

EVALUATION OF ARTIFICIAL DIETS FOR REARING *ANTHONOMUS TENEBROSUS*
(COLEOPTERA: CURCULIONIDAE), A POTENTIAL BIOLOGICAL CONTROL AGENT
OF TROPICAL SODA APPLE, *SOLANUM VIARUM*

By

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This research is dedicated to my mother Billie Taps and to my significant other, Ed Drevermann.

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Abstract of Thesis Presented to the Graduate School
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The host specificity of many weevils makes them ideal biological control agents of weeds. A flower bud weevil, *Anthonomus tenebrosus* Boheman, is currently being investigated as a biological control agent of tropical soda apple, *Solanum viarum* Dunal, an invasive weed that is spreading throughout much of the southeast United States. An efficient rearing system was developed for *A. tenebrosus*. This system allowed us to rear more than 30 generations in the laboratory with 7 – 8 generations occurring per year. Under these conditions, the egg, larval, pupal, and adult stages of the weevil were completed in 4.2 ± 0.9 , 10.7 ± 1.9 , 5.2 ± 1.2 , and 20.4 ± 8.3 days, respectively. Feeding and oviposition on artificial diets also was evaluated. An artificial diet suitable for adult *A. tenebrosus* was formulated by modifying an existing boll weevil (*A. grandis* Boheman) diet. The influence of diet components on feeding and oviposition of *A. tenebrosus* was investigated. Ten existing *Anthonomus* spp. diets were evaluated for diet presentation (color, shape, and texture), protein content, host plant material, and levels of carbohydrates, lipids, sterols, solasodine, proline, bee pollen, and anti-oxidation agents. The formation of cone shaped diet capsules and the addition of sucrose, host plant material, lipids, and proline elicited marked feeding responses. However, oviposition was and continues to be a

problem on the diet. We also examined whether larvae of *A. tenebrosus* could be reared on the same diet. Unfortunately, larval survivorship on the diet was poor. The adult diet did not provide sufficient nutrients or feeding stimulants for complete larval development. Additional research is needed to improve the production of these weevils on an artificial diet.

CHAPTER 1 REVIEW OF LITERATURE

This literature review will focus on the significance of invasive plant species in Florida and current strategies for invasive plant management. We will concentrate on one invasive species, *Solanum viarum* Dunal (tropical soda apple), and examine the possibility of controlling this pest by the introduction of the flower bud weevil *Anthonomus tenebrosus* Boheman, a potential biological control agent. Finally, the possibility of rearing this beneficial insect on an artificial diet will be considered.

Exotic Invasive Species in Florida

An exotic plant species is one that is native to somewhere else; it is not native to the area in which it has arrived (Frank 2000). In 1997, more than 900 of the over 3500 species of plants in Florida were considered exotic plant species (Schmitz et al. 1997; Ward 1989). By 2002, the Florida Exotic Pest Plant Council reported that more than 1300 exotic species were established and reproducing outside of cultivation in Florida (FLEPPC 2002). Although most of these exotic species apparently have little impact on Florida's ecosystems, some are considered to be highly invasive. Plants that are considered invasive are those that form self-sustaining and expanding populations within plant communities with which they were not previously associated. In their native environment, plants are often fed upon by natural enemies which help to limit their abundance. When plants are introduced into new areas, their natural enemies are generally left behind (Williams 1954). When conditions are favorable, introduced plants not accompanied by their natural enemies may increase and become problematic (Williams 1954; Harley and Forno 1992).

Weeds are those plants existing in places in which they are considered undesirable, or put simply, a plant in the wrong place (Adkins 1997; Monaco et al. 2002; O'Brien 1995; Sweetman 1958). Plants in many taxonomic groups have become weeds in a variety of habitats, including forest, agricultural, rangeland, and native ecosystems (Van Driesche and Bellows 1996). Plants may be regarded as weeds for economic or aesthetic reasons (Williams 1954). Invasive weeds can have a detrimental effect on the environment, reduce biodiversity by displacing native plants and wildlife, and alter natural ecological processes (Langeland 1998; FLEPPC 2007). They are often injurious to the economic scheme devised by humans (Sweetman 1958). Economic losses amount to billions of dollars each year due to invasive species (Hoddle 2002; Pimentel et al. 2005). According to FLEPPC, between the years of 1980 - 2002, more than \$240 million dollars was spent in Florida to control exotic invasive plant species.

Tropical Soda Apple (*Solanum viarum* Dunal)

Tropical soda apple, *Solanum viarum* Dunal, is a stout, woody shrub growing up to 2 meters in height and 1.5 meters in diameter. Leaves are ovate with sharp spines on both surfaces. Flowers are small with white petals and cream-colored anthers. It reaches full maturity and produces fruit 120 to 130 days after germination (Akanda et al. 1996). Flowering and fruit production of tropical soda apple in Florida occurs throughout the year but is concentrated from September to May in South Florida and from April to October in North Florida. Flower production throughout the year results in fruit of different maturities on a plant. Fruits are berries that are green with dark veining when immature and yellow when ripe. Each mature fruit contains 200 to 400 seeds. One tropical soda apple plant can produce over 125 berries with at least 75% seed germination (Mullahey et al. 1993). Although tropical soda apple spreads primarily from seed, it can regenerate new plants from roots. Plants that survive freezing in more

northern locations can resprout from root buds the following spring (Mullahey and Hogue 2003; Patterson et al. 1997).



Figure 1-1. Mature tropical soda apple.

Invasive plants share many characteristics including a wide environmental tolerance in germination and growing conditions; rapid growth to reproductive age; high seed production; seed dormancy; and a growth form suited for successful competition for resources (Gordon and Thomas 1997). Tropical soda apple possesses most of the characteristics exhibited by successful non-native invasive plant species. A perennial weed native to South America, tropical soda apple was first discovered in Glades County, Florida in 1988 (Coile 1993). This invasive weed forms dense monotypic stands by displacing the existing vegetation. Because of its ability to out-compete native vegetation, tropical soda apple has been listed by FLEPPC as a “Category 1” invasive species (FLEPPC 2007). Category 1 species are widespread in Florida and have the potential to invade and disrupt native plant communities (Gordon and Thomas 1997). It also was placed on the Florida Noxious Weed List and on the Federal Noxious Weed List (FDACS 1999; USDA-APHIS-PPQ 1999 - 2006). Soon after its discovery in Florida, tropical soda apple expanded its range into other eastern states, including Alabama, Georgia, Louisiana, Mississippi, North and South Carolina, Tennessee, and Pennsylvania (Medal et al. 1999; 2002a; 2002b; 2003;

USDA Plants Database). Infestations in more northern states have yet to reach the numbers seen in Florida (Medal et al. 1999; Mullahey and Hogue 2003).

One reason that tropical soda apple became an invasive weed is due to the ‘enemy escape hypothesis’, or lack of natural enemies to regulate its population growth (Williams 1954). While a few native arthropods and pathogens will attack this weed, those that do are generalists that use other resources in the environment, and thus will do little to control tropical soda apple (Cuda et al. 2004; Hoddle 2002). Tropical soda apple’s rapid spread in the southeastern United States can be attributed to its lack of natural enemies, favorable environmental conditions, seed dispersal by cattle and wildlife that feed on the fruits, the movement of contaminated sod or hay, and the plant’s great reproductive potential. Current estimates of tropical soda apple presence in Florida from all land uses is over 405,000 hectares (one million acres), and this invasive weed continues to spread to new areas within Florida (Cuda et al. 2004; Mullahey et al. 2003a). If not controlled, pasture production declines, resulting in lower stocking rates and lower forage quality (Mullahey et al. 2003b). The economic impact in Florida due to tropical soda apple infestations is estimated at \$6.5 to \$16 million annually (Thomas 2007). Tropical soda apple also may serve as host for insect pests such as the silverleaf whitefly (*Bemisia argentifolii* Bellows), Colorado potato beetle (*Leptinotarsa decemlineata* Say), tomato and tobacco hornworms (*Manduca quinquemaculata* Haworth and *M. sexta* Linnaeus), southern green stinkbug (*Nezara viridula* Linnaeus), tobacco budworm (*Heliothis virescens* Fabricius), mirid suckfly (*Tupiocoris notatus* Distant) and flea beetles (*Epitrix* spp.) (Medal et al. 2002b; Mullahey and Hogue 2003; Patterson et al. 1997). An additional negative impact is tropical soda apple’s ability to serve as an alternate host for diseases such as powdery mildew and various tomato, tobacco, and potato viruses that affect

solanaceous vegetable crops (Medal et al. 2002b; Mullahey and Hogue 2003; Patterson et al. 1997).

Based on the environmental variables most likely to affect the growth, reproductive development, and range expansion of tropical soda apple, Patterson et al. (1997) determined that it was unlikely that naturally occurring temperature and photoperiods in the United States would significantly limit the ability of tropical soda apple to grow or reproduce. Thus, the potential range of tropical soda apple is even greater than its current distribution. Growth projections indicate that the range of tropical soda apple can be expected to continue to increase throughout the southern United States. Akanda et al. (1996) reported seed germination over a wide range of soil pH, osmotic potential, and temperature, suggesting that seedlings could become established under a broad range of environmental conditions. For these reasons, actions should be taken as soon as possible to contain current infestations and limit further spread of this noxious weed (Patterson et al. 1997).

Integrated Pest Management

Humans have used various combinations of control techniques known as Integrated Pest Management (IPM) in attempts to suppress weed populations (Flint and van den Bosch 1977; Watson and Wymore 1989; Hoddle 2002). IPM is a sustainable approach to managing pests by combining biological, cultural, mechanical, chemical, and regulatory control methods in a way that maximizes beneficial plant health and minimizes pest outbreaks (Sweetman 1958). Actions are taken to control pest problems only when numbers exceed what is deemed to be an acceptable level. IPM practitioners strive to respond to pest problems with the most effective and lowest risk options available. A good weed control tactic should be safe for the environment, easy to use, cost-effective, and provide effective control (Watson and Wymore 1989).

The current suggested method for controlling tropical soda apple is by a combination of mowing and herbicides (Mullahey et al. 2003a; 2003b). Unfortunately, herbicide applications combined with mechanical practices often provide only temporary weed suppression for tropical soda apple at a cost estimated at up to US \$187.50 per ha to control dense infestations (Mullahey et al. 1996). In addition, mowing and chemical application are not always possible because of a lack of accessibility to infested areas (Medal et al. 1999). Despite aggressive control programs in Florida, tropical soda apple has become well established in the state and complete eradication of tropical soda apple is unlikely because of its high degree of tolerance to commonly used herbicides and its prolific seed production (Akanda et al. 1996). Not only are chemical or physical controls likely to be temporary and expensive, they require a continuous investment of human labor (Center et al. 1997).

Biological Control

The inability of chemical, physical, or mechanical methods to provide long-term control of tropical soda apple led to the investigation of potential biocontrol agents. Biological control is defined as the suppression of animal or plant pests by introduction and/or manipulation of natural enemies (O'Brien 1995). The aim of biological control programs is not to eradicate exotic weeds from invaded areas, but to regulate them at lower densities (Julien 1997; Monaco et al. 2002; Schooler et al. 2004; Piper 2004). The biological control agent suppresses the weed population, making it less abundant and thus less damaging than it would otherwise be (Hoddle 2002; Van Driesche and Bellows 1996).

Insects are considered to be a prime factor regulating the abundance of plants, and thus can be highly successful biological control agents. (Harley and Forno 1992). Biological control with specialist insect natural enemies can provide a long-term sustainable control strategy and offers

several advantages over other control methods (Hoddle 2002; Frank et al. 1997). Beneficial effects of biological control include lower weed numbers and damage, reductions in environmental contamination from overuse of pesticides including phytotoxicity and pesticide resistance, increased productivity of agricultural or recreational areas, and cost savings otherwise spent on pesticides, labor, and specialized equipment. In addition, the numbers and diversity of desirable species of plants increase once the weed is brought under control (Hoddle 2002; Van Driesche and Bellows 1996).

Sweetman (1958) stated: “The risk taken in introducing a biocontrol agent to attack a given noxious weed is closely related to the degree of specialization of the insect and to the botanical position of the weed in relation to economic plants”. The genus of tropical soda apple, *Solanum*, contains 33 species native to the United States, 27 of which are native to the Southeast. The family (Solanaceae) includes pepper (*Capsicum* spp.), tobacco (*Nicotiana* spp.), tomato (*Solanum lycopersicum* Linnaeus), eggplant (*Solanum melongena* Linnaeus), potato (*Solanum tuberosum* Linnaeus) and others. The great majority of species of plant-feeding insects are more or less restricted in their diets (Brown and Hyman 1995; Monaco et al. 2002; Sweetman 1958). Any biological control agent introduced to control tropical soda apple must be highly specific (Center et al. 1997). Once an insect is determined to be host specific, there is little likelihood of the insect evolving to feed on other plants (Brown and Hyman 1995; Monaco et al. 2002). The attributes of an effective natural enemy include a high searching capacity or ability to find the host at low densities; host specificity in its feeding; a high potential rate of increase including a short development time and high fecundity; and the ability to survive in all areas occupied by the host (Doutt and Debach 1964). These characteristics mean that the natural enemy is well adapted biologically, physiologically, and ecologically to the host (Doutt and DeBach 1964).

In May 2003, *Gratiana boliviana* Spaeth, a leaf-feeding beetle from South America, was the first insect released in Florida for biological control of tropical soda apple (Medal et al. 2003). In climates that are relatively lacking in abiotic stresses for plants, such as extreme cold and hot periods, a series of introduced herbivores may be needed to place enough cumulative stress on plants to reduce plant survival (O'Brien 1995; Van Driesche and Bellows 1996). The release of *G. boliviana* in Florida followed by the introduction of other potential biocontrol agents that significantly damage different plant parts (flowers, seeds, and stems) may reduce the competitive ability of tropical soda apple (Medal et al. 2002a).

Insect Rearing

One desirable characteristic of a biological control agent is the ability to be laboratory reared and ultimately, mass-produced. Insects have been reared for the benefit of humans for thousands of years. Silk technology and sericulture in China date back 4500 – 7000 years (Singh and Moore 1985). In the 1950s, the rearing of insects in the laboratory increased dramatically following the development of a successful phytophagous insect artificial diet by Beck et al. (1949). By the mid-1970s, most entomological research utilized laboratory reared insects (Dickerson and Leppla 1992).

The ability to produce large numbers of high quality natural enemies is necessary for the widespread adoption of a biological control program (Waage et al. 1985). The goal of a mass-rearing program is to produce the maximum number of insects as rapidly and inexpensively as possible without affecting their quality (Shimoji and Miyatake 2002). Successful rearing of these insects depends on sound knowledge of insect biology, behavior, and nutrition (Singh 1984). An understanding of the mating habits, oviposition periods, fecundity, longevity, sex ratio, environmental requirements, and feeding preferences of the insect is necessary in developing

rearing techniques (Singh 1984). Laboratory reared insects are now commonly used for research on insect ecology, behavior and physiology, screening pesticides, host plant resistance, sterile insect technique, etc.

***Anthonomus tenebrosus* Boheman**

The genus *Anthonomus* belongs to the family Curculionidae – the weevils. In general, weevils have a public image as being harmful, but those that attack weeds should be regarded as beneficial (Sweetman 1958; Frank et al. 1997). Weevils have been among the more successful of the biological control agents of weeds (O'Brien 1995). They are a very diverse group of insects that can be found in most habitats and geographical regions. They are the second most abundant phytophagous group, after the Cicadellidae (Homoptera) (Brown and Hyman 1995). Most weevils are specialists, feeding on one genus or species of plant (Brown and Hyman 1995; O'Brien 1995). Seven species of weevils have been released in Florida as biological control agents of weeds; none of these releases have been proven to harm any native species (Frank and McCoy 2007). Thirty-two species of *Anthonomus* weevils are known to be associated with plants of the family Solanaceae (Clark and Burke 1996). Host specificity studies with the flower bud weevil *Anthonomus tenebrosus* Boheman show it to be a promising candidate for biological control of tropical soda apple (Medal and Cuda 2001). The weevil's positive attributes include high fecundity, longevity, mobility, and a short overlapping generation time that facilitates increases in population numbers.

Little information is available in the scientific literature regarding *A. tenebrosus*. The native range of this weevil includes Argentina, Bolivia, Brazil, Paraguay, and Uruguay (Clark and Burke 1996; Cuda et al. 2004). *Anthonomus tenebrosus* is a small black weevil approximately 2 mm in length that feeds on tropical soda apple flowers and on the apical leaflets

of plants in times of flower shortage. Females deposit their eggs in the anthers of unopened flower buds. Developing larvae consume the contents of the flower bud. Attacked flower buds remain closed and eventually abort early, and thus do not set fruit. The biology of *A. tenebrosus* is expected to be similar to other *Anthonomus* species, the insect developing from an egg stage, to the larval stage consisting of three instars, a pupa or resting stage, and finally the adult stage.

An *A. tenebrosus* colony was established in April 2002 at the Division of Plant Industry quarantine facility in Gainesville, Florida. The colony has been maintained and supplemented with additional insects to increase both colony size and genetic diversity. Rearing procedures have evolved throughout this time period. A laboratory rearing method was developed for *A. tenebrosus*. This involved research on its basic biology, as well as investigating its ability to be reared on an artificial diet, in order to assess its potential as a biological control agent of tropical soda apple.

Artificial Diets

One of the barriers to the widespread implementation of biological control agents is the lack of suitable artificial diets for mass rearing of insects. An artificial diet is any diet that is not the natural food of the insect. Singh (1977) defined an artificial diet as “an unfamiliar food which has been formulated, synthesized, processed, and/or concocted by man, on which an insect in captivity can develop through all or part of its life cycle”. Bogdanov (1908) was the first to rear an insect entirely on an artificial diet. The insect was *Calliphora vomitoria* Linnaeus (blowflies) and the diet was a medium of peptone, meat extract, starch, and minerals (Singh 1977). The rearing of plant-feeding insects began with the artificial diet work of Beck et al. (1949) and many of the first diets for other insects were based on the diet of the European corn borer (*Ostrinia nubilalis* Hübner) (Vanderzant 1974).

The ultimate goal in diet development is to formulate an artificial food source that is both economical and nutritionally adequate for producing large numbers of healthy insects that are comparable to those feeding on their natural food plants (Adeyeye and Blum 1988; Brewer 1976). The development of an effective artificial diet requires a thorough understanding of the insect's biology, physiology, and behavior. The insect must be attracted to the diet and the diet must supply all the nutrients needed for normal growth and reproduction. Complex chemical cues often are found in the natural food source that stimulates food searching behavior and acceptance. Hence, in addition to defined nutrients, host plant extracts often are included in the artificial diet to supply a nutrient or a chemical feeding stimulant to encourage the desired host specificity of the insect (Blossey et al. 2000; Vanderzant 1974).

Artificial diets now are widely used for studying nutrition, testing compounds for physiological effects, maintaining insect colonies, and mass-producing insects for year round availability (Vanderzant 1974). Artificial diets have been used to rear multiple generations of insects and their formulations have been compiled by House (1967), House et al. (1971), and Singh and Moore (1985). Several artificial diets and rearing protocols are published for *Anthonomus* species (Earle et al. 1966; Gast and Davich 1966; Moore et al. 1967; Toba et al. 1969; Singh 1977; Sterling and Adkisson 1966; Vanderzant et al. 1958; 1959; 1961; 1963a; 1963b; 1963c; 1965; 1967; 1973). The study of existing literature on related *Anthonomus* species provides a starting point, as diets originally designed for rearing one insect species often can be modified for rearing other species (Adeyeye and Blum 1988). Considerable effort has gone into rearing *A. tenebrosus* adults on artificial diet because of the biological control potential of these insects.

