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INVESTIGATIONS OF SMALL HIVE BEETLE-YEAST ASSOCIATIONS

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by

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ABSTRACT

The small hive beetle is an invasive pest of honey bees from Africa which has recently spread to the US and Australia. While not a serious pest of honeybees in its native range, the small hive beetle is a major problem for beekeepers its invasive range, frequently causing hives to abscond. Recently, a yeast (*Kodamaea ohmeri*) has been found associated with the small hive beetle. When growing on pollen, this yeast produces an odor blend that is very attractive to the beetle. It has been hypothesized that the presence of *K. ohmeri* may be a key factor in severity of small hive beetle infestations. This dissertation explores the connection between the small hive beetle and its yeast in three areas. The first area is the growth of *K. ohmeri* in bee hives. It has been hypothesized that the presence of *K. ohmeri* in the bee hive causes attraction and aggregation of the small hive beetle. However, no evidence has been provided that *K. ohmeri* is capable of multiplying in the hive environment when small hive beetle infestations are low. This dissertation, then, examines the factors that affect growth of *K. ohmeri* in the bee hive, including water activity, nutrient availability, the presence of beetles, and the presence of bees. This dissertation documents *K. ohmeri* is capable of growing on bee bread from bee hives without beetles present, and that water activity is important to the growth of *K. ohmeri* on bee bread and pollen. Beetle frass, which contains a high quantity of *K. ohmeri*, may be the source of yeast inoculum. The second area of research concerns the specificity of the interaction between *K. ohmeri* and the small hive beetle. Several species of yeast occur naturally in bee hives or in beekeeper-added pollen supplements. This dissertation reports that these yeasts, as well as other yeasts associated with beetles and other insects are capable of producing beetle-attractive volatiles and attracting small hive beetle adults, implying that the small hive beetle's relationship with *K. ohmeri* is not an exclusive one, and the small hive beetle may be attracted to naturally occurring yeasts growing on pollen in the bee hive. Finally, the third area of research concerns the effect of *K. ohmeri* on the oviposition of the small hive beetle. Though *K. ohmeri* has been shown

to increase reproduction of small hive beetles on pollen or oranges, the mechanism for this increased growth is unknown. This dissertation finds that pollen fermented by *K. ohmeri* is a preferred oviposition substrate for small hive beetle females, though fermentation by *Saccharomyces cerevisiae* or *K. ohmeri* did not increase the rate of ovary development over that of control pollen. Preference was most likely mediated by gustatory or olfactory cues in the pollen. Beetles switched ovipositional preference to control pollen after 24 hour hours of oviposition. This switch in preference appeared to be caused by changes in the *K. ohmeri*-fermented substrate, since adding fresh *K. ohmeri*-fermented pollen after 24 hours restored preference. The presence of yeast in the hive may influence small hive beetle host selection and beetle reproduction in bee hives. In addition, this dissertation provides initial data on the mating biology of the small hive beetle, including first descriptions of mating behavior and reproductive structures. Initial data gathered in this study indicates that small hive beetles may require an extended mating period in order to be fertile, providing an opportunity for control by mating disruption strategies.

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"…A creature's pain by small or great;

The greatest being

Can have but fibres, nerves, and flesh,

And these the smallest ones possess,

Although their frame and structure less

Escape our seeing."

Thoughtless Cruelty by Charles Lamb

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Chapter 1

Introduction and Literature Review

"Where the bee sucks, there suck I"

Shakespeare, *The Tempest*

Abstract

The small hive beetle (*Aethina tumida* Murray) (Coleoptera: Nitidulidae) is a parasite and scavenger of honey bee hives. In its native range of sub-Saharan Africa, it is a minor pest, but since it was introduced to the US and Australia it has become a major pest, rapidly infesting hives and causing hives to collapse. Effective control options for this pest are few. More basic research is needed to understand why the small hive beetle is a pest and how it can be controlled. The small hive beetle morphology and life cycle is similar to other nitidulid beetles, but is uniquely adapted to life in the bee hive. The small hive beetle can feed and reproduce on many alternative diets, but consistently prefers to inhabit bee hives, though bee hives are presumably well-guarded. Honey bees can corral adult beetles into beetle prisons and remove eggs and larvae, but these resistance mechanisms appear to be largely ineffective against the small hive beetle. Imprisoned beetles can even solicit food from guard bees. Many nitidulids are associated with fungi, and the small hive beetle is no exception. The yeast *Kodamaea ohmeri* has been found consistently associated with the small hive beetle and produces beetle attractants including honey bee alarm pheromone components when growing on pollen. The purpose of this dissertation, then, is to describe the relationship between the small hive beetle and its yeast by answering the following research questions: 1) What are the factors effecting growth of *K. ohmeri* in the bee hive? 2) How

specific is the relationship between the small hive beetle and *K. ohmeri*? 3) Does *K. ohmeri* affect ovipositional preference or ovary development of the small hive beetle?

Introduction

The small hive beetle (*Aethina tumida* Murray) (Coleoptera: Nitidulidae) is a parasite and scavenger of western honey bee (*Apis mellifera*) hives. While in its native range of sub-Saharan Africa, it is a minor pest (Lundie 1940; Schmolke 1974), in the United States and Australia it has become a serious pest, rapidly infesting hives and causing hives to abscond (Ellis et al. 2003b; Elzen et al. 1999b; Elzen et al. 1999a; Hood 2000; Neumann et al. 2010). One mysterious aspect of the small hive beetle's biology is its tendency to aggregate in some hives or apiaries, causing major damage, while leaving other hives or apiaries relatively alone (Schmolke 1974; Spiewok et al. 2007). Since hive phenotype, including characteristics such as shade or open location, colony size, amount of brood, honey, and pollen, varroa control, hive entrance size, and screened bottom boards, does not seem to have any effect on the number of beetles infesting a colony (Ellis et al. 2003a; Ellis and Delaplane 2006; Neumann et al. 2010; Spiewok et al. 2008; Spiewok et al. 2007), it is thought that beetle aggregation is chemically mediated (Neumann and Elzen 2004; Spiewok et al. 2008; Spiewok et al. 2007). Several small hive beetle attractants have been identified. These include volatile compounds produced by honey bees (Graham et al. 2011b; Suazo et al. 2003; Torto et al. 2005), bumble bees (Graham et al. 2011b), honey bee and bumble bee colony components (Graham et al. 2011b; Suazo et al. 2003; Torto et al. 2005), fruit (P. Teal, personal communication), cider vinegar (Nolan and Hood 2008), and pollen fermented by a yeast carried by the small hive beetle (Benda et al. 2008; Nolan and Hood 2008; Torto et al. 2007c; Torto et al. 2007a). Interestingly, pollen fermented by beetle yeast produces an odor blend which contains honey bee alarm pheromone components such as isopentyl acetate and 2-heptanone

(Benda et al. 2008; Torto et al. 2007c; Torto et al. 2007a). The yeast responsible for this attractive blend has been identified as *Kodamaea ohmeri* (NRRL Y-27634) (Benda et al. 2008). This yeast has since been discovered in bumble bee hives as well (Graham et al. 2011a). It has been hypothesized that the presence of *K. ohmeri* in the bee hive causes attraction and aggregation of adult small hive beetles (Benda et al. 2008; Spiewok et al. 2007; Torto et al. 2007c). In addition, the presence of this yeast appears to increase small hive beetle reproduction (Arbogast et al. 2010; Arbogast et al. 2009). However, there are many unanswered questions about the small hive beetle and its relationship with *K. ohmeri*. Questions include: 1) What are the factors affecting growth of *K. ohmeri* in bee hives and production of attractants that could cause beetle aggregations? 2) Is the relationship between *K. ohmeri* and small hive beetle exclusive, or are other yeasts, such as those already present in the bee hive, capable of attracting the small hive beetle as well? 3) *K. ohmeri* has been documented to increase reproduction of the small hive beetle, but what is the mechanism for increased reproduction?

In this review, we will examine the literature surrounding the small hive beetle and its relationship with the yeast *K. ohmeri*. We will begin with a discussion of the small hive beetle's early history in Africa and subsequent worldwide range expansion and rise in status as a major pest of honey bees. Then we will examine in detail the adaptations and other factors that may be to blame for the small hive beetle's pest severity, including association with *K. ohmeri*. We will summarize what is known about the small hive beetle and the yeast *K. ohmeri*, making reference to other nitidulid-fungal interactions. Finally, we will suggest new lines of inquiry to understand the relationship between *K. ohmeri* and the small hive beetle and move toward more effective control methods for the small hive beetle.

The small hive beetle, not the large hive beetle: Identification and early description

In 1867 Andrew Murray described a new species of Nitidulid, *Aethina tumida*, from two dried specimens out of west Africa (Murray 1867). It wasn't until 1940, however, that A. E. Lundie described the biology of this beetle invading honey bee hives and named it the small hive beetle, to distinguish it from another hive beetle, *Oplostomus haroldi*, a large scarab that also invades honey bee hives in sub-Saharan Africa. Lundie described the small hive beetle as a nuisance pest of honey bee hives, particularly in weakened hives or stored honey supers, where feeding and frass of beetle larvae fermented the honey. Lundie did not consider the small hive beetle a major pest, and even mused about the beetle's advantages in preventing spread of American foulbrood to South Africa by scavenging dead or abandoned hives.

In 1974, *Aethina tumida* again received a modicum of attention when M.D. Schmolke, a student at the University of Rhodesia, produced a thesis on the small hive beetle. Schmolke carried out a number of experiments on the life cycle of the small hive beetle, its food requirements, distribution, methods of trapping adults and larvae, and control of larvae in the soil. Schmolke, like Lundie, emphasized that the small hive beetle was only a pest problem on weak hives or stored equipment. Again, the small hive beetle vanished into obscurity.

No further studies were published about the small hive beetle until 1998, when they were found destroying European honey bee hives in St. Lucie county, Florida (Elzen et al. 1999b; Sanford 1998). Later, it was discovered that the small hive beetle had been present in the United States since at least 1996, when it was intercepted but unidentified in Charleston, South Carolina (Hood 2000). From the southeast, where the majority of the US obtains bee packages every spring, the small hive beetle spread into 25 states by 2002 (Evans et al. 2003), and can now be found throughout the eastern United States, southern California, Hawaii, and in isolated locations in Canada (Connor 2011; Neumann and Ellis 2008). The small hive beetle has now established

itself in Australia as well, with disastrous results for beekeeping there (Gillespie et al. 2003; Neumann et al. 2010; Spiewok et al. 2007).

Pest status

Interest in the small hive beetle has increased since its introduction into the US, resulting in over 100 new papers on *Aethina tumida* published since 1998. This interest has been warranted. In the US and Australia, the small hive beetle has proven a much more serious pest than in Africa (Spiewok et al. 2007). Beetle populations can reach extremely high densities in European honey bee hives and can cause the collapse of entire apiaries (Sanford 1998). In 1998, beekeepers in Florida lost an estimated $3 million dollars to the small hive beetle (Ellis et al. 2002c). In a recent survey of winter colony losses, of 5,441 US beekeepers, 3.7% of the beekeepers who reported colony losses listed the small hive beetle as a major cause of colony mortality (VanEngelsdorp et al. 2012). In addition to colony losses, the small hive beetle has been shown to negatively affect flight activity and brood area of European honey bees (Ellis et al. 2003b). When combined with infestations of the parasitic mite *Varroa destructor*, the small hive beetle can significantly affect the thermoregulation of winter clusters of honey bees (Schäfer et al. 2011). Beetles can also directly harm bees by consuming eggs and brood (Ellis et al. 2002c; Elzen et al. 2000b; Schmolke 1974).

The small hive beetle is extremely mobile and has expanded its range with alarming speed since its introduction into the US. In Australia, infestations of the small hive beetle have been especially severe (Neumann et al. 2010; Spiewok et al. 2007). The small hive beetle now threatens to expand its range into Europe and New Zealand if more effective control methods are not found (Cuthbertson et al. 2008; Mostafa and Williams 2002; Murilhas 2004). Disturbingly,

the small hive beetle is also a biological vector of honey bee viruses associated with colony collapse disorder (Eyer et al. 2009).

Current control methods for small hive beetle

There are very few effective control methods that have been developed for the small hive beetle. Frustrated beekeepers are continually inventing new traps and treatments, but research has not yet proven the majority these control methods effective. Few pesticide treatment options exist. With the recent discussion of the possible side-effects of pesticides on honey bees, more research is needed to find effective biocontrol methods for the small hive beetle. Here we will discuss popular control methods and their effectiveness.

Sanitation

Sanitation has been the primary method of small hive beetle control for African beekeepers and is probably one of the simplest control methods for beekeepers outside of Africa as well. In the first description of the biology of the small hive beetle, Lundie (1940) advocated honey house sanitation as a key to controlling small hive beetle. Beekeepers in Africa mostly fear the beetle's effect on stored honey supers, so rapid extraction and rendering of wax cappings prevents the accumulation of material for the beetles to feed upon in the honey house. Lundie (1940) also noted that long-established apiaries in Africa were more likely to harbor high populations of the small hive beetle due to the beekeeper's failure to remove equipment left behind by absconded or dead hives. Schmolke (1974) echoed Lundie's (1940) assertion that honey house and apiary sanitation was a key to controlling small hive beetles. In a more recent

study by Spiewok et al. (2007), high beetle infestations were found in apiaries adjacent to honey houses with poor sanitation and high populations of beetle larvae.

Genetic resistance

The resistence of African subspecies of honey bees to small hive beetle is well known and will be discussed further later. Importation of African honey bee stocks is clearly not a viable control option for US and Australian beekeepers. The resistance of africanized bees to small hive beetle is currently unknown. However, hygienic strains of European bees are known to be resistant to other parasites such as varroa (Harbo and Harris 2005; Rinderer et al. 2003), and may offer some protection from small hive beetle as well. Russian honey bees, selectively bred for hygienic resistance to varroa, tracheal mites, and brood diseases, have been shown to have some resistance to small hive beetle when compared with parental Italian honey bee stock (Frake et al. 2009; de Guzman et al. 2010). The reason for the Russian honey bees' increased resistance is probably due to their effective removal of small hive beetle eggs and larvae, though Italian honey bees are also capable of removing eggs and larvae (de Guzman et al. 2008; de Guzman et al. 2006). Further research is needed to determine if small hive beetle resistant honey bee stocks will be an effective control method.

Chemical control methods

Shortly after the discovery of the small hive beetle damaging hives in Florida, an emergency-use label was issued for CheckMite +® (10% coumaphos, Bayer Corp.) to control small hive beetle in hives (Hood 2000). This product is contained in a plastic strip which is stapled underneath a piece of corrugated cardboard placed on the bottom to the hive. The small

crevices of the corrugated cardboard are alluring to the small hive beetle as a hiding place. When adult beetles enter these spaces, they contact the CheckMite® strip and die (Elzen et al. 1999b). A similar pesticide-filled crevice trap has been designed using fipronil-treated corrugated cardboard (Levot 2008). Pesticide-treated crevice traps have been shown to reduce adult small hive beetle populations, though many small hive beetles may avoid exposure because they hide in other parts of the hive and never contact the strip (Neumann and Hoffmann 2008). CheckMite can only be applied when bees are not producing a honey crop and is less effective at low temperatures when small hive beetles are inactive (Hood 2000). Beekeepers have modified the design of these crevice traps by loading easily obtained CD jewel cases with a pesticide solution containing fipronil and/or boric acid, however, the effectiveness of these home-brewed pesticide traps has not been thoroughly evaluated.

The small hive beetle can also be controlled by the addition of pesticides to the soil around the hive. The product labeled for this use is Gard Star® (40% permethrin, Y-Tex Corp.). Wandering larvae which exit the hive to search for a suitable pupation site contact the insecticide in the soil and die, effectively reducing the next generation of small hive beetles (Hood 2000). Several other chemicals have been tested for control of wandering larvae. Schmolke (1974) found that two organochloride insecticides and salt water were capable of killing wandering larvae in the soil. Lime and diatomaceous earth may also be effective against wandering larvae at high concentrations (Buchholz et al. 2009). However, soil treatments will only be effective when appropriately timed to when larvae are migrating (Pettis and Shimanuki 2000).

Other chemicals which have been tested against the small hive beetle are organic acids (acetic acid, formic acid, lactic acid, oxalic acid) and thymol. These products are already approved for use in bee hives to control varroa mite and wax moth. While some of these chemicals showed promising activity against the small hive beetle in laboratory studies, in field studies they failed to provide effective control (Buchholz et al. 2011; Schäfer et al. 2009).

