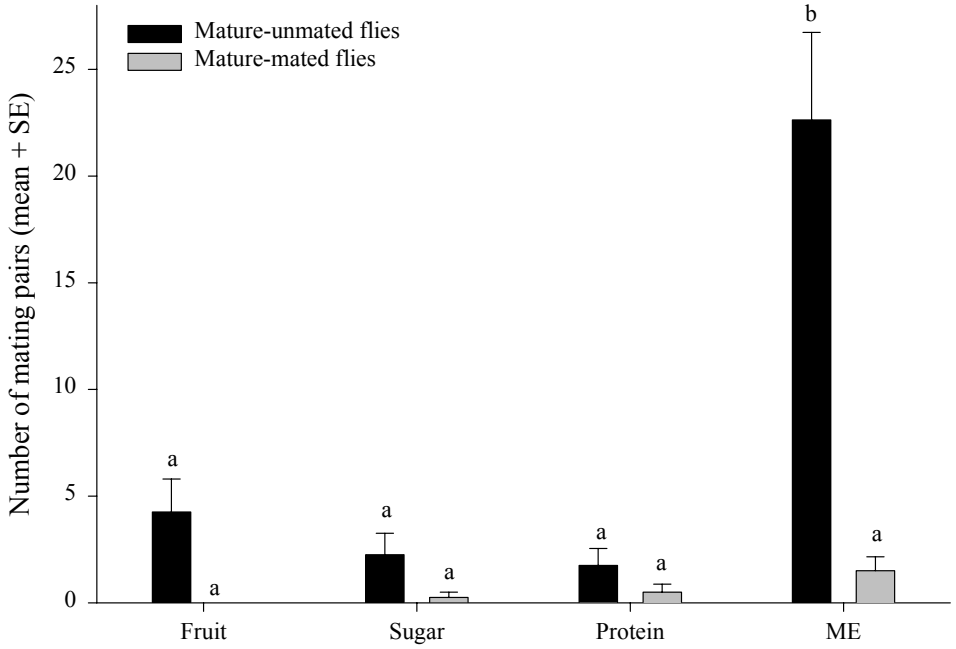
9.3.4. Mating and oviposition behaviour

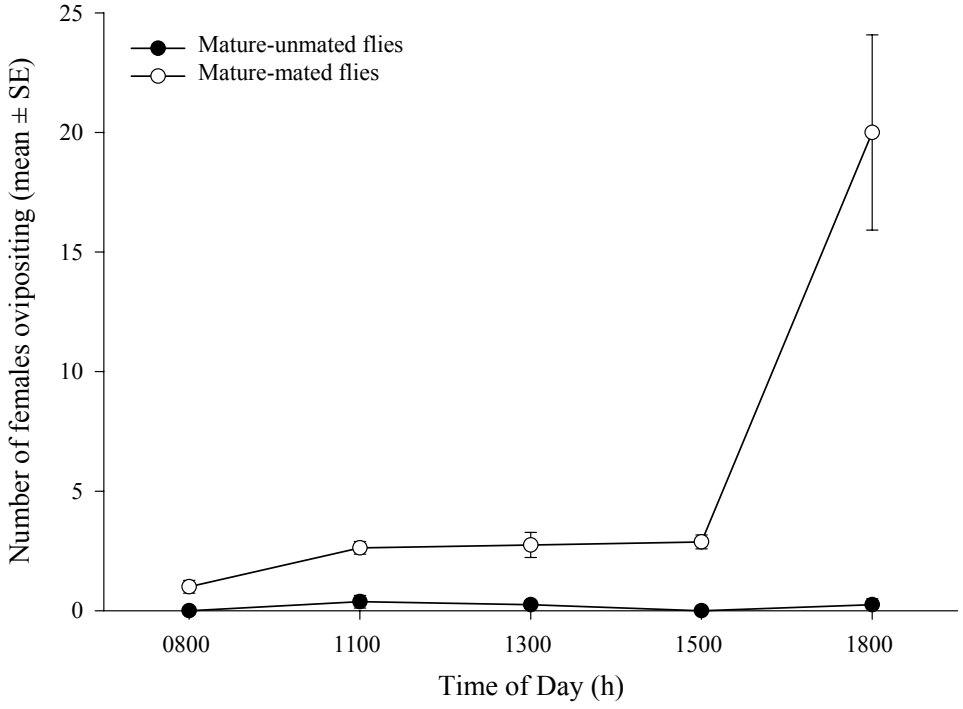
Analyses of number of mating pairs, among flies of the same physiological profile between resources, revealed that for MU flies a significantly greater proportion of flies were mating at ME than at the other resources ($F_{3, 28}$ = 13.779, p < 0.001, log (x+1) transformed data; Figure 9.4). There was no difference in the numbers of mating pairs between fruit, sugar and protein. Though the number of mating pairs observed in MM flies were significantly lower than in the case MU flies, the trends were similar with a greater proportion mating at ME than the other resources ($F_{3, 28}$ = 2.747, p = 0.062, log (x+1) transformed data; Figure 9.4).

Oviposition by MM flies showed a strong diurnal trend; with oviposition activity remaining relatively low until 1500h and significantly increasing at dusk (Figure 9.5).

Figure 9.4. (top) Mating behaviour of *Bactrocera cacuminata* in relation to different resources. Bars with the same letters indicate no significant difference (based on post hoc LSD tests) in number of mating pairs among flies of the same physiological profile between resources.

Figure 9.5. (bottom) Diurnal patterns in oviposition behaviour of female *Bactrocera cacuminata*.


Mature-unmated flies
Mature-mated flies
Number of mating pairs (mean + SE)
25
20
15
10
5
0
a
a
a
a
a
a
b
a
Fruit
Sugar
Protein
ME


Mature-unmated flies
Mature-mated flies
Number of females ovipositing (mean ± SE)
25
20
15
10
5
0
0800
1100
1300
1500
1800
Time of Day (h)

9.4 DISCUSSION

Understanding resource use by organisms is perhaps the most intriguing aspect of ecology (Andrewartha and Birch 1984). The behaviours of organisms are often attuned to the functional significance of resources to different life-history stages (Kitching 1977, Wiklund 1977). Understanding such age-structured resource use is vital to understanding the autecology of insects. Dacine species are believed to acquire resources vital for all life stages from a single location, *viz.* the larval host plant, hence making it the centre of dacine behaviour (Drew and Lloyd 1987, 1989, Metcalf 1990, Prokopy et al. 1991). When studies on the dacine *B. cacuminata* revealed a paucity of the vital behaviours of feeding and mating behaviours at the host plant (Raghu et al. 2002), the possibility that at least some dacine species partition their behaviour between spatially and temporally variable resources was considered to warrant further investigation (Chapter 5).

Data from this study clearly shows that, in a field-cage situation, *B. cacuminata* partitions its behaviour between spatially separated 'resources' over time. This partitioning of behaviour shows differences between flies of different physiological profiles that help elucidate variations in the ecology of this species over its adult life.

As anticipated, IM males and females spend a significant proportion of their time foraging for nutritional resources such as protein and sugar, with the number of individuals feeding increasing with time of day (Figures 9.1, 9.2). In contrast, MU individuals tended to forage in considerably smaller numbers on these resources. Feeding patterns in MM males on the other hand tended to resemble IM males with an increase in feeding behaviour leading up to dusk (Figure 9.2). As in many dacine species (Barton-Browne, 1957, Fay and Meats 1983, Mazomenos 1989), monandry appears to be the norm in this species, with mated females being less receptive to subsequent

copulation attempts, a fact borne out by the paucity of mating behaviour in MM flies (Figure 9.4). These patterns are consistent with predictions based on the known physiology of the fly (see Introduction).

9.4.1. Methyl eugenol as a mate rendezvous site

Use of the plant with ME showed a clear difference in relation to sex and physiology. Immature males are not as responsive as MU and MM males in terms of feeding on ME. While MU and MM males fed on ME in the first half of the day, their female counterparts fed at dusk. These trends are consistent with Fitt's (1981c) observations in *B. opiliae* and earlier studies in *B. cacuminata* (Chapter 5), that response to ME is closely related to sexual maturity.

Significantly, the pattern of response to ME substantiates Metcalf's hypothesis (Metcalf et al. 1979, Metcalf 1990, Metcalf and Metcalf 1992) that ME serves as a rendezvous stimulus in mate location. It is evident from the data that mature flies gathered at ME to mate (Figure 9.1, 9.4). This hypothesis has been discounted previously based on observations that male Dacinae are least responsive to lures during periods of peak sexual activity and that female flies seldom respond to them in natural environments (Brieze-Stegeman et al. 1978, Fitt 1981b, c, Shelly and Dewire 1994). However, female response in some of these studies was measured by visitation to insecticide-baited lure traps, which may have given anomalous results. If males are the first to arrive at the mate rendezvous site and a combination of the chemical stimulus in conjunction with male mating signals are the cues that female flies home in on to arrive at the mating site, then traps with lure and contact insecticide that kill flies is not the most appropriate method to assay female response. Alternately, if these phenyl propanoids are found relatively abundantly elsewhere in the environment then the probability of encounter of the sexes at a trap containing them would be significantly lower, than the combined probability of other locations bearing this resource.

The results of this study clearly show that though time of feeding on ME (morning) is not correlated with time of mating (dusk) (Figure 9.2), MU flies of both sexes orient towards this resource at dusk and a significantly higher proportion mate at the site with ME than the other sites (Figure 9.5), while their abundance at other resources declines simultaneously (Figure 9.1). This trend is further substantiated in the abundance and resting behaviour of MM males that increase at ME significantly at dusk (Figures 9.1, 9.3). This indicates that the polygamous males return to the potential mate rendezvous site (i.e. ME) at dusk, while the monandrous mated females are at the oviposition resource (i.e. the fruit) at dusk (Figures 9.1, 9.3, 9.5). These trends correspond with observations based on earlier field studies that indicate that mating does not occur at the host plant and, that at dusk, the females found on the host plant were there to oviposit (Figures 9.4, 9.5, Chapter 2, Raghu et al. 2002).

Fitt (1981a) discounted Metcalf et al.'s (1979) claims that ME was a rendezvous stimulus by stating, "although naturally occurring male attractants have been isolated from several plant species these plants are usually not hosts of the species attracted to them". However, an implicit assumption in this statement is that the larval host plant serves as the mating site for the sexually mature adults. As demonstrated by earlier work (Raghu et al. 2002, Chapter 2), this need not be the case in species such as *B. cacuminata*. This is further validated by results of this study that show that flies partition their behaviour between spatially separated resources.