Research Goals

The purpose of this research was to investigate the life history and laboratory rearing of a potential biocontrol agent, *A. tenebrosus*. Because an effective artificial diet is needed to produce healthy biological control agents, existing *Anthonomus* spp. diets were evaluated for rearing *A. tenebrosus* and the preferred artificial diet was modified to improve feeding.

CHAPTER 2
BIOLOGY AND REARING OF *ANTHONOMUS TENEBROSUS*, A POTENTIAL
BIOLOGICAL CONTROL AGENT FOR TROPICAL SODA APPLE, *SOLANUM VIARUM*

Introduction

Tropical soda apple (*Solanum viarum* Dunal) is a weed indigenous to South America (Medal et al. 2000; Nee 1991). It was first reported in Glades County, Florida in 1988 (Coile 1993), although its method of entry is unknown. Tropical soda apple has continued to spread throughout much of the eastern United States, including Alabama, Georgia, Louisiana, Mississippi, North and South Carolina, Tennessee, and Pennsylvania (Medal et al. 1999; 2002a; 2002b; 2003; USDA Plants Database), where it often forms monotypic stands that disrupt native plant communities and displace existing vegetation. This invasive weed also may serve as host to various insect pests and act as an alternative host for viruses that affect important solanaceous vegetable crops (Medal et al. 2002b; Mullahey and Hogue 2003; Patterson et al 1997).

The rapid spread of this plant in the United States can be partially attributed to its great reproductive potential and a lack of natural enemies. Control methods thus far have been mostly mechanical and chemical. Mowing and herbicides may control this weed in managed areas, but these methods are not feasible for large infestations or for infestations in natural areas. Because of its invasive qualities and limited control options, tropical soda apple has been targeted for biological control. Surveys of insects associated with tropical soda apple in South America were conducted in 1994 and 1996 (Medal et al. 1996; 2000). *Anthonomus tenebrosus* Boheman (Curculionidae), a flower bud weevil, was among the insect species collected. Based on its narrow field host range and good dispersal capability, *A. tenebrosus* was determined to be a potentially important natural enemy of tropical soda apple.

Anthonomus tenebrosus is a promising candidate for biological control of tropical soda apple (Medal and Cuda 2001). Native to Argentina, Bolivia, Brazil, Paraguay, and Uruguay

(Clark and Burke 1996), this small black weevil feeds on tropical soda apple flowers, flower buds, and on the apical leaflets of plants during times of flower shortage. Females deposit their eggs in the anthers of unopened flower buds. Developing larvae consume the contents of the flower bud. Attacked flower buds remain closed and eventually abort early. Similar to the situation with *A. grandis* Boheman (boll weevil), abscission of infested flower buds is thought to be linked to an unidentified protein released from 2nd and 3rd instars (Coakley et al. 1969; King and Lane 1969). This abscission of flower buds prevents flowering and eventual fruit set, limiting the reproductive ability of the plant.

A high degree of specificity and an ability to cause significant decline in fruit production indicate that *A. tenebrosus* might be a suitable biological control agent on tropical soda apple. However, except for a few taxonomic papers, little has been published in the scientific literature about *A. tenebrosus* and information on its biology is lacking. Our objectives were to develop a laboratory rearing procedure for *A. tenebrosus* and to investigate its basic biology.

Materials and Methods

Insects

The laboratory culture originated from adults collected in Buenos Aires, Argentina, South America. Insects were collected by collaborators at the USDA-ARS South American Biological Control Laboratory in Buenos Aires, Argentina and sent to the Division of Plant Industry, Florida Biological Control Laboratory in Gainesville, Florida. Insects were shipped immediately after collecting in insulated containers containing tropical soda apple cuttings.

The identification of *A. tenebrosus* was confirmed by Drs. Wayne Clark (Auburn University, AL) and Germano Neto (Universidade Federal do Paraná, Curitiba, Brazil). Voucher specimens were deposited at FDACS-DPI-Florida State Collection of Arthropods in Gainesville,

Florida. All rearing and developmental studies were conducted in quarantine at the Division of Plant Industry, Florida Biological Control Laboratory. The biology of *A. tenebrosus* was expected to be similar to other *Anthonomus* spp. such as the pepper weevil (*A. eugennii* Cano), apple flower bud weevil (*A. pomorum* Linnaeus), and boll weevil (*A. grandis* Boheman), which develop from eggs, undergo three instars, pupa, and eclose as an adult.



Figure 2-1. *Anthonomus tenebrosus* adult. Photo credit: Stephan McJonathan.

Tropical Soda Apple Production

Plant propagation began with the use of seeds obtained from mature tropical soda apple fruit collected in the field. Seeds were removed from mature fruit and allowed to dry on a flat surface for a week. Seeds were sown in a 27.94 cm by 53.34 cm by 7.62 cm (W:L:D) size seedling tray containing a 1:1 mixture of soil (Fafard Professional Formula 3B-Mix, BWI, Apopka, FL and Garden Plus Top Soil, Greenleaf Products, Inc., Haines City, FL) on benches placed outside under semi-shade conditions. Seedlings, which were watered every other day, were allowed to grow for 6 – 8 weeks before being transferred into 1 gallon pots containing the same 1:1 soil mixture previously described, as well as a sprinkling of time released fertilizer (Sta-Green Fruit, Vegetable and Flowering Slow Release Plant Food®). During summer months, 1 gallon pots were housed on benches within 182.88 cm by 365.76 cm by 365.76 cm (H:W:L)

screened cages with 32 X 32 mesh screening (Synthetic Industries, Gainesville, GA) that provided partial shading and reduced pest insect activity. During winter months, plants were moved into greenhouse space with artificial lighting (combination of Sylvania Fluorescent and Gro-Lux 40-watt bulbs for broad spectrum coverage). All 1 gallon pots were watered daily. Miracle-Gro Bloom Booster Flower Food® was applied every 2 weeks to increase flower production. A 1:1 ratio of Miracle-Gro Water Soluble All Purpose Plant Food® and Miracle-Gro Water Soluble Tomato Plant Food® was applied monthly. Sta-Green Fruit, Vegetable and Flowering Slow Release Plant Food® capsules were applied once every 3 months. From January to March, greenhouse plants were sprayed every 7 – 9 days with Benlate for control of powdery mildew. Malathion was sprayed as needed when populations of mirid suckfly (*Tupiocoris notatus* Distant) became large enough to damage flowers. Thuricide HPC® was sprayed approximately three times a year during July and August to control hornworms (*Manduca* spp.). New seedlings were planted every year and older plants destroyed after the new generation of plants was ready for use. These methods allowed for a continuous supply of vigorous plants.

Rearing Method

Rearing of *A. tenebrosus* began in April 2002. Weevils were raised in containers (Pioneer Packaging®, Dixon, KY) rather than on whole plants in order to conform to space limitations, to maximize the number of flowers exposed to the weevils, and to facilitate harvesting of the insects. The *A. tenebrosus* colony was maintained in round containers 25.24 cm in diameter by 9.04 cm high, with four 7 cm diameter screened vents along the sides of the container to allow for air exchange. Each container had a Viva® (Kleenex Brand) paper towel cut to fit snugly in the bottom of the container. A piece of egg carton (Bio-Serv®, Frenchtown, NJ) was cut to the same dimensions inside the container and sat on the paper towel. A varying number of 5.08 cm

to 7.62 cm length cuttings with apical leaves, flowers, and flower buds were cut from mature plants for feeding and oviposition by the weevils. The flower cuttings were placed in 29.57 ml (1 oz) creamer cups containing water and fitted with lids to prolong life of cuttings while preventing insects from drowning. The cuttings were inserted through a hole made in the middle of the lid with a leather hole punch (cups and lids from Fill-Rite, Newark, NJ). The cups containing tropical soda apple cuttings were placed inside the egg carton indentations. An 11.4 cm by 10.6 cm piece of Kimwipes® paper was placed in between the egg carton and each cup to allow for a snug fit. During times of flower shortage, 21 tropical soda apple cups were set up per container. When flowers were abundant, 12 tropical soda apple cups were set up along the edge of the container and loose cuttings, flowers, and flower buds were placed in the center of the tray on the paper towel. Regardless of the number of cups used, loose flowers were sprinkled inside the container along the edge to facilitate finding of flowers by weevils immediately after changing trays. Additional cuttings and loose flowers and flower buds were added along the edges and to the center of the containers every other day when available. Containers were housed in a Forma Scientific (Marietta, OH) growth chamber at a temperature of $21.7^{\circ} \pm 2^{\circ} \text{C}$, 53 - 56% RH, and a photoperiod of 16:8 (L:D). There were two 15-watt 18" GE Plant & Aquarium bulbs overhead and two additional bulbs attached under a shelf centrally within the growth chamber.



Figure 2-2. Tropical soda apple cup and tray set-up.

Each container held from 30 - 150 adult weevils (averaged 75 weevils) and was housed in a growth chamber for 7 days. The weevils were allowed to feed and oviposit on flowers and flower buds. Flower buds exposed to ovipositing adults were routinely dissected to determine development time of the different life stages of *A. tenebrosus*. After 7 days, the adults were removed from the container and placed in a new container with fresh tropical soda apple cuttings. Leaves were removed from the old cuttings and the cuttings, along with all loose buds and flowers, were placed inside the original container on a paper towel. This plant material was misted lightly with water 4 - 5 days a week to keep buds fresh and allow the immature stages to complete their development. High humidity was maintained to prevent bud drying. The old bud trays were supplied with a florist pick containing fresh plant material, providing a food source for emerging adults. Old bud trays were examined 5 days a week for emerging adults. New individuals were removed from the tray with a small sable haired brush and placed with the rest of the colony. Emergence from each old bud tray was recorded for later determination of developmental rates.

Results and Discussion

By dissecting tropical soda apple flower buds that had been exposed to adults, we were able to observe complete development of *A. tenebrosus*. The majority of observations occurred during summer months when flower buds were abundant.

Table 2-1. Duration of life stages of *A. tenebrosus* under laboratory conditions.

Stage	n	Range	Mean Number of Days (mean \pm SD)
Egg	30	3 – 5	4.2 \pm 0.9
Larva	30	7 - 13	10.7 \pm 1.9
Pupa	31	3 - 7	5.2 \pm 1.2
Egg to Adult	1050	11 - 69	20.4 \pm 8.3

The life cycle of *A. tenebrosus* begins when the female deposits an egg in a tropical soda apple flower bud. These oblong/oval off-white eggs are inserted into small feeding depressions that are chewed into the anthers by the adult female. Incubation of the egg takes 4.2 \pm 0.9 days (Table 2-1). Larvae are cream colored with a yellowish brown head capsule. They feed on the contents of the flower bud, with all development occurring inside the flower bud. This larval feeding prevents the flower bud from opening. There is usually one larva per flower bud, but occasionally two larvae can be found in a single flower bud. As larval feeding progresses, the flower bud detaches from the plant. The three larval instars are completed in 10.7 \pm 1.9 days (Table 2-1). Pupae resemble the adult in form. They are cream colored but darken shortly before emergence as an adult. The pupal stage is completed in 5.2 \pm 1.2 days inside the flower bud (Table 2-1). Emerging adults chew their way out of the spent flower bud. Development from egg to adult stage is completed in 20.4 \pm 8.3 days (Table 2-1). Longer development times are not

associated with seasonal differences as they occurred at all times of the year. Adults can live up to 7 months (210 days) under laboratory conditions.

We were able to determine the sex of adult *A. tenebrosus* without dissection of the genitalia. The female is often larger than the male in many insects but this character proved not to be accurate for *A. tenebrosus*. Size appears to be related to food abundance during development as opposed to differences due to sex. Males and females can be distinguished by separating the elytra and examining the last two abdominal tergites, which is similar to procedures for *A. pomorum* and *A. grandis* (Duan et al. 1999; Agee 1964). The last abdominal tergite of the male is about one-third the size of the last abdominal plate of the female. Approximately 50 dead weevils were sexed with this method with 100% accuracy, as verified by dissection. The use of carbon dioxide to anesthetize the weevils may allow for sexing of live weevils without causing injury.

More than 30 generations of *A. tenebrosus* have been reared in the laboratory since April 2002 with 7 - 8 generations occurring per year. The ability to produce large numbers of high quality natural enemies is necessary for the widespread adoption of a biological control program (Waage et al. 1985). Successful rearing of these insects depends on sound knowledge of insect biology, behavior, and nutrition (Singh 1984). The rearing methods described here for *A. tenebrosus* are for small scale laboratory rearing. We have made refinements in the rearing techniques throughout this study and expect these refinements to continue. *Anthonomus tenebrosus* were easily reared on tropical soda apple cuttings, completing development in about 20 days. Adults were long-lived with many surviving 7 months. They have many of the traits needed in a biological control agent including a short generation time, a high reproductive rate,

and being long-lived. *Anthonomus tenebrosus* appears to possess the qualities needed for a successful biological control agent of tropical soda apple.

CHAPTER 3
EVALUATION OF *ANTHONOMUS* SPP. ARTIFICIAL DIETS FOR ADULT FEEDING AND
OVIPOSITION

Introduction

Artificial diets often are used for mass-rearing insects. The first insect to be reared from egg to adult on an artificial diet was *Calliphora vomitoria* L. (Diptera: Calliphoridae), which was reared on a diet of meat extract, starch, peptone and mineral salts (Bogdanow 1908 [as cited in Singh and Moore 1985]). The rearing of plant-feeding insects on artificial diets began with the work of Beck et al. (1949), who developed a diet for the European cornborer (*Ostrinia nubilalis* Hübner) (Lepidoptera: Pyralidae) (Vanderzant 1974). This diet formed the basis for many subsequent phytophagous insect diets (Reinecke 1985). Since the success of *C. vomitoria* and the European cornborer, numerous species of insects have been reared on artificial diets for a variety of reasons including nutritional studies, testing compounds for physiological effects, maintaining colonies, and mass-producing insects for release programs (Vanderzant 1974).

Insects generally have the same basic nutritional needs as large animals (Dadd 1985). A nutritionally complete diet must contain a protein source that supplies most if not all of the ten essential amino acids, carbohydrate, fatty acids, cholesterol, various vitamins and minerals, and water (Vanderzant 1974). A successful artificial diet for insects is one that is nutritionally adequate to produce healthy insects that are comparable to those feeding on their natural food plants. Several insects have been mass-reared successfully on artificial diets and used for various insect-control programs. In the mid-1960s the boll weevil, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), was mass-produced on artificial diet by Gast and Davich (1966). Since that time, more than 28 species of Curculionidae have been reared successfully on artificial diets (Singh and Moore 1985).

Anthonomus tenebrosus Boheman feeds on the tender shoots, buds, and flowers of tropical soda apple (*Solanum viarum* Dunal). The flower buds also are used as oviposition sites. However, for rearing the insects, flowers would not be economical as oviposition sites. In order to produce large quantities of high quality weevils for effective weed biological control, it would be beneficial to develop an artificial diet. Our objective was to determine the most suitable artificial diet for rearing *A. tenebrosus* by investigating existing *Anthonomus* spp. diets and making modifications to achieve optimum feeding and oviposition.

The present work was carried out as part of an investigation of *A. tenebrosus*. A method of rearing the adult insects on artificial diet was desired in order to pursue the possibility of mass rearing the insects for a biological release program against tropical soda apple. In September of 2004, experiments were initiated to assess the suitability of artificial diets for feeding and reproduction of *A. tenebrosus* adults.

Materials and Methods

Insects

All diet studies were conducted from September 2004 to October 2006. The insects used in this study were obtained from a laboratory colony of *A. tenebrosus* maintained in quarantine at the Division of Plant Industry, Florida Biological Control Laboratory, Gainesville, Florida. Only actively moving weevils that were observed within the colony containers were selected for the diet development studies. This procedure helped to avoid using weak or old individuals.

Artificial diets

R. F. Moore (1985) provided a systematic approach to follow for selecting an initial artificial diet and its subsequent modifications to obtain optimal feeding and oviposition.

Existing *Anthonomus* spp. diet formulations were obtained from various sources (House 1967; House et al. 1971).

Diet ingredients were obtained from MP Biomedicals®, Bio-Serv®, and Fisher® Scientific. The compositions of the diets are listed in Table 3-1. Preparation of the diets was simplified by the incorporation of commercially prepared vitamin and salt mixtures. The agar was dissolved in distilled water by heating and allowed to boil for 1 minute. The agar was allowed to cool to 75° C before adding the pre-mixed dry ingredients. Microbial growth was inhibited by the addition of a 10g potassium sorbate and 100ml 95% ethyl alcohol mixture at 1.25ml/100g of diet. The pre-made inhibitor was added and all the ingredients were mixed thoroughly. After mixing, the diet was dispensed into 48 count well trays (Costar®, Fisher Scientific) or cone shaped molds within a laminar flow hood and housed in a sealed plastic container (Pioneer Packaging®, Dixon, KY), where they were allowed to gel before capping. Trays were wrapped in aluminum foil and were stored in a refrigerator until needed. All diet was used within 1 week from time of preparation.

The host plant material (tropical soda apple apical leaves, flowers, and flower buds) incorporated into the diets was prepared by lyophilization (freeze-drying) to stop enzyme reactions and the formation of toxic products. The freeze-dried plant material was ground to a fine powder in a coffee grinder (Mr. Coffee®, Sunbeam Products, Inc.). The powder was sifted through a 40-mesh strainer, and the strained powder was stored in airtight vials at room temperature and kept in a dark area until needed.

Antimicrobial inhibitors are added to artificial diets to suppress the growth of mold, yeast, and bacteria on the surface of the diet. In the absence of an inhibitor, artificial diets quickly became moldy. By using an inhibitor, we were able to slow the development of mold and finish

the 3-day testing without replacing the diet capsules with fresh ones. The antimicrobial agents, methyl-p-hydroxybenzoate and potassium sorbate, have been tested and proven safe for use with the boll weevil, *A. grandis* (Hedin et al. 1974). In earlier tests, a methyl-p-hydroxybenzoate, sorbic acid, and acetone mixture was used as an antifungal agent. During later tests, this was replaced with a potassium sorbate and ethyl alcohol mixture. The inhibitor was relatively effective in controlling contamination and was deemed non-toxic to *A. tenebrosus* in the amounts used here. Although rarely done, the diets could be altered to pH 5 to achieve a greater reduction in microbial contamination and prolong the lifespan of the diet capsules (Cohen 2004).

Initiation of Diet Experiments

Unsexed adults were collected from the existing colony. Adults were transferred with a sable haired brush to the same round containers 25.24 cm in diameter by 9.04 cm high, with four 7 cm diameter screened vents along the sides of the container to allow for air exchange (Pioneer Packaging®, Dixon, KY). In this case, each container had an opaque lid so that the weevils were encouraged to feed rather than to migrate toward the light. Approximately 30 weevils were placed in each container. Each container also had a Viva® paper towel cut to fit snugly in the bottom of the container. A piece of plastic carpet protector (Surface Source Carpet Protector, LG Sourcing, Inc., North Wilkesboro, N.C.) was placed upside down in each container with the spikes pointing upward. Diet capsules were impelled on the spikes to prevent the capsules from rolling around in the containers. The containers were held at a temperature of $25^{\circ} \pm 3^{\circ}$ C, 61 – 75% RH and kept on a 16:8 (L:D) photophase.