Preliminary experiments with several other chemical insecticides and insect growth regulators have shown that the small hive beetle is very susceptible to these insecticides (Kanga and Somorin 2011). Certainly these merit further investigation for the development of new chemical control methods. New chemicals will provide more control options, but until the problem of chemical placement to maximize contact with the small hive beetle is resolved, chemical control methods will continue to be largely ineffective.

Attract-and-kill trapping

Trapping has been used as an effective control method for other nitidulid beetles (Bartelt and Hossain 2006; Hossain et al. 2006; James et al. 1998). Typically, these traps are baited with a combination of a fermenting substrate such as bread dough or rotten fruit and nitidulid aggregation pheromones. For the small hive beetle, no aggregation pheromone has been found, but the small hive beetle can be trapped with several baits, including pollen fermented by *Kodamaea ohmeri* (Arbogast et al. 2007; Nolan and Hood 2008; Torto et al. 2010a), cider vinegar (Nolan and Hood 2008), and fruit (Eischen et al. 1999). Some in-hive traps have been shown to significantly reduce adult beetle numbers (Nolan and Hood 2008; Torto et al. 2007b). These traps have no non-target effects, are inexpensive, and require no chemical pesticides. However, as these traps require frequent checking and re-baiting, they are not yet practical for commercial beekeeper use.

Alternative hive entrances

Use of alternative hive entrances for small hive beetle control has also been investigated. The hypothesis behind this control method is that a narrow, tube-shaped entrance will allow

guard bees to more effectively prevent the ingress of hive invaders like the small hive beetle. There has been some indication that these alternative entrances may be effective in reducing the number of adult small hive beetles in the hive (Ellis et al. 2002a). However, entrance reduction has side-effects of decreasing brood production and increasing debris and water build up in the hive, side-effects which are only partially mitigated by the use of a screened bottom-board (Ellis et al. 2003a; Ellis et al. 2002a). Another study found no effect of alternative entrances on small hive beetle populations, but confirmed the side-effects on brood production (Hood and Miller 2005). At this time, alternative entrances are not recommended as a small hive beetle control measure.

Life cycle of the small hive beetle

Eggs and larvae

Despite the wealth of recent studies on the small hive beetle and its critical status as an invasive pest with few viable control options, many questions remain unanswered about the basic biology of the small hive beetle. The life cycle of the small hive beetle, however, has been described reasonably well. The eggs are pearly white, 1.4 mm long by 0.26 mm wide, laid in small crevices in irregular masses (Lundie 1940). Larvae hatch from their eggs via a longitudinal slit. Larvae are white to yellow, with regularly spaced orange spines along the body. The head and legs are also orange. The small hive beetle has three larval instars which differ in size, but little in appearance (de Guzman and Frake 2007), except for neonates, whose heads are particularly large for their bodies and bear more spines or hairs than older larvae (Lundie 1940). The time spent in the egg and larval period is affected by temperature (de Guzman and Frake 2007; Meikle and Patt 2011). However, Lundie, Schmolke, and recent studies disagree about the

length of time spent in the egg and larval period, even at the same temperatures (de Guzman and Frake 2007; Haque and Levot 2005; Meikle and Patt 2011; Muerrle and Neumann 2004). Humidity, which is important for egg viability, may play a role in larval development (Ellis and Ellis 2010). It is clear that the small hive beetle grows quickly at temperatures between 25°C and 34°C, hatching at a minimum of 21 hours and developing within as little as 4.5 days to their maximum larval weight (Meikle and Patt 2011). The minimum temperature for egg survival and larval development is 10°C to 13°C (Meikle and Patt 2011). Larval weight and development is also highly affected by competition between larvae (Meikle et al. 2012). However, cannibalism does not appear to occur among crowded larvae (Meikle et al. 2012).

Wandering stage and pupation

After achieving their maximal weight, small hive beetle larvae cease feeding and begin a wandering stage, seeking a place to pupate. Wandering larvae have been noted to wait until early evening to emerge from the hive, possibly to avoid predators (Schmolke 1974). Larvae can travel long distances (at least 30 meters) in search of moist, loose soil as a pupation site (Schmolke 1974). Larvae fail to pupate in dry soils, and seek to pupate at soil depths that maintain soil moisture even during dry periods (Ellis et al. 2004b; Torto et al. 2010a). The wandering stage can extend for more than a month if larvae are unable to find a suitable pupation site (Ellis et al. 2004b; Schmolke 1974). Once burrowed into the soil at a depth of 3 to 25 cm (de Guzman et al. 2009; Pettis and Shimanuki 2000; Torto et al. 2010a), larvae construct a pupation cell, smoothed with a saliva-like secretion (de Guzman and Frake 2007). Pre-pupae appear short and stout compared with wandering larvae (de Guzman and Frake 2007; Schmolke 1974). Small hive beetles spend 16-23 days in the soil, the females developing slightly faster than males (Ellis et al. 2004b; Schmolke 1974). The pearly white pupae mature from all-white to various stages in which

eyes darken first, followed by mandibles and wings (Schmolke 1974). Teneral adults are light brown, darkening gradually over time (Lundie 1940).

Adults

After emergence, small hive beetle adults are positively phototactic, possibly to facilitate their emergence from the soil (Schmolke 1974). Newly emerged adults have been observed to linger in the soil as if waiting to attain sexual maturity or some host cue to trigger dispersal (Cuthbertson et al. 2008; de Guzman and Frake 2007). One anecdotal report from Lundie alleges that newly emerged beetles disperse in swarms (Tribe 2000), though no further evidence of this behavior has been collected. Location of a suitable hive as a host appears to be preceded by long-range dispersal (Neumann et al. 2012; Spiewok et al. 2008). Selection of a host hive is likely chemically mediated, and may be influenced by the presence of yeast in the hive (Benda et al. 2008; Graham et al. 2011b; Neumann et al. 2012; Torto et al. 2007c).

Mating and oviposition

Little is known about the mate location or mating behavior of the small hive beetle. Beetles have been observed to mate in the hive as well as in laboratory rearing conditions (Neumann et al. 2001a; Neumann et al. 2001c). No pheromones for mate location have been reported from the small hive beetle, though many nitidulid beetles produce aggregation pheromones (Bartelt and Hossain 2010). It is thought that host-locating cues may be equivalent to mate-finding cues for the small hive beetle (Benda et al. 2008). Oviposition has been reported to begin within 24 hours to 7 days post-emergence (de Guzman and Frake 2007; Lundie 1940). Female small hive beetles alternate between periods of egg laying and inactivity, laying eggs for

up to 4 months at an average rate of 13 eggs per female per day (Schmolke 1974). However, total eggs produced by a single female varies considerably, from zero to 4,000 eggs in a lifetime (Arbogast et al. 2010; Meikle and Patt 2011).

Pest adaptations

Clearly, the small hive beetle is capable of rapid population growth due to its high fecundity and short life cycle. In Africa, the small hive beetle is capable of up to 5 generations in a single year (Lundie 1940). Even in strong hives where mass reproduction of beetles is hindered by the presence of bees, cryptic, low-level reproduction of small hive beetles is possible (Arbogast et al. 2012; Spiewok and Neumann 2006a). The pest severity of the small hive beetle on European bees is due to a multitude of additional factors. These factors, which we will examine in detail, include pest adaptations of the beetle: its anatomy, host manipulation, diet flexibility, and overwintering behavior. Other factors are also at work, including the beetle's invasive nature and differences between European and African honey bee races.

Anatomy of the small hive beetle

The small hive beetle is built like a small tank. A smooth, limuloid body shape coupled with the ability to withdraw its appendages and grip tightly onto a surface make it almost impossible to dislodge the small hive beetle (Neumann and Elzen 2004). Indeed, while adult small hive beetles are frequently attacked by worker bees, they are rarely ejected from the hive (Atkinson and Ellis 2011b; Elzen et al. 2001; Schmolke 1974). Small hive beetle larvae and eggs are less fortunate. These are quickly eaten or jettisoned from the hive by workers, even when hidden in capped cells containing honeybee pupae (Ellis and Delaplane 2008; Neumann and

Härtel 2004; Schmolke 1974). For this reason, the small hive beetle typically lays its eggs in crevices of the hive (Lundie 1940; Schmolke 1974). In strong hives, larvae likewise keep to the edges and bottom of the hive to avoid ejection (Schmolke 1974; Spiewok and Neumann 2006a).

In regards to adult body shape, the small hive beetle is quite ordinary among the Nitidulidae (Parsons 1943) and well adapted to surviving hive defenses. Several other nitidulid species have been found inhabiting honey bee hives (Ellis 2008; Neumann and Ritter 2004). None of these appear to reproduce on hive components such as honey, pollen, and brood (Ellis 2008; Neumann and Ritter 2004), though a cryptophagid beetle has been observed reproducing at a low level in bee hives in Jordan (Haddad 2008). That these beetles are tolerated in the hive is curious, since honey bee hives rarely host symbionts (Ellis and Hepburn 2006; Kistner 1982). These other beetles which have a similar anatomy to the small hive beetle are able to invade bee hives but not successfully reproduce, so we must look to other adaptations of the small hive beetle and its host to understand why the small hive beetle is so successful where others fail (Ellis and Hepburn 2006).

Host manipulation

One striking adaptation of the small hive beetle is its ability to extort food from honey bee workers through trophallaxis. This behavior has been observed numerous times when beetles are confined in beetle "prisons" lined with propolis and guarded by worker bees (Ellis et al. 2002d; Neumann et al. 2001b). Beetles mimic the behavior of other worker bees soliciting trophallaxis, and are frequently rebuffed by their prison guards. The occasional success allows adult beetles to survive their imprisonment to escape later when hive defenses relax. It is unknown how exactly the small hive beetle accomplishes this host manipulation (Ellis 2005). Other parasites of social insects frequently make use of chemical mimicry in addition to

behavioral mimicry to avoid host defenses. It is unknown if the small hive beetle uses chemical mimicry in addition to behavioral mimicry (Ellis and Hepburn 2006), though beetle-associated yeast *K. ohmeri* may affect bees since it produces honey bee alarm pheromone components (Benda et al. 2008; Torto et al. 2007c). Honey bees recognize the small hive beetle as an invader and attack it more often than other insects that do not threaten the hive, so presumably the small hive beetle does not produce chemicals to appease hive defenders and prevent attacks (Atkinson and Ellis 2011b). In fact, the exact opposite has been suggested – that the small hive beetle elicits confinement behavior in order to be fed by honey bees (Atkinson and Ellis 2011a).

Diet flexibility

In addition to adaptations to evade hive defenses and manipulate their hosts, the small hive beetle has demonstrated the ability to avoid eradication by feeding on alternative diets when honey bee hives are unavailable. The small hive beetle has been known to reproduce on diets of grapes (Arbogast et al. 2009; Buchholz et al. 2008; Schmolke 1974), oranges (Arbogast et al. 2009), grapefruit (Eischen et al. 1999), banana (Buchholz et al. 2008; Keller 2002), mango (Buchholz et al. 2008; Keller 2002), pineapple (Keller 2002), avocado (Eischen et al. 1999), Kei apples (Ellis et al. 2002c), cantaloupe (Arbogast et al. 2009; Eischen et al. 1999; Keller 2002), artificial diet for *Manduca sexta* (Keller 2002), pollen substitute patties (Hood 2009), and sugar-vegetable shortening patties (Elzen et al. 2002; Westervelt et al. 2001). In addition, during cryptic, low-level reproduction in strong bee hives, the small hive beetle may be able to subsist on hive debris (Spiewok and Neumann 2006a). Indeed, the Nitidulidae is a family with diverse dietary habits, including many scavenging species with little diet specificity (Habeck 2002). While the small hive beetle is able to reproduce on these alternative diets in the absence of honey bee hives, they still maintain a preference for hive components and reproduce more on such diets

(Arbogast et al. 2010; Buchholz et al. 2008; Ellis et al. 2002c). Diets which include *K. ohmeri* are especially beneficial (Arbogast et al. 2010; Arbogast et al. 2009), though it is unclear if *K. ohmeri* has a direct effect on ovary development or oviposition in the small hive beetle.

The small hive beetle is also able to infest bumble bee (Ambrose et al. 2000; Neumann et al. 2012; Spiewok and Neumann 2006b; Stanghellini et al. 2000) and stingless bee colonies (Greco et al. 2010; Halcroft et al. 2011; Mutsaers 2006; Neumann et al. 2012). The small hive beetle's catholic tastes ensure that it is not hindered by the constant moving of hives in commercial beekeeping operations. In Florida, field populations of small hive beetles may be trapped at considerable distance from existing managed and feral colonies (Arbogast et al. 2009). However, it is unknown to what extent the small hive beetle makes use of alternative diets in the field. Even if small hive beetles fail to reproduce on alternative diets in the field, adults may persist on hive remnants. Adult small hive beetles can live for 5 months on comb with honey alone, and over a month on empty brood comb (Ellis et al. 2002c).

Overwintering behavior

The small hive beetle's resilience extends beyond their dietary flexibility. Even though the small hive beetle is native to the warm climate of sub-Saharan Africa, it is capable of overwintering with honey bees in more temperate climates (Pettis and Shimanuki 2000; Schäfer et al. 2011; Schäfer et al. 2010). Larvae cannot tolerate low temperatures and can be controlled in stored equipment by freezing (Hood 1999). The minimal temperature for larval development has been calculated to be 10°C (Meikle and Patt 2011). The small hive beetle adult, however, enters thermoregulatory honey bee clusters as the temperature descends (Atkinson and Ellis 2012). Beetles will even share cells with honey bee workers (Ellis et al. 2003c). It is unclear how the small hive beetle evades honey bee defenses during clustering, though recent research indicates

that less than 17% actually succeed in overwintering in strong honey bee clusters (Schäfer et al. 2010). In weakened hives, as many as 300 small hive beetles have been observed occupying a winter cluster of bees (Pettis and Shimanuki 2000). The small hive beetle is certainly capable of surviving in cold climates, and may expand its established range to include Canada, where importation of bees from Australia has allowed the small hive beetle to gain a foothold (Clay 2006; Lounsberry et al. 2010).

Invasive species

We have examined many factors that cause the small hive beetle to be, in some ways, an ideal pest species. A high reproductive rate, rapid dispersal, dietary and climatic flexibility, and parasitic host manipulation all contribute to their great success in their introduced range. Like many invasive species, however, the small hive beetle is at best a minor pest in its native range (Elzen et al. 2000a; Lundie 1940; Schmolke 1974; Spiewok et al. 2007). Lack of competitors and natural enemies are often cited for the severity of invasive pest species (Lockwood et al. 2007). In the case of the small hive beetle, few other honey bee nest parasites exist to compete with them. The large hive beetle, *Oplostomus haroldi*, is also known to occupy the same hives and is attracted to similar host volatiles, but no evidence has been presented that these species are in competition (Torto et al. 2010b). The bee louse, *Braula* sp., likewise co-exists in bee hives and its larvae also feed on pollen in the hive (Hepburn and Radloff 1998a). Though the bee-louse is found throughout Africa, its interaction with the small hive beetle is unknown. It is also unknown to what extent the small hive beetle interacts with the wax moth, *Galleria mellonella*, a frequent co-inhabitant of weak honey bee hives, which, unlike the other potential competitors, may be found in their invasive range as well (Lundie 1940). More research is needed to understand the interaction between the small hive beetle and its competitors in the hive.

Exotic species often escape natural enemies that keep their populations in check in their native range (Keane and Crawley 2002; Torchin et al. 2003). Little is known about the natural enemies of the small hive beetle (Lundie 1940; Schmolke 1974). In lab colonies, storage mites (*Caloglyphus hughesi*) can feed on all stages of the small hive beetle (Strauss et al. 2010), and *Aspergillus* sp. can infect wandering larvae and pupae (Ellis et al. 2004d). Unidentified pathogenic fungi have also been known to interfere with lab rearing (Lundie 1940; Muerrle and Neumann 2004). Small hive beetle larvae are also susceptible to generalist entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* (Muerrle et al. 2006). Entomopathogenic nematodes are capable of infecting wandering larvae and pupae as well (Cabanillas and Elzen 2006; Ellis et al. 2010). Recently, field populations of the small hive beetle in Mississippi have been found naturally infected with unidentified entomopathogenic nematodes (de Guzman et al. 2009). The only documented natural predator of the small hive beetle in Africa is the ant *Pheidole megacephala*, which preys upon wandering larvae (Torto et al. 2010a). More research is needed to discover other predators, parasites, or pathogens providing biological control of the small hive beetle in their native range.