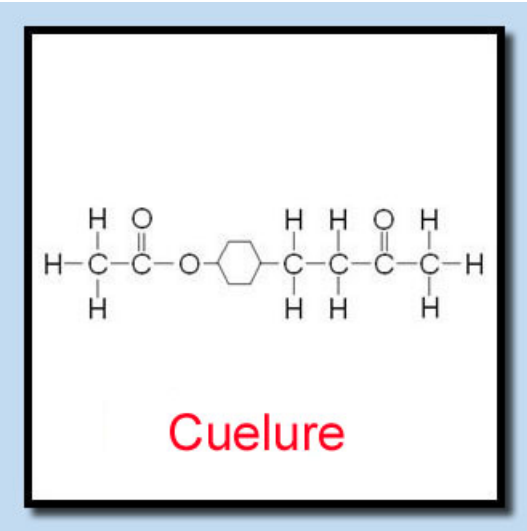
The alternative explanation suggested by Fitt (1981b) was that ME may be a pheromone precursor. However, the principal substance released by *B. cacuminata* at the time of sexual activity is the spiroacetal, 1,7-dioxaspiro[5.5]undecane (Krohn et al. 1991), one of a class of compounds long suspected to be significant as pheromones (Metcalf 1990, Fletcher et al. 1992). Recent chemical ecology literature on the biosynthetic pathways of

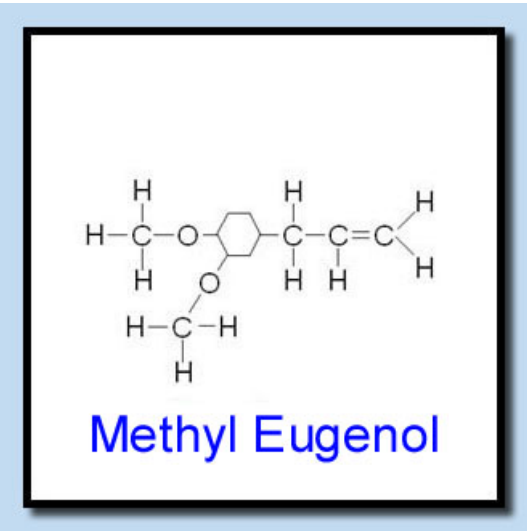
these spiroacetals (Krohn et al. 1991, Fletcher and Kitching 1995, Fletcher et al. 2002) shows that their synthesis is independent of ingestion of ME. This suggests that ME does not play a role in the male pheromone system of *B. cacuminata*.

This study represents the first direct test of Metcalf's hypothesis that phenyl propanoids act as mate rendezvous sites for dacine flies. While my results strongly support his hypothesis, similar behavioural observations need to be made with natural sources of these resources, to see if the patterns observed in the present study are consistent. A vital first step in this process would the documentation of the availability, distribution and abundance of these resources in the fly's habitat. Then the validity of these results can be tested by observations in the natural habitat of the flies and at natural scales of spatial distribution of these resources.

Chapter Ten

Functional significance of phytochemical lures to dacine fruit flies: An ecological and evolutionary synthesis





10.1 INTRODUCTION

The identification of bombykol in 1959 as a pheromone emitted by female moths is often credited as being a key stimulus to the field of chemical ecology (Karlson and Butenandt 1959, Mori 1997). However, nearly a century prior to the chemical characterization of the pheromone, the French naturalist Jean-Henri Fabre had made observations that male moths flew considerable distances, attracted to a female moth caged in his laboratory. In fact Fabre had speculated that the females were emitting something that was attracting the males (Fabre 1912). Similarly keen, albeit serendipitous observation, was also significant as a forerunner to the discovery of attractants used widely in dacine research today.

Nearly half a century prior to the characterization of bombykol, Howlett (1912) discovered that the citronella oil used by a neighbour to repel mosquitoes was actually attracting a dacine pest species, *Bactrocera zonata* Saunders. A subsequent study (Howlett 1915) demonstrated that the phenolic, methyl eugenol (ME) was the active constituent in citronella oil attractive to flies. A similar accident led to the discovery that kerosene was attractive to the Mediterranean fruit fly, *Ceratitis capitata* Weidemann (Severin and Severin 1913) and the subsequent systematic search and determination of attractants for this tephritid species (Cunningham 1989a). While these discoveries and the resultant synthetic production of 'lures' are of tremendous applied entomological value (Metcalf and Metcalf 1992), the proximate (ecological) and ultimate (evolutionary) functional significance of these chemicals remain largely unresolved.

In this chapter, I review the significance of the group of plant-derived secondary chemicals collectively known to tephritid biologists as parapheromones or 'lures' (Cunningham 1989a). First I present the biosynthetic pathways that lead to the formation of these chemicals.

I then elaborate on the two principal hypotheses, *viz*. the ancestral host hypothesis and the sexual selection hypothesis, invoked in explaining dacine response to lures. The ancestral host hypothesis provides an evolutionary or ultimate explanation for dacine response to lures, while the sexual selection provides an ecological/ behavioural or proximate explanation to dacine response to lures. They are therefore not alternatives to each other. Rather, they may collectively help explain the ecological and evolutionary significance of dacine lures. I synthesize the known information to evaluate the evidence in support of the two hypotheses, both in the context of past research on the Dacinae and the findings of the current thesis.

10.2 BIOSYSNTHESIS OF LURES

Plant metabolism is broadly classified into primary metabolism, involving those biochemical processes directly supporting growth, development and reproduction, and secondary metabolism, encompassing those processes not directly involved in the aforementioned processes. The products of secondary metabolism are usually more restricted in occurrence or distribution (Figure 10.1, Edwards and Gatehouse 1999). The use of the term 'secondary' does not imply a hierarchy of importance to plant function, as illustrated by the variety of roles played by secondary compounds in plant defense and the facilitation of pollination (Swain 1977, McKey 1979, Haslam 1995, Berenbaum and Zangerl 1996). Likewise, the term 'primary' is unnecessarily restrictive as primary metabolites may play roles normally considered the domain of secondary compounds (Berenbaum 1995). The application of these labels is often the result of historical precedent, rather than as a result of physiological reasoning (Berenbaum 1995, Haslam 1995, Edwards and Gatehouse 1999).

Secondary chemicals permeate the external surface of plants in various conspicuous (e.g. waxes, odours, resins) and not so conspicuous

forms and mediate the interaction of plants with other components of their environment (Haslam 1995). The secondary chemicals that dacine biologists are most familiar with are those that are used as attractants/ lures used in the monitoring and management of pest fruit flies.

The two principal dacine lures are 4-(*p*-acetoxyphenyl-2-butanone) and 4-allyl-1,2-dimethoxy-benzene, commonly known as cuelure and methyl eugenol (ME) respectively (Cunningham 1989a, b). These chemicals belong to the class of organic compounds based on a C_6–C_3 skeleton referred to as phenyl propanoids (Friedrich 1976). The shikimic acid/ shikimate pathway is the main biosynthetic route by which these aromatic compounds are produced from carbohydrates in plants (Figure 10.1, Herrmann 1995a,b, Matsuki 1996, Hermann and Weaver 1999). An end product of the shikimate pathway is the aromatic amino acid phenylalanine that serves as the precursor to phenyl propanoids in biological systems (Herrman 1995a,b, Haukioja et al. 1998, Herrmann and Weaver 1999, Schmid and Amrhein 1999).

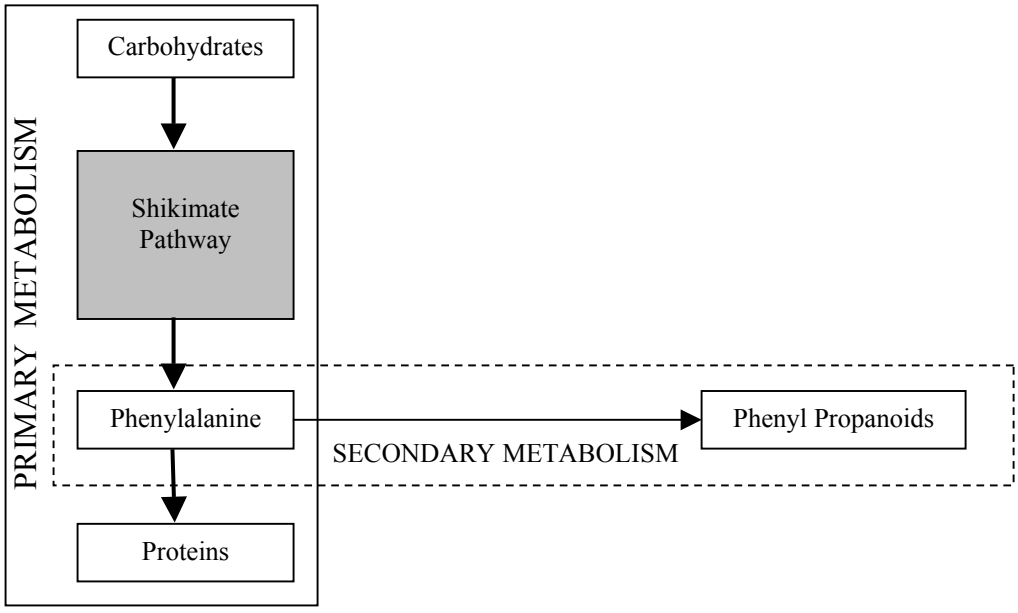


Figure 10. 1. Schematic illustration of origin of secondary metabolic pathway that leads to synthesis of dacine attractants (adapted from Haukioja et al. 1998).

The synthesis of dacine lures from *p*-hydroxycinnamic acid (or *p*-coumaric acid), derived from phenylalanine, is fairly well understood (Geismann and Crout 1969, Friedrich 1976, Metcalf 1979, Metcalf and Metcalf 1992). The SCoA derivative of *p*-hydroxycinnamic acid serves as the starting point for cuelure synthesis. Conjugation with Malonyl CoA, decarboxylation, oxidation and dehydrogenation results in the formation of 4-(*p*-hydroxyphenyl-2-butanone), commonly known as raspberry ketone. Acetylation of raspberry ketone results in the formation of 4-(*p*-acetoxyphenyl-2-butanone), cuelure (Figure 10.2). Cuelure is not known to occur in nature and is only found in its analogous form as raspberry ketone.

The synthesis of ME from *p*-hydroxycinnamic acid is achieved through a process of reduction of the –COOH group, hydroxylation and subsequent O-methylation (Figure 10.3).

Dacine attractive phenyl propanoids are known to occur in several plant groups among the monocots (Figure 10.4) and the eudicots (Figure 10.5).