Adults were observed daily and records were maintained for feeding, oviposition, number of eggs laid, diet contamination, and weevil mortality. The diets were evaluated on the basis of the number of feeding occurrences. We defined a feeding occurrence as any time a weevil was

seen on or beside a diet capsule with its rostrum touching the diet. A minimum of 10 observations were taken at least 2 hours apart over a 3-day period.

Starvation tests

Our preliminary observations showed that in starvation tests, newly emerged adults could live an average of 6 days without food or water. If they were given water, they could live an average of 9.5 days. If adults were allowed to feed for 1 week and then starved, they could live an average of 12.3 days. If adults were allowed to feed for 1 week but thereafter given only water, they lived an average of 26.1 days. From these observations, we concluded that diet tests could run for 3 days without weevils dying from starvation.

Statistics

The data were analyzed as a completely randomized design, using the General Linear Model procedure (SAS, PROC GLM, 2004). If the factorial effects were found to be significant through ANOVA, then these data were subjected to LSD multiple comparison procedures at the 5% significance level.

Table 3-1. Composition of artificial diets investigated for laboratory rearing of *A. tenebrosus*.
 The 10 diets tested were: A) Earle et al. 1966 adult, B) Vanderzant and Davich 1958, C) Moore et al. 1967 adult, D) Vanderzant et al. 1959 (diet b), E) Vanderzant 1967, F) Sterling and Adkisson 1966, G) Gast and Davich 1966 larval, H) Vanderzant et al. 1959 (wheat germ diet), I) Earle et al. 1966 larval, and J) Toba et al. 1969 larval.

Ingredients (grams)	Earle et al. 1966 adult	Vanderzant and Davich 1958 both	Moore et al. 1967 adult	Vanderzant et al. 1959 (diet b) adult	Vanderzant 1967 both	Sterling and Adkisson 1966 both	Gast and Davich 1966 larval	Vanderzant et al. 1959 (wheat germ) adult	Earle et al. 1966 larval	Toba et al. 1969 larval
wheat germ		2			3			4		3.56
casein				3	3.5			2		4.15
sugar/sucrose	4	5	5	2	3.5	1.7	2.33	1	2	4.15
salt	0.67	1	0.75	0.75	1		0.33	0.75	0.67	1.19
choline chloride	0.05	0.1		0.1	0.1		0.08	0.1	0.1	1.19
cholesterol	0.05	0.05	0.05	0.05	0.05	0.01	0.1	0.05	0.05	
agar	1.5	2.6	3	2	2.5	1.3	3.5	2	3	2.96
water	80	79	85	100	85	71	117	100	80	103
vitamins	0.01	1	2	1	1	2.6	1.3	1	0.01	0.4
TSA (1:1 fl/leaves)	2.73	3.06	3.15	3.28	3	2.48	3.87	3.33	2.67	3.71
egg albumen			3			0.8				
Alphacel			4			1.3				0.6
brewer's yeast		1				1.3	1.17			
oils/lipids		0.5	1	0.25		1.3				
inositol	0.02				0.04				0.02	
ascorbic acid	0.2		0.1	0.05	0.4				0.1	0.5
alfalfa meal										1.78
Aureomycin										0.02
powdered cellulose		4								
soybean protein	2.5	5					3.5		1	
l-cysteine hydrochloride		0.05								
glycine		0.1								

* Lipids - 1:1 ratio of linseed oil and vegetable oil

** Inhibitor mixture – 10g potassium sorbate and 100ml 95% ethyl alcohol

Results

Existing *Anthonomus* Artificial Diets

Most plant-feeding insects have similar gross nutritional requirements since the basic chemical composition of their tissues and their metabolic processes are similar (Chapman 1998). For this reason, diets originally designed for rearing one insect species often can be modified for rearing other species with some degree of success. With this in mind, we exposed 10 existing *Anthonomus* spp. diets to *A. tenebrosus* to observe feeding and ovipositional behavior. The diets included an arbitrary 3g of tropical soda apple flowers and apical leaves. Figure 3-2 illustrates the feeding observations obtained.

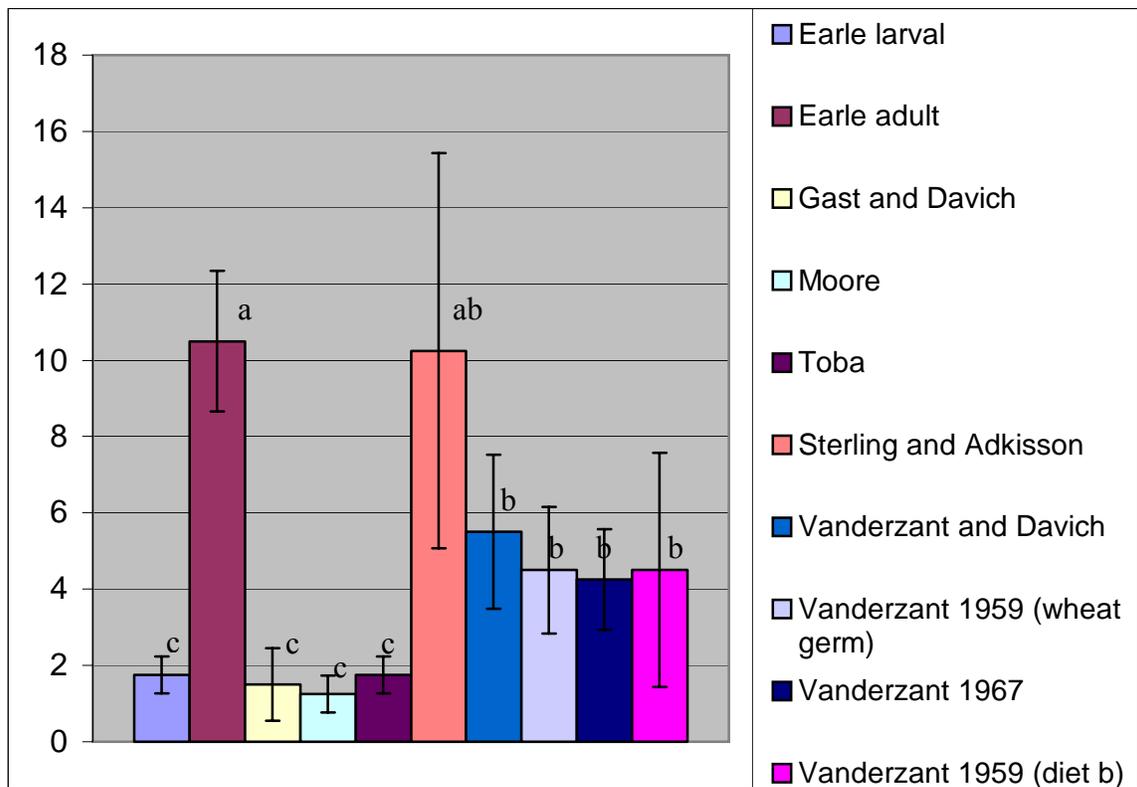


Figure 3-2. Existing *Anthonomus* spp. diets with 3g tropical soda apple flowers and apical leaves (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

Statistical differences were observed amongst some of the diet treatments. The Earle adult was the preferred diet with 23.08% of the feeding occurrences, followed by the Sterling diet with 22.53% of the feeding occurrences. The Moore diet received the least feeding occurrences (2.75%). Testing of the 10 existing *Anthonomus* spp. diets was repeated without the addition of tropical soda apple. Figure 3-3 depicts the results of this second test.

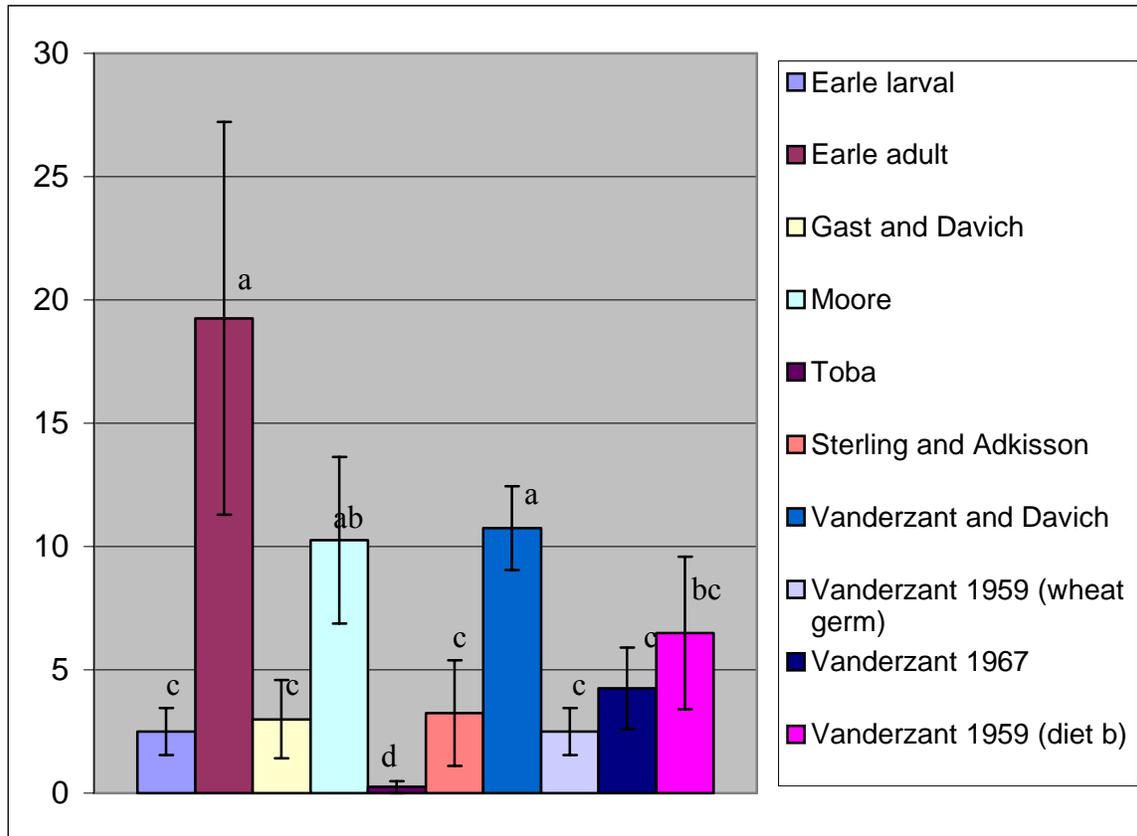


Figure 3-3. Existing *Anthonomus* spp. diets without tropical soda apple (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

Statistical differences were observed between some of the treatments. The Earle adult diet was the preferred diet with 30.80% of the feeding occurrences. The Toba diet received the fewest feeding occurrences (0.40%).

The Earle adult diet was the preferred diet in tests both with and without tropical soda apple in the diet. The protein source in the Earle adult diet is soybean. Soybean protein flour is rich in all 10 essential amino acids (Cohen 2004), the same essential amino acids that Vanderzant (1973) determined *A. grandis* required for continuous egg formation throughout the adult life. Ultimately, a large-scale mass rearing program for the boll weevil was accomplished using a soybean protein diet (Vanderzant and Davich 1958; 1961; Gast and Davich 1966). Thus, the choice of a soybean protein diet is appropriate for *A. tenebrosus*. Additionally, the pH of the Earle adult diet is approximately 6.0. This pH is similar to the pH range of sap expressed from fresh tropical soda apple leaves and stems. The Earle adult diet was selected as the baseline control diet for all further diet modification comparisons.

Diet Presentation (color and shape)

Plants provide adult insects with a variety of resources such as food, mating encounter sites, ovipositional sites, and shelter. The importance of visual cues in host selection/detection behavior has been reported in many phytophagous insects (Beck 1965; Prokopy and Owens 1983). Visual perception of intensity and color contrasts, shape, and pattern disruption may all be important to detection of plant structures (Prokopy and Owens 1983; Thorsteinson 1960). Particular physical conditions, including texture, may be required to elicit feeding behavior (Cohen 2004; Reinecke 1985). If one is attempting to improve an artificial diet, then it stands to reason that diet presentation, shape, texture, and color, should be examined.

Color plays an important part in recognition and acceptance of foods by insects (Bernays and Chapman 1994; Cohen 2004). Insects search from a distance for their host plant primarily on the basis of hue and intensity of reflection (Prokopy and Owens 1983). While color does not

account for the host specificity of an insect, it does exert an influence on the early stages of orientation (Beck 1965).

The majority of research dealing with color vision in insects has been on those insects deemed to be pests of agriculture. For example, Zehnder and Speese (1987) demonstrated that Colorado potato beetle (*Leptinotarsa decemlineata* Say) adults, common pests of potato and other solanaceous plants, are capable of spectral discrimination. They are visually attracted to a specific light wavelength that is reflected from the foliage of their host plant.

Unlike foods for human consumption, enhancement of artificial diet acceptance by use of coloring additives is relatively untested. We postulated that *A. tenebrosus* would find artificial diet capsules more quickly, possibly increasing feeding and/or oviposition events, if the artificial diet was the color of tropical soda apple foliage. The Earle adult artificial diet is a light beige color, similar to the color of the anthers of tropical soda apple flowers. To mimic green foliage, green food color was added to the artificial diet. Both a control (beige) and a green diet were exposed to the weevils. The 2 treatments were: A) beige diet capsules and B) green diet capsules.



Figure 3-4. Testing of green and beige diet capsules.

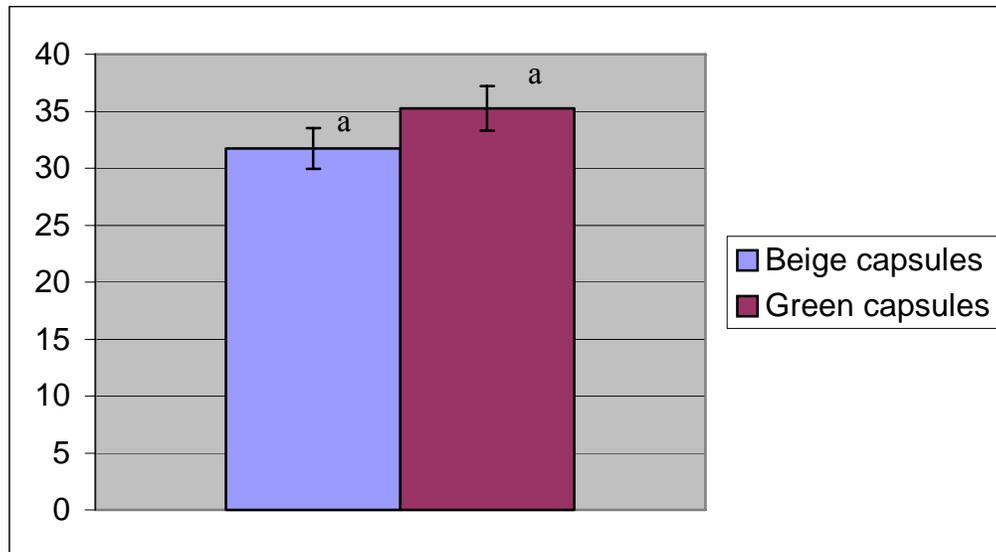


Figure 3-5. Diet presentation: color testing (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

As seen in Figure 3-5, there was no statistical difference between the two diet treatments. 47.39% of all feeding occurrences were on beige capsules and 52.61% were on green capsules. Prokopy and Owens (1983) stated that insects search from a distance for specific hues known to be that of their host plant. Under testing situations, the weevils were in close proximity to the diet capsules, as testing occurred in relatively small containers, which may explain why the ‘long distance’ search behavior described by Prokopy and Owens (1983) was not detected.

When locating host plants from a distance, insects search for a specific hue or spectral reflectance associated with their host plant. Once the insect is on the host foliage, they locate flower buds for feeding and oviposition sites primarily through bud shape and size (Prokopy and Owens 1983). Prokopy and Owens (1983) noted that, “Insects appear to respond to objects on the basis of the object matching a specific predetermined template of stimulus perception, and they seem to ignore stimuli that do not conform.”

Vanderzant and Davich (1961) devised tests to determine if diet shape contributed to the adult boll weevil's selection of diet. Based on their observations of feeding and eggs laid, they determined that the boll weevil preferred curved rather than flat surfaced diets. Weevils fed and oviposited as well on the cylindrically shaped diet capsules as they did on cotton buds. Eggs were laid on these diet capsules even in the presence of cotton squares. Shape of the artificial diet was deemed very important in obtaining eggs from the boll weevil.



Figure 3-6. *Anthonomus tenebrosus* on tropical soda apple flowers.

Anthonomus tenebrosus feeds almost exclusively on the flowers and flower buds of tropical soda apple. We hypothesized that if given a choice, the weevils will search for flowers of characteristic host shape in order to feed. Preliminary tests using empty gelatin capsules (size 00) (Eli Lilly®, Indianapolis, IN) to which diet was added were unsuccessful, as the diet would melt the plastic capsules. In further attempts of mimicking the form and size of the tropical soda apple flower buds, Costar® 96, 48, and 24 count well culture plates (Fisher® Scientific) were used as molds, creating cylinder shaped diet capsules, and the diets were exposed to the weevils. The larger diet capsules (24 count well plates) did not resemble the shape of flower buds. The

smallest capsules (96 count well plates) were closest to actual tropical soda apple flower size, but tended to dry out within 18-24 hours. Additional trials dipping the diet capsules in paraffin or wrapping the capsules in Parafilm® proved unsuccessful, as the weevils did not respond to the diet inside. Diet capsules were made using the 48 count well plates as molds until additional attempts to more closely mimic flower buds were made.

Using a 12.7mm glass and tile bit, we drilled 24 holes in a piece of ultra high molecular polyethylene plastic (101.6 mm by 177.8 mm by 25.40 mm [WxLxH]). Because of the unique shape of the glass and tile bit, the holes were cone shaped, similar to the host plant flowers. We ran tests to determine the extent to which the 48-count well culture plate or cone shape diet capsules were attractive to *A. tenebrosus*. The 2 treatments were: A) well shaped capsules and B) cone shaped capsules.



Figure 3-7. Well shaped and cone shaped diet capsules.

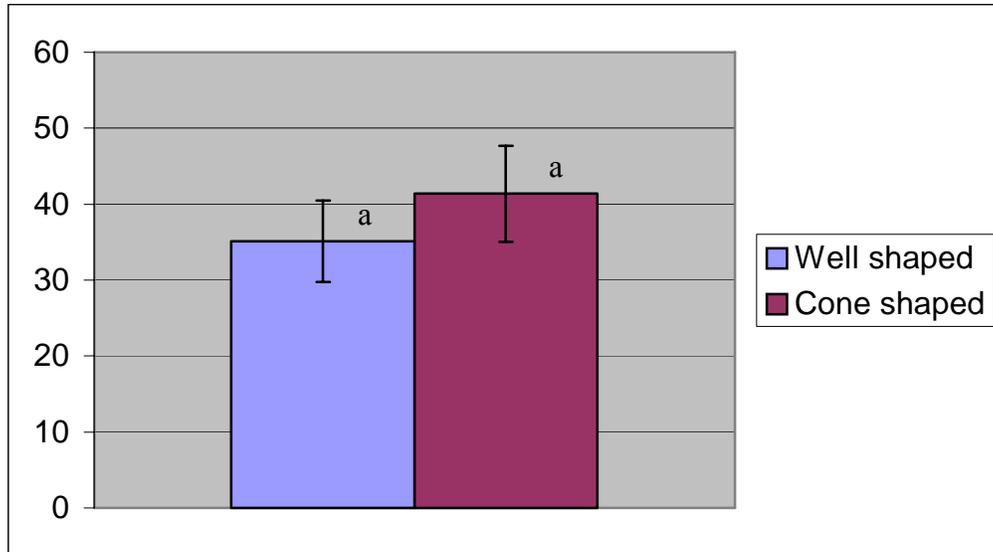


Figure 3-8. Diet presentation: capsule shape – test 1 (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

There was no statistical difference observed between the treatments. The cone shaped diet capsules received 54.08% of the feeding occurrences, while the well shaped diet capsules received 45.92%. Testing was replicated to confirm the previous results.