European honey bees versus African honey bees

Probably the most important factor contributing to the pest severity of the small hive beetle is their introduction to a new host subspecies. The western honey bee, *Apis mellifera*, is composed of many diverse subspecies. Common African honey bee subspecies in the small hive beetle's native range include *scutellata*, *capensis*, *monticola*, *litoria*, and *adansonii*. In its introduced range in the US and Australia, European honey bee subspecies *mellifera*, *linguistica* and *carnica* are common. These subspecies have very different behavior and biology. In general, the African subspecies are more aggressive, hoard fewer resources such as honey and pollen, and

abandon their hives or migrate much more frequently than European subspecies (Hepburn and Radloff 1998b). These characteristics, which we will examine in detail, make the African subspecies more resistant to the small hive beetle.

Aggressive behavior

Aggressive behavior is one of the most striking adaptations of the African subspecies of the western honey bee, especially for beekeepers inspecting their hives. The aggressive behavior of these subspecies is not limited to their tendency to sting beekeepers, however. African subspecies are considered very efficient in defending their hive from predators, removing hive pests, and addressing other threats to hive health such as pathogens (Fazier et al. 2010; Hepburn and Radloff 1998a; Human et al. 2011). When it comes to the small hive beetle, African subspecies react more strongly to adult beetles than European subspecies (Elzen et al. 2001), though both African and European subspecies remove eggs and larvae at high efficiency (Ellis et al. 2004a; Ellis and Delaplane 2008; Neumann and Härtel 2004), and both confine adult beetles in beetle prisons (Ellis 2005; Ellis et al. 2004c; Ellis et al. 2003d; Neumann et al. 2001b). Since both African and European subspecies exhibit similar defensive behaviors toward the small hive beetle, it is thought that a difference in degree rather than kind accounts for behavioral resistance of African subspecies to the small hive beetle (Ellis et al. 2003d).

Reduced hive stores

African subspecies, due to the warm climate and their tendency to migrate or abscond frequently, typically do not store as much honey or pollen as European subspecies which must

store large quantities of honey to survive the winter (Hepburn and Radloff 1998c). The small hive beetle tends to reproduce well on such stored provisions, so it is likely that reduced hive stores prevents massive reproduction of the small hive beetle on African subspecies (Lundie 1940; Neumann and Elzen 2004; Schmolke 1974). Quantity of hive stores has not been shown to correlate with beetle populations in individual hives (Spiewok et al. 2007). However, the quantity of abandoned hive stores after absconding would directly affect the quantity of small hive beetles produced in the next generation (Neumann and Elzen 2004).

Absconding and migration

Along with lower quantities of hive stores in general, African subspecies reduce the amount of food available to scavenging small hive beetles by practicing "prepared absconding" or migration. Absconding is a behavior almost entirely unique to African subspecies (Hepburn et al. 1999; Hepburn and Radloff 1998c). Unlike reproductive swarming, during absconding the entire hive, including the queen, will vacate the hive. There exists a great deal of variation in absconding behavior, ranging from sudden and unprepared events which leave brood and hive stores behind to planned events where the queen ceases egg laying and the hive waits for developing brood to emerge before absconding (Hepburn and Radloff 1998c). It is thought that the latter form of absconding, especially when it occurs in concert with seasonal patterns of food availability, should be considered migration (Hepburn and Radloff 1998c; McGlynn 2012; McNally and Schneider 1992). Such migratory nest movement could help break the life cycle of the small hive beetle, or at least force the beetle to find alternative diets (Neumann and Elzen 2004), since little food remains in the abandoned hive.

All of these differences between European and African honey bee subspecies may contribute to the overall low beetle infestations in African honey bee subspecies compared with

European subspecies. The beetle has been introduced into a few areas with extensive Africanization (bees which are hybrids between African and European subspecies) (Neumann and Ellis 2008). However, it is still unknown what effect the small hive beetle has on Africanized bees. An additional factor that is likely to play a role is that European honey bees, along with other pollinators, are experiencing declines world-wide due to a variety of causes, some of which are still unknown (Carreck and Neumann 2010; Dainat et al. 2012; VanEngelsdorp et al. 2012; 2007).

Association with *Kodamaea ohmeri* as another possible pest adaptation

We have examined many factors that contribute to the small hive beetle's severity as a pest of honey bees. An additional factor that demands investigation is the small hive beetle's association with a yeast, *Kodamaea ohmeri*. When growing on pollen, this yeast has been found to produce beetle attractants (Benda et al. 2008; Torto et al. 2007c; Torto et al. 2007a). Interestingly, many of the attractants produced by *K. ohmeri* are identical or similar to honey bee alarm pheromone components (Torto et al. 2007c; Torto et al. 2007a). The importance of this yeast association is still unknown. First we will review the literature existing on the yeast *K. ohmeri* and its association with the small hive beetle and then examine other systems to understand some general patterns of nitidulid-fungal interactions in order to inform further research.

Small hive beetle and *K. ohmeri*

K. ohmeri was first reported by Torto et al. (2007b), as a fungal associate of the small hive beetle producing beetle attractants when growing on pollen. This study actually began as an

effort to discover aggregation pheromones that might be produced by the small hive beetle. Previous work showed that the small hive beetle was attracted to volatile compounds from honey bees and hive products (Elzen et al. 1999b; Suazo et al. 2003; Torto et al. 2005). Aggregation of the small hive beetle in bee hives, however, suggests that the small hive beetle uses aggregation pheromones which are also commonly produced by other nitidulids (Neumann and Elzen 2004). Torto et al. (2007c) reported that indeed, the small hive beetle was attracted to other small hive beetles on honey bee comb. Combs occupied by honey bees were no more attractive to the small hive beetle than comb occupied by other small hive beetles. Bee-collected pollen dough fed upon by the small hive beetle was also attractive. But rather than an aggregation pheromone, these findings led the researchers to identify a yeast found on small hive beetle larvae which produced beetle-attractants when growing on pollen. This suggestive study showed that the small hive beetle is extremely sensitive to volatile components of the yeast-fermented pollen blend, including isopentyl acetate, 2-heptanone, methyl benzoate, and alarm pheromone mimics such as ethyl esters. So sensitive, that the small hive beetle antennae could detect honey bee alarm pheromone isopentyl acetate at a much lower level than honey bees themselves. A later follow-up study (Torto et al. 2007a) showed that beetle attractants were produced by pollen-honey mixture fed upon by beetles for 1-7 days, regardless of beetle sex. After 14 days of feeding, attraction was reduced, likely due to the presence of 2-phenylethanol in beetle frass.

Small hive beetle-conditioned pollen-honey mixture has since been successfully used to monitor for beetles using aerial traps (Arbogast et al. 2007). Pollen dough fermented by pure culture of *K. ohmeri* has been used in the same manner (Arbogast et al. 2009). Pollen dough fermented by *K. ohmeri* has also been used successfully to trap small hive beetles from honey bee hives in the US and Africa (Nolan and Hood 2008; Torto et al. 2010a; Torto et al. 2007b). There is indication that such traps can provide control of the small hive beetle (Nolan and Hood 2008;

Torto et al. 2007b). Researchers have obtained a patent for use of in-hive traps baited with *K. ohmeri*-fermented pollen dough (Teal et al. 2006).

The yeast *K. ohmeri* appears to be quite attractive to the small hive beetle and certainly has potential for use as a trapping lure. However, the attraction of the small hive beetle to yeast has also suggested several hypotheses to explain the unique aspects of hive infestations by the small hive beetle. *K. ohmeri* growing on pollen stores in the hive has been hypothesized to attract small hive beetles to hives (Benda et al. 2008; Torto et al. 2007c), influence small hive beetle aggregation in certain hives over others (Benda et al. 2008; Spiewok et al. 2007; Torto et al. 2007c), increase absconding behavior by producing alarm pheromones (Benda et al. 2008; Torto et al. 2007c), and increase reproduction of the small hive beetle (Arbogast et al. 2009; Benda et al. 2008). Presence of *K. ohmeri* in bumble bee hives uninfested by the small hive beetle (Graham et al. 2011a) fits with the hypothesis that both bees and beetles could be responsible for initial inoculation of hive stores with *K. ohmeri*, followed by growth of yeast on hive stores, which would presumably attract more small hive beetles, creating an aggregation effect. The yeast *K. ohmeri* has also been shown to increase small hive beetle reproduction on different diets (Arbogast et al. 2009), though it is unknown whether this increase in reproduction on yeast-inoculated diets is due to increased oviposition on inoculated diets or increased larval survival as in the case of pollen versus orange diet (Arbogast et al. 2010). This reproductive benefit also fits with the hypothesis that *K. ohmeri* is important for the massive reproduction of the small hive beetle in weak or absconded hives (Benda et al. 2008).

These hypotheses regarding the relationship between the small hive beetle and *K. ohmeri* demand further research. If *K. ohmeri* is indeed as important to the small hive beetle as these hypotheses assume, control of *K. ohmeri* could provide an effective method of small hive beetle control. Without waiting for a verdict on the exact role of *K. ohmeri* in infestations of the small hive beetle, some researchers have already begun to investigate methods of controlling *K. ohmeri*.

These initial experiments suggest that acetic acid and formic acid, already used to protect bee hives from wax moth and *Varroa* mite, may have potential for controlling *K. ohmeri* as well (Schäfer et al. 2009).

Fungal associations among the Nitidulidae

Understanding the relationship between *K. ohmeri* and the small hive beetle and its role in beetle infestations is of major importance to beetle control. If we turn to the existing literature on insect-fungal associations, we find that insects are associated with an amazing array of fungi. Their interactions are likewise diverse, exhibiting the full range of pathogenic, parasitic, and mutualistic relationships (Blackwell and Vega 2005). If we restrict ourselves to relationships between fungi and beetles in the Nitidulidae, we find a consistent positive relationship, at least for the beetles. Here we will examine these relationships in more detail.

Nitidulids are a diverse group of beetles with an extraordinary range of feeding habits. Mycetophagy is considered to be the ancestral state for the entire group (Habeck 2002; Kirejtshuk 1997; Lawrence 1991), but modern nitidulids feed on fungi, carrion, flowers, fruit, fermenting tree sap, various aphids or scale insects, decaying plant parts, stored grains, and several are inhabitants of ant and bee nests (Habeck 2002; Lawrence 1991; Parsons 1943). Many nitidulids are associated with yeasts and other characteristic fungi (Lachance et al. 2001; Lawrence 1991; Miller and Mrak 1953; Suh et al. 2006). However, the nature of these associations is often not well understood (Suh and Blackwell 2005; Vega and Dowd 2005). Here we will discuss three nitidulid-fungal interactions which have received attention in the literature.

Nitidulids and Ceratocystis *spp.*

Oak mat wilt fungus *Ceratocystis fagacearum* and pineapple disease of sugarcane *Ceratocystis paradoxa* are well known nitidulid-vectored plant pathogens. Nitidulids are significantly attracted to these fungi in the field and consume the fungi itself as well as transmitting it to new hosts (Chang and Jensen 1974; Kyhl et al. 2002; Lin and Phelan 1992; Skalbeck 1976). *C. fagacearum* produces a very different volatile blend than other non-insect-vectored fungi from a similar environment, and attracts significantly more nitidulids than these other species (Lin and Phelan 1992), apparently by mimicking fruity volatiles released by ripening or fermenting fruits. Chang and Jensen (1974) showed that nitidulid larvae reared on cane juice diet inoculated with *C. paradoxa* performed better than those reared on cane juice diet alone, indicating that fungus-feeding beetles derive a nutritional benefit.

The relationship between *Ceratocystis* spp. and nitidulids may be mutualistic, but it is a facultative rather than obligate relationship. The beetles which transmit *Ceratocystis* spp. fungi can live on other fruit and plant substrates (Lin and Phelan 1992). *Ceratocystis* spp. can also be transmitted by other beetles and *Drosophila* sp. (McMullen et al. 1960).

Nitidulids and Fusarium *spp.*

Nitidulids are also important vectors of mycotoxin-producing fungi (*Fusarium* spp.) on corn. *Fusarium* spp. were consistently isolated from larvae, pupae, and newly emerged adults of a nitidulid *Glischrochilus quadrisignatus* feeding on corn ears buried by tillage, indicating that nitidulids could be important in re-inoculation of new crops with *Fusarium* pathogens (Windels et al. 1976).

Nitidulids are attracted to *Fusarium moniliforme* (Hepperly and Rodriguez-Cancel 1987) and *Fusarium verticillioides* (Ako et al. 2003) on corn. Like other nitidulid-fungal associates, *F. verticillioides* produces typical nitidulid attractant volatiles, as well as some additional phenolic compounds (Bartelt and Wicklow 1999). These compounds attract *C. humeralis* and *C. dimidatus* which vector the fungus (Ako et al. 2003; Bartelt and Wicklow 1999; Zilkowski et al. 1999). *F. verticillioides* also increases fecundity of *C. dimidiatus* and shortens larval development time on corn (Ako et al. 2003). *C. dimidiatus* has an extraordinary ability to detoxify mycotoxins associated with *Fusarium*, suggesting that this species has adaptations specifically for feeding on *Fusarium*-infected corn (Dowd and Van Middlesworth 1989).

Nitidulid-*Fusarium* interactions appear to follow the same pattern as interactions with *Ceratocystis* fungi. The fungi provide nutrition for the beetles while the beetles provide transmission to new substrates for the fungi. The relationship is not obligate, since nitidulids which vector *F. verticilliodes* can feed on a variety of other diets. *F. verticilliodes*, likewise, can be transmitted by other insects such as *Helicoverpa armigera* and *Ostrinia nubilalis* (Darvas et al. 2011). Though the relationship is facultative, *C. dimidatus*'s ability to detoxify mycotoxins seems to indicate some level of coevolution between nitidulids and *Fusarium* spp.

Nitidulids are also capable of transmitting *Aspergillus* spp., another mycotoxin-producing group of fungi on corn (Lussenhop and Wicklow 1990). Little is known about this interaction, but apparently *Aspergillus flavus* produces beetle toxins in its sclerotia which protect these overwintering structures from feeding by *C. hemipterus* (Wicklow et al. 1988). These toxins are not produced in the mycelia or conidia, facilitating dispersal by beetles which feed on these structures while protecting vital overwintering inoculum.

Nitidulids and yeasts

Yeasts are a ubiquitous group of unicellular fungi which reproduce by budding. However, many yeasts can switch between single-celled budding phases to pseudohyphal or hyphal phases (Sudbery et al. 2004). Yeasts are important symbionts for a number of insects, including *Drosophila* spp. (Gilbert 1980; Starmer and Fogleman 1986) and many beetles such as the cigarette beetle, *Lasioderma serricorne* (Dowd and Shen 1990), and wood-feeding ambrosia beetles (Batra 1966; Batra 1963), as well as several other species of beetles feeding on trees (Crowson 1981). These mutualisms can take on many forms, but frequently the benefit to the insect is micronutrient enrichment, detoxification of plant allelochemicals and/or digestion of complex polysaccharides (Martin 1987; Vega and Dowd 2005), while the benefit to the yeast is transmission to new hosts (Ganter 2006; Gilbert 1980).

Crop-damaging nitidulids in the genus *Carpophilus* are consistently attracted to yeast-inoculated fruit and grain substrates (Blackmer and Phelan 1991; Dowd and Bartelt 1991; Miller 1952; Miller and Mrak 1953; Nout and Bartelt 1998; Wildman 1933). The volatiles responsible for nitidulid attraction are a blend of common fermentation volatiles including ethanol, acetaldehyde, ethyl acetate, 1-propanol, 2-methyl-1-propanol, 3-methyl-1-butanol, and 2-methyl-1-butanol (Bartelt and Hossain 2010). These attractive volatiles overlap with those produced by ripening fruits, so it is difficult to determine if beetles are attracted to yeasts or to fruit, since the presence of yeasts on fruit may simply amplify the existing volatile signal of the fruit (Phelan and Lin 1991). Nout and Bartelt (1998) hypothesize that naturally-occurring yeasts on corn may be the reason for increased attraction of nitidulids to damaged ears of corn, where yeasts have proliferated.