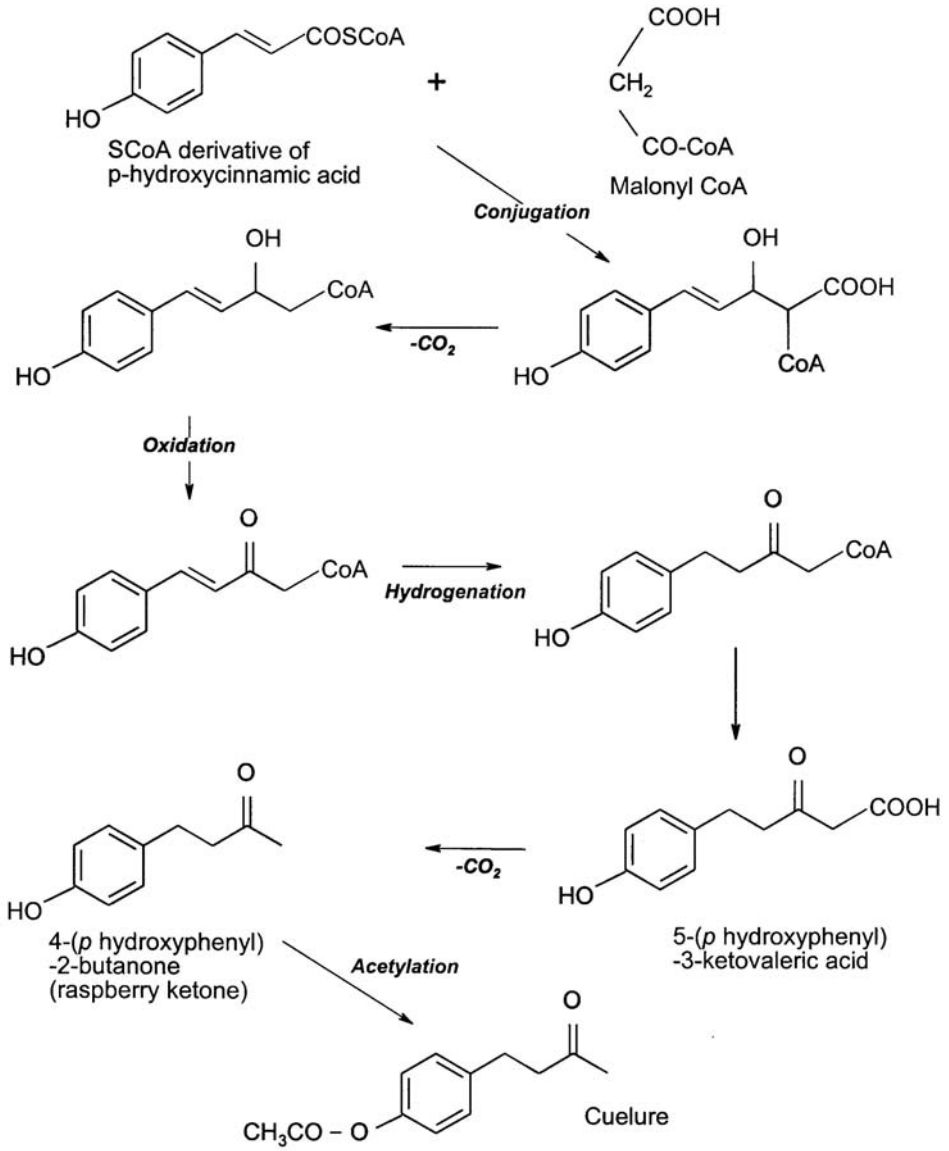


Figure 10.2. Hypothesized biosynthetic pathway for raspberry ketone and cuelure (Adapted from Geismann and Crout 1969, Friedrich 1976, Metcalf 1979)

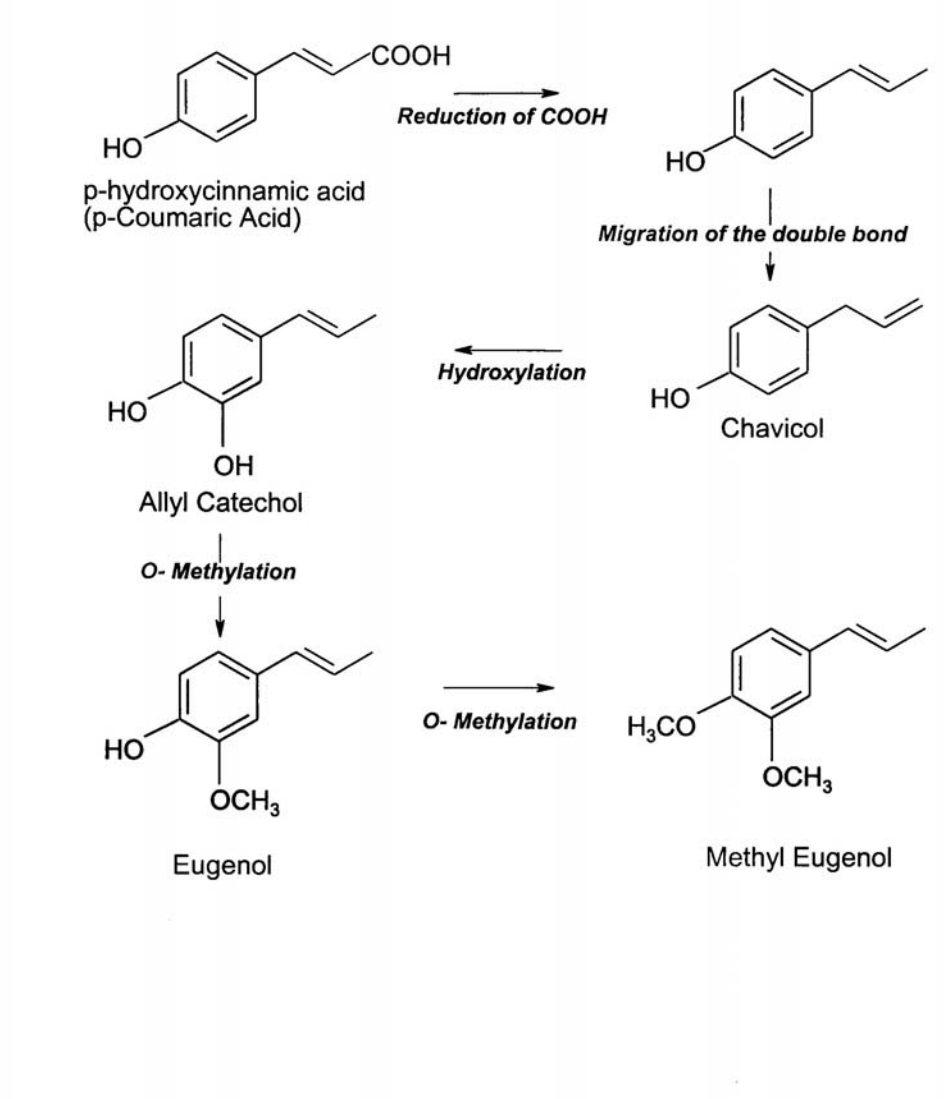


Figure 10.3. Hypothesized biosynthetic pathway for methyl eugenol (Adapted from Geismann and Crout 1969, Friedrich 1976, Metcalf 1979).

Figure 10.4. Cladogram of "primitive"/ basal angiosperms highlighting Orders in which phenyl propanoids attractive to dacine fruit flies are present. Red asterisks represent methyl eugenol and its derivatives and green asterisks represent raspberry ketone and its derivatives.

(Angiosperm phylogeny from Judd et al. 1999, data for distribution of dacine attractants from Nursten 1970, van Buren 1970, Fletcher et al. 1975, Thien et al. 1975, Honkanen et al. 1980, Hirvi et al. 1981, Gallois 1982, Hirvi and Honkanen 1984, Lewis et al. 1988, Marco et al. 1988, Metcalf 1990, Metcalf and Metcalf 1992, Knudsen et al. 1993, Fletcher and Kitching 1995, Dudareva et al. 1999)

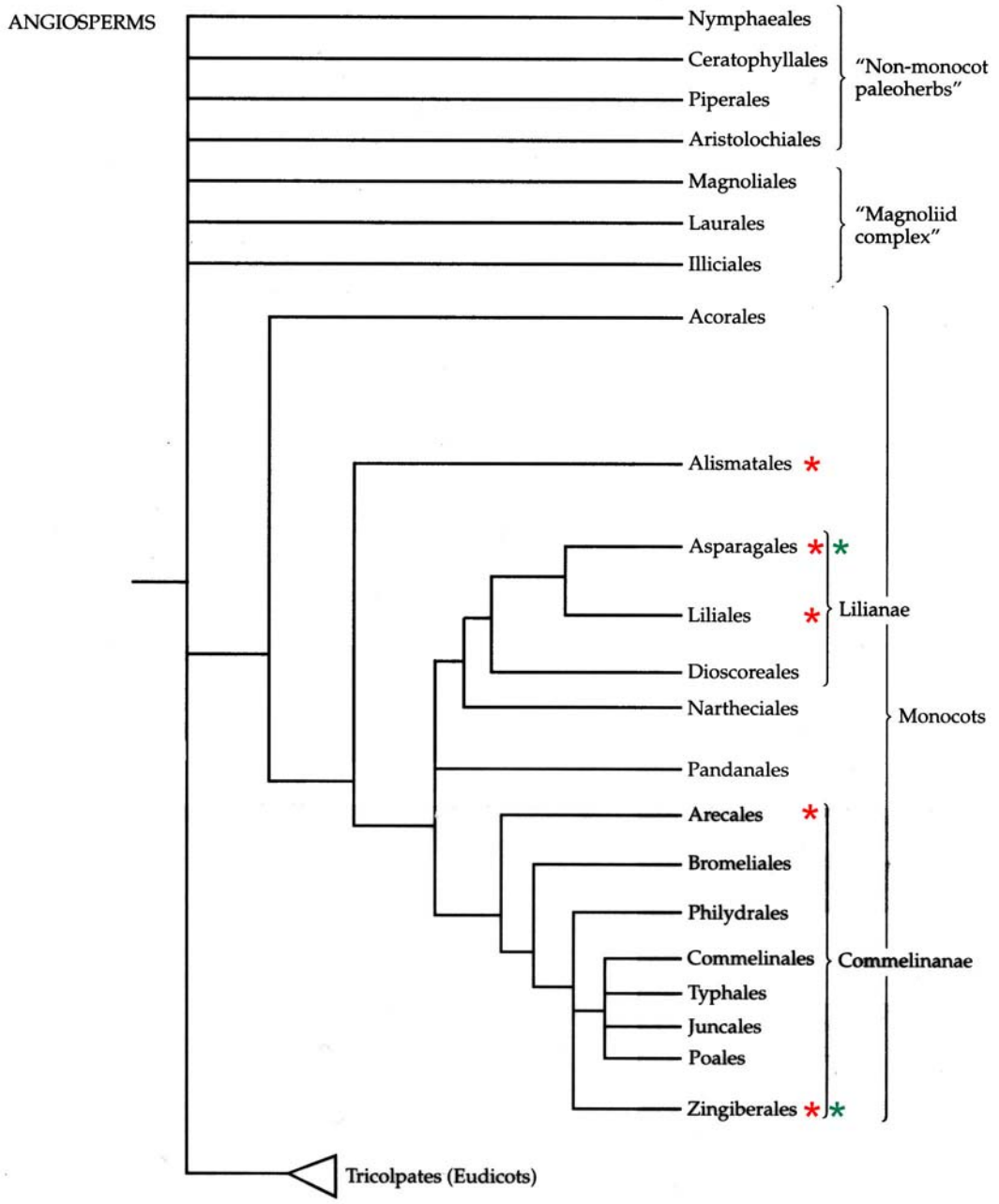
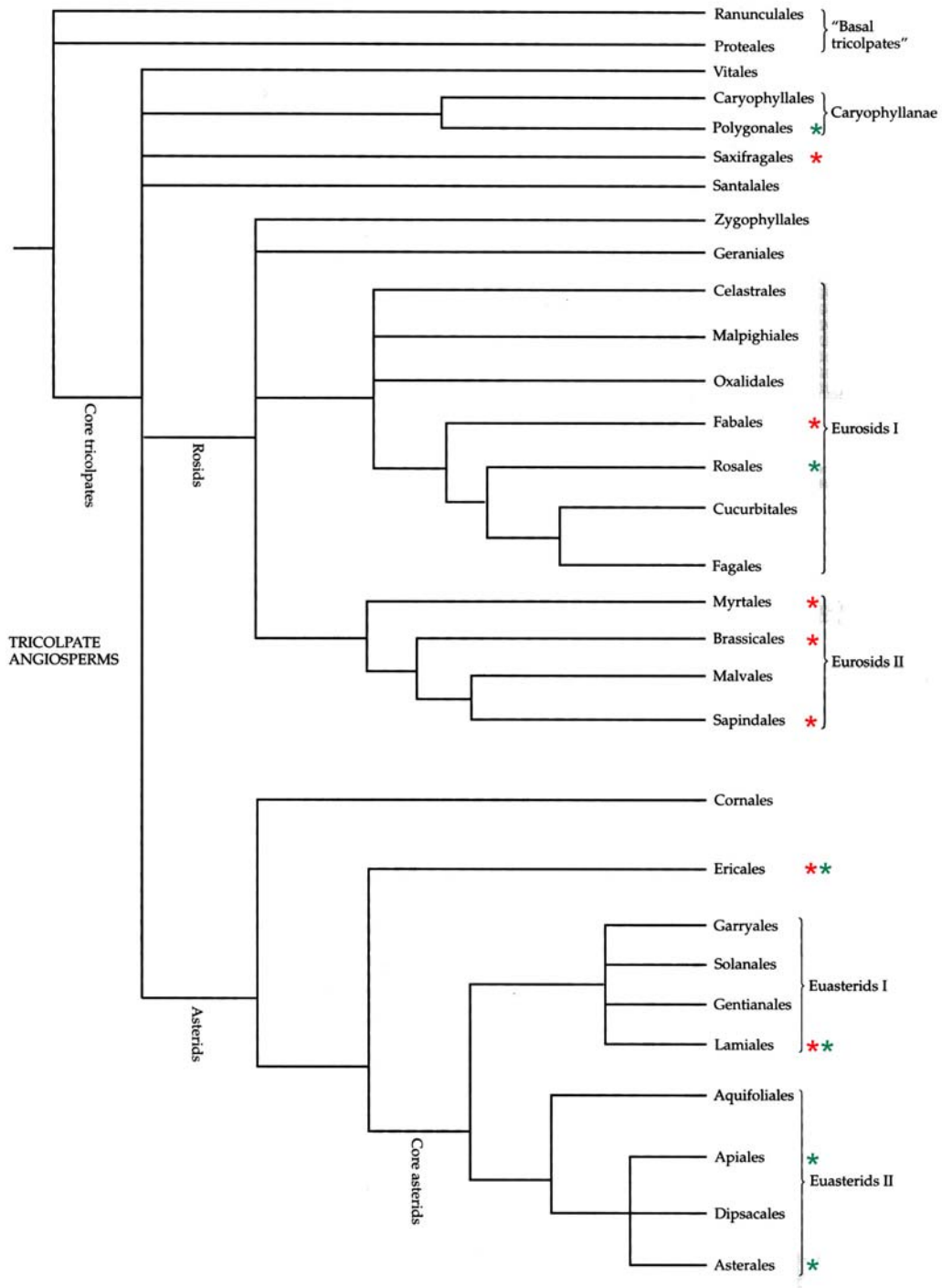