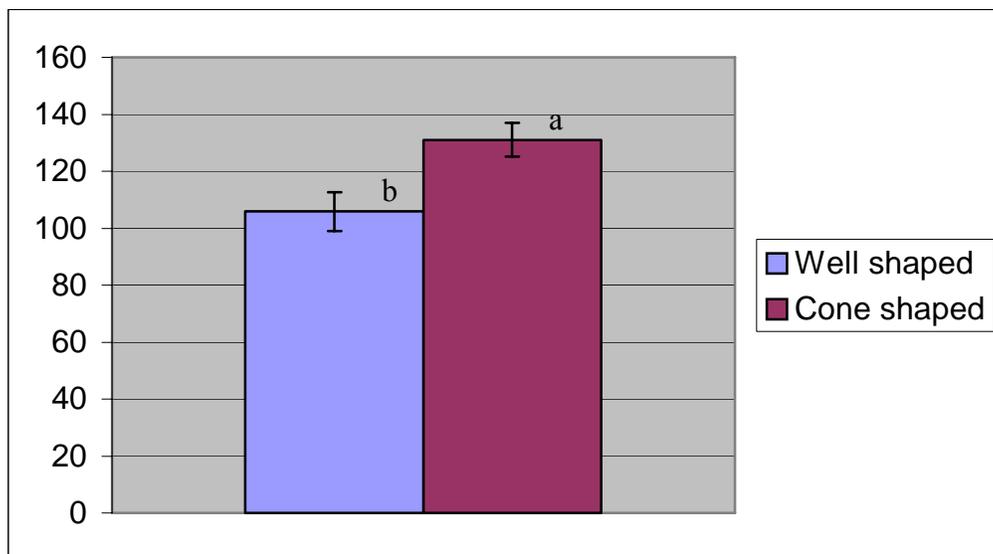


Figure 3-9. Diet presentation: capsule shape – test 2 (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

There was a statistically significant difference between the two diet treatments. The cone shaped capsules received 55.30% of the feeding occurrences, while the well shaped capsules received 44.70%. Cone shaped diet capsules were deemed the most attractive equivalent of tropical soda apple flower buds. No eggs were laid on the diet capsules during either capsule shape test.

Gelling Agents

The physical properties of an artificial diet, or texture, may determine acceptability by an insect. One must begin by devising a diet that possesses an acceptable texture that satisfies the insects' natural instinct to chew. A major problem in devising diets for phytophagous insects is to prepare a diet that is solid yet has high water content (Vanderzant 1974). Gelling agents render a high water content mixture into a solid and prevent settling of the diet components (Cohen 2004). Additionally, they are a way of altering shape, texture, and firmness of the diet, which may ultimately determine acceptability by the feeding insect (Cohen 2004; Lindig 1984; Reinecke 1985). Texture and firmness are particularly important to weevils that use their mouthparts to excavate ovipositional chambers in their hosts (Reinecke 1985).

When an artificial diet must be moist yet firm enough to support the feeding insect, gels such as agar, gums, and carrageenan are frequently used. A number of gelling agents were tested in varying concentrations in order to determine the best suited for *A. tenebrosus* artificial diet rearing. The 4 treatments were: A) diet made with 0.6g agar, B) diet made with 1.3g agar, C) diet made with 2.0g agar, and D) diet made with 1g carrageenan.

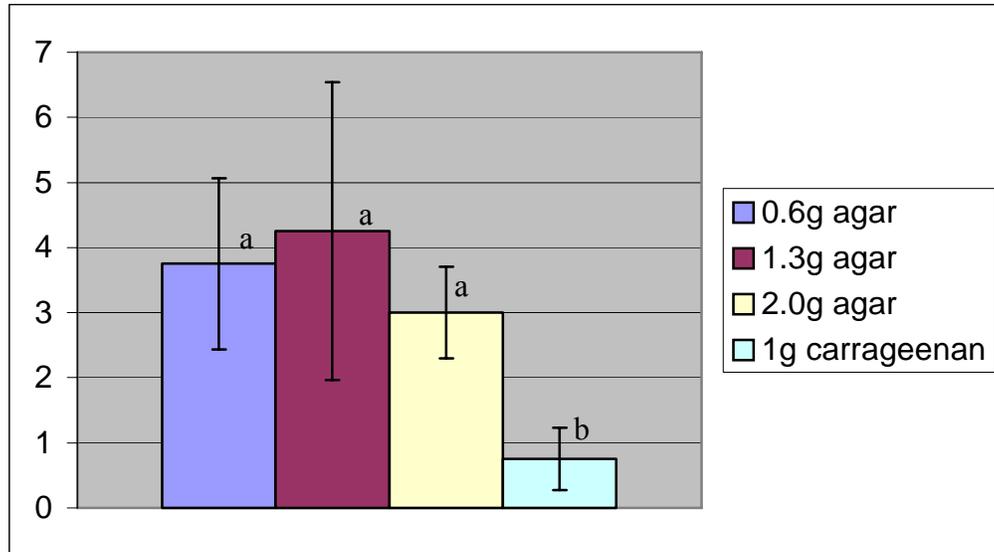


Figure 3-10. Gelling agents – test 1 (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

Although statistical differences were detected amongst some of the diet treatments, testing was aborted due to an unusually high number of weevil deaths. The physical properties of the 1g carrageenan diet were not favorable for feeding. The 1g carrageenan diet was a soft gel-like consistency that would not solidify into capsules. The weevils often became stuck to the diet and died. Testing of gelling agents resumed using different concentrations of both agar and carrageenan. The 4 treatments were: A) diet made with 0.5g agar, B) diet made with 1.5g agar, C) diet made with 2.5g agar, and D) diet made with 2.5g carrageenan.

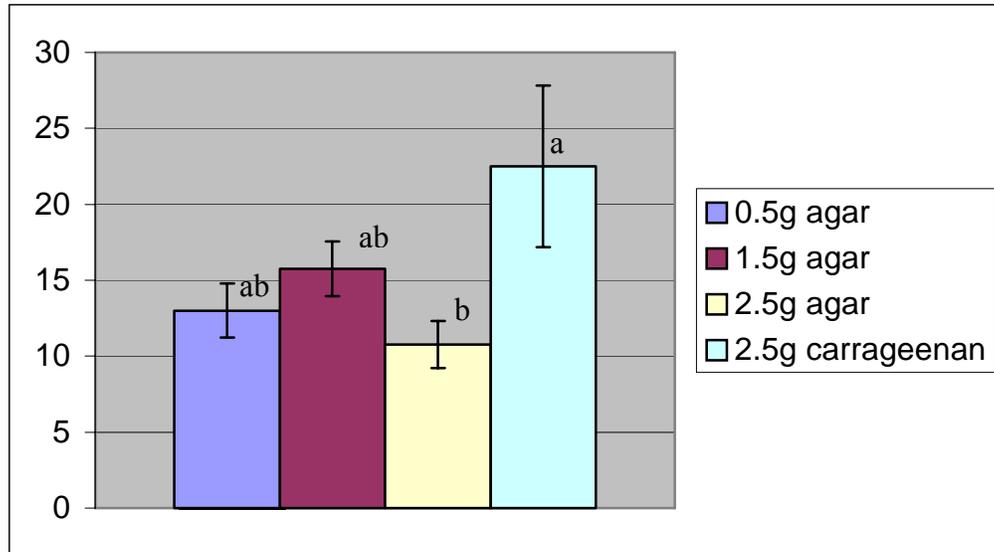


Figure 3-11. Gelling agents – test 2 (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

Statistical differences amongst the diet treatments were observed. The 2.5g carrageenan diet received the most feeding occurrences (36.29%), while the 2.5g agar received the least (17.33%). To confirm results, additional testing was conducted. The 5 treatments were: A) diet made with 1.5g agar, B) diet made with 2.5g carrageenan, C) diet made with 3.5g carrageenan, D) diet made with 1g/1g carrageenan/locust bean gum, and E) diet made with 2g/1g carrageenan/locust bean gum.

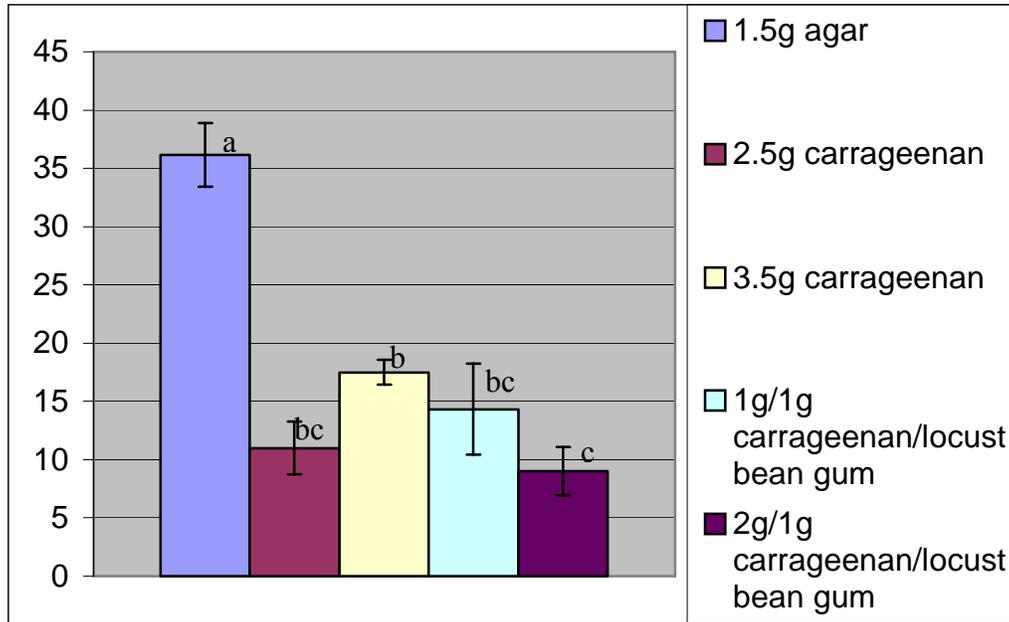


Figure 3-12. Gelling agents – test 3 (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

Statistical differences were observed amongst the diet treatments. The 1.5g agar treatment had the highest number of feeding occurrences (41.09%) and was determined to be the best gelling agent. The combination of carrageenan and locust bean gum proved to be unsuccessful due to syneresis (water seeping from the diet), which can potentially lead to drowning of the insects. The weevils had difficulty climbing up the carrageenan and locust bean gum capsules because of this water seepage. Because these results conflicted with the previous gelling agent test, a final test was performed. Tropical soda apple (TSA) was included in some of the diet treatments. The 6 treatments were: A) diet made with 1.5g agar and 3g TSA, B) diet made with 2.5g carrageenan and 3g TSA, C) diet made with 3.5g carrageenan and 3g TSA, D) diet made with 1.5g agar, E) diet made with 2.5g carrageenan, and F) diet made with 3.5g carrageenan.

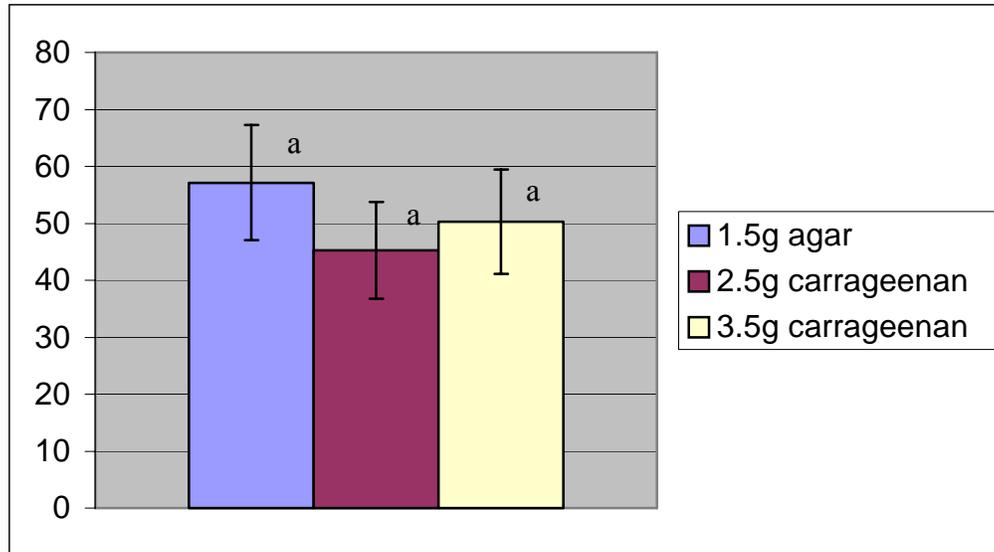


Figure 3-13. Gelling agents – test 4 (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

There were no statistical differences observed between the treatments. The 1.5g agar treatment received 37.43% of the feeding occurrences; the 2.5g carrageenan treatment received 29.65%; and the 3.5g carrageenan treatment received 32.93%. The 1.5g agar treatment had the highest number of feeding observations. The original Earle diet contained agar; agar remained the gelling agent of choice.

Protein

Insects acquire essential amino acids from ingested proteins. Thus, protein is extremely vital and makes up a significant portion of the diet (Gast and Davich 1966). Proteins may even serve as phagostimulants for some insects (Nation 2002). The protein source in the Earle adult diet is soybean. Soybean flour has a high protein, lipid, and vitamin content. It is rich in all the essential amino acids: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Cohen 2004; Gast and Davich 1966). Although most

commonly used in diets for Lepidoptera, soybean flour has also been used successfully for many coleopteran diets (Gast and Davich 1966).

Most insects probably have an optimal level of protein required in the diet for best growth and reproduction, but this varies widely depending on the species, the age, and the sex of the insect (Chapman 1998; Nation 2002). As adults, females have a higher need than males for dietary protein to mature their ovaries and eggs (Chapman 1998; Nation 2002). To ascertain the optimal level of protein for *A. tenebrosus*, we increased the protein in the Earle adult diet to a maximum of 15% to establish if there was sufficient protein for optimal adult feeding. Figure 3-14 presents the results obtained with diets in which the protein was increased. The 7 treatments were: A) diet with normal 4.4% protein, B) diet with 5% protein, C) diet with 7% protein, D) diet with 9% protein, E) diet with 11% protein, F) diet with 13% protein, and G) diet with 15% protein.

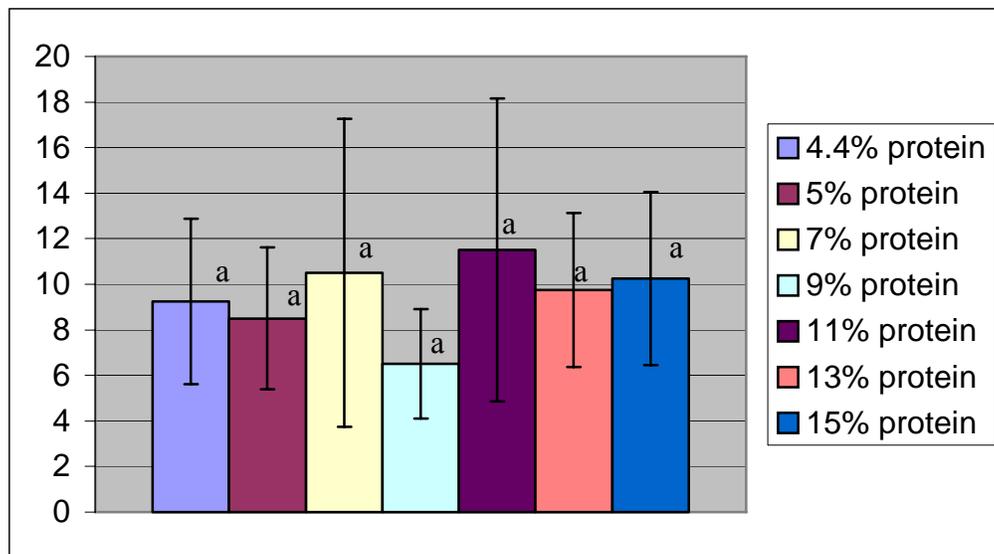


Figure 3-14. Protein testing (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

There were no statistical differences between diet treatments. The 11% protein diet received 17.36% of the feeding occurrences, while the original 4.4% protein diet received 13.96%. We concluded that additional protein did not influence feeding of *A. tenebrosus* adults. Many insects are known to select a diet that approximates an optimal balance of the major components (Chapman 1998). We suspect that the weevils ingested sufficient levels of protein, simply by eating more of the diet.

There are a number of protein sources that can be used in insect diets. In addition to soybean, casein, wheat germ, and egg albumin also are used as sources of protein in artificial diets. Although all three proteins were found in at least one of the 10 *Anthonomus* diets originally tested, it would be of interest to know the extent to which additional types of protein in the Earle adult diet influence feeding.

Host Plant Material

The complex interactions between phytophagous insects and their hosts are the results of a long and continuing evolutionary process (Beck 1965; Prokopy and Owens 1983). Host specificity of phytophagous insects is often mediated by host-produced odors, chemical attractants, and feeding stimulants (Beck 1965; Dorskotch et al. 1970; Maxwell et al. 1963b; Thorsteinson 1960). The exact function of these secondary plant substances in plants is largely unknown (Yamamoto and Fraenkel 1960), but many induce insects to feed. These substances are usually present in small amounts and are frequently restricted to a group of related plants; they appear to serve as indicators of appropriate food.

The inclusion of host plant material, as fresh or dried ground whole plants, plant parts, or extracts, reinforces the insect's specialized feeding habits that steer it to certain plant species (Cohen 2004; Reinecke 1985). It may be impossible to get insects to feed on what would

otherwise be a suitable diet without the presence of token stimuli, including olfactory and gustatory cues stemming from the host plant (Cohen 2004). Vanderzant and Davich (1958) found that adult boll weevils fed readily but did not oviposit on artificial diet unless cotton plant extracts were added. Oviposition of the tobacco hornworm (*Manduca sexta* Linnaeus) did not occur on artificial diets in the absence of host plants (Yamamoto and Fraenkel 1960). Yamamoto and Fraenkel (1960) speculated that for oviposition and normal feeding, olfactory and gustatory stimuli common only to solanaceous plants were involved.

Solanaceous plants are widely known for their toxic qualities derived from a diverse suite of alkaloids, glycosides, and steroids (Brown, Jr. 1987; Schreiber 1968; Ripperger and Schreiber 1981; Ripperger 1998). Earlier studies have shown that incorporating fresh plant material that may break down and form additional toxic products may be inappropriate. For example, Vanderzant and Davich (1961) found that fresh cotton plant parts added to artificial diet made it repellent to the boll weevil. Thus, tropical soda apple young leaves, flowers, and flower buds were freeze-dried before incorporating into the diets. To investigate the effect of host plant material on feeding and oviposition of *A. tenebrosus*, 3g of flowers and flower buds, or 3g of leaves were added to the Earle adult diet. The 3 treatments were: A) control diet with no host plant material, B) diet with 3g TSA leaves, and C) diet with 3g TSA flowers/flower buds.