Beetle preferences for specific yeasts are influenced by a combination of the sugar composition of the substrate and fermentation capabilities of the yeasts. Nout and Bartelt (1998)

found that, of the 49 species of yeasts tested, yeasts which were capable of fermenting sugars found in corn typically produced nitidulid-attractive volatiles. Irregular distribution of these sucrose and maltose-fermenting yeasts has been proposed to account for the aggregation of nitidulid beetles in certain damaged ears of corn but not in other nearby damaged ears (Nout et al. 1997). *Carpophilus hemipterus* shows a preference for *Saccharomyces cerevisiae*-inoculated substrates over *Candida krusei*-inoculated substrates (Blackmer and Phelan 1991). This difference may be attributed to the ability of *S. cerevisiae* to ferment a larger variety of sugars than *C. krusei* (Suihko and Mäkinen 1981). Bread dough or other sugar source fermented with *S. cerevisiae* attracts a broad range of nitidulids (Bartelt and Hossain 2010; Blackmer and Phelan 1995; Skalbeck 1976). Skalbeck (1976), for example found that *S. cerevisiae* was a superior attractant for 10 species of nitidulids found in deciduous forests in Minnesota.

Attractancy of general yeast volatiles is synergized by the presence of nitidulid aggregation pheromones, which exhibit cross-attraction of species (Bartelt and Hossain 2010; Bartelt and Hossain 2006; James et al. 1998; Kyhl et al. 2002; Lin and Phelan 1991). Blackmer and Phelan (1992), citing cross-attraction of acorn-specialist nitidulid *Stelidota octomaculata* and fruit generalist *Stelidota geminata* to fruit, fungal, and acorn volatiles, have suggested that nitidulids may be chemical 'generalists.' Specialization, they contend, is in the beetle's behavior such as propensity to fly to food volatiles, rather than in its response to specific host volatiles (Blackmer and Phelan 1992; Blackmer and Phelan 1995).

While it is well established that many nitidulids respond to volatiles produced by yeasts, further information about the relationship between nitidulids and yeast is difficult to come by. Miller and Mrak (1953) showed that yeasts were digested in the gut of *C. hemipterus*, providing evidence that these beetles were both attracted to yeasts and using yeast for food. Some authors have contended that polyphagous nitidulids are in fact feeding on the yeasts associated with various fruits and other substrates rather than the fruits themselves (Dowd and Bartelt 1991;

Miller 1952; Miller and Mrak 1953). While no publications previous to recent studies of *K. ohmeri* and the small hive beetle (Arbogast et al. 2009) have documented increased growth or reproduction of nitidulids on yeast-inoculated substrates, Blackmer and Phelan (1991) hint that there is a benefit for *Carpophilus* larvae feeding on yeast-inoculated substrates.

These findings suggest a mutualistic relationship between yeasts and nitidulids, or at least a positive one for the nitidulids. The yeasts could derive a benefit from being spread to new hosts, but it is difficult to ascertain if the fungi actually derive a benefit in the absence of quantitative assessment. Unlike economically important plant pathogens, which cause significant damage to crops and require monitoring for disease incidence, yeasts are cryptic. Lack of specificity in these nitidulid-yeast relationships is evidence of a facultative relationship between the nitidulids and their associated yeasts. Unlike relationships between nitidulids and *Fusarium* spp. or *Ceratocystis* spp., however, it is unknown to what extent nitidulids associate with yeasts throughout their life cycle. It is possible that nitidulids, like many beetle species, are always associated with a characteristic yeast community of yet unknown function (Suh and Blackwell 2005).

Small hive beetle and *K. ohmeri*, directions for research

Most fungal associations within the Nitidulidae appear to have the hallmarks of mutualism. However, our understanding of nitidulid-yeast interactions is still limited. As we explore the relationship between *K. ohmeri* and the small hive beetle, three main areas of concern present themselves, which we will examine in detail.

Growth of **K. ohmeri** *in hives and attraction of the small hive beetle*

It has been hypothesized that *K. ohmeri* may play a role in attracting the small hive beetle to bee hives and causing aggregations of beetles. This hypothesis is very similar to that of Nout and Bartelt (1998), who posited that the irregular distribution of nitidulid-attractive yeasts on damaged ears of corn contributed to the aggregations of nitidulids observed in the corn field. Aggregation pheromones could explain the aggregation of multiple beetles on the same ear of corn, but presence of yeast may explain how beetles chose which ears of corn to initially infest. Likewise, the presence of *K. ohmeri*, either introduced to the hive by bees, beekeepers, contaminated equipment, or the small hive beetle, may influence initial attraction to a hive. Graham et al. (2011) have already documented the presence of *K. ohmeri* in bumble bee hives uninfested by the small hive beetle, lending evidence to the assertion that *K. ohmeri* could be transmitted in the absence of the small hive beetle. In fact, *K. ohmeri* is a yeast which has been isolated from a number of microhabitats, including flowers (Rosa et al. 1999), and may have multiple routes of transmission to bee hives. In order to test the hypothesis that *K. ohmeri* is important for small hive beetle attraction and aggregation, the factors necessary for growth of *K. ohmeri* in the hive environment must be assessed. Water activity, nutrient availability, competition with microbes in bee bread, beetle presence, and bee presence may all affect the growth of *K. ohmeri* in the hive and subsequent attraction of the small hive beetle.

Species specificity

Since *K. ohmeri* is the primary yeast isolated from the small hive beetle (Benda et al. 2008), and is identical with yeast associated with the small hive beetle in its native range (Torto et al. 2007c), research has naturally focused on this yeast species alone. Benda et al. (2008),

however, noted that several other fungi were isolated from the small hive beetle. The role of these fungi is still unknown. From what we have learned by examining the literature on the nitidulids and their interactions with yeast, we can assume that many other species of yeast may attract the small hive beetle. Various yeasts have frequently been isolated from bee bread in honey bee hives as well as in honey bee workers when under stress or fed antibiotics (Gilliam 1979; Gilliam et al. 1974; Gilliam 1973). Yeast is considered to play a role in the conversion of raw bee-collected pollen into bee bread, the primary source of protein for honey bees (Gilliam 1979). Yeast also contains important micronutrients which may be vital for honey bee nutrition (Pain and Maugenet 1966). Two species of yeasts may be expected to inhabit bee hives naturally. One is *Candida magnoliae*, which dominates the yeast flora of finished bee bread (Gilliam 1979). Bakers' or brewers' yeast *S. cerevisiae* is also present in the bee hive in the form of protein patties used as a food supplement. These patties have been noted to attract the small hive beetle in the hive and serve as an excellent larval food source (Hood 2009). However, high levels of fungicides can be found in bee bread of modern hives, which may reduce the number of yeasts in the hive (Mullin et al. 2010). A study of the range of the small hive beetle's attraction to yeasts will allow researchers to predict the effects of changing hive yeast species composition on small hive beetle populations.

Effects of **K. ohmeri** *on oviposition of the small hive beetle*

Small hive beetle reproduction can proceed rapidly and lead to extraordinarily high levels of adults and larvae, causing hives to abscond (Neumann and Elzen 2004; Wenning 2001). This rapid population buildup and "slime" in the hive has been associated with the presence of *K. ohmeri* (Benda et al. 2008). Arbogast et al. (2009) has reported a high level of beetle reproduction on *K. ohmeri*-inoculated pollen dough and oranges compared with uninoculated substrates. The

reason for this increased reproduction is unknown, however. As discussed above, fungi associated with nitidulids have a consistently positive effect on beetle growth and reproduction. Research on the ovipositional preferences of small hive beetle females and the influence of diet on ovary development will offer insight into the rapid population growth of small hive beetles in bee hives and may allow for the creation of control methods which target small hive beetle reproduction.

Conclusions

In conclusion, research on the relationship between *K. ohmeri* and the small hive beetle is a priority for controlling the small hive beetle and an opportunity understand beetle-yeast interactions. The small hive beetle is very unique. Though it is capable of reproducing on many alternative diets, the small hive beetle prefers to associate with well-guarded honey bee nests. Several beetles have transitioned from mycophagous to predatory feeding habits during their evolutionary history. Most of these predatory beetles from mycophagous lineages feed upon sap-sucking hemipteran insects such as aphids and scales. It has been hypothesized that fungi such as sooty molds associated with these prey insects are the connection between these two divergent feeding habits (Leschen 2000). If this pattern can be applied to small hive beetles, the evolution of their relationship with bees may have been influenced by their preference for fungi producing a chemical blend with similarities to honey bee alarm pheromone. If yeast formed the link, then, between the small hive beetle and honey bees, perhaps it will be the key to breaking the link between them.

Chapter 2

Factors affecting growth of *Kodamaea ohmeri* associated with the small hive beetle (*Aethina tumida*) in western honey bee (*Apis mellifera*) hives

"Stolen waters are sweet, and bread eaten in secret is pleasant."

Proverbs 9:17

Abstract

Kodamaea ohmeri is a yeast associated with the small hive beetle (*Aethina tumida*) which has been hypothesized to cause small hive beetle attraction and aggregation in bee hives. However, it is currently unknown if this yeast is capable of growing in bee hives apart from severe small hive beetle infestations. The effects of water activity, bee bread, and beetle presence on growth of *K. ohmeri* were assessed in the laboratory, while field experiments attempted to show increased attraction of the small hive beetle to bee hives inoculated with yeast in both Pennsylvania and Kenya. Water activity had a strong effect on yeast growth, allowing growth on pollen to occur at 0.9 but not at 0.7 A_w. *K. ohmeri* was able to grow on bee bread without beetles present, and beetles were effective inoculators of the bee bread. Beetle frass, however, contained 10 times more yeast per gram than the inoculated bee bread. Small hive beetles carry *K. ohmeri* throughout their life cycle, so even newly emerged beetles may be capable of transmitting *K. ohmeri*. Field experiments were inconclusive, but it is possible to conclude that high humidity and high beetle populations offer the optimal conditions for growth of *K. ohmeri* in bee hives.

Introduction

The small hive beetle (*Aethina tumida* Murray) (Coleoptera: Nitidulidae) is a parasite and scavenger of honeybee (*Apis mellifera*) hives. While it is a minor pest in its native range of sub-Saharan Africa (Lundie 1940; Schmolke 1974), in the United States and Australia it has had a serious impact, rapidly infesting hives and causing bees to abscond (Elzen et al. 1999b; Elzen et al. 1999a; Neumann et al. 2010). One mysterious aspect of the small hive beetle's biology is its tendency to aggregate in some hives or apiaries, causing major damage, while leaving other hives or apiaries relatively unharmed (Neumann and Elzen 2004; Schmolke 1974; Spiewok et al. 2007). Hive phenotype and habitat do not correlate with small hive beetle numbers (Ellis and Delaplane 2006; Spiewok et al. 2007), so it is thought that beetle aggregation is chemically mediated, perhaps by aggregation pheromones produced by the beetles (Neumann and Elzen 2004; Spiewok et al. 2007). In an effort to find these aggregation pheromones or other chemical cues that can cause aggregations of beetles, several small hive beetle attractants have been identified. The beetles are attracted to volatile compounds produced by honey bees, bumble bees, honey bee and bumble bee colony components (Graham et al. 2011b; Suazo et al. 2003; Torto et al. 2005). However, they are also attracted to fruit (P. Teal, personal communication), and pollen fermented by a yeast carried by the small hive beetle (Benda et al. 2008; Torto et al. 2007c; Torto et al. 2007a). Interestingly, pollen fermented by beetle yeast produces an odor blend which contains honeybee alarm pheromone components such as isopentyl acetate and 2-heptanone (Benda et al. 2008; Torto et al. 2007c; Torto et al. 2007a). The yeast responsible for this attractive blend has been identified as *Kodamaea ohmeri* (NRRL Y-27634) (Benda et al. 2008). This yeast has since been discovered in bumble bee hives as well (Graham et al. 2011a). Since no aggregation pheromones have been discovered, and *K. ohmeri* is a consistent small hive beetle symbiont, it has been hypothesized that the presence of *K. ohmeri* in the bee hive causes attraction and

aggregation of adult small hive beetles in certain hives over others (Benda et al. 2008; Spiewok et al. 2007; Torto et al. 2007c). Currently, however, it is unknown to what extent this yeast colonizes honey bee hives without a high infestation of small hive beetles.

Colonization of bee hives by *K. ohmeri* is potentially affected by factors in two general categories: general yeast growth factors and system-specific factors. Yeast growth, like that of many microorganisms, is highly moisture-dependent. Ambient humidity varies by location, climate, weather, and season, which will affect the water activity of any unsealed substances in the bee hive. However, honey bees are known to regulate humidity in their nests at least to some degree (Human et al. 2006), so the appropriate humidity may already be present in a well-maintained bee hive. Water activity, defined as the relative humidity of the air above a substance at a given temperature is a standard measurement of how susceptible a substance is to spoilage by microbial growth (Scott 1957). Yeasts usually require a water activity of at least 0.8 to grow, though some osmophilic species have been observed to grow at water activities as low as 0.62 (Corry 1978). The growth response of *K. ohmeri* at different water activities is currently unknown.

Nutrient availability is likewise, an important factor governing the growth of all microorganisms including yeasts. Pollen is a heterogeneous, nitrogen-rich food source for bees (Haydak 1970; Herbert and Shimanuki 1978; Stanley and Linskens 1974). Bees rarely consume pollen directly, but instead convert it into bee bread (Herbert and Shimanuki 1978; Pain and Maugenet 1966). Bee bread differs from bee-collected pollen in acidity due to the activity of lactobacilli, which retards the growth of spoilage microorganisms but allows for the growth of yeasts (Gilliam 1979; Herbert and Shimanuki 1978; Loper et al. 1980; Standifer et al. 1980; Vásquez and Olofsson 2009). *K. ohmeri* produces the most beetle attractive volatiles while growing on pollen compared with other substrates (Benda et al. 2008; Torto et al. 2007c; Torto et

al. 2007a), but the ability of *K. ohmeri* to grow on bee bread is currently unknown, since existing studies have made use of bee-collected pollen and commercially available pollen patties (5% bee-collected pollen) rather than bee bread.

In addition to water activity and nutrient availability, colonization of bee hives by *K. ohmeri* may be affected by several system-specific factors. Currently, it is unknown if small hive beetles harbor yeast throughout their life cycle or if it is acquired from the environment after emergence. If the small hive beetle carries the yeast throughout its life cycle, it is likely to be able to transmit it much more effectively than if it is acquired later. Fungi which are vectored by an insect, such as *K. ohmeri* vectored by the small hive beetle, are continuously spread to new food sources and may even derive benefit from the insect's feeding behavior (Gilbert 1980). Since *K. ohmeri* is associated with major infestations of small hive beetle and the accompanying gooey "slime" which coats the frames, it is possible that *K. ohmeri* benefits from the presence of the beetle (Benda et al. 2008). The effect of bees on the growth of *K. ohmeri* in the bee hive is also unknown. *K. ohmeri* has been reported in bumble bee hives uninfested by the small hive beetle, and may thus be transmitted by bees (Graham et al. 2011a). The honey bee hive is an environment with a unique microbial flora which is maintained in balance by antimicrobials in honey, propolis, pollen as well as symbiotic microbes carried by the bees themselves (Anderson et al. 2011). Though relatively little is known about the regulation of microbial flora of honey bee hives, it is thought that a hive with the appropriate microbial balance could be very difficult for rogue microbes such as *K. ohmeri* to invade (Anderson et al. 2011; Gilliam 1997).

The purpose of this study, then, is to explore the factors affecting the colonization of bee hives by *K. ohmeri*, including general yeast growth factors of water activity and nutrient availability on bee bread, and system specific factors of beetle presence and the natural hive environment including honey bees.

Methods

Water activity tests

Kodamaea ohmeri isolated from the small hive beetle was obtained from the ARS culture collection (NRRL accession no. Y-27634) and maintained in 15% glycerol at -80°C. Yeast was transferred from glycerol stocks into sterile sabouraud dextrose broth with 1% yeast extract (SDBY) (Atlas 2004) and incubated in a shaker-incubator at 28°C to a high cell density corresponding to a colony count of approximately 1×10^5 cfu/ml prior to use in water activity tests.