ANGIOSPERMS
Nymphaeales
Ceratophyllales
Piperales
Aristolochiales
"Non-monocot paleoherbs"
Magnoliales
Laurales
Illiciales
"Magnoliid complex"
Acorales
Alismatales *
Asparagales * *
Liliales *
Dioscoreales
Lilianae
Nartheciales
Monocots
Pandanales
Arecales *
Bromeliales
Philydrales
Commelinales
Typhales
Juncales
Poales
Commelinanae
Zingiberales * *
Tricolpates (Eudicots)

Figure 10.5. Cladogram of tricolpate (eudicot) angiosperms highlighting Orders in which phenyl propanoids attractive to dacine fruit flies are present. Red asterisks represent methyl eugenol and its derivatives and green asterisks represent raspberry ketone and its derivatives.
(Angiosperm phylogeny from Judd et al. 1999, data for distribution of dacine attractants from Nursten 1970, van Buren 1970, Fletcher et al. 1975, Thien et al. 1975, Honkanen et al. 1980, Hirvi et al. 1981, Gallois 1982, Hirvi and Honkanen 1984, Lewis et al. 1988, Marco et al. 1988, Metcalf 1990, Metcalf and Metcalf 1992, Knudsen et al. 1993, Fletcher and Kitching 1995, Dudareva et al. 1999).


Ranunculales
Proteales
"Basal tricolpates"
Vitales
Caryophyllales
Polygonales
Caryophyllanae
Saxifragales
Santalales
Zygophyllales
Geraniales
Celastrales
Malpighiales
Oxalidales
Fabales
Rosales
Cucurbitales
Fagales
Eurosids I
Myrtales
Brassicales
Malvales
Sapindales
Eurosids II
Cornales
Ericales
Garryales
Solanales
Gentianales
Lamiales
Euasterids I
Aquifoliales
Apiales
Dipsacales
Asterales
Euasterids II
TRICOLPATE ANGIOSPERMS
Core tricolpates
Rosids
Asterids
Core asterids

10.3 ECOLOGICAL AND EVOLUTIONARY BASIS OF DACINE ATTRACTANCE TO 'LURES'

The biological basis for the attractance of chemicals used as lures has intrigued several researchers since their discovery by Howlett (1912). The different explanations put forward fall within two broad categories. One school of thought (Metcalf 1979, 1987, Metcalf et al. 1979, 1981, 1983) was interested in explaining the evolutionary origin of dacine response to these plant-derived chemicals (i.e. ultimate function) and hypothesized that lures functioned as kairomones. The contemporary approach to dacine attractance to lures is that these chemicals are pheromone precursors that play a proximate role in the sexual behaviour of dacine fruit flies (Shelly and Dewire 1994, Shelly et al. 1996a, b, Nishida et al. 1997, Tan and Nishida 1998, Shelly 2000). In this section I elucidate these two hypotheses.

10.3.1. Ultimate explanations – 'Ancestral host hypothesis'

Metcalf (1979, 1987, 1990, Metcalf and Metcalf 1992) erected this hypothesis to explain the strong response of several dacine species to one of two naturally occurring phenyl propanoids, i.e. raspberry ketone or ME. The ancestral habit of Dacinae is believed to be saprophagy and they are hypothesized to have developed an association with rotting fruits (Rohdendorf 1974, Labandeira 1997). Therefore, coumaric acid and its derivatives in rotting fruit probably served as a kairomone regulating the behaviour of ancestral dacines. The positive response of *Bactrocera cucurbitae* Coquillett to *p*-hydroxycinnamic acid (or *p*-coumaric acid) and the absence of a similar response by *Bactrocera dorsalis* Hendel (an ME responding fly) to the same, suggested that the chemoreceptors in *B. cucurbitae* are more ancient (Metcalf et al. 1983). Based on this evidence Metcalf suggested that species responding to raspberry ketone were more closely related to the ancestral dacines that evolved in association with plants containing cinnamic acid derivatives. Subsequent evolution of oxygenase enzymes in plants resulted

in the transformation of *p*-hydroxycinnamic acid into raspberry ketone and methyl eugenol (Figures 10.2, 10.3). The processes of acetylation and methylation rendered these derived aromatics lipophilic, and they were subsequently integrated into essential oils. Adaptation of the antennal chemoreceptors of dacines through small mutational changes to these new substances is hypothesized to have followed (Metcalf et al. 1979, 1981, 1983). This coevolutionary process is believed to have led to the diversification of dacines in association with the diversification of essential oils in angiosperms (Metcalf 1979, 1990; Figures 10.4, 10.5). The term 'ancestral host hypothesis' for Metcalf's hypothesis was suggested by Tallamy et al. (1999).

Metcalf (1987, 1990, Metcalf and Metcalf 1992) briefly discussed the proximate significance of these chemicals in the behavioural ecology of dacines, arguing that they were principally kairomones, possibly serving as an aggregation chemical for the location of mates or as oviposition stimulants in females (Metcalf et al. 1983). Howlett (1915) had made a similar speculation about the functional significance of these chemicals.

10.3.2. Proximate explanations – Sexual selection by female choice

A hypothesis that has been erected in place of the ancestral host hypothesis contends that these phenyl propanoids are precursors to the male sex pheromone and have a role to play in the sexual behaviour of dacines (Fitt 1981b, c). Female dacines have been demonstrated to have the ability to discriminate between potential mates indicating that sexual selection could be operating in this group (Poramarcom and Boake 1991). Sexual selection by female choice has subsequently been invoked as the explanation for the attraction of dacine fruit flies to lures (Shelly and Dewire 1994, Shelly and Villalobos 1995, Shelly et al. 1996a, b, Nishida et al. 1997, Tan and Nishida 1998, Shelly 2000).

Dacine parapheromones elicit strong anemotaxis in male flies and, at least in some species, an equally strong chemotactic feeding response (Meats and Hartland 1999, Meats and Osborne 2000). Metabolites of these chemicals are then integrated into the rectal gland of adults (Fletcher 1968, Nishida et al. 1988, 1993, 1997), an organ considered to play a role in the synthesis of the male sex pheromone (Fletcher 1968, Nation 1981, Koyama 1989).

Feeding on lures enhances mating success, with females preferentially mating with lure-fed males over unfed males (Shelly and Dewire 1994, Shelly et al. 1996, Nishida et al. 1997, Tan and Nishida 1998, Shelly 2000). Trends in mating success are not as strong in cuelure responding flies in comparison to ME responding flies (Shelly and Villalobos 1995) and were not apparent in the ME responding *B. cacuminata* (Chapter 7). However, there are no observable benefits to females mating with lure-fed males in terms of fecundity or subsequent egg hatch (Shelly 2000). Shelly and Dewire (1994) and Shelly (2000) have suggested that by preferentially mating with lure-fed males, a female may be serving to increase the odds that her sons have a higher ability to forage for these chemicals and subsequently have an enhanced mating success. In sexual selection terms, response to lures could therefore be a trait under runaway selection, where female choice confers her sons an advantage in sexual competition whilst the benefits of choice in the context of offspring viability are arbitrary (Andersson 1994). Additional benefits to flies that have fed on these lures such as defense against predators (i.e. allomonal function) have been suggested (Nishida and Fukami 1990, Tan and Nishida 1998, Tan 2000). These proximate pharmacophagous functions are therefore believed to be maintaining the strong response of male dacines to cuelure and ME.