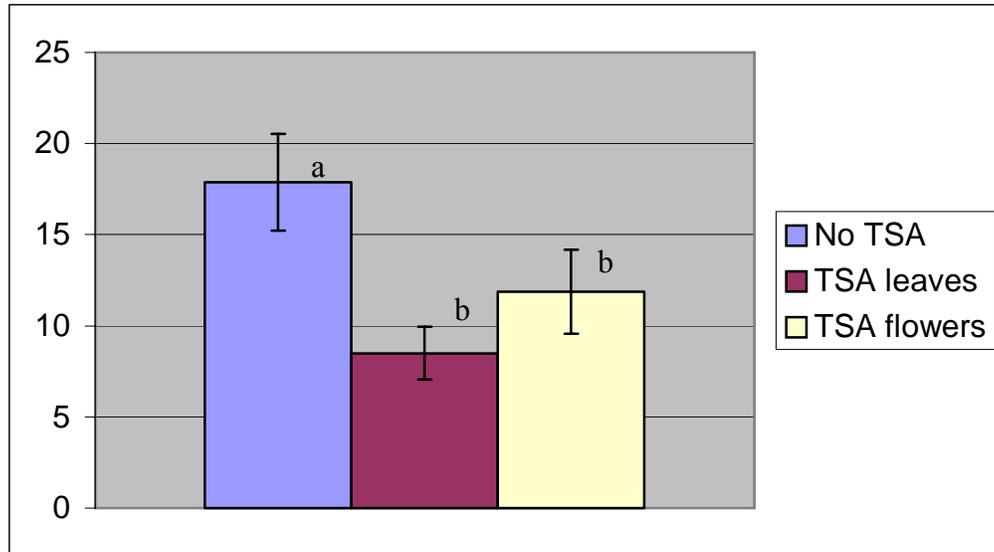


Figure 3-15. Host plant material – test 1 (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

Statistical differences were detected. The diet treatment containing no host plant material received 46.73% of the feeding occurrences; the 3g leaves treatment received 22.22%, and the 3g flowers/flower buds treatment received 31.05%. Adding 3g of host plant material (per 100g diet) to the diet treatments made them less acceptable. The slight acceptance of the diets with 3g of tropical soda apple may have been due to feeding deterrents. Repellents may be present in the freeze-dried tropical soda apple along with nutritive and nonnutritive feeding stimulants. Maxwell et al. (1963a; 1963b) noted that feeding and oviposition of the boll weevil were reduced when leaf powder in excess of 1g per 100g diet was used. The cotton plant has both attractants and repellants to the boll weevil (Maxwell et al. 1963a; 1963b) and these latter compounds may have been responsible for the reduced feeding. Since a compound can show a positive effect on feeding at one concentration but be inhibitory at another (Reinecke 1985), additional tests were run varying the host plant material amounts. The 7 treatments were: A) control diet with no host plant material, B) diet with 0.2% leaves, C) diet with 0.5% leaves, D) diet with 1.0% leaves, E)

diet with 0.2% flowers/flower buds, F) diet with 0.5% flowers/flower buds, and G) diet with 1.0% flowers.

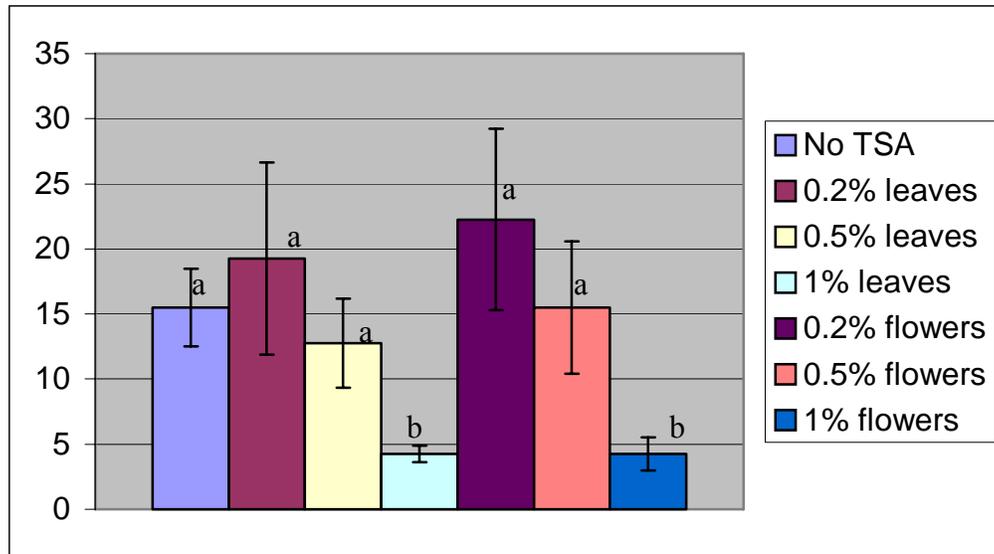


Figure 3-16. Host plant material – test 2 (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

Statistical differences were detected amongst some of the diet treatments. The 0.2% leaves diet (20.53%) and the 0.2% flowers diet (23.73%) received the most feeding occurrences. Both these diets had the least amount of tropical soda apple, yet had the most feeding observations. The 1% leaves and 1% flowers treatments were the least attractive to the weevils, each receiving only 4.53% of the feeding occurrences. The amount of tropical soda apple in the diet was reduced once more and the test was run again. The 7 treatments were: A) diet with 0.05% flowers/flower buds, B) diet with 0.1% flowers/ flower buds, C) diet with 0.2% flowers/ flower buds, D) diet with 0.2% stamens, E) diet with 0.05% leaves, F) diet with 0.1% leaves, and G) diet with 0.2% leaves.

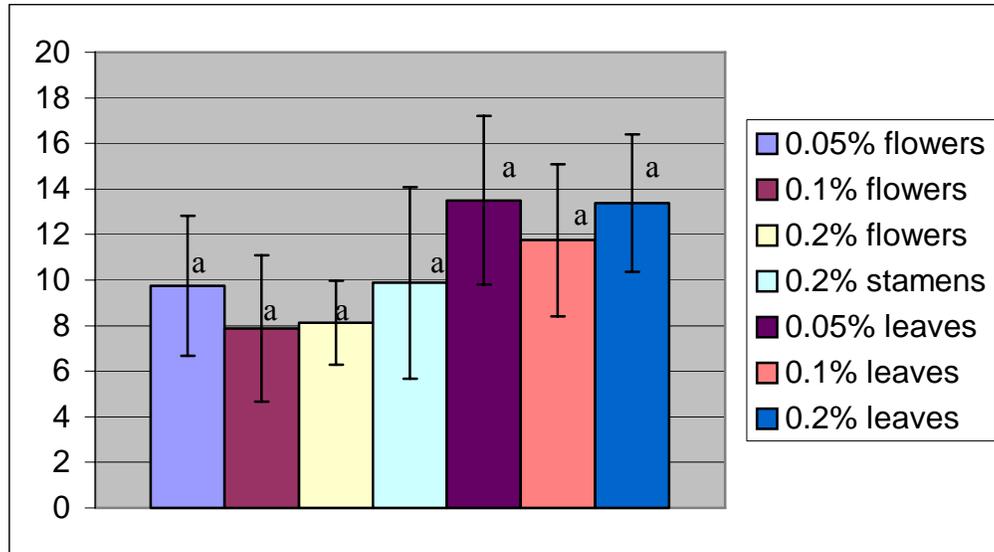


Figure 3-17. Host plant material – test 3 (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

There were no statistical significances between the diet treatments, although more feeding occurrences were seen on the 0.05% leaves diet (13.13%) and 0.2% leaves diet (18.01%). A final test was performed to help clarify the results. The 8 treatments were: A) control diet with no plant material, B) diet with 0.05% flowers/flower buds, C) diet with 0.1% flowers/flower buds, D) diet with 0.2% flowers/flower buds, E) diet with 0.2% stamens, F) diet with 0.05% leaves, G) diet with 0.1% leaves, and H) diet with 0.2% leaves.

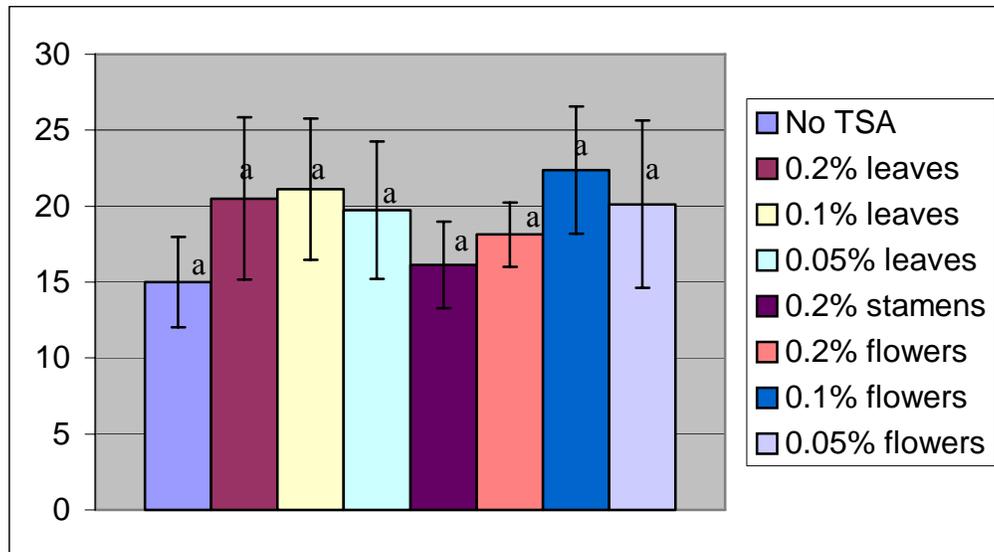


Figure 3-18. Host plant material - test 4 (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

There was no statistical difference among the diet treatments. The 0.1% flowers diet had the most feeding (14.61%), while the no TSA diet had the least amount of feeding (9.80%). Overall, consumption of the artificial diet was increased to some extent by the addition of tropical soda apple leaves and flowers/flower buds. Testing using the same treatments was repeated numerous times with 0.1% - 0.2% leaves and flowers/flower buds alternating as the diet with the most feeding observations. The optimal level of host plant material was determined to be between 0.1% and 0.2%. Henceforth, a 0.1% level of freeze-dried plant material was adopted for all subsequent diets. There was a slight increase in ovipositional behavior on diet capsules with tropical soda apple, but there were no eggs laid

The presence of feeding stimulants and absence of feeding deterrents are important factors for inducing insects to consume artificial diets (Nation 2002). As Dethier et al. (1960) reported, “The same compound may release nonlocomotory reactions at one concentration, true attraction at another, and repellence at another.” Tropical soda apple was both an attractant and a repellent

to *A. tenebrosus*. Larger amounts of tropical soda apple in the diet were repellent to the insects, with smaller amounts being more acceptable and even preferred over diets without host plant material. Previous attempts with tropical soda apple and agar diet capsules proved unsuccessful. The weevils refused to feed on the hard capsules. By experimentally demonstrating the weevils' preference for minimal amounts of tropical soda apple, it seems likely that the larger amount of plant material used in the initial diets tested contained an abundance of chemical deterrents that the weevils simply could not overcome.

The role of plant-borne alkaloids in the resistance of *Solanum* species to insect attack has been well studied. A number of alkaloids exert adverse effects on feeding. Beck (1965) determined that several alkaloids had adverse effects on larval feeding of the Colorado potato beetle (*L. decemlineata*). Analysis of tropical soda apple flowers and leaves for their alkaloid content would be valuable and may shed light on possible deterrents and attractants found in the plant.

Carbohydrates

Carbohydrates are the major energy source for most insects (Nation 2002), and are important components of artificial diets. Insects use carbohydrates as building materials and as fuels (Chapman 1998; Cohen 2004). Although lipids and proteins can be used as alternative energy sources, insects generally survive better when supplied carbohydrates (Nation 2002; Reinecke 1985). A number of carbohydrates are also phagostimulatory including glucose, fructose, and sucrose (Cohen 2004; Nation 2002; Reinecke 1985; Thorsteinson 1960). Once contact with the diet has occurred, the maintenance of feeding often can depend on the presence of one of these feeding stimulants.

All sugars are not interchangeable; sugars that can be used by some insects may not be useable by others (Cohen 2004). The use of different carbohydrates depends on the insect's ability to absorb different compounds and the possession of enzyme systems capable of introducing these substances into the metabolic processes (Chapman 1998).

Sugars, in particular, are known to affect feeding by the adult boll weevil (Vanderzant and Davich 1961). Among sugars tested for the boll weevil, fructose and sucrose produced the best growth, while glucose was less effective (Vanderzant 1965). Lindig (1984) attributed Vanderzant's results to an increased gustatory response induced by sucrose. Haynes and Smith (1992) also found that the longevity of adult boll weevils could be extended by diets supplemented with sucrose. To investigate the carbohydrate preference by *A. tenebrosus*, various sugars were added to the Earle adult diet. The 3 treatments were: A) diet made with 4.4% sucrose, B) diet made with 4.4% fructose, and C) diet made with 4.4% glucose.

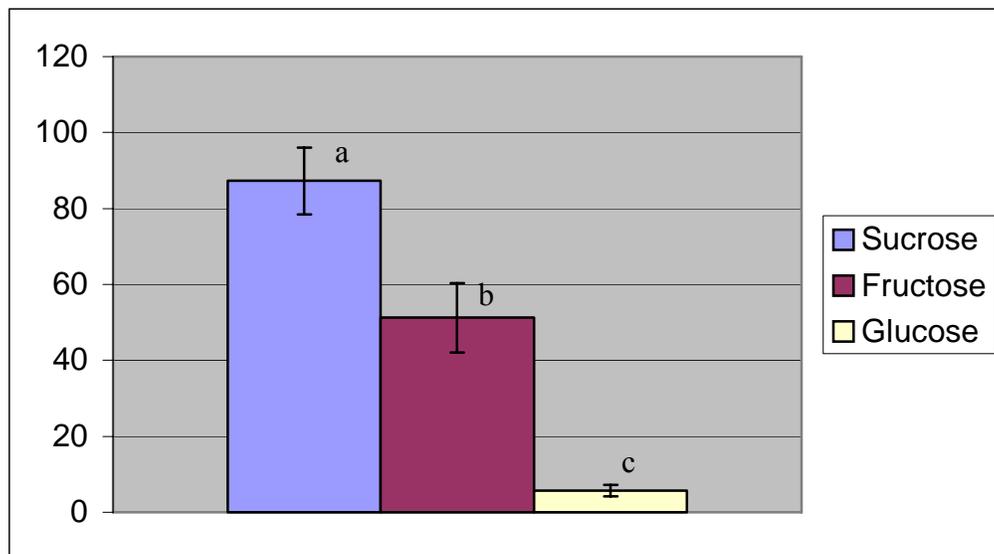


Figure 3-19. Carbohydrate testing (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

Statistical significances were detected among the diet treatments. Sucrose in the diet was preferred, receiving 60.49% of the feeding occurrences. On the other hand, glucose had an almost deterrent effect in the concentration tested here, receiving a mere 3.99% of the feeding occurrences.

While the soybean flour found in the Earle adult diet is high in protein and lipids, its carbohydrate content is lower than some other protein sources (Cohen 2004), so having additional carbohydrates in the diet is a necessity. An insect might also fail to develop on diet because its food intake is abnormally low because a phagostimulant essential for normal feeding, such as sucrose, is deficient (Reinecke 1985). Ascertaining appropriate concentrations required by the insect can be difficult. While most insects grow better as the proportion of carbohydrate is increased (Chapman 1998), there is a limit to which this holds true. When sugars were increased above 3 to 5 grams per 100 grams of diet, growth of the boll weevil was retarded (Vanderzant 1965). Optimal levels are most likely going to be those most similar to the insect's natural food (Chapman 1998). We tested various concentrations of sucrose to determine the amount most stimulatory for *A. tenebrosus*. The 4 treatments were: A) diet with normal 4.4% sucrose, B) diet with 6% sucrose, C) diet with 8% sucrose, and D) diet with 10% sucrose.

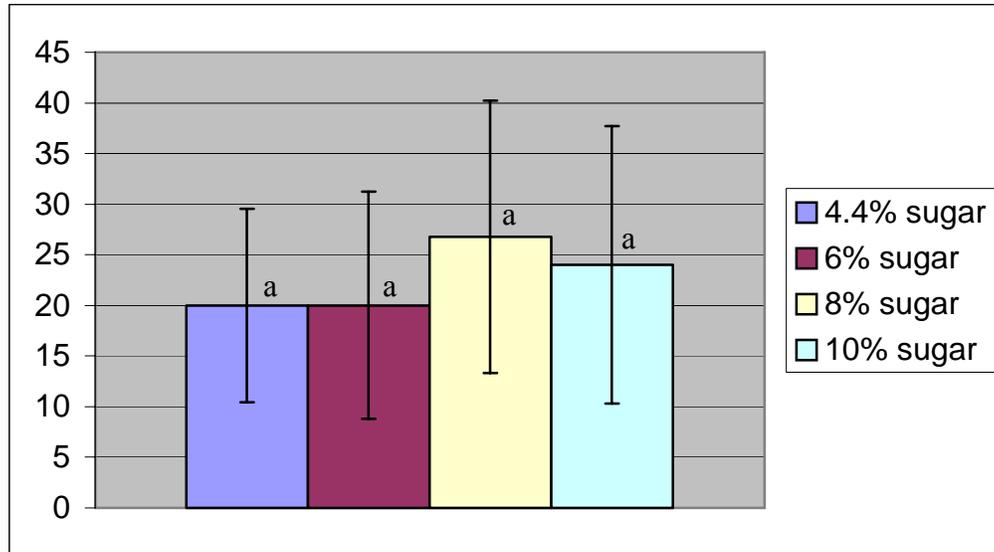


Figure 3-20. Sucrose concentrations – test 1 (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

The original 4.4% sucrose concentration received 22.04% of the feeding occurrences. In comparison, the 8% sucrose diet received 29.48% and the 10% sucrose diet received 26.45% of the feeding occurrences. Although there were no statistical differences between treatments, there was a tendency for consumption of the artificial diet to increase slightly by the addition of 8% and 10% sucrose. A second test was performed using the same sucrose treatments along with some additional concentrations. The 6 treatments were: A) normal 4.4% sucrose, B) 5.54% sucrose, C) 6% sucrose, D) 7% sucrose, E) 8% sucrose, and F) 10% sucrose.

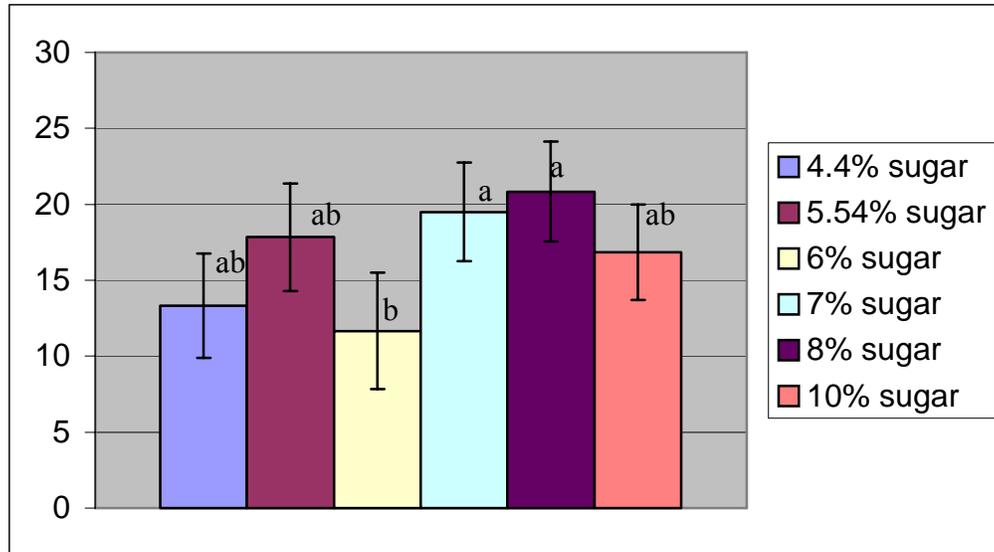


Figure 3-21. Sucrose concentrations –test 2 (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

Statistical differences were detected amongst some of the diet treatments. The 8% sucrose treatment had the highest number of feeding occurrences (20.83%). Although no eggs were laid by *A. tenebrosus* in the presence of diet with increased sucrose levels, the sucrose content was nevertheless increased to 8% for all future diets.