Sterile pollen was prepared by gamma irradiating (10kGy) commercially available bee-collected pollen (Brushy Mountain, Moravian Falls, NC) at the Penn State Radiation Science and Engineering Center. Sterile pollen was mixed with sterile distilled water to a cookie-dough consistency then rolled out into a 3 mm-thick patty between two sheets of ethanol-washed acetate film. Rounds measuring 1cm in diameter were cut out using a ethanol-washed cork-borer and soft touch forceps. Rounds were transferred to individual 1.5-ml sterile eppendorf tubes placed in racks. Racks of tubes containing pollen dough rounds were placed into humidity chambers in an incubator at 28°C. Inside these humidity chambers, a saturated solution of either sodium chloride (70% humidity) or potassium sulfate (90% humidity) in distilled water was used to control the interior humidity. The solution filled the bottom 1.5 inches of the chamber, and a wire rack was used to elevate the tubes above the solution. Humidity was checked using a temperature and humidity logger (HOBO) set to record the temperature and humidity once every hour throughout the experiment. Tubes of pollen dough were left with lids open and the water activity of the pollen dough was allowed to equilibrate with that of the chamber over 24 hours. After this period of equilibration, yeast was added at a concentration of 3×10^4 cfu per tube. Samples were taken at

0, 12, 24, and 48 hours after yeast was added to pollen. Four samples were taken for each treatment and time point. To quantify yeast present in the samples, 500µl of sterile saline (0.085% NaCl) was added to each tube and the entire contents of the tube was homogenized with a vortexer. When all the pollen was in suspension, 200µl of the mixture was transferred to a sterile 96-well plate which was used to carry out multiple serial dilutions in sterile saline (Thiaucourt and Dí Maria 1992). From the 96-well plate, 7µl droplets of each dilution of each sample were plated in a grid pattern on selective media (acidified yeast-malt agar with 0.35% sodium propionate added). Plates were inverted and placed in an incubator at 28°C. Yeast colonies were counted 36 to 48 hours after plating. When possible, yeast quantity was calculated using the dilution where the colonies numbered no less than 3 and no more than 300 and where the colony count agreed with the count of at least one other dilution in the series.

For statistical analysis a t-test was performed on the log-transformed yeast concentration at each time point.

Growth of *K. ohmeri* on bee bread

Kodamaea ohmeri isolated from the small hive beetle was prepared as above to obtain a homogenous liquid culture with a high cell density.

Frames containing bee bread were frozen at -20°C at the end of the 2009 field season from beehives which were previously uninfested by the small hive beetle. Push-in cages (similar to those used by Ellis and Delaplane (2008)) were constructed from wide-mouth mason jar rings (8.25 cm diameter). Rings were pushed into the combs and sealed with melted beeswax applied with an electric batik pen. Each push-in cage ring was closed with a wide mouth mason jar lid modified with a rectangular screened opening for ventilation and screwed down with 4 small screws through holes drilled into the lid and ring (Figure 2-1). Within each push-in cage, yeast

treatments were applied to every pollen-containing cell. Yeast treatments consisted of either the application of 10 µl of yeast solution of a known cell concentration in sterile saline or 10 µl sterile saline as a control. The resultant concentration of yeast was approximately 30 cfu/cell in the yeast treatments, as determined by plating. Frames were placed into an incubator at 33°C and 90-95% relative humidity. Samples were taken after 48 hours of incubation. Samples were taken by coring 8 pollen-containing cells with a clean, ethanol-washed cork-borer 4 mm in diameter. The cork-borer was washed in sterile water and ethanol between each sample. Cores of pollen were transferred to a 96-well plate where each sample was homogenized with a clean, ethanol-washed glass stir-bar. The glass stir-bar was washed in sterile water and ethanol between each sample. Pollen homogenate from each cell was serially diluted and plated as described above on acidified YM agar with 0.35% sodium propionate. Colony counts were quantified as above. This experiment was repeated twice for a total of 4 push-in cages and 4 bee bread frames per treatment.

The effect of beetles on yeast growth was tested by the following method. Within the experiment described above, additional push-in cages were set up with beetle treatments or beetles in addition to yeast. Beetle treatments consisted of the addition of 100 third-instar larvae. Samples were taken as above, except that prior to taking bee bread samples, larvae and any larval frass was aspirated from the surface of the bee bread. In addition, larval frass samples were collected from all cages and weighed to compare concentration of yeast in bee bread and beetle frass. Frass samples and bee bread sample weights were only taken from the second replicate experiment.

Log-transformed yeast concentration per cell at 48 hours was compared between treatments and replicates with a general linear model followed by Tukey-Kramer separation of means. Log-transformed concentration of yeast per gram of bee bread and frass was compared with a t-test.

Figure 2-1: Push-in cage used for studies of growth of *K. ohmeri* on bee bread.

Yeast quantification on beetles

Yeasts on small hive beetle adults and larvae were quantified in similar ways. Ten beetles were randomly selected from a pool of synchronously aged individuals and placed into 10 ml of sterile saline (0.85% NaCl) on ice. Beetles were then vortexed for 30 seconds in the saline. Beetles were removed from the saline with clean, ethanol-washed forceps and placed into 10 ml of 70% ethanol on ice. The saline wash was then collected and 200µl of the wash was transferred to a sterile 96-well plate which was used to carry out multiple serial dilutions in sterile saline for plating. After vortexing in 70% ethanol for 30 seconds, beetles were washed twice with 10 ml of sterile water to remove remaining ethanol. Beetles were exposed to ethanol for approximately 5 minutes total. Beetles were again washed in 10ml of sterile saline and this wash was transferred

to a sterile 96-well plate for serial dilution and plating to confirm that the yeast remaining on the exterior of the beetles was below the detection threshold. After washing, the beetles were transferred to 5ml of cold sterile saline in a glass tissue-grinder and ground until tissues were homogenized. Pieces of exoskeleton were filtered out and the homogenate kept on ice until transferred to a sterile 96-well plate for serial dilution and plating.

From the 96-well plate, 7µl droplets of each dilution of each sample were plated on selective media (acidified yeast-malt agar with 0.35% sodium propionate added) and quantified as above for bee bread samples. A total of 8 replicates consisting of 10 pooled individuals were carried out for second and third instar larvae. Pupae had 9 replicates, newly emerged adults had 11, while wandering larvae only had 4 replicates.

Mean yeast per insect internally and externally in each stage was analyzed using a general linear model after log transformation.

Field experiments

The growth of *K. ohmeri* in bee hives and attraction of the small hive beetle to yeast-treated hives was tested in two field experiments in Pennsylvania and Kenya.

Pennsylvania Beehives

A field in Mount Union, PA was subdivided into 2 mini-apiaries placed approximately 100 meters apart in order to minimize drift of worker bees between apiaries. Mini-apiaries contained 12 hives each. Each mini-apiary was surrounded by electric tape fencing (Kencove Inc.) electrified with a solar charger as a bear deterrent. Packages of bees were installed on new wooden hive bodies with beeswax-coated all-plastic frames. Bees were fed sugar syrup with

Fumagilin B (25mg/L Medivet) at the beginning of the season to prevent infection with the microsporidian *Nosema*, a common honey bee parasite. No effort was made to control for *Varroa* mites. One apiary was designated as the control apiary and one was designated as the yeast-treated apiary.

Pennsylvania bee hives and treatments

Yeast treatments were accomplished by incubating one frame with open brood and bee bread from each hive in a sealed hive box with several hundred hive beetles until frames were fully covered with beetle larvae, frass, and yeast-containing slime (2 weeks at 28°C). The beetle-infested frames were then removed from the incubator box and all of the adult small hive beetles were collected from the frames with an aspirator before the frames were transferred back into their original hives. In control hives, a single brood frame was removed from each hive and held at room temperature, then transferred back to the original hive. Presence of the yeast was verified by swabs of the frame surface plated on acidified yeast-malt extract agar with 0.35% sodium propionate added. Yeast identity was later confirmed with PCR primers specifically designed to detect *Kodamaea ohmeri* (Benda et al, unpublished). Adult lab-reared small hive beetles (approximately 200) were released the same day as the treated frames were re-introduced into the hives. The point of release was equidistant from the two treatment apiaries.

Pennsylvania data collected

In Pennsylvania, pieces of white corrugated plastic (Coroplast, Dallas TX) measuring 50 cm by 10 cm were used to trap and monitor beetles. The beetle traps were placed flat on the bottom-board so that they could be removed from the entrance swiftly and tapped into a light

colored plastic bin to count the number of beetles in the trap. After the beetles were counted, they were placed back in the hive. Bee bread of control and yeast-treated hives was sampled 1 week after treatments. For the control hives, bee bread was sampled by collecting 10 cores of pollen as above from two frames in the center of the hive. In yeast-treated hives, 10 cores were taken from two frames in the center of the hive and 10 additional cores were taken from the beetle-fermented frame introduced 1 week before. Collected bee bread was weighed, homogenized in 1 ml of sterile saline, then diluted and plated on YM agar. Yeast colonies were counted after 24 hours of growth at 28°C. Quantity of yeast present in yeast-positive samples from control and yeast-treated hives was compared with a t-test.

Kenya bee hives and treatments

In February 2009, 16 nucleus colonies of *Apis mellifera scutellata* were established at the icipe Duduville campus near Nairobi, Kenya. Hives had 3 to 9 frames occupied at the time of the experiment. Each hive was randomly assigned to one of four treatment groups: Yeast, Queenless, Yeast and Queenless, and Control, for a total of 4 hives per treatment. Yeast-treated hives were separated from untreated hives by at least 85 meters to minimize worker drift. For queenless treatments, the queens were removed from the hives at the time of treatment. For yeast treatments, a mix of commercially available pollen patty (Global Patties, Butte, Montana), water, and stored dry *Kodamaea ohmeri* isolated from the small hive beetle was incubated at 30 °C for 3 days before use. After fermentation, approximately 60 ml of the fermented pollen substitute was spread onto 1 frame in each of the yeast-treatment hives. Control hives were inspected at the same time as treatment hives to control for hive disturbance.

Kenya data collection

Beetles were counted in the hives before treatments and 1 week after treatments by the following method. First the hive was disassembled and all of the components were stacked on the lid. Beetles were first counted on the bottom board and removed with an aspirator. After all beetles were collected from the bottom board, the frames and sides of the hive body were inspected for beetles, followed by the inner cover and lid of the hive. After counting and removing all the beetles, the beetles were then released back into the hive. Data loggers (HOBO) were introduced into the hives to monitor temperature and humidity during the data collection period. Loggers were pushed into the wax of empty cells on a frame as near to the center of the hive as possible. A fine-meshed aluminum screen was glued over the humidity sensor and duct tape was added around the other openings in the logger housing to prevent bees from destroying the sensor with propolis. Yeast was quantified as above before treatments and 1 week after treatments. Differences in beetle numbers between the 4 treatments were analyzed with ANOVA.

Results

Water activity tests

Quantity of yeast in 70% and 90% humidity treatments (Aw 0.7 and 0.9, respectively) remained similar for 24 hours of fermentation. However, by 48 hours, the quantity of yeast per tube in the 70% humidity chamber decreased while the quantity of yeast per tube in the 90% humidity chamber had increased and was statistically different from the 70% humidity treatment ($p < 0.0005$). Figure 2-2 shows plots of the mean yeast quantity over time for 70% and 90% humidity treatments.

Growth of *K. ohmeri* on bee bread

After 48 hours of fermentation, yeast quantity in the sterile saline-treated control bee bread cells was still below the detection threshold in both replicates of the experiment. After 48 hours, the quantity of yeast in all treatments (except control) had increased. However, there were no significant differences in yeast quantity between yeast-, beetle-, and beetle-and yeast- treated bee bread cells (GLM replicate*treatment $p = 0.408$) (see Figure 2-3). Samples of larval frass from beetle- and beetle-and-yeast-treated cages contained a high level of yeast compared with bee bread ($p = 0.001$) (Figure 2-4).

Yeast quantification on beetles

Beetles were found to harbor yeast throughout their life cycle, including pupae and newly emerged adults. Figure 2-5 displays the result of quantification of both external and internal yeast on second-instar, third-instar, and wandering larvae, pupae, and newly emerged adults. Larvae of all stages harbored a significantly higher quantity of yeast per insect than pupae or newly emerged adults. Internal and external yeast quantities were similar in all stages except for wandering larvae, where external yeast surpassed internal yeast in number.

Pennsylvania field data

No beetles were detected in the hives until after the lab-reared beetles were released. One week after initial release, beetles were detected in both yeast-treated and control hives. At one week, the total number of beetles detected in the yeast-treated apiary was greater than that of the control apiary. Two weeks after initial release, there were more total beetles in the control hives than the yeast-treated hives. Table 1-1 presents this data in dot-plot form, where each dot

represents a single hive with a certain number of beetles. Both 1 week and 2 weeks after initial release of beetles, half of the hives in the yeast-treated apiary contained zero beetles. Statistical analysis of this data was impossible due to the low number of beetles recovered.

One week after introduction into the bee hives, yeast was still detected on fermented frames from 8 of 10 yeast-treated hives. Control frames from yeast-treated hives (frames that did not have yeast added directly but may have been inoculated indirectly by bees), in contrast, harbored little yeast one week after yeast introduction into hives. Only 1 of 10 yeast-treated hives showed any evidence of bees moving yeast from yeast-inoculated frames to these control frames. In the control apiary, yeast was detected in 5 of 13 hives, however, it is unknown if this yeast was introduced by beetles or bees. Quantity of yeast detected in control apiary hives was significantly less than the quantity of yeast recovered from fermented frames from the yeast-treated hives ($p = 0.03$), indicating that inoculation with *K. ohmeri* was successful.

Kenya Field data

Most hives experienced a decrease in beetles through the experiment (Figure 2-6). Treatment was not a significant factor influencing before-treatment and after-treatment beetle counts (ANOVA $p = 0.140$). Likewise, the amount of yeast quantified in the bee bread in the hives decreased between the first and second sampling (Table 2-3). In yeast-treated hives, yeast was detected in only 2 of the 8 hives one week after treatment. The initial yeast counts of Kenyan hives were, on average, lower than control yeast counts from Pennsylvania hives (t-test, $p = 0.019$). The temperature in Kenyan bee hives ranged from an average daily maximum of 34.9°C to an average daily minimum of 30°C. Humidity in Kenyan bee hives ranged from an average daily maximum of 69% to an average daily minimum of 42%.

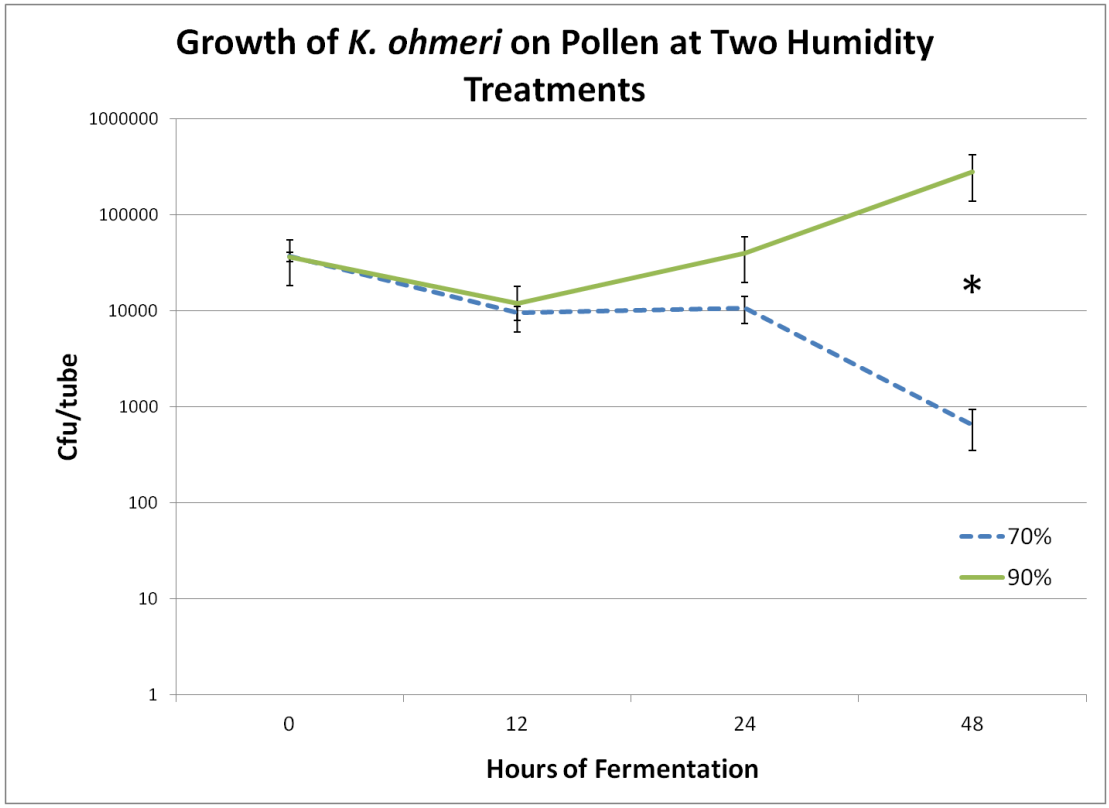

Figure 2-2: Growth of *K. ohmeri* on pollen at 70% or 90% humidity. Asterisk indicates significant difference between treatments at this time point ($N = 4$, $p < 0.0005$).