10.4 SYNTHESIS – EVALUATING THE EVIDENCE

In this section I evaluate the evidence for and against the two above-mentioned hypotheses. These fall under four main categories, each of which is addressed individually below.

10.4.1. Male-baised response to synthetic lures

(*Evidence against ancestral host hypothesis; Neutral for sexual selection hypothesis*)

Traps baited with phenyl propanoids lures are the principal method of assessing population dynamics of dacine fruit flies, are vital tool in taxonomic surveys and are used extensively in quarantine surveillance. While the response of virgin females to these chemicals is documented in a few species (see later section), the evidence from such surveys is that the synthetic dacine lures are principally male attractants, with females rarely trapped (Metcalf et al. 1979, Metcalf and Metcalf 1992).

While Metcalf's coevolutionary explanation may account for the origin of dacine response, it fails to account for the current sex-biased response to lures. If these chemicals serve as mating aggregation stimuli, then one would anticipate a more regular encounter of female flies in trapping surveys. Possible explanations for why we do not observe female response to lures in traps are explored below.

Lures are not mating aggregation stimuli – Metcalf's proximate explanation may be wrong and that these lures do not serve a role as a mate rendezvous stimulus or female attractant in most species. Very few studies have documented such a role for dacine attractants (Fitt 1981b, Chapter 9).

Sensory thresholds – Concentrations of these volatiles from natural sources are not known. It is almost certain that the dose of the pure form of

lure used in traps, usually 5ml, well exceeds the natural volumes of these substances and the sensory thresholds of dacines (Metcalf 1987). If females have a lower threshold of response than males, they may be distributed at the periphery of the odour plume gradient from the traps and as a result may never enter the trap. But such a difference in sensory thresholds is unlikely if these chemicals play a role in bringing the sexes together for mating.

Disruption of the mate recognition system – The entire mating systems of most dacines are unknown. Lure-baited traps incorporate a contact insecticide (usually 1ml of the organophosphate, malathion). If males are the first to arrive at the mating rendezvous site based on olfaction, then any subsequent male signals (visual, olfactory or auditory), that may form part of the signal-response chain in the fly's mate recognition system (*sensu* Paterson 1993), will be absent following the males' poisoning. Alyokhin et al. (2001) reported that in Malathion baited traps, males were killed on contact prior to exhibition of any calling behaviour.

Females may orient to a mate rendezvous site by a combination of olfactory and visual stimuli, while males may only use the former. The absence of a visual stimulus at a standard ME trap (as was used by Brieze-Stegeman et al. [1978]) may therefore impede orientation of females towards lures. Meats and Osborne (2000) found that orientation to lures in male *B. cacuminata* was enhanced by combining olfactory and visual cues, but used a glass cover slip and cotton wick as their visual cues. A similar experiment comparing natural concentrations of lures with and without natural visual stimuli (e.g. natural concentration of lure only vs. flower emitting the compound) may help elucidate this aspect.

Female response to lures in specific circumstances

While the strong male-biased response in trapping surveys strongly counters Metcalf's hypothesized proximate function of lures as mating rendezvous stimuli, female flies do respond to lures in specific circumstances. Steiner et al. (1965) reported the appearance of female *B. dorsalis* flies in traps at the end of male annihilation programs when males from the population were depleted. Allan Allwood (pers. comm.) reported a similar phenomenon in the fruit fly eradication program on Nauru when females of the mango fruit fly, *Bactrocera frauenfeldi* (Schiner) outnumbered the males in cuelure-baited traps when populations were low. Such female response has also been documented in the related tephritid species, *C. capitata* (Nakagawa et al. 1970). More recently, both sexes of the univoltine Chinese citrus fruit fly, *Bactrocera* (*Tetradacus*) *minax* Enderlein, have been documented as responding to ME at the time of their life cycle when they are just reaching sexual maturity (R.A.I. Drew and C. Dorji– unpublished data, pers. comm.). Such behaviour suggests a functional role for these lures to female Dacinae.

Evidence from *B. cacuminata* (Chapter 9) clearly suggests that ME functions as a mate rendezvous stimulus. Such response is not unique. Virgin females of *Bactrocera opiliae*, *B. aquilonis* May and *B. tenuifascia* May all respond to their respective phenyl propanoid lures at times of day corresponding with peak periods of sexual activity (Fitt 1981b).

10.4.2. Response to other related phenyl propanoids

(*Evidence in support of ancestral host hypothesis; Neutral for sexual selection hypothesis*)

The two major dacine lures (i.e. cuelure and ME) attract over a 100 species of dacine flies each (Table 10.1, R.A.I. Drew – pers. comm.). However, roughly a third of all dacine species have no known lure record (Table 10.1, Metcalf and Metcalf 1992), suggesting that there are perhaps other phenyl propanoids

that would be more attractive to these dacines than the two widely used lures. This is substantiated by the fact that certain dacine species respond to related phenyl propanoids, but neither to cuelure nor methyl eugenol. Examples of these include the response of *Dacus vertebratus* Bezzi to methyl *p*-hydroxybenzoate (Hancock 1985) and *Bactrocera latifrons* Hendel to α-ionol (Metcalf and Metcalf 1992).

Perhaps the most telling supporting evidence for a kairomonal basis dacine response to phenyl propanoids comes from the fact that the benzyl acetate (a benzenoid derived from phenyl propanoids by the loss of C_8–C_9 carbons [Dudareva et al. 1999]) is attractive to *B. dorsalis* (an ME responding fly), *B. cucurbitae* (a cuelure responding fly) and *Ceratitis capitata* (a tephritid responding to α-copaene from *Angelica archangelica*) (Lewis et al. 1988). Benzyl acetate occurs in many plants including *Spathiphyllum cannaefolium* and several orchid species (Dodson and Hills 1966, Dodson et al. 1969, Lewis et al. 1988). Zingerone (4-(4-hydroxyphenyl-3-methoxyphenyl)-2-butanone), synthesized by the methoxylation of raspberry ketone, occuring naturally in ginger and in the orchid species *Bulbophyllum patens* and *B. cheiri*, is another phenyl propanoid that attracts both culure and ME responding dacines (Tan and Nishida 2000, Tan et al. 2002). The generic response by dacine flies to these chemicals indicates that their receptors are attuned to the basic phenyl propanoid structure, in accordance with Metcalf's hypothesis (see Section 10.2.1.).

10.4.3. Anomalous lure records

(*Evidence neutral for ancestral host hypothesis and sexual selection hypothesis*)

Though there is a firm belief that dacines respond to either ME or cuelure (Metcalf and Metcalf 1992), there are several records in the literature of flies responding to both chemicals (see for e.g. *B. latilineata*, *B. furfurosa* and *B. melanotus* in Drew 1989b). In each of these cases the response to both lures is

dismissed as contamination or incorrect record of the lure. However, the presence of these and other similar anomalous records in the taxonomic literature warrants specific research to clarify if certain species indeed do respond to both lures. Only then can we fully evaluate the validity of Metcalf's hypothesis that dacine chemosensory receptors evolved in association with these phenyl propanoids.

Table 10.1. Summary of lure response in Australasian Dacinae
(Data from Drew 1989b)

Genus	***Subgenus***	***ME***	***CUE***	***Not known***
Bactrocera	*Afrodacus*	0	4	3
	Bactrocera	40	90	51
	Gymnodacus	0	1	2
	Notodacus	1	0	0
	Polistomimetes	3	0	5
	Trypetidacus	1	0	0
	Hemisurstylus	0	0	1
	Hemizeugodacus	0	0	3
	Melanodacus	0	0	2
	Queenslandacus	0	0	1
	Austrodacus	0	0	1
	Diplodacus	0	0	1
	Heminotodacus	0	0	1
	Hemiparatridacus	0	0	1
	Javadacus	2	0	1
	Niuginidacus	0	1	0
	Papuadacus	0	1	0
	Paradacus	0	2	2
	Paratridacus	2	0	4
	Sinodacus	0	10	3
	Zeugodacus	0	13	7
Dacus	*Callantra*	2	4	4
	Dacus	0	8	1
	Didacus	0	4	1
	Semicallantra	1	1	1
Paracallantra		0	0	1
TOTAL		52	139	97

10.4.4. Dacine mating behaviour

(*Evidence in support of sexual selection hypothesis; Neutral for ancestral host hypothesis*)

The absence of phenyl propanoids attractive to dacines in their current host plants, in conjunction with the view that the host plant serves as a mating site for dacines, has led to the rejection of ancestral host hypothesis by dacine biologists (Fletcher et al. 1975, Fitt 1981b). The alternate explanation, that feeding on these phytochemicals serves to enhance mating success, has hence gained prominence in the recent dacine literature. The strongest support for the sexual selection hypothesis comes from work done on some of the major pest species amongst the Dacinae.

Male dacines have a rectal gland that is hypothesized to be significant in the synthesis of the male sex pheromone (Nation 1981). Upon ingestion of lures, male Dacinae accumulate metabolites derived from these chemicals in the rectal gland that are subsequently released as a volatile emission at dusk (Nishida et al. 1988, 1993, 1997), coinciding with the period of peak sexual activity.

Shelly and Dewire (1994) demonstrated that feeding on synthetic ME enhanced mating competitiveness in *B. dorsalis* up to 30 days after a single exposure. Such mating benefits have also been demonstrated in *Bactrocera philippinensis* Drew and Hancock (Shelly et al. 1996). For the cuelure feeding melon fly, *Bactrocera cucurbitae* (Coquillett), a similar augmentation of mating success has been recorded, albeit not as strong as in the case of the ME responding species (Shelly and Villalobos, 1995). Tan and Nishida (1998) and Shelly (2000) have recently demonstrated similar advantages in mating behaviour for *B. dorsalis* after feeding on natural sources of methyl eugenol. Evidence to the contrary comes from *B. cacuminata*, where feeding on methyl eugenol does not appear to confer any mating advantage (Chapter 7).

10.4.5. Defensive role

(*Evidence in support of sexual selection hypothesis; Neutral for ancestral host hypothesis*)

In addition to mating benefits, the potential role of ME as an allomone has been recently explored. Nishida and Fukami (1990), Tan and Nishida (1998) and Tan (2000) have reported that feeding on methyl eugenol renders flies 'distasteful' to the house gecko and sparrow. Pairs in copula are usually stationary and hence vulnerable to predation and female flies may preferentially mate with male flies that have fed on methyl eugenol to mimise predation risk. Although predation was not explicitly measured, studies in *B. cacuminata* showed that survival of ME-fed flies was not enhanced in the presence of predators (Chapter 8). However, studies on allomonal benefits have to date not been undertaken in the natural environmental of the fly species in the presence of natural predators.

10.4.6. Botany and plant biochemistry

(*Evidence in support of ancestral host hypothesis; Neutral for sexual selection hypothesis*)

The origin of angiosperms in the Cretaceous is the outcome of perhaps the greatest botanical evolutionary innovation, the origin of the carpel to protect the genetic material of the plant (Stewart 1983). Though the origin of insects preceded the origin of flowering plants by over 200 million years, their coevolutionary associations/ interactions with insects (herbivores and pollinators) were significant in enabling them to occupy their current dominant position in the terrestrial world (Crepet and Friis 1987, Friis and Crepet 1987, Judd et al. 1999). The evolving angiosperms, however, would also have contended with pathogenic microorganisms, the truly ancient and dominant life forms on earth (Niklas 1982).