Lipids

Lipids function as building blocks of cell membranes, hormones, nutrient transporters, sources of energy, and as structural material for building other molecules (Cohen 2004; Vanderzant and Richardson 1964). They also may serve as phagostimulants for some insects (Cohen 2004; Nation 2002).

Polyunsaturated fatty acids (linoleic and linolenic) are most commonly found to be essential in insects (Reinecke 1985). Vanderzant and Richardson (1964) showed that boll weevil adults must have fat for optimal and continued oogenesis, with linolenic acid being the most

critical. In general, coleopterans show slow growth and a decrease in adult fecundity in response to a deficiency in polyunsaturated fatty acids (Nation 2002).

It stands to reason that feeding and oviposition would be improved if the insects were fed a combination of fatty acids more nearly like those in their host plant. Several oils are commonly used in artificial diets to provide essential fatty acids, including corn, linseed, olive, peanut, rapeseed, safflower, and sunflower (Reinecke 1985). A 1:1 ratio of linseed oil and vegetable oil (made from soybean oil) was chosen as the source of lipids for testing. Linseed oil is a linolenic acid, and soybean oil is a complex mixture of five fatty acids (palmitic, stearic, oleic, linoleic, and linolenic acids). In an attempt to determine the optimal amount of lipids for *A. tenebrosus*, varying amounts of lipids were incorporated into the diet. The 4 treatments were: A) diet with no lipids added, B) diet with 1% lipids added, C) diet with 3% lipids added, and D) diet with 5% lipids added.

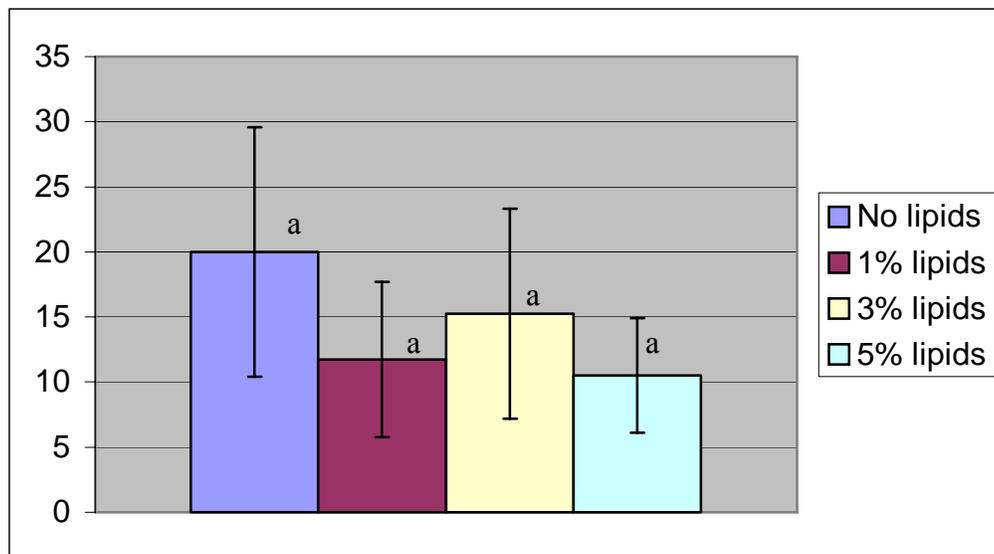


Figure 3-22. Lipid concentrations – test 1 (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

There were no statistical differences observed between the treatments. 34.78% of the feeding occurrences were on the diet with no added lipids. When only given a choice between relatively high lipid concentrations, the weevils preferred the diet with no lipids added. The test was run again with smaller amounts of lipids; lipids varied between 0.1% and 1.0%. The 5 treatments were: A) diet with no lipids added, B) diet with 0.1% lipids added, C) diet with 0.4% lipids added, D) diet with 0.7% lipids added, and E) diet with 1.0% lipids added.

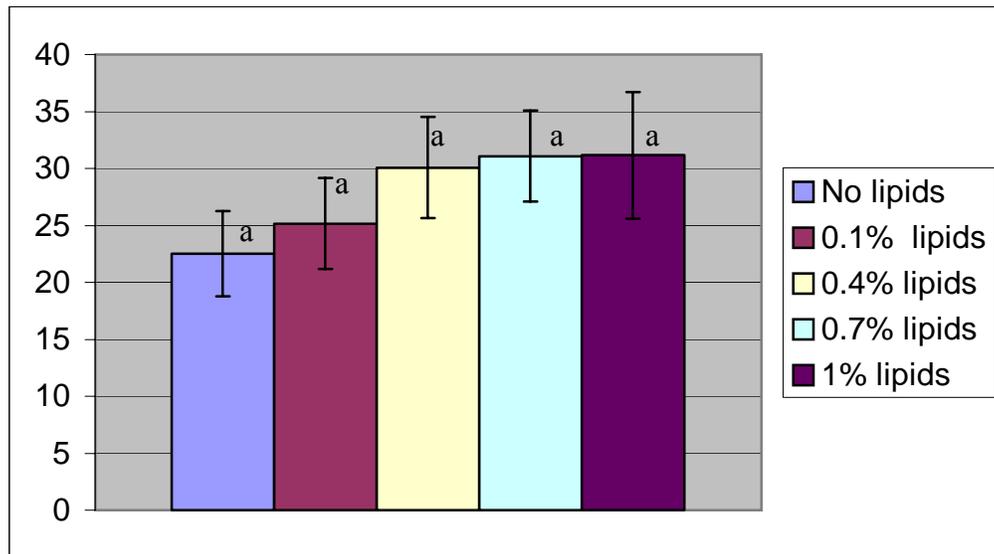


Figure 3-23. Lipid concentrations – test 2 (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

There were no statistical significances, although the no lipids treatment and 1% lipids treatment approached statistical significance ($p=0.0751$). The diet treatment with no lipids received 16.07% of the feeding occurrences; the 0.1% lipids diet received 17.98%; the 0.4% diet received 21.49%; the 0.7% lipids diet received 22.20%; and the 1% lipids diet received 22.26%. In general, consumption of the diet increased as lipid concentration increased. We chose the treatments with the highest number of feeding observations to use in subsequent diets. The

optimal level of oil was determined to be between 0.4% and 1.0%. A 0.7% level was adopted for routine use in subsequent diets.

Sterols

Specific lipids required by virtually all insects are sterols (Chapman 1998; Nation 2002). Sterols may serve as important phagostimulants for various insects (Nation 2002; Reinecke 1985). Sterols also function as building blocks of cell membranes; hormones including the synthesis of ecdysoid growth regulators; and give cellular membranes specialized characteristics, especially with regard to import and export of materials into and out of cells (Chapman 1998; Cohen 2004; Reinecke 1985). Some adult insects need sterols to produce the normal number and hatching of eggs (Nation 2002). A deficiency of sterols in the diet of adult boll weevils resulted in a decline in egg production as well as in egg hatch (Vanderzant and Richardson 1964), followed by death (Earle et al. 1966). While an insect can metabolize excess sterols for energy or for building carbohydrates, it cannot reverse the process and build sterols from carbohydrates. Because of their inability to synthesize sterols, insects must obtain them from their food.

All insects require a source of dietary sterols; yet because it is difficult to dissolve sterols, they are often omitted or provided in the wrong form in artificial diets (Cohen 2004). Dietary requirements for sterols often are closely correlated with sterols present in the natural food material (Vanderzant 1974). Although cholesterol has been detected in small quantities in some plants, many insects cannot metabolize it (Svoboda 1984). Phytophagous insects may perform better with plant sterols such as β -sitosterol, campesterol, or stigmasterol. We investigated whether the substitution of cholesterol for a plant sterol would improve *A. tenebrosus* feeding on the artificial diet. The 3 treatments were: A) diet made with cholesterol, B) diet made with β -sitosterol, and C) diet made with no sterol added.

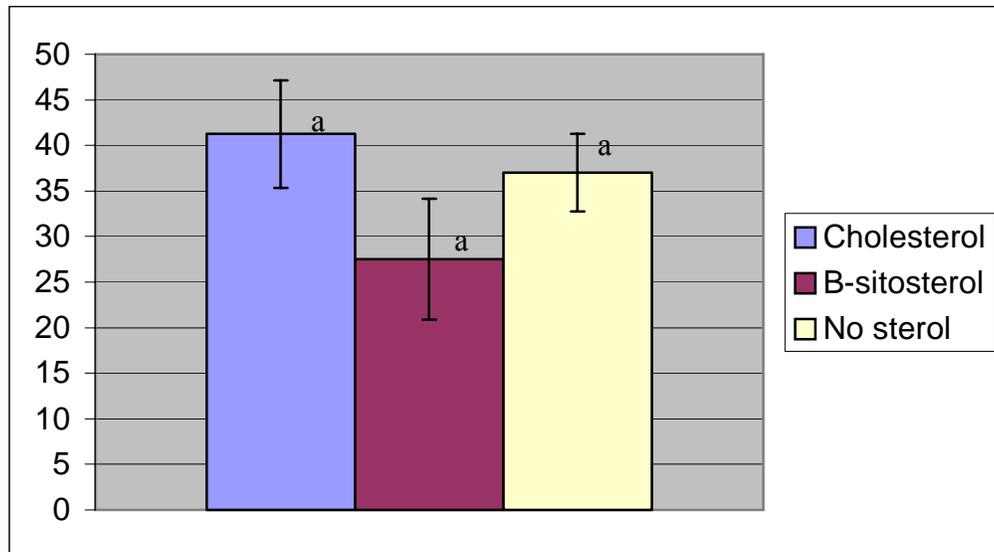


Figure 3-24. Sterol testing (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

There are no statistical differences observed between the treatments. The cholesterol diet received 39.01% of the feeding occurrences; the β -sitosterol diet received 26%; and the diet with no sterol received 34.99%. Surprisingly, *A. tenebrosus* preferred no sterol over the plant sterol β -sitosterol in the diet. The sterol content found in tropical soda apple is unknown. The addition of host plant material may have provided sufficient sterol in the diet treatment where no sterol was added. We chose the treatment with the highest number of feeding occurrences (cholesterol) to use in subsequent diets.

Solasodine

An exceptionally diverse suite of alkaloids, glycosides, and steroids occur in plants of the family Solanaceae (Brown, Jr. 1987; Schreiber 1968; Ripperger and Schreiber 1981; Ripperger 1998). The inability of most insects to sequester Solanaceae alkaloids is an indication that the proper physiological machinery to deal with these plant toxins has not yet evolved (Hsiao and

Fraenkel 1968). Those insects that can successfully overcome the alkaloid defenses often become specialists (Brown, Jr. 1987; Hsiao and Fraenkel 1968). Host specificity is based on the presence of secondary chemicals, such as essential oils, alkaloids, and glycosides that are restricted to this group of plants. During the course of evolution, many insect species were able to overcome plant resistance imparted by the secondary chemicals and began to utilize them as indicators of appropriate food (Beck 1965; Cohen 2004; Nation 2002). While many of these plant secondary compounds serve no known function in the metabolism of insects, they often evoke feeding and oviposition in insects that consume them (Cohen 2004; Deahl et al. 1993; Yamamoto and Fraenkel 1960). Two examples of this are the tobacco hornworm (*M. sexta*) and the Colorado potato beetle (*L. decemlineata*). A glycosidic substance from solanaceous plants was found to stimulate the feeding of both these insects (Yamamoto and Fraenkel 1960).

Anthonomus tenebrosus feeds extensively on the flowers, flower buds, and young leaves of tropical soda apple. Solasodine is a steroidal alkaloid (glycoalkaloid without a sugar) found in the foliage of numerous solanaceous plants. Solasodine is concentrated primarily in the developing fruits of tropical soda apple with mere traces of the steroidal alkaloid occurring in the foliage. Besides its value as a precursor for synthesis of steroidal drugs, solasodine has been shown to act as an antifungal agent and to regulate insect growth (Harborne 1986; Nawloka et al. 2003; Weissenberg et al. 1986). Work by Kuhn and Low (1955) put our concerns of safety to rest. Kuhn and Low (1955) showed that solasodine was noninhibitory to Colorado potato beetle because of its lack of sugar moieties required to produce deterrent effects. More recently, Alzerreca and Hart (1982) found that solasodine had no negative affect on snails (*Lymnaea cubensis* Pfeiffer and *Biomphalaria glabratus* Say).

Glycoalkaloids synthesized in the foliage of various *Solanum* species were tested by Sanford et al. (1996) in synthetic diets for their effects on potato leafhopper adults, *Empoasca fabae* (Harris), at concentrations of 0.03%, 0.09% and 0.27%. The concentrations chosen were based on the work of Deahl et al. (1993), who reported the foliage glycoalkaloid concentrations for 60 wild *Solanum* species.

The presence of secondary plant compounds is often useful in promoting optimal feeding of insects on artificial diet (Nation 2002; Reinecke 1985). Because solasodine is concentrated in the reproductive parts of tropical soda apple, solasodine may influence *A. tenebrosus* feeding and oviposition. Our objective was to determine what effect solasodine would have on feeding behavior and oviposition of *A. tenebrosus* when incorporated in an artificial diet. Following the work of Sanford et al. (1996), we tested solasodine in the artificial diet at concentrations of 0.03%, 0.09%, and 0.27%. The 7 treatments were: A) diet with no solasodine or TSA, B) diet with 0.03% solasodine and 3g TSA, C) diet with 0.09% solasodine and 3g TSA, D) diet with 0.27% solasodine and 3g TSA, E) diet with 0.03% solasodine, F) diet with 0.09% solasodine, and G) diet with 0.27% solasodine.

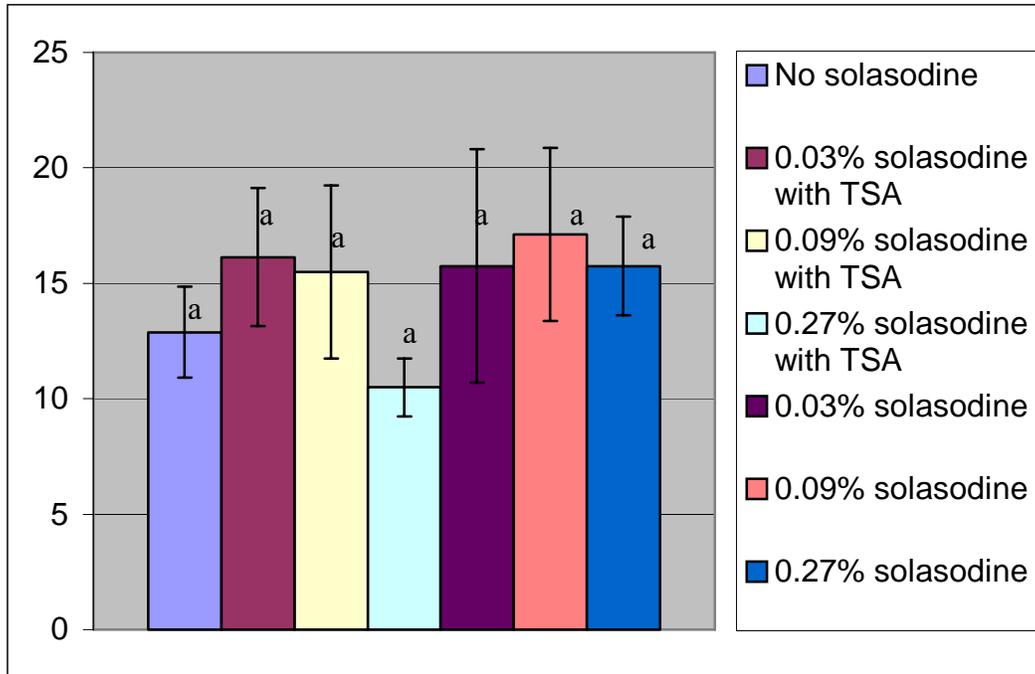


Figure 3-25. Solasodine testing (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

No statistical differences were detected amongst the diet treatments. The highest number of feeding observations was seen on the 0.03% solasodine and TSA diet (15.56%). The fewest feeding occurrences were on the 0.27% solasodine and TSA diet (10.13%). Oviposition behavior was observed only in the presence of fresh capsules. No eggs were laid during the 72-hour exposure period. One death occurred during the first 24 hours of testing but appeared to be from natural causes. *Anthonomus tenebrosus* showed a degree of tolerance to solasodine incorporated in artificial diets. Solasodine added to the artificial diet did not act as a feeding attractant or deterrent to adults. Weevils were observed equally on all diet treatments. We concluded that the addition of the solasodine alkaloid did not improve the attractiveness of the diet to the weevils under the experimental conditions used.

Solasodine also has been shown to act as an antifungal agent, defending plants against microbial infection (Harborne 1986; Dahlman and Hibbs 1967). Because the introduced adults

were not free from surface contamination, fungal growth on the diet treatments became evident before the experiments terminated. Therefore the fungal growth-retardant effect of solasodine was not confirmed under the current testing conditions.

Proline

Amino acids are used by insects to build required proteins. Of the 20 known amino acids, only 10 are considered to be essential to insects. The other amino acids can often be synthesized from the 10 essential ones. Proline is the most common of the non-essential amino acids. In some insects, proline is an important energy source and is necessary for good growth and survival.

Cohen (2004) and Reinecke (1985) suggested that some amino acids, like proline, may serve as feeding stimuli. Proline is unique among the amino acids because insects have the ability to taste it (Gardener and Gillman 2002). Active selection for diets containing high proline levels has been observed for a number of phytophagous insects (Behmer and Joern 1994; Heron 1965; Cook 1977; Haglund 1980). Carter et al. (2006) showed that honey bees prefer nectars rich in the amino acid proline. Behmer and Joern (1994) determined that females of *Ageneotettix deorum* (Scudder) (Orthoptera: Acrididae) and *Phoetaliotes nebrascensis* (Thomas) (Orthoptera: Acrididae) displayed preferences for diets high in proline and exhibited an increased feeding response as the proline level increased. In Dadd's (1985) work on *A. grandis*, he determined that proline was required in artificial diet to produce optimal growth.

Amino acids are abundant in both pollen and nectar (Gardener and Gillman 2002; Kaczorowski et al. 2005), with proline being one of the most abundant amino acids identified (Baker 1978; Gardener and Gillman 2001; Gilliam et al. 1980; Zhang et al. 1982), sometimes accounting for up to 70% of total free amino acids (Zhang et al. 1982). Carter et al. (2006) indicated that nectar of ornamental tobacco (Solanaceae), a plant closely related to tropical soda

apple, to be extremely rich in proline. From the previous studies, it is not unreasonable to expect proline to be a prominent amino acid in tropical soda apple and be a key factor in the selection of tropical soda apple flowers and flower buds by *A. tenebrosus*.