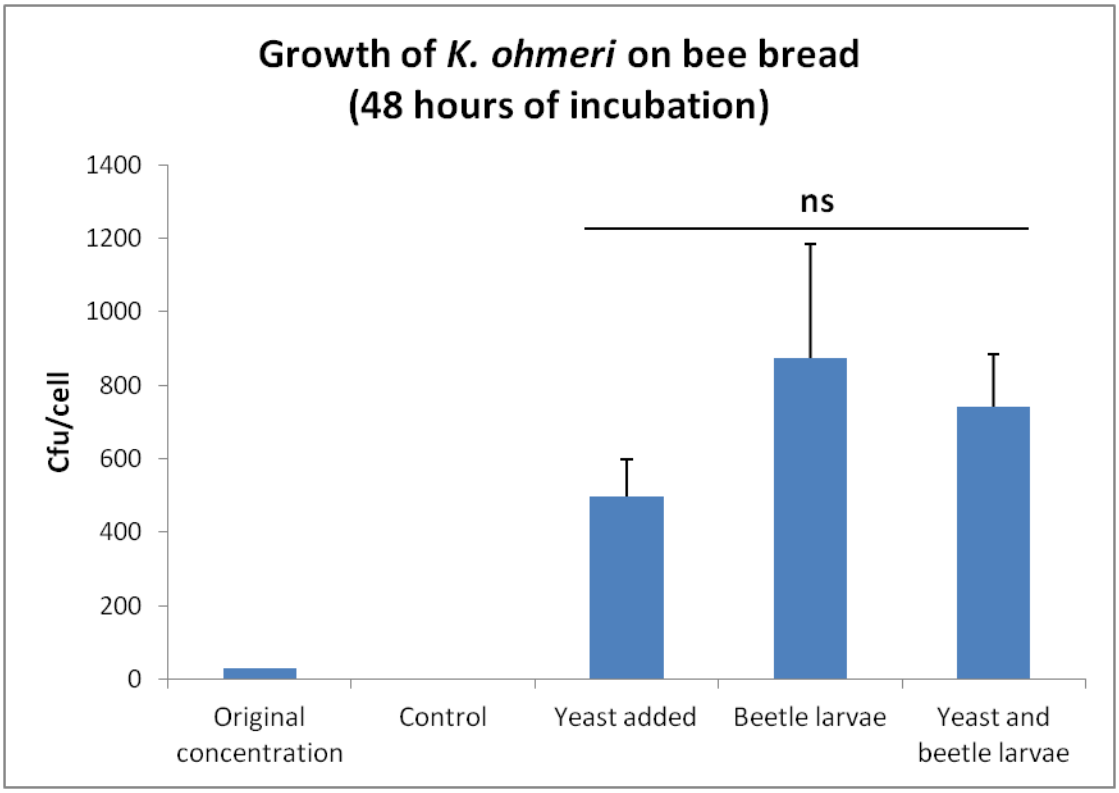

Figure 2-3: Growth of *K. ohmeri* on bee bread after 48 hours of incubation. Original concentration bar represents the 30 cfu/cell added at the beginning of the experiment. No yeast was detected in control cells treated with sterile saline. There was no significant difference between concentration of yeast in bee bread cells treated with 30 cfu/cell, 100 beetle larvae, or both yeast and beetle larvae (GLM $p = 0.408$).

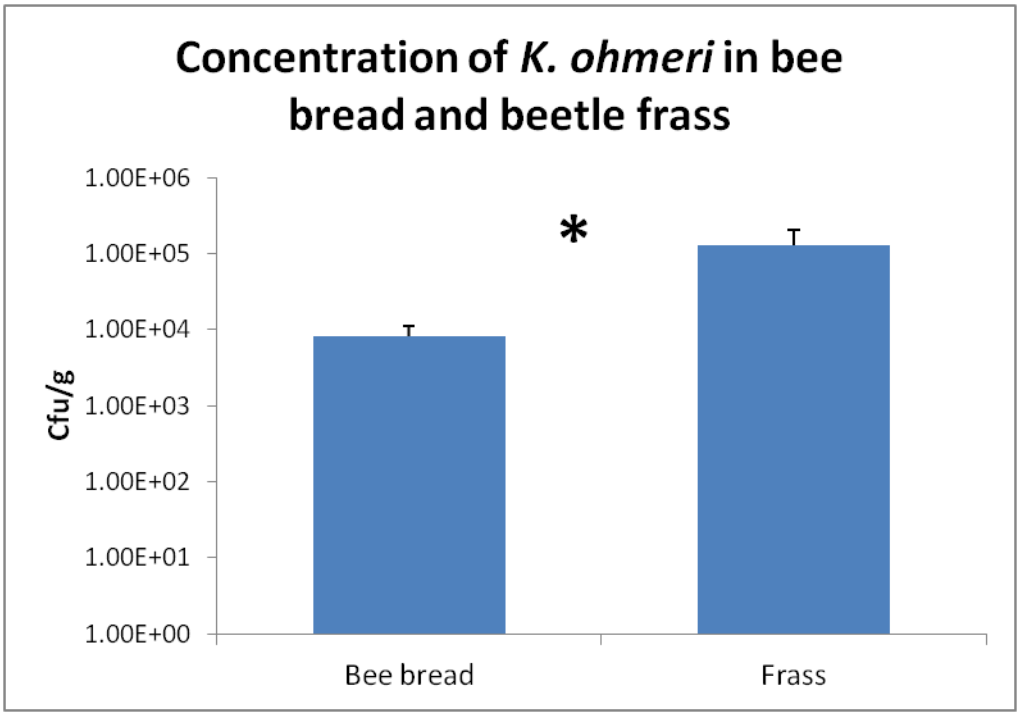

Figure 2-4: Concentration of *K. ohmeri* per gram of bee bread or frass. Concentration per gram of frass was significantly higher than concentration per gram of bee bread $p = 0.001$.

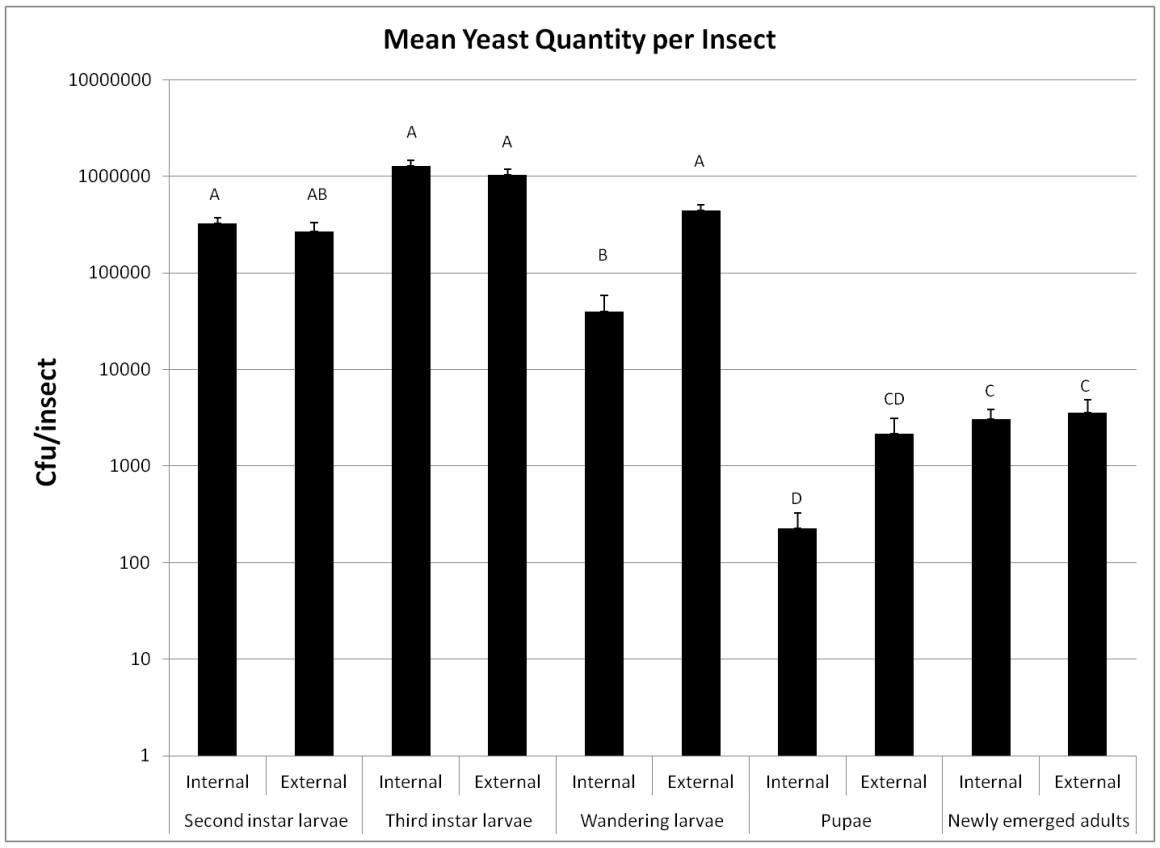

Figure 2-5: Mean yeast quantity per insect of various life stages of the small hive beetle. Bars with the same letter are not significantly different (GLM $p > 0.05$).

Table 2-1: Dot plot of number of beetles in control and yeast-treated hives 1 week and 2 weeks after release of lab-reared beetles in Pennsylvania field experiment. Each dot represents a single hive with a beetle count corresponding to the number to the left.

Week 1				Week 2			
Control		Yeast		Control		Yeast	
Number of beetles	Frequency	Number of beetles	Frequency	Number of beetles	Frequency	Number of beetles	Frequency
0	••••••	0	••••••	0	•	0	••••••
1	••••••	1	•••	1	•	1	••••
2		2	•	2	•••	2	
3		3		3	•	3	
4		4	•	4	••••	4	
5		5	•	5		5	
6		6		6		6	•
7		7		7	•	7	
8		8		8		8	
9		9		9		9	
10		10		10	•	10	

Table 2-2: Average yeast concentration per gram of bee bread (cfu/g) in Pennsylvania hives 1 week after introduction of fermented frames.

Control hives	**Yeast-treated hives**	
	Control frames	**Fermented frames**
$8.12 \times 10^2 \pm 3.93 \times 10^2$ **a**	4.37×10^3 *	$1.21 \times 10^5 \pm 8.5 \times 10^4$ **b**
5 of 13 samples positive for yeast	1 of 10 samples positive for yeast	8 of 10 samples positive for yeast
Means followed by different letters are significantly different by t-test ($p < 0.05$). Asterisk indicates single data point.		

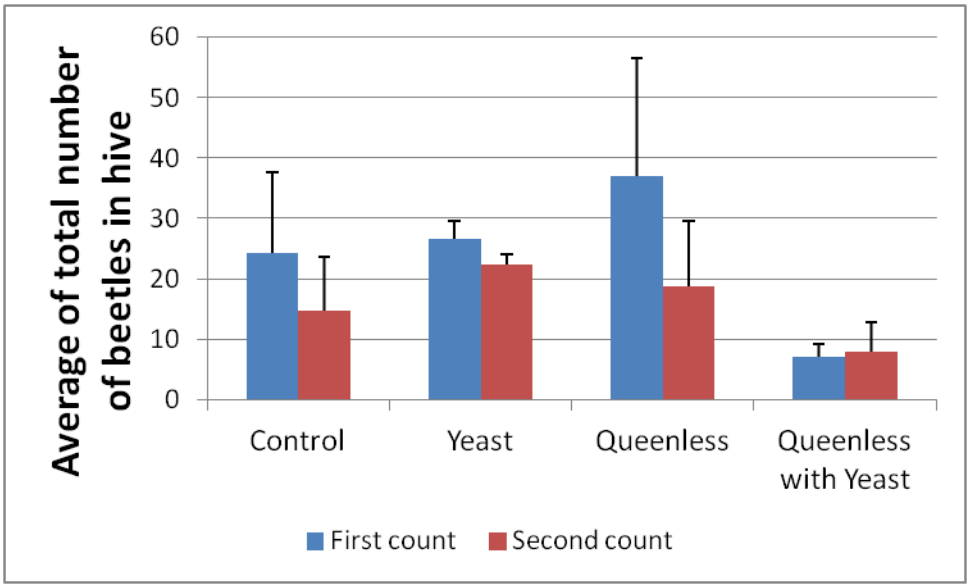

Figure 2-6: Average number of total beetles per hive in four treatment groups in Kenyan hives. The blue bar represents the first count, before treatments were performed. The red bar represents the number of beetles in the second count, taken one week after treatments were performed. N=4 hives per treatment, error bars indicate standard error.

Table 2-3: Average concentration of yeast per gram of bee bread in Kenyan hives

Hive treatment	First count	Second count
Control	69 ± 20	37
	3 of 8 hives positive for yeast	1 of 8 hives positive for yeast
Yeast-treated	185 ± 9	150 ± 50
	3 of 8 hives positive for yeast	2 of 8 hives positive for yeast

Discussion

These results offer much valuable information about the constraints on growth of *K. ohmeri* in bee hives. Water activity is a major factor. Since bee bread is normally uncapped and permeable, the water activity of bee bread will fluctuate with the ambient humidity. The water activity necessary for growth of *K. ohmeri* on pollen is above 0.7 which may be an improbable

value for many seasons and locations where average ambient humidity is low. For example, hive humidity never reached above 70% in Kenyan hives in the field experiments, so water activity of bee bread in these hives could not be expected to be above 0.7. It would be valuable to know if *K. ohmeri* is capable of growth at water activities between 0.9 and 0.7 to predict if *K. ohmeri* may be expected to grow at various humidity levels encountered by bee hives. However, most yeasts require water activities above 0.8 (Corry 1978).

While water activity was a major constraint on the growth of *K. ohmeri*, nutrient availability was not limiting. At appropriate water activity, bee bread and pollen were nutritionally sufficient for the growth of *K. ohmeri*. Beetle frass was found to contain a significantly higher concentration of yeast than bee bread, however. It is possible that, for *K. ohmeri*, beetle-digested bee bread is nutritionally superior to bee bread itself. Alternatively, the high level of *K. ohmeri* in the frass may simply be a product of the high levels of yeast found in feeding larvae of the small hive beetle.

Having considered general yeast growth factors of water activity and nutrient availability, we now turn to consider the system-specific factors governing the growth of *K. ohmeri* in bee hives. Frass of beetle larvae fed on bee bread was high in *K. ohmeri*, but the presence of beetle larvae did not increase the growth of *K. ohmeri* on bee bread itself. This is intriguing, since many reports have stated that beetle larvae are associated with the yeast-filled "slime" of heavily infested hives (Lundie 1940; Neumann and Elzen 2004; Schmolke 1974). This slimy hallmark of severe beetle infestation was not observed in any of the bee bread experiments, either with or without beetle larvae. Apparently, slime requires more than just beetle larvae and yeast to form. Lundie (1940) noted that beetle feeding caused honey to "weep" from the combs, so perhaps the missing element in slime formation is honey or nectar dripping onto the comb surfaces.

Beetle larvae did not significantly increase the growth of *K. ohmeri* already on bee bread or cause slime formation, but beetle larvae were efficient at inoculating bee bread with *K. ohmeri*.

This is probably due to the high levels of yeast associated with small hive beetle larvae. While Nitidulids are consistently associated with yeasts (Lachance et al. 2001; Miller and Mrak 1953; Suh et al. 2005; Suh et al. 2006; Suh et al. 2004b), to the author's knowledge, this is the first study that has documented the persistence of a single species of yeast throughout the life cycle of a Nitidulid. The ability to persist throughout the life cycle of the beetle, including the reorganization of the pupal phase may indicate a coevolved relationship between the small hive beetle and *K. ohmeri*. During the pupal stage, holometabolous insects completely reorganize their internal organs, including the midgut epithelium (Hakim et al. 2010), where *K. ohmeri* likely occurs. In mosquitoes, it has been shown that the larval gut contents and degraded larval midgut epithelium becomes encased in a meconium peritrophic membrane which appears to prevent contamination of the adult midgut with bacteria present in the larval midgut (Moll et al. 2001; Moncayo et al. 2005). However, bacterial endosymbionts are preserved and even increase in titer during metamorphosis of the ant *Camponotus floridanus* (Stoll et al. 2010). *K. ohmeri* titer did not increase during the pupal stage, but did increase slightly in unfed, newly emerged adults. It is possible that *K. ohmeri* remains viable in the meconium of pupae and rather than being excreted, re-colonizes the gut of newly emerged adults. Alternatively, newly emerged adults could acquire *K. ohmeri* from consuming their pupal skin, since *K. ohmeri* also persists on the external surfaces of beetle pupae.