Microorganisms may have been the *agents provacateurs* for the original diversification of phenyl propanoids by facilitating the production of allelochemicals in plants that subsequently influenced insect-plant interactions (Berenbaum 1988). Therefore, 'ancestral' phenyl propanoids or precursors to dacine attractants may have evolved as defense chemicals to protect early flowers from bacteria and fungi and their contemporary counterparts may continue to play such an antibiotic role (Walker 1975, Herrewijn et al. 1995, Janssen et al. 2002). The presence of these chemicals in floral volatiles has been noted from ancient groups such as the Aspargales (Orchidaceae, albeit a rapidly evolving group) and Alistamales, and from more recent groups such as the Myrtales (Onagraceae) and Asterales (Figures 10.4, 10.5), indicating such an ancient origin of these chemicals.

Flower feeding by adults was a habit in ancient Diptera (Syrphidae, Culicidae, Tipulidae, Mycetophylidae, Empedidae, Bombylidae, Anthomyiidae and some Muscidae) (Van der Pijl 1960, 1961, Rohdendorf 1974, Crepet and Friis 1987, Labandeira 1997) and they were associated with the pseudoflowers of the Gnetophytes, principally for the consumption of the amino acids and polypeptides in the nectar and served as incidental pollinators (Crept and Friis 1987, Harrewijn et al. 1995, Gardner and Gillman 2002). This incidental pollination is an indication of the progression from an anemophilous to a zoophilous pollination syndrome (Van der Pijl, 1961, Crepet and Friis 1987, Labandeira 1997). The origin of the angiosperm nectaries in the late Creataceous (Friis and Crepet 1987), highlighting the success of this pollination syndrome, coincides with the origin of the tephritids and dacines in the early Tertiary (Rohdendorf 1974, Metcalf and Metcalf 1990, Labandeira 1997).

The benefits of an antibiotic effect and the incidental pollination by primitive Diptera could have thus enabled the sustained production of phenyl propanoids. Dudareva et al. (1999) have recently demonstrated that

the process of synthesis of ME is restricted to the epidermal cells of the petal tissue, with the mRNAs coding for the biosynthetic enzymes detected in petal cells just prior to the opening of the flower. The emission of ME peaks at anthesis and gradually declines subsequently. Such a *de novo* synthesis supports the notion that these chemicals could have evolved for antibiotic protection of genetic material and subsequently facilitated pollination, i.e. pollination was an exaptation. Such biochemical exaptations are not uncommon in chemicals evolved for plant defense (Armbuster et al. 1997). Since flowers of several 'ancient' plants also function as mating rendezvous sites and adult feeding sites for insects (Pellmyr and Thien 1986), response to flowers by olfaction would thus have been maintained in flower visitors, including dacines (Tan and Nishida 2000, Clarke et al. 2002, Tan et al. 2002).

The stage would have thus been set for the types of coevolutionary processes envisaged by Metcalf. Diversification in phenyl propanoids would have resulted in associated changes in dacine chemoreceptors. The presence of these chemicals in fruits (Nursten 1970, van Buren 1970) may have subsequently facilitated the exploitation of the fruit resources by female flies for larval development. The olfactory receptors thus "tuned" to these phenolics would explain the attractance of dacines to these chemicals.

A key difficulty, however, with accepting coevolution as an explanation for dacine lure response, is that there are no obvious phylogenetic patterns in the distribution of these phenyl propanoids in plants (Figure 10.4, 10.5). This may be an artefact of the paucity of sampling for these volatiles, rather than a true absence of pattern. Furthermore, while it is conceivable that dacines evolved in response to plant chemistry (based on the receptor sensitivity) and that such response was maintained by a role played by these chemicals as sex pheromones and/ or allomones, the benefits to the plant of interacting with fruit flies in unclear. Only if the fitness benefits to plants emitting these volatiles can be demonstrated (as has

been suggested by Tan and Nishida [2000] and Tan et al. [2002]), can we invoke coevolution as an explanation.

10.4.7. Dacine pheromone chemistry

(*Evidence for and against sexual selection hypothesis; Neutral for ancestral host hypothesis*)

A difficulty with the hypothesis that these phenyl propanoids function as precursors to dacine sex pheromones is that if this were the case, by definition of the unique nature of pheromones, one would anticipate there should be over a 100 unique derivations, or at least in blends, for each of these chemicals. Rectal gland composition, at the time of peak sexual behaviour, needs to be examined to evaluate if such species-specific variations in lure metabolites exist.

The role of a long range dacine sex pheromone has been attributed to a class of compounds significantly different from phenyl propanoids, i.e. spiroacetals (Haniotakis et al. 1977, 1986, Bellas and Fletcher 1979, Francke et al. 1979, Baker et al. 1980, Mazomenos and Haniotakis 1981, Baker and Bacon 1985, Baker and Herbert 1987, Kitching et al. 1989, Mazomenos 1989 [and references therein], Perkins et al. 1990, Krohn et al. 1991, Stok et al. 2001, Fletcher et al. 2002). They appear to be more likely candidates as components of the sex pheromone, as has been demonstrated in the case of *B. oleae* (Haniotakis et al. 1977, Baker et al. 1980, Mazomenos 1989). *Bactrocera cacuminata* produces spiroacetals independent of exposure to methyl eugenol (Krohn et al. 1991, Fletcher et al. 2002, S. Raghu and C.J. Moore – unpublished data). These chemicals, in association with N-alkylacylamides that function as short-range aphrodisiacs (Bellas and Fletcher 1979, Metcalf 1990), warrant further investigation. Spiroacetals and amides are distinctly different from the plant-based phenyl propanoids and bioassays in the laboratory and field will clarify their role in the mating system of dacines.

Although chemical evidence suggests that spiroacetals are more likely candidates for pheromones, we need to reconcile the fact that lure metabolites are recovered from the rectal gland (see section 10.3.2.). Bellas and Fletcher (1979) have shown that amides from dietary leucine accumulate in the rectal glands of *Bactrocera tryoni* Frogatt and these amides are released at dusk. A similar dietary influence on the terpene composition of the rectal gland has been documented in *Bactrocera passiflorae*, by changing the food source from papaya (*Carica papaya*) to rose-apple (*Syzigium* sp.) (Fletcher et al. 1992). Recent observations in *B. cacuminata* show that ME metabolites are given off by males in the middle of the day and hence not restricted to dusk, when mating occurs in this species (S. Raghu and C.J. Moore – unpublished data). Since dacines ingest phenyl propanoid lures, the presence of their metabolites in the rectal gland may be a simple dietary consequence.

Alternately, these metabolites and spiroacetals may both be components of the pheromone blend of fruit flies. The relative concentrations of these two chemicals may be a component of the mate recognition system of a particular species. If the pheromone blend of a species had a higher concentration of ME metabolites than spiroacetals, then feeding on the synthetic methyl eugenol lure may result in the enhanced mating success documented in certain Dacinae (e.g. Shelly and Dewire 1994, Shelly 2000). Alternately, if the metabolites were not critical in the pheromone or mate recognition system, we may anticipate that they not be any significant effect of feeding on the lure on mating success, as is the case in *B. cacuminata* (Chapter 7).

10.5. CONCLUSION – GAPS IN THE KNOWLEDGE

Imagine a Martian peering through a window at a writer whose papers are disturbed by a willful breeze. The Martian sees him solve the problem by taking out his pocket-watch and using it to restrain the sheets. Think of the problem facing the Martian when, having managed to get hold of the watch, he tries to work out the rationale of its design while believing its function is that of paperweight!

H.E.H. Paterson (1993)

We are in the position of the proverbial Martian. Having chanced upon this incredibly useful toolkit of chemicals in attracting dacine flies, we are faced with the puzzle of explaining their 'function' in the context of dacine ecology and evolution. We need to exercise caution about confusing proximate (ecological/ behavioural) and ultimate (evolutionary) functions of dacine lures. The ancestral host hypothesis is an ultimate explanation of the origin of the lure response, while its proximate function may be in the mating behaviour of fruit flies. So, as mentioned earlier, the two hypotheses outlined above are not logical alternatives to one another and need to be explored independently. It is entirely possible for these chemicals to have the same ultimate function for Dacinae, while having different proximate functions in different dacine species. While there is evidence in support of both hypotheses, considerably greater research is required to explain the phenomenon of lure response in Dacinae.

10.4.1. Testing ultimate hypotheses

Dacine phylogeny

Despite the extensive taxonomic treatment that the Dacinae have received, dacine systematics is still rudimentary (Drew and Hancock 2000, White 2000) and the morphogenetic cladistic treatment of this group of insects is still

preliminary (Graham et al. 1998, Muraji and Nakahara 2001). This is one of the key gaps in our knowledge that is impeding our understanding of the evolutionary significance of lures. Such a genealogical treatment will enable us to determine if response to phenyl propanoids/ cinnamic acid derivatives is plesiomorphic (an ancestral trait) or synapomorphic (shared derived character). If lure response is plesiomorphic then inferences cannot be made about evolutionary relationships among dacine taxa based on lure response, in the manner they currently are in dacine taxonomy (Drew and Hancock 2000, White 2000). Alternately, if it is synapomorphic then this will enable us to test Metcalf's hypothesis based on receptor evolution in the different monophyletic groups. Recent research in this regard on a small sample of *Bactrocera* species indicates that lure response is labile with response to cuelure as the ancestral trait (Smith et al. 2002, 2003). Lure response has been lost on multiple occasions and response to ME has evolved independently several times (Smith et al. 2002, 2003).

Distribution of lures in relation to plant phylogeny

There are no clear phylogenetic patterns evident from the distribution of dacine attractants amongst the plant orders (Figure 10.4, 10.5). But this may be a result of paucity of information, rather than any true evolutionary pattern. Based on the available information, these chemicals are haphazardly distributed among many clades (Figures 10.4, 10.5), with some plant orders having both dacine lures (e.g. Asparagales, Zingiberales and Ericales). Further plant chemosystematic information (Harbourne and Turner 1984) and subsequent investigation of congruence between dacine phylogeny in relation lure response and the distribution of these chemicals in relation to host plants will enable us to test for any coevolutionary association.

A key difficulty in this regard is that designation of plants as hosts for dacines (and phytophagous insects in general) is determined by their ability to support larval development (e.g. Drew 1989b). But adult dacines respond

to plants that emit these phenyl propanoid volatiles, independent of their ability to sustain larvae (Fletcher et al. 1975, Shelly 2000, Tan and Nishida 2000, Clarke et al. 2002, Tan et al. 2002). Therefore, adult flies need not be restricted to larval host plants (Chapters 2, 9) and may indeed have other ecological roles. Preliminary investigations in this regard suggest that this may indeed be the case with adult dacines playing a role in pollination (Tan and Nishida 2000, Tan et al. 2002). Further research in natural systems may shed light on functional roles played by adult dacines. Any investigation of coevolution must therefore explore the congruence between the phylogeny of plants emitting volatile lures to which adult dacines respond, and dacine phylogeny.