Chapman (1998) declared that non-essential amino acids like proline may comprise over 50% of the total amino acids necessary to produce optimal growth on an artificial diet. To investigate the effect of proline on feeding and oviposition of *A. tenebrosus*, proline was added to the artificial diet in varying levels. The 6 treatments were: A) diet with no proline, B) diet with 0.02g proline, C) diet with 0.07g proline, D) diet with 0.14g proline, E) diet with 0.34g proline, and F) diet with 0.54g proline.

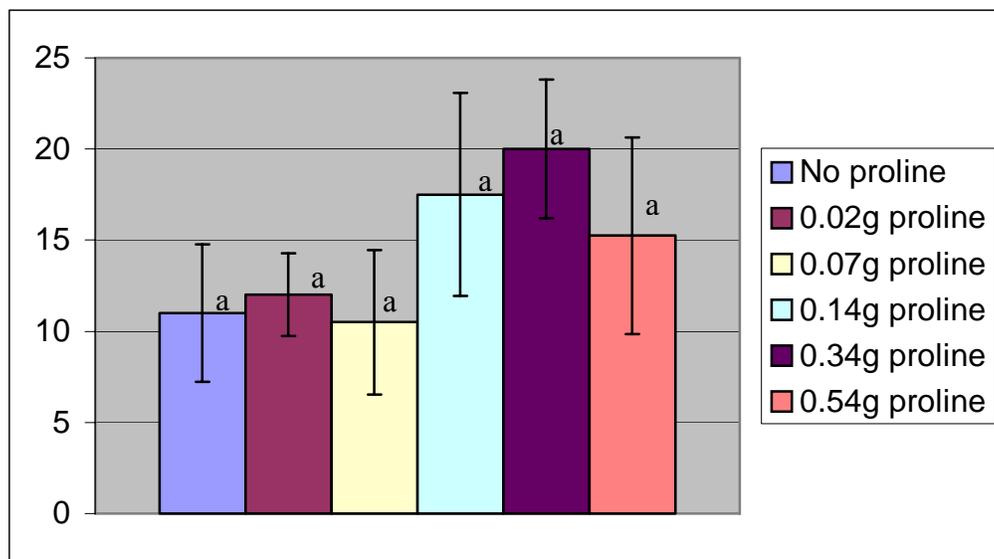


Figure 3-26. Proline – test 1 (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

Although there were no statistical differences observed between treatments, more feeding was observed on those diets containing higher proline concentrations. The 0.34g proline diet received the most feeding occurrences (23.19%). No eggs were laid in the presence of proline in the diets. Additional tests were run with greater concentrations of proline added to the diet. The 4

treatments were: A) diet with no proline, B) diet with 0.20g proline, C) diet with 0.40g proline, and D) diet with 0.60g proline.

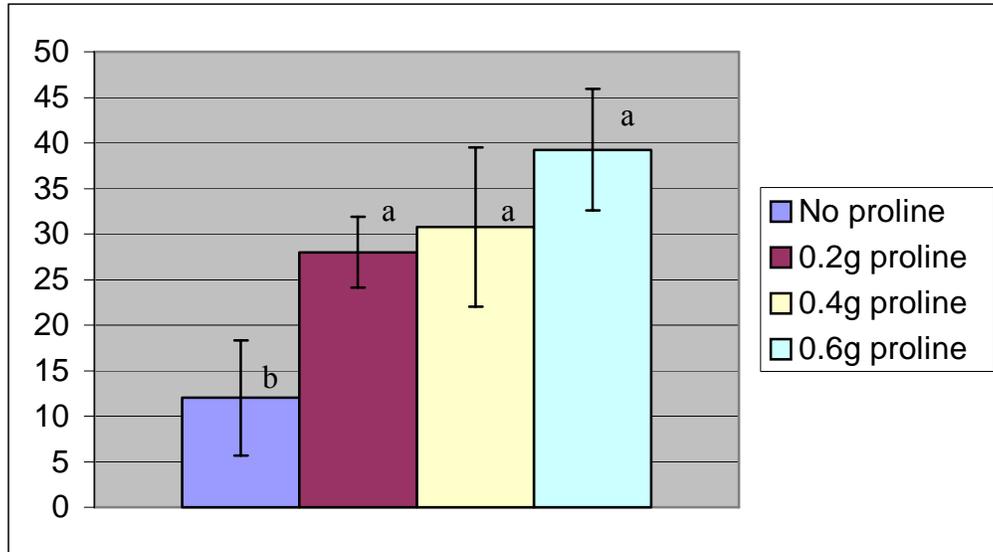


Figure 3-27. Proline – test 2 (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

Statistical significances were detected among the diet treatments. Weevils exhibited a slight preference for the 0.60g proline diet, with 23.64% of the feeding occurrences associated with this diet. Another experiment was run with additional proline concentrations tested. The 7 treatments were: A) diet with no proline, B) diet with 0.20g proline, C) diet with 0.40g proline, D) diet with 0.60g proline, E) diet with 0.80g proline, F) diet with 1.0g proline, and G) diet with 1.20g proline.

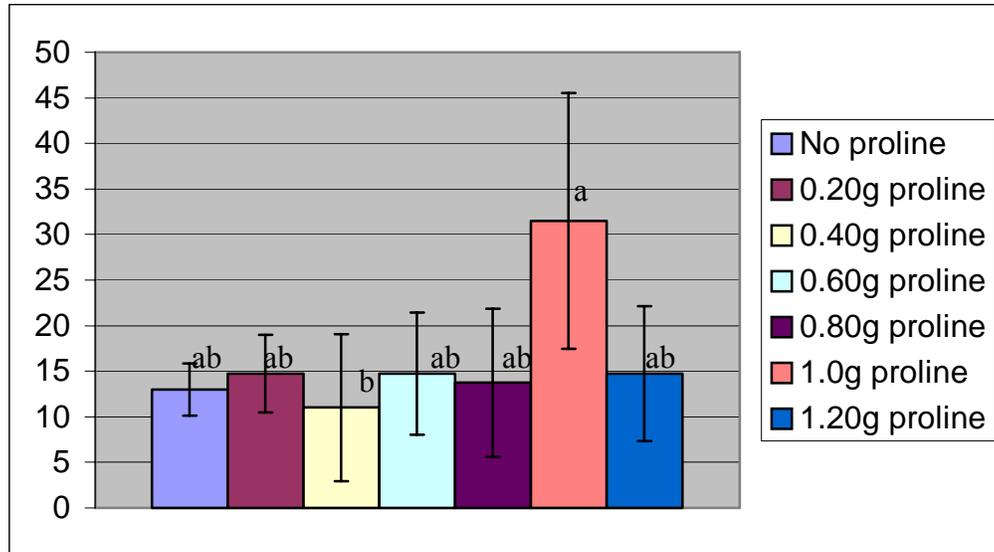


Figure 3-28. Proline – test 3 (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

Statistical significances were detected among some of the diet treatments. The 1.0g proline diet received 27.75% of the feeding observations. Testing was repeated using the same proline concentrations, but freeze-dried tropical soda apple was added to the diets. The 7 treatments were: A) diet with no proline with TSA, B) diet with 0.20g proline and TSA, C) diet with 0.40g proline and TSA, D) diet with 0.60g proline and TSA, E) diet with 0.80g proline and TSA, F) diet with 1.0g proline and TSA, and G) diet with 1.20g proline and TSA.

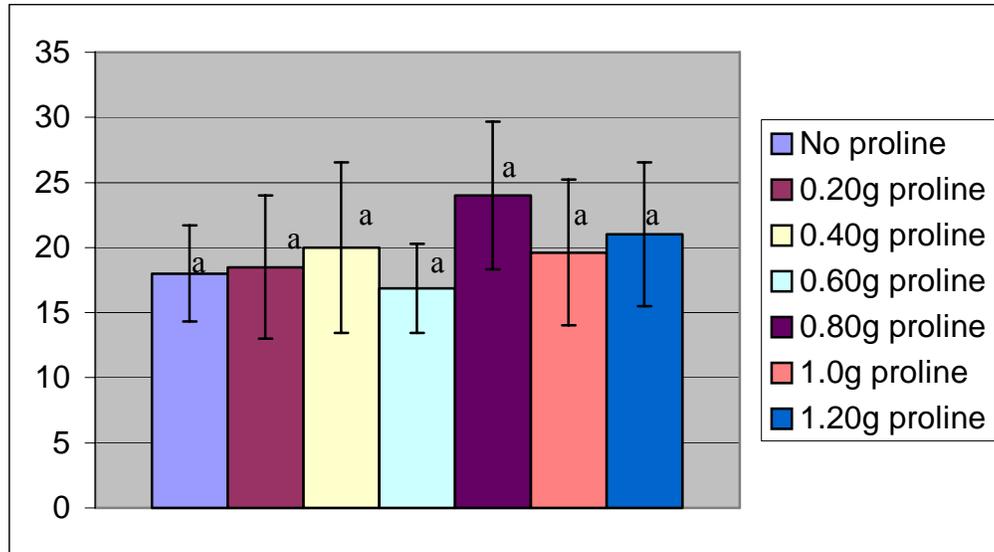


Figure 3-29. Proline – test 4 (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

Although there were no statistical differences observed between treatments, the 0.80g proline diet received a larger proportion (17.39%) of the feeding occurrences. Proline did not have a significant phagostimulatory effect when added to the artificial diet. However, treatments containing proline were chosen more frequently than the control treatments throughout all of the proline tests. As the weevils appeared to be somewhat attracted to proline, 0.8 - 1g of proline was used in subsequent diets.

In an earlier study, Behmer and Joern (1994) determined that females of *A. deorum* and *P. nebrascensis* preferred diets high in proline and theorized the difference between male and female grasshoppers response to proline was due to differences in the nutritional requirements associated with reproduction. *Anthonomus tenebrosus* adults used in our tests were of mixed sex. A preference for proline in the diet may have been significant had only females been used.

Bee Pollen

Pollen has been shown to be an important dietary component to many flower-feeding herbivores (Cook et al. 2004; Gardener and Gillman 2002; Kevan and Baker 1983); many insects require it for survival and maturation of eggs. Pollen provides a variety of essential nutrients, including protein, starches, sterols, lipids, carbohydrates, vitamins, and minerals (Roulston and Cane 2000).

Schmidt and Hanna (2006) and Standifer et al. (1970; 1978) greatly enhanced consumption of artificial diet by the honey bee (*Apis mellifera* Linnaeus) by adding pollen to the diets. Vanderzant et al. (1959b) found that pollen from different families of plants in amounts of 0.1 to 5g per 100g of artificial diet stimulated feeding and oviposition of the boll weevil (*A. grandis*). In another study, Haynes and Smith (1992) determined that the longevity of adult boll weevils could be extended by diet supplementation with pollen.

Schmidt and Hanna (2006) showed that pollen contained phagostimulants that may serve as attractants to some insects and enhance feeding. Because *A. tenebrosus* feeds primarily on the anthers of tropical soda apple flowers, the addition of bee pollen in the artificial diet may affect the weevil's feeding behavior. In an attempt to induce better feeding and oviposition on the artificial diet via the addition of nutrients and phagostimulants found in pollen, we tested pollen concentrations of 0.5g - 1.0g per 100g diet. The 3 treatments were: A) diet with no pollen, B) diet with 0.5g pollen, and C) diet with 1.0g pollen.

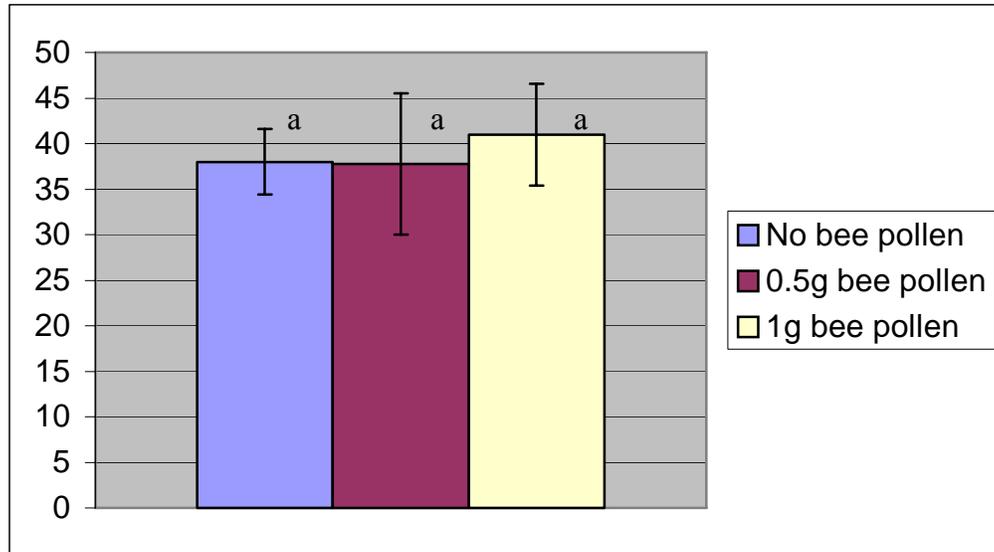


Figure 3-30. Pollen testing (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

No statistical differences were observed among the diet treatments. Feeding occurrences were essentially the same on each of the diets. The diet with no pollen received 32.55% of the feeding; the 0.5g pollen diet received 32.33%; and the 1g pollen diet received 35.12%. Although the insects exhibited a slight preference for the 1.0g bee pollen diet, there was no noticeable change in oviposition or number of eggs laid by the weevils.

While pollen feeding has been shown to influence both the longevity and total fecundity of some weevils, pollen had little influence on feeding behavior and oviposition in *A. tenebrosus*. It is important to emphasize that pollens from different plant species vary widely in their acceptability as nutrients and feeding stimulants. Variations in pollen composition may be a way of attracting particular insect species. Insects that specialize on only a few host plant species may undergo optimal development and reproduction only on pollen from their specific hosts (Millar et al. 2003). The bee pollen used in this test was from an unknown source. Incorporating pollen into the artificial diet collected solely from solanaceous plants may prove to be more effective.

Anti-Oxidation Testing

Ascorbic acid (vitamin C) is one of the most abundant compounds in the green tissues of plants. Ascorbic acid is found in many plants which suggests that this vitamin may play a vital role in the development and reproduction of phytophagous insects (Vanderzant et al. 1962; 1974). Ascorbic acid is required for normal growth and development of some insects, particularly phytophagous insects (Beck et al. 1968; Vanderzant and Richardson 1963c). Ascorbic acid also serves as a phagostimulant (Beck 1965; Thorsteinson 1958), an antioxidant, and is involved with some of the processes of cuticular sclerotization (Chapman 1998; Cohen 2004; Vanderzant et al. 1962).

When artificial diets contain grains or seeds as the main diet components, as opposed to green parts of plants, they must often be supplemented with ascorbic acid (Cohen 2004). Ascorbic acid is both a nutrient for the insect and an antioxidant in artificial diets, preventing degradation of the diet. The deterioration of diet components by oxidation is thought to decrease the palatability and nutritional quality of the diet (Cohen 2004). The incorporation of ascorbic acid in a diet slows down this oxidation process.

Vitamin C is quite susceptible to degradation, especially when it is in a solution, exposed to heat, light, oxygen, or free radicals (Cohen 2004). Decomposition of ascorbic acid in diets under rearing conditions makes it difficult to determine the exact needs of the insects (Vanderzant et al. 1962). Vanderzant et al. (1962) determined that ascorbic acid was an indispensable nutrient for the boll weevil. Boll weevil adults that fed on diets deficient in ascorbic acid laid fewer eggs with lower hatch rates. Diets containing 0.1 or 0.2 g ascorbic acid per 100 g of diet increased egg hatch rates to 75% or more (Vanderzant et al. 1962). Ascorbic acid deficiency in insects tends to result in molting cycle failure. On the other hand, high dietary

concentrations of vitamin C have been shown to slow development of the cotton bollworm (*Heliothis armigera* Hübner) and cause changes in sex ratios in silkworms (*Bombyx mori* Linnaeus) (Reinecke 1985).

The incorporation of freeze-dried tropical soda apple leaves, flowers, and flower buds into the *A. tenebrosus* diet accelerates the oxidation of the diet. Diet with host plant material turns brown shortly after exposure to air; enzymes in the host plant material are thought to cause this oxidative browning. We were concerned that the oxidation was decreasing the palatability and nutritional quality of the diet. Ultimately, browning causes the loss of ascorbic acid. Browning can temporarily be halted in potatoes by the addition of lemon juice to reduce the pH. In preliminary tests, the addition of lemon juice (ReaLemon®, Stamford, CT) to the *A. tenebrosus* diet in increments from 5 to 35 drops per 100g of diet proved to be ineffective.

The most common chemical method of controlling the enzymatic browning is the addition of more ascorbic acid. We tested the effectiveness of varying levels of ascorbic acid in slowing the oxidation or browning of the diet. Figure 3-31 presents the results obtained with diets with additional ascorbic acid. The 5 treatments were: A) control diet with 0.2g ascorbic acid, B) diet with 0.4g ascorbic acid, C) diet with 0.6g ascorbic acid, D) diet with 0.8g ascorbic acid, and E) diet with 1.0g ascorbic acid.

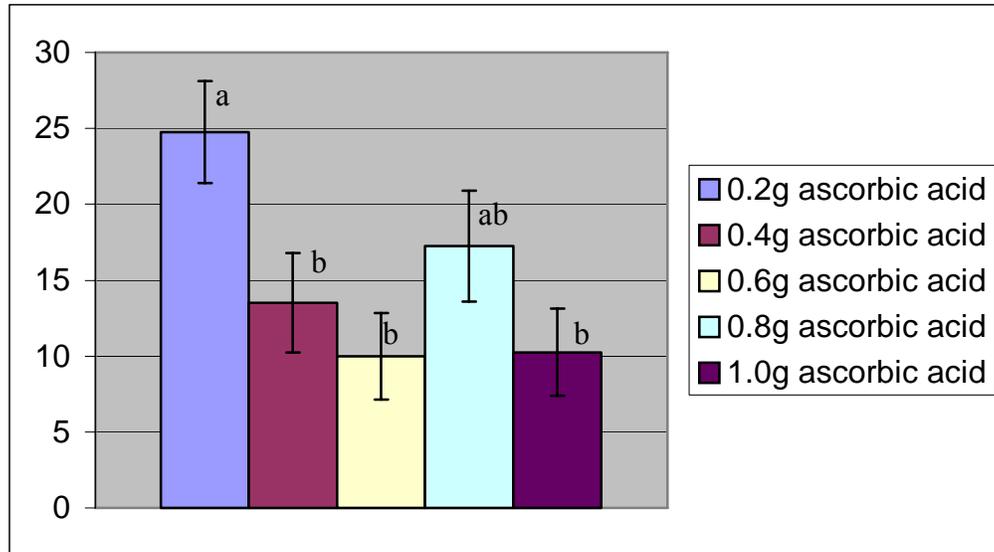


Figure 3-31. Ascorbic acid testing (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

Statistical differences were detected among the diets. The 0.2g ascorbic acid diet received the highest amount of feeding (32.67%), while 0.6g ascorbic acid received the least amount (13.20%). Higher concentrations of ascorbic acid appeared to be detected by the weevils and avoided in favor of the 0.2g ascorbic acid diet. Three days after the experiment was initiated, the diet capsules with increased ascorbic acid remained lighter in color than the control diet (0.2g ascorbic acid), signaling less oxidation. The addition of ascorbic acid was effective in slowing browning of the diet capsules but also was repellent to the weevils.