Data from the field experiments tend to support the laboratory findings that water activity and beetle presence are key factors in growth of *K. ohmeri*. Yeast growth in both Kenyan and US bee hives was limited and did not proliferate extensively beyond the point of inoculation. In Kenya and the US, bees were observed eating the fermented substrate, and may have thus sanitized the hive. Humidity was low in Kenyan bee hives, making the probable water activity of bee bread in these hives well below the threshold for growth of *K. ohmeri*. The humidity in US bee hives during the experiment is unknown, but the ambient humidity in Mount Union, PA

during the weeks of the experiment fluctuated between an average daily maximum of 94% and an average daily minimum of 54% (Weather Underground), potentially permitting the growth of *K. ohmeri*.

Despite the high concentration of *K. ohmeri* on fermented frames in Pennsylvania bee hives and a humidity range that permitted the growth of *K. ohmeri*, there was very little recruitment of small hive beetle adults to yeast-treated hives. It is possible that *K. ohmeri* does not play as significant a role in attraction and aggregation of the small hive beetle as previously thought. Alternatively, fluctuations in humidity or control of hive humidity may have limited the ability of *K. ohmeri* to produce attractive volatiles. In the case of *Aspergillus ochraceus*, the water activity necessary for the production of secondary metabolites is higher than the minimum necessary for growth (Pardo et al. 2004). More research is needed to determine the conditions necessary for production of attractive volatiles by *K. ohmeri* in bee hives.

While more research is certainly needed to determine the role of *K. ohmeri* in attraction and aggregation of small hive beetles in bee hives, the results of these studies suggest that growth of yeast in the hive would be greatest when humidity is high and beetles are present. These results are consistent with the observation that small hive beetle presents a more serious pest problem in the humid southern US than other areas with lower average humidity and beetle numbers (Spiewok et al. 2007).

Chapter 3

Small hive beetle attraction to volatiles produced by diverse yeasts growing on pollen and liquid media

"Don't you know that a little yeast leavens the whole batch of dough?"

I Corinthians 5:5-7

Abstract

Kodamaea ohmeri is a yeast associated with the small hive beetle (*Aethina tumida*). When growing on pollen, this yeast produces an attractive odor blend containing chemical components of the honey bee alarm pheromone. It has been hypothesized that *K. ohmeri* growing on pollen in the hive could cause attraction of the small hive beetle and aggregations in bee hives. However, several yeasts are also capable of producing similar volatile blends, and several yeasts have been consistently isolated from honey bee hives. However, it is unknown if any of these other yeasts are attractive to the small hive beetle when growing on pollen or liquid media. Six yeasts diverse yeasts were assessed for production of attractive volatiles on pollen and liquid media. Pollen and liquid media fermented by the six yeasts were then compared to unfermented substrates for attractiveness to the small hive beetle. All six yeasts, including ubiquitous *Saccharomyces cerevisiae* and lacewing-associated *Candida pimensis* produced similar volatile blends when growing on pollen or liquid media and were attractive to the small hive beetle. These results indicate that the relationship between *K. ohmeri* and the small hive beetle is not very specific and that many yeasts, including those already in the bee hive may attract the small hive beetle.

Introduction

The small hive beetle (*Aethina tumida* Murray) (Coleoptera: Nitidulidae) is a parasite and scavenger of western honey bee (*Apis mellifera*) hives. While in its native range of sub-Saharan Africa, it is a minor pest (Lundie 1940; Schmolke 1974), in the United States and Australia it has become a serious pest, rapidly infesting hives and causing bees to abscond (Elzen et al. 1999b; Elzen et al. 1999a; Neumann et al. 2010). It is thought that beetle aggregation is chemically mediated (Neumann and Elzen 2004; Spiewok et al. 2007). Pollen fermented by a yeast carried by the small hive beetle is highly attractive to the beetle and may play a role in beetle aggregations (Benda et al. 2008; Torto et al. 2007c; Torto et al. 2007a). Interestingly, this beetle yeast, identified as *Kodamaea ohmeri*, produces an odor blend which contains honeybee alarm pheromone components such as isopentyl acetate and 2-heptanone when growing on pollen (Benda et al. 2008; Torto et al. 2007c; Torto et al. 2007a). However, little is known about the relationship between the small hive beetle and *K. ohmeri*.

Understanding the relationship between *K. ohmeri* and the small hive beetle and its role in beetle infestations is of major importance to beetle control. Nitidulids are a diverse group of beetles with an extraordinary range of feeding habits. Mycetophagy is considered to be the ancestral state for the entire group (Habeck 2002; Kirejtshuk 1997; Lawrence 1991), but modern nitidulids feed on fungi, carrion, flowers, fruit, fermenting tree sap, aphids and scale insects, decaying plant parts, stored grains, and several are inhabitants of ant and bee nests (Habeck 2002; Lawrence 1991; Parsons 1943). Many nitidulids are associated with yeasts and other characteristic fungi (Lachance et al. 2001; Lawrence 1991; Miller and Mrak 1953; Suh et al. 2006). However, the nature of these associations is often not well understood (Suh and Blackwell 2005; Vega and Dowd 2005). Nitidulids are attracted to volatiles produced by plant pathogenic fungi such as *Ceratocystis* spp (Chang and Jensen 1974; Lin and Phelan 1992) and *Fusarium*

spp., (Ako et al. 2003; Windels et al. 1976), which the beetles feed upon as well as transmit. Nitidulids are also frequently attracted to yeast-fermented substrates (Blackmer and Phelan 1991; Dowd and Bartelt 1991; Miller 1952; Miller and Mrak 1953; Nout and Bartelt 1998; Wildman 1933). Some of these fungi provide a nutritional benefit for the beetles (Ako et al. 2003; Blackmer and Phelan 1991; Chang and Jensen 1974), but the relationship is facultative, since nitidulids can feed on many alternative substrates with or without fungi (Lin and Phelan 1992; Nout and Bartelt 1998).

Volatiles responsible for attraction of nitidulids to fungi are common fermentation volatiles such as ethanol, acetaldehyde, ethyl acetate, 1-propanol, 2-methyl-1-propanol, 3-methyl-1-butanol, and 2-methyl-1-butanol (Bartelt and Hossain 2010). Blackmer and Phelan (1992), citing cross-attraction of acorn-specialist nitidulid *Stelidota octomaculata* and fruit generalist *Stelidota geminata* to fruit, fungal, and acorn volatiles, have suggested that nitidulids may be chemical 'generalists.' Specialization, they contend, is in the beetle's behavior such as propensity to fly to food volatiles, rather than in its response to specific host volatiles (Blackmer and Phelan 1992; Blackmer and Phelan 1995).

While nitidulids may be chemical 'generalists,' some specificity of attraction is known to occur. Beetle preferences for specific yeasts, for example, are influenced by a combination of the sugar composition of the substrate and fermentation capabilities of the yeasts. Nout and Bartelt (1998) found that, of the 49 species of yeasts tested, yeasts which were capable of fermenting sugars found in corn typically produced nitidulid-attractive volatiles. Irregular distribution of these sucrose and maltose-fermenting yeasts has been proposed to account for the aggregation of nitidulid beetles in certain damaged ears of corn but not in other nearby damaged ears (Nout et al. 1997). *Carpophilus hemipterus* shows a preference for *Saccharomyces cerevisiae*-inoculated substrates over *Candida krusei*-inoculated substrates (Blackmer and Phelan 1991). This difference may be attributed to the ability of *S. cerevisiae* to ferment a larger variety of sugars

than *C. krusei* (Suihko and Mäkinen 1981). Lin and Phelan (1992) found that oak wilt fungus *Ceratocystis fagacearum*, which is primarily transmitted by nitidulid beetles, produces volatiles which attract its vectors, while other wind- or water-dispersed fungi did not produce these attractive volatiles.

Since *K. ohmeri* is the primary yeast isolated from the small hive beetle (Benda et al. 2008), and is identical with yeast associated with the small hive beetle in its native range (Torto et al. 2007c), research has naturally focused on this yeast species alone. Benda et al. (2008), however, noted that several other fungi were isolated from the small hive beetle. The role of these fungi is still unknown. Since nitidulids have a generalist response to fungal volatiles, we can assume that many other species of yeast may attract the small hive beetle. Various yeasts have frequently been isolated from bee bread in honey bee hives as well as in the guts of honey bee workers when under stress or fed antibiotics (Gilliam 1979; Gilliam et al. 1974; Gilliam 1973). Yeast is considered to play a role in the conversion of raw bee-collected pollen into bee bread, the primary source of protein for honey bees (Gilliam 1979). Yeast also contains important micronutrients which may be vital for honey bee nutrition (Pain and Maugenet 1966). Two species of yeasts may be expected to inhabit bee hives naturally. One is *Candida magnoliae*, which dominates the yeast flora of finished bee bread (Gilliam 1979). Bakers' or brewers' yeast *S. cerevisiae* is also present in the bee hive in the form of protein patties used as a food supplement. These patties have been noted to attract the small hive beetle in the hive and serve as an excellent larval food source (Hood 2009). However, high levels of fungicides can be found in bee bread of modern hives, which may reduce the number of yeasts in the hive (Mullin et al. 2010). A study of the range of the small hive beetle's attraction to yeasts will allow researchers to predict the effects of changing hive yeast species composition on small hive beetle populations. The purpose of this study, then, is to examine a diversity of beetle-associated and non-beetle-associated yeasts, including two yeast species typically found in bee hives, for the ability to

produce volatiles which attract the small hive beetle in an effort to shed light on the relationship between the small hive beetle and *K. ohmeri*.

Methods

Insects

A small hive beetle colony was established from beetles collected from beehives in Florida and Pennsylvania and maintained for 2 years on moistened bee-collected pollen (Brushy Mountain, Moravian Falls, NC) at 30 ± 3°C. Adults were maintained in 2-quart plastic canisters (Rubbermaid® 12 × 18.5 cm) at an average density of 50-200 beetles per canister. Canisters were filled so beetles in a single canister emerged within 0-5 days of each other. Beetles laid eggs between two plastic diet cup lids snapped together. Eggs and larvae were transferred to moistened bee-collected pollen and fed until they reached the wandering phase. Wandering larvae were then transferred to moistened sand placed in plastic, rectangular containers (Glad® 3.07L, 24 × 24× 10cm) or pupation. After emergence, beetles were collected with an aspirator and introduced into new plastic canisters.

Yeasts selected for comparative study

The yeasts selected for comparative study are listed in Table 3-1. Yeasts were obtained from the USDA ARS culture collection (Peoria, IL) and Louisiana State University culture collection (Baton Rouge, LA) and maintained in 15% glycerol at -80°C according to standard techniques.

Yeast cultures on liquid media and pollen

Yeasts were transferred from glycerol stocks into sterile sabouraud dextrose broth with 1% yeast extract (SDBY) (Atlas 2004) and incubated in a shaker-incubator at 28°C to a high cell density corresponding to a colony count of approximately 1×10^5 cfu/µl. These liquid cultures were used for both volatile sampling and behavioral bioassays. Sterile pollen was prepared by gamma irradiating (Gamma Cell 220 Excel Irradiator, dose: 10 kGy) commercially available bee-collected pollen (Brushy Mountain, Moravian Falls, NC) at the Penn State Radiation Science and Engineering Center. To obtain fermented pollen, sterile pollen (16g) was placed in a petri dish and 10 ml of yeast culture was added. Pollen dishes with yeast were incubated in a humid container at 28°C for 3 days before use, when preliminary experiments showed that volatile production was high and stable. For controls, sterile SDBY and sterile pollen mixed with 10 ml of sterile SDBY were used.

Headspace volatile sampling

Volatile collection and analysis

Volatiles were collected by enclosing petri dishes containing SDBY or pollen in a chamber, pushing purified air into the chamber, and pulling the air through an adsorbent to trap the volatile organic compounds. Volatile collection chambers consisted of a modified Pyrex bottle (7 L) with a Teflon stopper with an inlet for charcoal filtered air (1 L/min) and an aluminum guillotine at the bottom of the chamber (Heath, 1994). Fabricated filter traps were attached to vacuum (0.5 L/min) via ports at the bottom of the chamber. The filter traps were constructed as described previously (Heath, 1994). Each filter trap contained an adsorbent (30 mg, Super Q, 80/100 mesh, Altech Associates Inc., State College, PA). After placing dishes in the

glass chambers, volatiles were allowed to concentrate in the headspace with the air off for 1 hour. Volatile chemicals were collected on 1 filter from each chamber for 2 hours at 25°C. To analyze the emitted volatiles, filter traps were eluted with 150 µl, 1:1 v/v mixture of hexanes (J.T. Baker) and dichloromethane (Honeywell) and internal standard was added (20 µl, 50 ng/µl butyl butyrate). Samples (1 µl injection volume, splitless) were analyzed with a gas chromatograph-flame ionization detector (GC-FID Agilent 6890N) fitted with an SPB-1000 column (30m × 0.25mm ID × 0.25µm, Supelco, Bellfonte, PA) with helium as the carrier gas at an average linear flow velocity of 21 cm per second. The oven program was 50°C, 1 min; 10°C/min to 190°C; and held for 5 min. Data were acquired and peak areas integrated with MSD ChemStation software (Agilent, 2007). Identified compounds were quantified by comparing the peak areas of the compounds of interest with the peak area of the internal standard, butyl butyrate. Compound identity was determined by comparing mass spectra and retention times of collected volatiles with standards. In addition, spectra were compared to the National Institute of Standards and Technology (NIST) mass spectral library (2002). For analysis, a gas chromatograph-mass spectrometer (GC-MS Agilent 7890A, 5975C) in electron ionization mode was used. Oven program and injection parameters of the GC-MS were the same as above. The GC-MS was fitted with a SPB-1000 column (30m × 0.25mm ID × 0.25µm, Supelco, Bellfonte, PA) with helium as the carrier gas at an average linear flow velocity of 33 cm per second.

Statistical analysis of volatile profiles

Twenty volatile components, a subset of the total volatiles detected by the GC-FID, was selected for quantitative analysis based on highest percent of total volatiles. Normality was confirmed by examining a histogram of the residuals. Mean total volatiles and mean quantities of each volatile component were tested for significant differences by 1-way ANOVA, and means

were then compared by Tukey-Kramer method (Minitab® 16.2.2). Additionally, multivariate statistics were applied to the volatiles produced by yeasts growing on pollen to visualize any groupings (PCA Minitab® 16.2.2).

Behavioral assays

Bioassay Chambers

Attraction of adult beetles to volatiles produced by yeasts was assessed using a still-air choice chamber constructed from 5cm plastic petri dishes and 5-ml glass vials (Figure 3-1). Two equally spaced 5mm holes were punched in the center of the bottom of the petri dish. Then, a black cap from the 5-ml vial was inverted and attached to the bottom of each hole with hot glue. Another vial cap was glued into place below, so that the tops of the caps were glued to one another and a vial could be inserted into the lower cap. Then a dark chamber was created below each of the two holes in the petri dish. A stainless steel screen mesh round (100 mesh, 0.5 inch diameter, Small Parts Inc.) was inserted into lower vial cap so that volatiles from the vial below could escape into the chamber above but would not allow the beetles to pass into the vial and make contact with the substrate in the vial. Twelve holes were punched in the lid of the petri dish with a hot syringe needle to allow volatiles to escape the chamber and avoid saturating the chamber with volatiles from both sides.

Figure 3-1: Still-air bioassay chamber for assessing adult small hive beetle preferences.

Attraction Bioassays

Clean 5-ml glass vials were filled with 0.4 ml of liquid media or pollen and screwed into assay chambers. Chambers were placed in an incubator at 30°C and 60% humidity for 30 minutes to equilibrate. Then, beetles were added, one per chamber, and the chambers were returned to the incubator. As Schmolke (1974) reported, at 2 weeks old, adults had transitioned from positive phototaxis to negative phototaxis. In these experiments, 2-week-old beetles were found to be negatively phototactic even under red light conditions and consistently chose to hide in one of the two small dark chambers below the petri dish rather than stay exposed in the petri dish above. Beetle choices were recorded at 15 and 30 minutes, then the chambers were removed from the incubator and left in the light at room temperature and humidity for 15 more minutes and choices

were recorded again. Beetles were able to move in and out of the holes in the chambers at will. Twenty chambers were monitored at a time, and the experiment was repeated 3 times for a total of at least 60 beetles and three replicate days. Between experiments, vials were discarded and chambers were washed in tap water, distilled water, and 95% ethanol and allowed to dry before using again.