10.4.2.Testing proximate hypotheses

Female response to lures

Female response to lures has been poorly studied. With the exception of this thesis, only one other study explicitly examined female response to these phenyl propanoids (Fitt 1981a). Assaying the receptor sensitivity of female flies to these chemicals, will enable the test of the hypothesis that female flies have a different sensory threshold of response to lures than males. In males, quantifying behavioural and electroantennographic responses to a series of closely related chemicals, incorporating systematic changes in molecular shape and size and associated changes in polarity and lipophilicity of interactive groups, enabled 'mapping' of receptor sites (e.g. Metcalf et al. 1979, 1981, 1983, Metcalf 1987). In dacines that use these chemicals are mate rendezvous stimuli (e.g. *B. cacuminata*) females may have similar receptor site geometry to conspecific males in relation to the respective lure.

Behaviour and consequences in relation to natural sources of lures

The concentration of chemicals used to assay response to lures, or in field research, is likely to be much higher than concentrations in natural sources.

Investigation into the natural concentrations of these volatiles is preliminary (e.g. Dudareva et al. 1999, Pichersky and Gershenzon 2002). Future ecological and behavioural investigations need to take into account the natural concentrations and mechanisms of release of these volatiles. Further investigations of the behavioural consequences of feeding on natural sources of these chemicals, such as those of *Bactrocera dorsalis* feeding on exudates from flowers of *Fagraea berteriana* (Nishida et al. 1997) and *Cassia fistula (*Shelly 2000), and the response to several *Bactrocera* species to orchids of the genera *Bubophyllum* (Tan and Nishida 2000, Tan et al. 2002), need to be undertaken to unravel the functional significance of these chemicals (Landolt and Philips 1997).

While allomonal benefits of feeding on lures are an exciting development (Nishida and Fukami 1990, Tan and Nishida 1998, Tan 2000), such benefits need to be determined for predators in natural systems. Only through the assessment of the physiological and behavioural consequences of dacine ingestion of natural doses of these chemicals, can we truly understand any biological significance they may have.

Dacine pheromones

Our understanding of the functional significance of dacine pheromones and their components is still too preliminary to conclusively evaluate the role of lures of botanical origin in dacine mating systems. Analysis of rectal gland composition of males sampled in the wild will, in conjunction with bioassays of different constituents, be crucial in evaluating the relative significance of phenyl propanoids, spiroacetals and amides in the mating systems of dacine fruit flies. More significantly, what role pheromones play in courtship of dacine species needs to be thoroughly investigated, given the poor understanding of the specifics of dacine mating behaviour.

Role of phenyl propanoids in plant biology

The distribution and physiological basis of the synthesis of these chemicals within plants is only now being explored (e.g. Dudareva et al. 1999, Pichersky and Gershenzon 2002). Further assessment of the role they play in the plant's biology (e.g. antibiotic defense of structures they are released from, attractant for pollinators) may aid clarification of the functional significance of these chemicals.

The spectacular success of dacine attractants in pest management has in some ways impeded our understanding of any biological role they may have. Only an integrative biochemical, botanical and entomological approach, while acknowledging the idiosyncrasies of species, will help unravel the functional significance of phytochemical lures to dacine fruit flies.

Chapter Eleven

GENERAL DISCUSSION

11.1 GENERAL DISCUSSION

Generalizations of knowledge derived from a few species, commonly pests in modified environments, have often been made to encompass a very diverse and highly speciose group of insects, the Dacinae. Such inductive reasoning is not uncommon in biological research given the complex nature of problems that biologists face. However, the significance of periodic, explicit, hypothetico-deductive testing cannot be understated as such tests bring to attention the limits and shortcomings of our generalizations. Using the dacine species, *Bactrocera cacuminata* I have tried to do this for those aspects of its autecology that relate to adult resources.

I began by explicitly testing the prevailing paradigm in dacine ecology, that the host plant serves as the centre of dacine activity, mediated by mutualistic associations with fruit fly-type bacteria (Chapter 2). Contrary to predictions, in the natural habitat, the host plant in this species appeared to only play a role in oviposition site, i.e. it functioned exclusively as a larval host plant. Even in disturbed habitats, the paucity of key adult behaviours, such as mating on this plant was striking (Chapter 3). This meant that adult flies were probably utilizing other components of their habitat, i.e. resources vital to their life history requirements.

Assessing the physiological status of flies in relation to the resources that dacine flies require, to meet physiological demands (sugar, protein, methyl eugenol and the host plant), could explicitly test if the host plant principally serves only as an oviposition site. Hence, based on related research in other dacine species (Drew 1969, Fletcher et al. 1978), I developed a method to predict the physiological status of adult *B. cacuminata* at different resources (Chapter 4). Using this method to assess the physiological and nutritional status of flies arriving at these resources in the field (Chapter 5), I discovered that only sexually mature and mated females were responding to

the host plant, while any males at the host plant were sexually immature. This confirmed my hypothesis that the host plant served primarily as an oviposition site. In addition, the physiological and nutritional status of flies revealed that sexually mature males, with high nutritional reserves, were located at methyl eugenol. This indicated that the methyl eugenol was perhaps a significant resource in the context of the reproductive behaviour of this species. This stimulated my curiosity, as mating behaviour was notably absent at the host plant (Chapter 2) and I explored the functional significance of this chemical in the biology of *B. cacuminata*.

The current hypothesis of the role of phenyl propanoids, such as methyl eugenol, is that they function as a pheromone precursor chemical. Response to these chemicals is also hypothesized to be a trait under sexual selection. In order to investigate if this was the function of methyl eugenol in the case of *B. cacuminata*, I investigated the feeding behaviour (Chapter 6) and associated reproductive (Chapter 7) and physiological consequences (Chapter 8). While methyl eugenol functioned as a strong phagosimulant for this species, eliciting recurrent feeding behaviour (Chapter 6), it did not have the strong mating benefits suggested in the case of other dacine species (Chapter 7) and neither did I detect any physiological/ nutritional benefit of feeding on this chemical (Chapter 8).

If methyl eugenol did not function as a pheromone precursor in *B. cacuminata* then why were sexually mature males responding to this chemical? Metcalf (1979, 1990) and Metcalf and Metcalf (1992) postulated that these chemical lures were ancestral host locating kairomones, but proximately served as a mating rendezvous stimulus that brought the sexes together at the time of mating. This hypothesis had been rejected as female flies almost never respond to traps baited with these chemicals and, in dacine species that mated at the larval host plant, these phenyl propanoids had seldom been detected at the host plant. Given that mating was a rarity at the

host plant in the case of *B. cauminata*, this hypothesis could explain the response of sexually mature males to this chemical (Chapter 5). I ran a field-cage experiment, spatially separating resources (host plant, methyl eugenol, sugar and protein), to explicitly test Metcalf's hypothesis in *B. cacuminata* (Chapter 9). Results from this study clearly demonstrated that methyl eugenol was functioning as a mate rendezvous stimulus for *B. cacuminata*.

To further understand the ecological and evolutionary basis of dacine response to phenyl propanoids, I synthesized the literature to evaluate the relative significance of the two hypotheses (i.e. sexual selection [pheromone precursor] vs. Metcalf's hypothesis) proposed to explain the basis for dacine lure response (Chapter 10). Evidence from botanical and plant biochemistry literature is supportive of Metcalf's coevolutionary hypothesis and this may provide a common evolutionary (ultimate) link across the Dacinae. However, there is conflict over explanations for the current (/proximate) use of these chemicals by dacines and it may that it varies across more recent lineages within the Dacinae.

11.2 REVISION OF LIFE HISTORY OF *BACTROCERA CACUMINATA*

Based on the results of this thesis it is evident that *Solanum mauritianum* serves principally as a larval development resource for *B. cacuminata*. Upon emerging from pupae underneath the larval host plant, teneral adults forage for other resources to attain sexual maturity. Significant among these include sugars to fuel foraging behaviour and protein to promote sexual development. Sugars in the form of homopteran honeydew and fruit exudates (including those from *S. mauritianum* fruit) are the two likely sources of this resource for *B. cacuminata* (Fletcher 1987). Protein is possibly acquired from feeding on phylloplane bacteria and bird faeces (Drew et al. 1983, Courtice and Drew 1984, Fletcher 1987). Both sugar and protein resources need not be restricted to the larval host plant and this may explain

the paucity of adult feeding behaviour observed at the host plant in this study. Source and location of sugar and protein resources were not explicitly tested in this thesis.

Sexually mature flies then forage for a mating site. The stimulus that enables the orientation of conspecific mates to this site appears to be the presence of the phenyl propanoid, methyl eugenol. If *S. mauritianum* also gives off these volatiles during a phase of its life cycle, then it may serve as a mating site during that phase. However, given that these chemicals occur widely in the plant kingdom it is likely that mating is not restricted to the host plant. Once mated, gravid females forage for an oviposition resource, i.e. fruiting *S. mauritianum* in which to lay eggs, thus re-initiating the life cycle. Males probably return to methyl eugenol (mating site) on multiple occasions to mate.

11.3 GAPS IN THE KNOWLEDGE

"We shall not cease from exploration and the end of all our exploring will be to arrive where we started and know the place for the very first time."

T. S. Eliot (1968; *Little Gidding*)

The autecology of *Bactrocera cacuminata* is far from resolved. In testing some of the central hypotheses about adult Dacinae, this thesis has revealed several interesting questions that require further investigation.

11.3.1. Interactions with the larval host plant

While this thesis has examined the use of the host plant in relation to the adult life stage of *B. cacuminata*, the nature of dacine host use over the lifetime of the larval host plant is unclear. The physiological status of the larval host plant may influence the physiological profile of flies that visit the plant. Drew and Lloyd (1987) explored the use of the host plant over the

fruiting cycle of the host plant, but if the plants release phenyl propanoids during anthesis (see Chapter 10) then the flowering stage may be significant. I am currently discussing the possibilities of doing headspace analysis of the flowers of *S. mauritianum* to assess the volatiles being released by them (C. J. Moore – pers. comm.). If the flowers do release methyl eugenol, then whether the larval host plant serves as a mate rendezvous site during the flowering stage needs to be examined.

Another key question in relation to the physiological status of the larval host plant is the phenomenon of post-teneral dispersal by emergent flies. Teneral dacines are hypothesized to experience a strong endogenous drive to disperse away from the emergence site (i.e. larval host plant), a hypothesized adaptation to avoid the density-dependent effects of intraspecific competition (Fletcher 1974a, b, 1989). While this is a plausible mechanism in plants that fruit *en masse*, the validity of this explanation in the context of plants like *S. mauritianum* that fruit continuously with asynchronous ripening among fruits within individual clusters, is debatable. Whether *B. cacuminata* possess an endogenous post-teneral dispersal, despite the continuous availability of a larval resource, needs further examination.