Original vs Improved Diet

Anthonomus tenebrosus showed improved feeding when artificial diet was formulated into cone shaped capsules and lipids, proline, tropical soda apple, and sucrose were added to the Earle adult diet. To confirm this fact, we exposed 2 diets to the weevils: A) the original Earle adult diet and B) our improved adult diet.

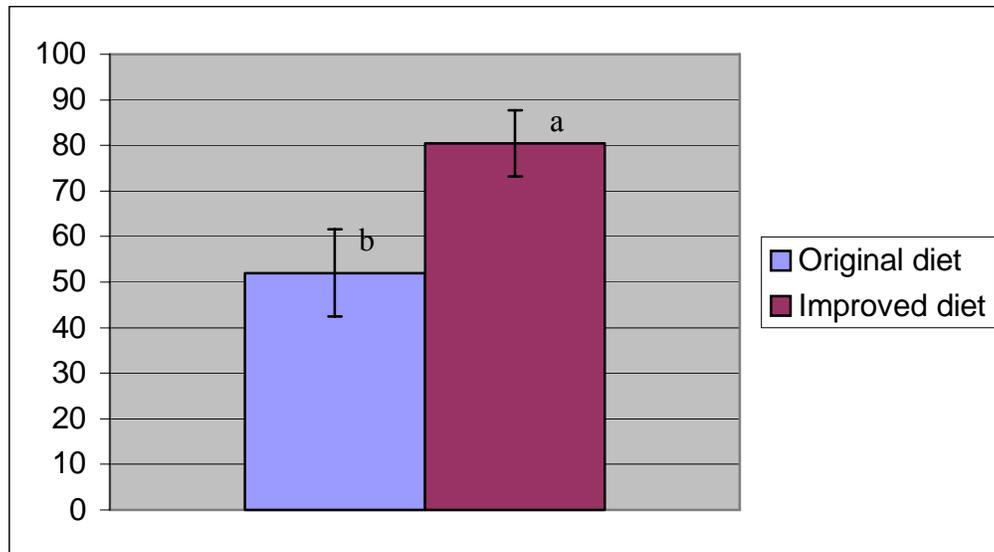


Figure 3-32. Original diet vs improved diet testing (mean feeding occurrences \pm standard error). Columns having the same lowercase letters are not significantly different using LSD with $p=0.05$.

A statistical significance was detected between the two diet treatments. The improved diet received a higher proportion (68.87%) of the feeding occurrences, while the original Earle diet received 31.13% of the feeding occurrences. Feeding was more than doubled, rising from 300 feeding events to over 700. The weevils clearly showed a preference for the improved diet. While feeding was greatly increased on the improved diet, no eggs were laid on either artificial diet. The inability to elicit oviposition by *A. tenebrosus* while on artificial diet remains a problem.

Summary and Conclusions

Several insects have been mass-reared successfully on artificial diets and used for various insect-control programs. For this reason, experiments were initiated to assess the suitability of artificial diets for feeding and reproduction of *A. tenebrosus* adults. The Earle adult diet was found to be the best one of those tested for rearing the flower bud weevil *A. tenebrosus*.

Additional experiments were conducted to determine the combination of ingredients and amounts in the diet that would produce a suitable diet for rearing these insects. Results showed that the diet could be improved, particularly in respect to feeding, by the formation of cone shaped diet capsules and the addition of sucrose, host plant material, lipids, and proline. Additional protein, solasodine, pollen, and ascorbic acid did not appear to improve the quality of the diet.

A more detailed investigation and analysis of tropical soda apple apical leaves, flowers, and flower buds is necessary. It is our belief that tropical soda apple possesses both deterrents and attractants to *A. tenebrosus*. Larger amounts of tropical soda apple in the diet had a repellent effect on the insects. Consumption of the artificial diet was increased by the addition of leaves, flowers, and flower buds in small concentrations alternating between 0.1% - 0.2%. Analysis of tropical soda apple flowers and leaves would prove beneficial and may shed light on possible deterrents and attractants.

The ultimate goal is to produce high quality insects comparable to those feeding on their natural food plants. Despite the increased feeding on the improved diet, oviposition remained low. Although the improved Earle adult diet is inferior to natural flowers and flower buds, the artificial diet is sufficient as a supplemental food source when there is an inadequate amount of host plant material available or as a food source for *A. tenebrosus* during shipment. Additional tests using more insects and possibly other modifications of this diet should be continued in order to discover a more nutritionally adequate diet for mass-rearing *A. tenebrosus*. Each major ingredient in the diet should be studied to determine how it affects feeding and oviposition. Further studies are needed to identify the combination of ingredients capable of supporting complete development of *A. tenebrosus*.

CHAPTER 4
EVALUATION OF THREE ARTIFICIAL DIETS FOR THE LABORATORY REARING OF
ANTHONOMUS TENEBROSUS LARVAE

Introduction

An attempt was made to rear *A. tenebrosus* on several artificial diets because of its biological control potential. Diet testing with adult *A. tenebrosus* resulted in the development of a diet that the weevils readily fed upon but failed to recognize as a suitable oviposition site (see Chapter 3). Preliminary testing of larvae on a few existing *Anthonomus* spp. diets was performed with some success. Up to 30% of second and third instars developed into adults during preliminary testing using a Moore et al. (1967) modified diet. Ideally, an artificial diet for rearing *A. tenebrosus* would be suitable for both larvae and adults, with adults depositing their eggs on the diet used for rearing the larvae. With this in mind, we wanted to determine if *A. tenebrosus* larvae would develop successfully on the modified *A. grandis* (boll weevil) artificial diet preferred by adults (Earle et al. 1966).

To assess the feasibility of rearing *A. tenebrosus* larvae on the modified Earle et al. (1966) adult diet, three diet treatments were evaluated to determine their ability to sustain the growth and development of larvae. We compared larval development on (a) the modified Earle et al. (1966) adult diet, (b) the same diet with the addition of host plant material, and (c) our improved adult diet. We evaluated the suitability of the artificial diets by following growth and other biological parameters including percent pupation, pupal development, and percent adult emergence.

Materials and Methods

Insects

The insects used in this study were obtained from a laboratory colony of *A. tenebrosus* maintained at the Division of Plant Industry, Florida Biological Control Laboratory, Gainesville, Florida. The procedures for maintaining the weevil colony were described in Chapter 2. To initiate the diet experiments, flower buds exposed to unsexed adults were dissected and larvae collected. Fifteen larvae were used per diet treatment.

Artificial diets

The ingredients for the three diets tested for larval development were obtained from MP Biomedicals®, Bio-Serv®, and Fisher® Scientific. The composition of the diets are listed in Table 4-1. The preparation of the diets was simplified by the incorporation of commercially prepared vitamin and salt mixtures. The agar was dissolved in distilled water by heating and allowed to boil for 1 minute. The agar was allowed to cool to 75° C before adding the pre-mixed dry ingredients. Microbial growth was inhibited by the addition of a potassium sorbate and 95% ethyl alcohol mixture at 1.25 ml/100 g of diet. The pre-made inhibitor was added and all the ingredients were mixed thoroughly. After mixing, the larval diet was dispensed into 29.57 ml (1 oz) creamer cups (10 ml of diet per cup) beneath a laminar flow hood. The cups were transferred to a sealed plastic container (Pioneer Packaging®, Dixon, KY), where they were allowed to gel before capping individual cups with lids (cups and lids from Fill-Rite, Newark, NJ). Extra diet cups were stored in a refrigerator and were used within 2 weeks from time of preparation.

Host plant material (tropical soda apple flowers and flower buds) was added to two of the three diets. The flowers were freeze-dried and ground to a fine powder in a coffee grinder (Mr. Coffee®, Sunbeam Products, Inc.). The powder was sifted through a 40-mesh strainer. The

strained powder was stored in airtight vials at room temperature and kept in a dark area until needed.

Table 4-1. Composition of three artificial diets investigated for laboratory rearing of *A. tenebrosus* larvae. A) Modified Earle (1966) adult diet, B) Modified Earle (1966) adult diet with tropical soda apple flowers and flower buds, C) Improved Earle (1966) adult diet with tropical soda apple flowers and flower buds.

Ingredients (grams)	A	B	C
1. Agar	3	3	3
2. Water	160 ml	160 ml	160 ml
3. Sucrose	8	8	14.44
4. Vanderzant vitamins	0.02	0.02	0.02
5. Inositol	0.04	0.04	0.04
6. Choline chloride	0.10	0.10	0.10
7. Wesson salt mixture	1.34	1.34	1.34
8. Soybean protein	5	5	5
9. Cholesterol	0.10	0.10	0.10
10. Ascorbic acid	0.4	0.4	0.4
11. TSA flowers	--	0.18	0.18
12. Lipids *	--	--	1.28 ml
13. Proline	--	--	2
14. Inhibitor mixture **	2.50 ml	2.50 ml	2.50 ml

* Lipids - 1:1 ratio of linseed oil and vegetable oil

** Inhibitor mixture – 10g potassium sorbate and 100ml 95% ethyl alcohol

Rearing methods

First and second instars were collected from dissected tropical soda apple flower buds. During preliminary tests, larvae were washed in a 3% bleach solution before being transferred to the diet. High mortality occurred with this process, so it was eliminated. A small hole was made in each diet cup. Larvae (one larva per cup) were transferred with a sable haired brush to the small hole made in the diet; each larva was placed inside the small hole made in the diet. Diet cups were placed inside a sealed plastic container (Pioneer Packaging®, Dixon, KY) with a green opaque lid and sides so that larvae were encouraged to feed rather than to migrate toward

the light. The diet cups were maintained at a temperature of $24^{\circ} \pm 2^{\circ} \text{C}$, 43 – 47% RH and a 11:13 (L:D) photophase. Larvae and pupae were observed daily and records were maintained for development, mortality, and diet contamination. Diet cups with visible contamination from microbial growth were discarded immediately and the larvae transferred to fresh diet cups. Larvae were fed *ad libitum* until they pupated. Larvae and pupae were observed once every 24 hours. Third instars often encapsulated themselves in the diet by moving diet to cover the top of the hole and creating a mound. A small portion of the mound covering was removed to allow for observations.



Figure 4-1. Diet cups for larval testing.

Results and Discussion

Survival on all three diets was low (Table 4-2). Highest survival to the pupal stage occurred on the modified Earle et al. (1966) diet with host plant material (Diet B). Highest survival to the adult stage also was observed on Diet B. Larvae and pupae survived an average of 7.2 ± 2.7 days (n=15) on the modified Earle et al. (1966) adult diet (Diet A), 6.5 ± 2.4 days (n=15) on the same diet with host plant material (Diet B), and 7.0 ± 2.8 days (n=15) on the improved diet with host plant material (Diet C). Due to high mortality, testing was discontinued and no further replications were run.

Table 4-2. Growth performance of flower bud larvae on three artificial diets.

Treatment	n	% Survival to Pupa	% Pupa Surviving to Adult	% Survival to Adult
A. Earle adult diet	15	20	0	0
B. Earle adult diet with flowers	15	40	50	20
C. Improved Earle diet with flowers	15	20	33.3	6.7

While the flower buds that *A. tenebrosus* consumes in its natural environment are ideal for rearing these insects, they may be difficult to obtain year-round, particularly during winter. Tropical soda apple flower production declines significantly from October to mid-March in North Florida. Greenhouse rearing is labor intensive and requires a large amount of space and an extensive system of artificial lighting. An artificial diet that would produce insects of quality comparable to those feeding on their natural food plants and that could be supplied year-round would be beneficial for rearing the weevils. Artificial diets have been successfully developed for other *Anthonomus* weevils, including *A. grandis* (boll weevil) and *A. eugenii* (pepper weevil) (Earle et al. 1966; Gast & Davich 1966; Moore et al. 1967; Toba et al. 1969; Singh 1977; Sterling & Adkisson 1966; Vanderzant et al. 1958; 1959; 1961; 1963a; 1963b; 1963c; 1965; 1967; 1973).

Earle et al. (1966) found that the nutritional needs of *A. grandis* adults were more critical than those of larvae. They found that only minor changes in the adult boll weevil diet were required to produce a diet that was suitable for larval development. Diet testing on *A. tenebrosus* adults produced a diet that adults readily fed upon (see Chapter 3). Because the ultimate goal is to devise one diet that is satisfactory for both larval development and adult reproduction, we investigated the extent to which three modified Earle et al. (1966) diets would be nutritionally adequate for rearing *A. tenebrosus* larvae. We found that larval and pupal survival on all three diets was poor.

Our previous findings with adults indicated that the addition of a small amount of host plant material to the diet enhanced feeding, while larger amounts were repellent (see Chapter 3). Diets B and C included 0.18g freeze-dried tropical soda apple flowers and flower buds and had the highest percentage of larvae surviving to adults. Thus, a successful larval diet will most likely include a certain amount of freeze-dried tropical soda apple to promote feeding activity and development, but we were unable to determine the specific quantity in this preliminary study.

The fact that no adults were produced on Diet A may indicate the absence of appropriate nutritional requirements or feeding stimulants. On the basis that Diet B produced the most weevils, this diet is the best of the three tested for rearing flower-bud larvae, although the 20% survival is lower than in preliminary tests with other diets. Due to the small sample size tested here, further testing of Diet B is recommended.

During earlier starvation studies, larvae survived an average of 4.4 ± 1.8 days ($n=11$). Larvae survived between 5.75 – 7 days on the three artificial diets tested here. This suggests that larvae were able to obtain some nourishment from the artificial diets. Vanderzant et al. (1959) stated that the quality and quantity of nutrients are important for larvae whose growth demands a greater supply of nutrients than required by adults. The modified Earle et al. (1966) diet may not provide sufficient nutrients for optimal development. Weevils that successfully developed were smaller than those produced on flower buds. Alternatively, nutrients may be available but larval feeding may be limited due to a lack of feeding stimulant or a repellent in the diet. Although the proportion of diet-reared larvae reaching the adult stage was generally low, results indicated that laboratory populations of *A. tenebrosus* could be maintained on diets for a short time until plant material became available.

More work is needed to improve the production of these weevils on an artificial diet. Further study should include testing larval survival on other *Anthonomus* spp. artificial diets. Formulations for many of these diets have been compiled by House (1967), House et al. (1971), and Singh and Moore (1985). Only after a better diet is identified, should steps be taken to improve development and feeding on the diet. Young larvae tend to drown in the sticky diets. Excessive moisture in the diet is a concern that will need to be addressed in the future to prevent larval drowning. Once a diet is found suitable for rearing *A. tenebrosus* larvae, comparisons of growth and other biological parameters of the insects reared on the diets should be made with insects reared on the natural host plant. Ultimately, an increased availability of adults as a result of rearing on an artificial diet will prove beneficial in a biological control program.

CHAPTER 5 SUMMARY AND CONCLUSIONS

Successful biological control programs often depend on our ability to rear high quality natural enemies and establish colonies in the laboratory. A rearing system was developed for *A. tenebrosus* that produced more than 30 generations in the laboratory on tropical soda apple plant cuttings. Prior to this study, little was known about the biology of *A. tenebrosus*. We determined that the weevil developed from egg (4.2 ± 0.9 days), underwent three instars (10.7 ± 1.9 days), pupa (5.2 ± 1.2 days), and eclosed as an adult in a total of 20.4 ± 8.3 days.

The mass production of insects on artificial diets has proven beneficial in biological control programs. Diets are now widely used for laboratory rearing and many are being used successfully for the mass production of insects. We evaluated existing *Anthonomus* spp. artificial diets to determine their ability to sustain growth and development of *A. tenebrosus*. We attempted to develop an artificial diet that would supply nutrients for adults and be acceptable as oviposition sites for females. The second part proved to be most difficult. Although the adults readily fed on the diet, very few eggs were produced. Weevils apparently recognized the artificial diet as a food source but not as an oviposition substrate.

The development of a successful artificial diet should include not only the chemical composition of the diet, but also physical characteristics that influence feeding and oviposition. Subtle differences in texture, nutrient imbalances, phagostimulants, and deterrents all can influence insect performance (Scriber and Slansky 1981). Various materials such as protein, host plant material, carbohydrates, lipids, sterols, solasodine, proline, and pollen were added and tested in the diet, along with an examination of physical characteristics such as color, shape, and texture. In most cases, performance of the weevil on the diets was marginal. Although sample sizes for these studies were relatively small, statistical differences were detected among some of

the diets tested. Our results showed that consumption of the artificial diet was significantly increased by the formation of cone-shaped diet capsules and the addition of sucrose, lipids, proline, and host plant material. However, we were unable to provide the appropriate oviposition cues.

Secondary compounds in the insect's host plant often stimulate oviposition. However, the incorporation of host plant material to the artificial diet failed to elicit ovipositional response by the weevil. By using freeze-dried tropical soda apple powder from flowers, buds, and leaves, we provided a source of protein and other nutrients that were not foreign to the insects. Yet, in the larger amounts tested, tropical soda apple actually was shown to be a deterrent. In order to promote oviposition on artificial diets, there needs to be an investigation of the nature of plant-related cues that promote egg-laying behavior. Failure to get maximum oviposition for adults may be explained by: 1) improper balance of nutrients, 2) low intake of food because of deficient phagostimulants essential for normal feeding, or 3) deterrents present in the diet that inhibit feeding. Even though *A. tenebrosus* survived on the improved Earle adult diet, efforts to improve both nutrient content and feeding stimulants should increase general vigor and perhaps oviposition.

Difficulties in rearing phytophagous insects on artificial diets may be due in part to the difficulties encountered in devising a medium that possesses a texture acceptable to an insect normally feeding on living plants (Beck et al. 1949). When an insect's natural food is unavailable, their willingness to accept an artificial food source is a test of its survivability (Haynes and Smith 1992). *Anthonomus tenebrosus* adults were able to survive on the artificial diet but did not fully accept the diet as a substitute for their natural host plant.

The artificial diet developed here for maintaining adults of *A. tenebrosus* in the laboratory failed to provide the conditions suitable for complete larval development. While the adult diet will require further refinements to improve oviposition, testing of diets for larvae has just begun. Testing of all 10 existing *Anthonomus* spp. diets will prove a good starting point before attempting any improvement modifications.

Finally, many new areas of investigation have been opened by findings of this research. These areas include isolation and chemical identification of both the deterrent and feeding stimulant in tropical soda apple apical leaves, flowers, and buds, and the use of this information in improving development, feeding, and oviposition on an artificial diet. Lastly, optimal feeding, growth, and reproduction of *A. tenebrosus* requires nutrient levels in the diet to be properly balanced; the optimal balance of diet components is generally adapted to the natural food of the insect (Chapman 1998). Chemical analyses of tropical soda apple apical leaves, flowers, and buds may provide clues to the proper proportion of amino acid, water, carbohydrate, lipid, fatty acid, sterol, sugar, mineral, and any potential token stimuli required in the artificial diet for optimal rearing of *A. tenebrosus*.

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BIOGRAPHICAL SKETCH

Bobbie Jo Davis was born and raised in Cincinnati, Ohio. She moved to Florida in 1996 to further her education. She earned her Associate in Arts degree from Pasco-Hernando Community College in 1998. She went on to earn a Bachelor of Science degree in wildlife ecology & conservation from the University of Florida in 2001. Taking some time to consider her career options, she worked a series of temporary jobs in varying environmental fields. One such temporary job was a six-week internship in the Entomology Department at the University of Florida rearing insects and studying artificial diets. This internship sparked her interest in insects and their role in the environment and eventually led her to pursue a Master of Science degree with emphasis on biocontrol. Upon graduating from UF, she hopes to obtain a fulfilling career in an environmental or animal related field.