Solanum mauritianum is an introduced plant species to which *B. cacuminata* appears to have adapted. What is its role in habitats where it co-occurs with endemic hosts (*Elaeocarpus* and *Disoxylum* sp.)? Are the patterns of use of *S. mauritianum* similar to those on endemic hosts? How recent is the association between *S. mauritianum* and *B. cacuminata*? Addressing these questions will enable the clarification of the value of the larval host plant as a resource to *B. cacuminata*.

11.3.2. Other resources in the environment and dacine foraging behaviour

In addition to the larval host plant and methyl eugenol, *B. cacuminata* requires other resources for its survival and reproduction. Protein is a vital resource needed by adult flies. While sources of protein such as bacteria, and other nitrogenous sources such as homopteran honeydew and bird faeces have been suggested as being significant to adult dacines (Drew et al. 1983, Courtice and Drew 1984, Bateman 1972, Fletcher 1987), the distribution of these resources in the environment of the fly and their relative importance as a protein source to *B. cacuminata* requires further investigation. To what extent individuals will disperse in their foraging efforts for these resources can be examined by mark-release-recapture studies with a known experimental distribution of these resources. This may shed light on the dispersal behaviour of the adult fly as well.

11.3.3. Mating behaviour of *Bactrocera cacuminata*

One of the key gaps in the knowledge is the lack on information on the specific aspects of the mating system of dacine fruit flies. While general mating patterns such as lekking have been suggested for some dacines (e.g. *B. dorsalis* [Shelly and Kaneshiro 1994]), the different components of the specific-mate recognition system of dacine species (*sensu* Paterson 1993) are far from clear. Further work in this area needs to elucidate the habitat cues that bring the sexes together, elaborate on the functional significance of rectal gland constituents (including methyl eugenol metabolites and spiroacetals), investigate other courtship cues (e.g. auditory signals) and explore other physiological aspects of insemination and fertilization process. Only then can we understand the coadapted signal-response chain between the sexes in *B. cacuminata* that results in successful fertilization.

11.4 THE NEED FOR AN AUTECOLOGICAL APPROACH TO DACINE ECOLOGY

Despite the extensive focus of research on dacine fruit flies on economic significance, few systems have been intensively investigated to unravel functional associations between individuals of a species and components of their habitat with which they interact. In part, this is the result of the approach to dacine ecology. As is the case with many economic insects, emphasis is made on controlling populations of pests below economic thresholds. Therefore, the emphasis in understanding the demographics and associated patterns has precluded the detailed exploration on specific aspects of adult behaviour. Perhaps Elton's general remark of the state of zoology summarizes the situation in dacine ecology best.

"…definition of habitats, or rather the lack of it is one of the chief blind spots in Zoology"

C.S. Elton (1966)

Considering that this remark was made almost four decades ago, it is a stark reminder of the constraints of the demographic approach in understanding the fundamental aspects of biology of the Dacinae.

Difficulties of interpreting the population dynamics of species often relate to the vagueness of non-operational concepts such as habitat or environment (Peters 1991, for dacine examples of see Raghu et al. 2000, Raghu and Clarke 2001). Identification of specific components of an organism's environment that serve as key resources for its survival and reproduction is therefore critical to understanding patterns seen at the population level. This task is the domain of the autecological approach.

Key functional questions that can be addressed by adopting an autecological approach to the Dacinae include,

1. In the natural environment of the fly, what resources are critical for the survival of the different life history stages and the reproduction of the species?
2. What is the availability of these resources (i.e. the nature of the spatial and temporal distribution of these resources in relation to dacine life history) and how does this vary between modified (e.g. orchard) environments versus natural environments?
3. What are the patterns of resource use by the different fly life history stages, between the different resources?
4. Are patterns of resource use and associated physiological consequences, species-specific or are there any general patterns across species?

In this thesis I asked some of these questions in the context of a non-pest species. Asking these questions in economically significant species such as *B. tryoni* or *B. dorsalis* could help us in their management. Identification of resources for adult flies may help enhance the efficacy of sterile male release programs, for example, by ensuring that that their resource requirements are adequately met. Such research may also help in identifying if the dacine species in question has a resource based mating strategy (e.g. at methyl eugenol in *B. cacuminata*). If so, then release of sterile males at this resource could provide significantly greater success than the ad hoc release of males into the environment.

Development of a female lure is a key priority in dacine research. Investigation of sex-specific resource requirements may shed light on this. The response of mature virgin *B. cacuminata* females to methyl eugenol is an exciting observation as it indicates that the possibility of developing female attractants from this group of plant derived chemicals. Successful

development of such attractants could significantly minimise fruit fly damage by capturing females before they become gravid.

Hence it is evident that adopting such a functional/ autecological approach would not only facilitate a clearer understanding of dacine ecology and evolution, but also provide the vital knowledge to help achieve successful management of the few pest dacines.

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Appendix

PUBLICATIONS COMMUNICATED/ PUBLISHED DURING PH.D. CANDIDACY

A. Publications related to the Ph.D. thesis

Peer-reviewed articles

1. **Raghu**, S., Hulsman, K. Clarke, A.R. and Drew, R. A. I. 2000. A rapid method of estimating abundant fruit fly species (Diptera: Tephritidae) in modified Steiner traps. *Australian Journal of Entomology* 39, 15–19.♣
2. **Raghu**, S., Clarke, A.R. Drew, R. A. I. and Hulsman, K. 2000. Impact of habitat modification on the distribution and abundance of fruit flies (Diptera: Tephritidae) in south-east Queensland. *Population Ecology* 42, 153–160.♣
3. **Raghu**, S. and Clarke, A.R. 2001. Distribution and abundance of *Bactrocera bryoniae* (Tryon) in three different habitat-types in South-eastern Queensland, Australia. *International Journal of Ecology and Environmental Sciences* 27, 179–183. ♣
4. **Raghu**, S., Clarke, A.R. and Bradley, J. 2002. Microbial mediation of fruit fly – host plant interaction. Is the host plant the "centre of activity"? *Oikos* 97, 319–328.
5. **Raghu**, S., Clarke, A.R. and Yuval, B. 2002. Investigation of physiological consequences of feeding on methyl eugenol by *Bactrocera cacuminata* (Diptera: Tephritidae). *Environmental Entomology* 31, 941–946.
6. Drew, R.A.I. and **Raghu**, S. 2002. The fruit fly fauna (Diptera: Tephritidae: Dacinae) of the rainforest habitat of the Western Ghats, India. *Raffles Bulletin of Zoology* 50, 327–352.
7. **Raghu**, S. and Lawson, A.E. Feeding behaviour of *Bactrocera cacuminata* (Hering) on methyl eugenol: a laboratory assay. *Australian Journal of Entomology* (in press).

♣ These papers stem from a previously examined M.Sc. (Environmental Management) thesis.

8. **Raghu**, S. and Clarke, A.R. Sexual selection in a tropical fruit fly: role of a plant derived chemical in mate choice. *Entomologia Experimentalis et Applicata* (in press).
9. **Raghu**, S., Halcoop, P. and Drew, R.A.I. Apodeme and ovarian development as predictors of physiological status in *Bactrocera cacuminata* (Hering) (Diptera: Tephritidae). *Australian Journal of Entomology* (in press).
10. **Raghu**, S. and Clarke, A.R. Spatial and temporal partitioning of behaviour between resources by adult dacine: Direct evidence for methyl eugenol as a mate rendezvous site. *Physiological Entomology* (in press).

Publications in review

11. **Raghu**, S. and Clarke, A.R. Influence of microclimate and structural attributes of the host plant on the abundance and behaviour of a tephritid fly. *Journal of Insect Behaviour* (in review).
12. **Raghu**, S., Yuval, B. and Clarke, A.R. Physiological and energetic status of *Bactrocera cacuminata* at different resources: Evidence for spatial and temporal partitioning of behaviour by adult flies? *Physiological Entomology* (in review).
13. Putulan, D., Clarke, A.R., **Raghu**, S., Sar, S. and Drew, R.A.I. Fruit and vegetable movement on domestic flights in Papua New Guinea and the risk of spreading fruit flies (Diptera: Tephritidae). *Plant Protection Quarterly* (in review).

Conference Publications *(Abstracts of presented papers)*

1. **Raghu**, S. 1999. Suburbia as an optimal habitat for pest fruit fly species: implications for quarantine and pest management. 30th AGM and Scientific Conference of the Australian Entomological Society, Canberra, Australia, September 28 to October 2, 1999, pp. 26.
2. **Raghu**, S., Clarke, A.R., Drew, R.A.I. and Huslman, K. 2000. Impact of habitat modification on the distribution and abundance of fruit flies in S.E. Queensland. ESA 2000 Ecological Society of Australia Inc. Annual Conference, La Trobe University, Melbourne, Australia, November 29 to December 1, 2000, pp. 77.
3. **Raghu**, S. 2002. Sexual selection is a tropical fruit fly: role of a plant-derived chemical in mate choice. Fifth International Congress of Dipterology, University of Queensland, Brisbane, Australia, September 29 to October 4, 2002, pp. 201.

B. Other publications

1. Raman, A., **Raghu**, S. and Sreenath, S. 2000. Integrating environment, education, and employment for a sustainable society: an HRD agenda for developing countries. *Current Science* 78, 101–107.
2. **Raghu**, S. 2000. Book Review – Ecological Entomology. 2nd ed. C. B. Huffaker and A. P. Gutierrez. John Wiley & Sons, New York. 1999. *Australian Journal of Entomology* 39, 49–50.
3. **Raghu**, S. 2000. Book Review – Evolutionary Ecology across Three Trophic Levels. Goldenrods, Gallmakers, and Natural Enemies. W.G. Abrahamson and A.E. Weis. Monographs in Population Biology, Volume 29, Princeton University Press, Princeton, New Jersey. 1997. *Australian Journal of Entomology* 39, 95–96.
4. **Raghu**, S. 2000. Book Review – Biology and Behaviour of Phytophagous Arthropods in Synthetic Environments. R. Beiderbeck and A. Raman. Special Issue of the International Journal of Ecology and Environmental Sciences, Volume 25, Issue 3, International Scientific Publications. 1999. *International Journal of Ecology and Environmental Sciences* 26, 83–85.
5. **Raghu**, S. 2000. Where have all the developing country editions gone? *Bulletin of the Ecological Society of America* 81, 106–107.
6. **Raghu**, S. 2000. Insect collection in the tropics: obsessions, myths and realities. *Antenna* 24, 135–140.
7. **Raghu**, S. 2001. Is ecology a profession and is it certifiable? *Bulletin of the Ecological Society of America* 82, 102–103.
8. **Raghu**, S. and Raman, A. 2001. Insect collection in the tropics: There is always more than one side to a story. *Antenna* 25, 43–47.

The Road goes ever on and on
Down from the door where it began.
Now far ahead the Road has gone,
And I must follow, if I can,
Pursuing it with eager feet,
Until it joins some larger way
Where many paths and errands meet.
And whither then? I cannot say.

Frodo Baggins
J.R.R. Tolkien – *Lord of the Rings*