Analysis

Beetles that were never seen entering either of the holes in the chamber were considered to have made no choice and thus were eliminated from the analysis. When a beetle entered both sides of the choice chamber during the experiment, the last choice was taken. The number of beetles choosing each side was analyzed for significant difference from 1:1 using an exact binomial test.

Table 3-1: Yeasts used in this study.

Species	NRRL	Description
Kodamaea ohmeri	Y-27634	Isolated from larvae of the small hive beetle *Aethina tumida* Murray (Coleoptera: Nitidulidae)
Kodamaea ohmeri	Y-27716	Isolated from gut of *Carpophilus* sp. (Coleoptera: Nitidulidae) ex *Pisolithus tinctorius*, Baton Rouge, Louisiana, USA
Metschnikowia similis	Y-27627	Isolated from *Conotelus* sp. (Coleoptera: Nitdulidae) ex *Ipomoea indica* (Convolvulaceae), Guanacaste Province, Costa Rica
Candida magnoliae	Y-2024	Magnolia flower
Candida pimensis	Y-27619	Isolated from female Green lacewing (*Chrysoperla carnea*, Neuroptera: Chrysopidae), Tucson, Arizona, USA
Saccharomyces cerevisiae	Y-12632	Beer, Oranjeboom Brewery, Rotterdam, Netherlands

Results

Headspace volatile analysis

Table 3-2 contains the detailed analysis of the volatile data collected from six yeasts growing on pollen. Table 3-3 contains the detailed analysis of six yeasts growing on SDBY. Figure 3-2 shows a graph of the first principal component vs. second principal component of the volatiles produced by yeasts growing on pollen. While some species groupings are evident, there are several outlier observations and groupings overlap broadly. However, ANOVA revealed that, within the pollen treatments, there were some minor differences between the volatile profiles of different yeasts. *S. cerevisiae* exhibited a unique unidentified compound with a retention time of 12.7 minutes, and a high quantity of isobutyric acid, while *C. pimensis* samples differed from the rest because they did not contain any 2,3-butanediol. As expected, the two strains of *K. ohmeri* produced the same volatile compounds in statistically similar quantities. Volatile profiles of beetle associated yeasts *K. ohmeri* (NRRL Y-27634), *K. ohmeri* (NRRL Y-27716), and *M. similis* (NRRL Y-27627) were compared as a group with volatile profiles of non-beetle-associated yeasts *C. magnoliae* (NRRL Y-2024), *C. pimensis* (NRRL Y-27619), and *S. cerevisiae* (NRRL Y-12632) as a group (Table 3-4). When ANOVA was performed on volatile components of these two groups of yeasts, the quantities of only three compounds, 2-hydroxy-3-butanone, isobutyric acid, and unknown 2 were significantly different. The compounds produced by yeasts growing on SDBY represented a small subset of the compounds produced by yeasts growing on pollen. These compounds were present in much lower quantities in the headspace of the SDBY treatments than the pollen treatments, and frequently fell below the limit of detection.

Behavioral assays

Beetles were significantly attracted to all six yeasts when growing on pollen ($p \leq 0.0001$) and SDBY ($p \leq 0.0001$) over control pollen and SDBY. Figure 3-3 and Figure 3-4 display the results of these behavioral assays. Beetles were significantly attracted to control pollen over control SDBY ($p < 0.0001$), and exhibited a preference for yeast growing on pollen over yeast growing on SDBY for all yeasts ($p \leq 0.021$) except *C. pimensis* (Figure 3-5). When beetles were offered a choice between *K. ohmeri* (NRRL Y-27634) isolated from SHB and *S. cerevisiae* (NRRL Y-12632), beetles did not exhibit a preference ($p = 0.435$) (data not shown).

Discussion

Contrary to our expectations, all of the yeasts in this study produced similar volatile blends when growing on pollen. Many components of these volatile blends have been identified from the headspace of nitidulid attractants such as fermenting fruit and bread dough (Bartelt and Hossain 2006; Dowd and Bartelt 1991; Lin and Phelan 1991; Nout and Bartelt 1998; Phelan and Lin 1991) or nitidulid-pollinated flowers (Jürgens et al. 2000). These components include isopentyl alcohol, 2,3-butanediol, 3-hydroxy-2-butanone, phenylethyl alcohol, isopentyl acetate, ethyl hexanoate, ethyl octanoate, isobutyric acid, ethyl decanoate, butyric acid, and benzyl alcohol. Only three compounds were consistently different between the volatile profiles of beetle-associated yeasts and non-beetle-associated yeasts. Two of these, isobutyric acid and 2-hydroxy-3-butanone, are known nitidulid attractants (Dowd and Bartelt 1991; Nout and Bartelt 1998), but only 2-hydroxy-3-butanone was produced in greater quantities by beetle-associated yeasts than non-beetle-associated yeasts. Since selection would be high for obligate beetle-associated fungi to produce an odor blend to attract beetles, the lack of unique volatile blends produced by beetle-

associated yeasts in this study suggests that none of these yeasts are obligate beetle-associates. Indeed, *K. ohmeri* has been reported from diverse substrates such as bumble bee colonies (Graham et al. 2011a), cassava roots (Ferreira et al. 2010), and fermenting cocoa beans (Daniel et al. 2009). *K. ohmeri* is also considered an emerging human pathogen (Al-Sweih et al. 2011).

Nout and Bartelt (1998) reported that yeasts which ferment the primary sugars available in corn are more likely to produce nitidulid attractants. Primary sugars in bee-collected pollen are fructose, glucose, and sucrose (Serra Bonvehí and Escolà Jordà 1997). *M. similis* and *C. pimensis* ferment glucose, but not sucrose (Lachance and Bowles 2004; Suh et al. 2004a). *S. cerevisiae*, *C. magnolia*, and *K. ohmeri* ferment glucose and sucrose. All of the yeasts which ferment glucose typically also ferment fructose (Konno et al. 1985), so all of the yeasts tested have the ability to ferment the major sugars in bee-collected pollen with the exception of sucrose by *M. similis* and *C. pimensis*, which may account for the very similar volatile blends produced by the yeasts on pollen.

Volatile profiles of the different yeasts in this study were similar, and the small hive beetle's response was likewise very similar, showing significant attraction to all yeasts growing on pollen or SDBY over control pollen or SDBY. Only *C. pimensis* failed to attract the small hive beetle more on pollen than on SDBY, though it is unclear why this should be so. On SDBY, *C. pimensis* produced no isobutyric acid, and a low level of 2-hydroxy-3-butanone compared with other yeasts. Small hive beetles were also attracted to gamma-sterilized bee-collected pollen over sterile SDBY. It is likely that this pollen had undergone some preliminary fermentation by bacteria that occur in the honeybee gut (Gilliam 1997; Olofsson and Vásquez 2008; Vásquez and Olofsson 2009). While the genus *Aethina* is composed primarily of mycetophagous and anthophilious species (Kirejtshuk and Lawrence 1999), the small hive beetle has never been reported to frequent flowers or reproduce on flowers (Buchholz et al. 2008). Low-level

fermentation by bee-associated microbes may therefore account for the attraction of beetles to control pollen.

The catholic response of the small hive beetle to yeast volatiles in this study and previous studies (Nout and Bartelt 1998) suggests that they do not have a highly obligate relationship with any specific yeasts. However, these behavioral assays were designed to be very sensitive and can only speak to short-range attraction. More research is needed to confirm whether volatile blends produced by diverse yeasts are also attractive as long-range host-finding cues. It is also unknown if all of these yeasts are nutritionally equivalent for small hive beetle larvae. However, these results suggest that the small hive beetle responds to more general yeast and pollen cues than a species-specific blend. If aggregation of small hive beetles in hives is mediated by microbial volatiles, the whole microbial community of the hive may play a role. Recently, several researchers have pointed out that the microbial community of the honey bee hive is tremendously complex and may be more important to honey bee health than previously thought (Anderson et al. 2011; Hamdi et al. 2011; Martinson et al. 2011; Runckel et al. 2011; Vásquez and Olofsson 2009). Most of these studies have focused on the bacterial community, though many yeasts (such as *C. magnoliae*) have been reported from bee hives and honey bees (Gilliam 1979; Gilliam et al. 1974; Gilliam 1973), and *S. cerevisiae* is routinely added to pollen patties. Understanding hive yeast communities may, therefore, also be important to improving honey bee health as well as controlling small hive beetle. Practically, our findings imply that effective fermented pollen baits for small hive beetle traps may be formulated with readily available yeasts such as *S. cerevisiae*. Commercially-available pollen patties containing *S. cerevisiae* may in fact be already acting as small hive beetle attractants in the field.

Table 3-2: Headspace volatiles collected from six yeasts growing on pollen

Component[a]	µg produced per 2 hours[b]						
	Pollen Control	***K. ohmeri* (Y-27634)**	***K. ohmeri* (Y-27716)**	***M. similis* (Y-27627)**	***C. pimensis* (Y-27619)**	***C. magnoliae* (Y-2024)**	***S. cerevisiae* (Y-12632)**
Unknown 1 (5.85)	-[c]	8.1 ± 0.84	8.7 ± 2.7	4.7 ± 0.82	4.8 ± 1.5	6.6 ± 1.0	5.7 ± 0.80
Unknown 2 (6.04)	-	1.3 ± 0.17**a**	1.0 ± 0.29**ab**	0.38 ± 0.07**c**	0.35 ± 0.09**bc**	0.65 ± 0.09**bc**	0.58 ± 0.06**bc**
Unknown 3 (6.23)	-	0.98 ± 0.10**a**	0.95 ± 0.27**ab**	0.39 ± 0.06**c**	0.41 ± 0.09**bc**	0.66 ± 0.10**abc**	0.59 ± 0.06**abc**
Isopentyl acetate (6.94)	-	0.33 ± 0.06	0.23 ± 0.08	0.12 ± 0.05	0.51 ± 0.37	0.33 ± 0.07	0.33 ± 0.04
Isopentyl alcohol (8.10)	-	6.4 ± 0.96	5.2 ± 1.3	3.5 ± 1.1	7.6 ± 5.5	6.1 ± 1.5	5.4 ± 0.69
Ethyl hexanoate (8.44)	-	3.4 ± 0.59	2.6 ± 0.75	2.5 ± 0.43	2.5 ± 0.72	3.4 ± 0.79	3.6 ± 0.55
2-Hydroxy-3-butanone (9.42)	-	4.2 ± 0.71**a**	4.0 ± 1.1**a**	2.6 ± 0.40**ab**	1.7 ± 0.46**ab**	0.74 ± 0.59**b**	2.5 ± 0.50**ab**
Unknown 4 (12.6)	-	2.5 ± 0.34	2.7 ± 0.76	1.9 ± 0.32	1.7 ± 0.42	2.4 ± 0.38	2.3 ± 0.35
Ethyl octanoate (11.1)	-	2.2 ± 0.36	1.8 ± 0.42	1.6 ± 0.27	1.7 ± 0.48	1.5 ± 0.37	2.9 ± 0.37
Benzaldehyde (12.6)	5.5 ± 2.6	-	-	-	-	-	-
Unknown 5 (12.7)	-	-	-	-	-	-	9.8 ± 1.8
Isobutyric acid (13.0)	-	1.7 ± 0.23**b**	1.4 ± 0.28**b**	0.96 ± 0.10**b**	0.88 ± 0.10**b**	2.0 ± 0.21**b**	4.0 ± 0.54**a**
2,3-Butanediol (13.2)	-	6.6 ± 0.95**a**	5.5 ± 1.8**ab**	1.6 ± 0.27**b**	-	3.7 ± 0.45**ab**	5.9 ± 0.85**a**
Ethyl decanoate (13.6)	-	3.9 ± 0.59	3.3 ± 0.73	3.0 ± 0.31	2.8 ± 0.37	2.5 ± 0.39	4.4 ± 0.50
Butyric acid (13.7)	15 ± 3.3	8.7 ± 0.94	11 ± 2.6	13 ± 1.1	15 ± 2.4	10 ± 1.2	8.7 ± 1.2
Unresolved 5-6C acids (14.2)	18 ± 3.8	14 ± 1.3	15 ± 2.3	14 ± 0.87	14 ± 1.1	15 ± 1.0	13 ± 1.3
Ethyl phenylacetate (15.5)	-	1.4 ± 0.28	1.3 ± 0.37	1.1 ± 0.20	1.0 ± 0.19	1.3 ± 0.28	1.1 ± 0.18
Hexanoic acid (16.2)	3.0 ± 0.97**a**	1.1 ± 0.24**b**	1.4 ± 0.20**ab**	2.0 ± 0.30**ab**	2.2 ± 0.10**ab**	1.4 ± 0.23**ab**	1.3 ± 0.21**b**
Benzyl alcohol (16.8)	-	1.7 ± 0.30	1.6 ± 0.52	1.4 ± 0.18	1.7 ± 0.23	1.6 ± 0.28	0.86 ± 0.11
Phenylethyl alcohol (17.3)	-	5.0 ± 0.92**ab**	4.2 ± 1.2**abc**	1.3 ± 0.29**c**	3.7 ± 2.4**abc**	7.0 ± 0.39**a**	3.2 ± 0.45**bc**
Total volatiles	56 ± 12	86 ± 7.7	81 ± 16	63 ± 6.2	71 ± 7.0	74 ± 5.8	86 ± 9.5

[a] In order of elution during gas chromatography on SPB-1000 column, compound names followed by average retention time.

[b] Values are the mean ± standard error of 5-15 replicate pollen preparations. Values followed by the same letter are not significantly different at the 0.05 level from other values in the row. Rows with no letters indicate that all values are not significantly different.

[c] Dashes indicate compounds that were not quantified either because the quantity fell below the level of detection, or in the case of benzaldehyde, the peak was unresolvable when yeast was present.

Table 3-3: Headspace volatiles collected from six yeasts growing on SDBY.

Component[a]	μg produced per 2 hours[b]						
	SDBY Control	***K. ohmeri* (Y-27634)**	***K. ohmeri* (Y-27716)**	***M. similis* (Y-27627)**	***C. pimensis* (Y-27619)**	***C. magnoliae* (Y-2024)**	***S. cerevisiae* (Y-12632)**
Unknown 1 (5.85)	-[c]	0.11	-	-	0.16 ± 0.07	-	0.07
Isopentyl acetate (6.94)	-	-	-	0.04	0.58 ± 0.27	0.17 ± 0.04	0.39 ± 0.15
Isopentyl alcohol (8.10)	-	29 ± 5.9**ab**	26 ± 4.9**ab**	12 ± 2.0**b**	19 ± 8.3**ab**	11 ± 5.0**ab**	41 ± 13**a**
Ethyl hexanoate (8.44)	-	-	-	-	-	-	0.09
2-Hydroxy-3-butanone (9.42)	-	2.1 ± 0.93	1.2 ± 0.23	1.7 ± 0.47	0.28 ± 0.13	-	0.52 ± 0.31
Unknown 4 (12.6)	-	0.06	-	0.03	-	-	0.05
Ethyl octanoate (11.1)	-	-	-	-	-	-	0.19 ± 0.02
Isobutyric acid (13.0)	-	0.34 ± 0.19**b**	0.20 ± 0.02**b**	0.33 ± 0.05**b**	-	3.3 ± 0.76**a**	0.34
Butyric acid (13.7)	-	-	-	0.45 ± 0.003	-	-	-
Unresolved 5-6C acids (14.2)	-	0.25	-	-	-	8.0 ± 1.71	0.88
Hexanoic acid (16.2)	-	-	-	-	-	0.25 ± 0.05	0.19
Benzyl alcohol (16.8)	-	0.20	-	0.14 ± 0.07	0.04	-	0.06
Phenylethyl alcohol (17.3)	-	1.4 ± 0.51**ab**	0.66 ± 0.17**ab**	0.32 ± 0.09**b**	1.0 ± 0.42**ab**	3.0 ± 1.5**a**	0.80 ± 0.13**ab**
Total volatiles	1.3	35 ± 4.5**ab**	30 ± 4.5**ab**	17 ± 2.6**b**	25 ± 10**ab**	27 ± 8.8**ab**	46 ± 13**a**

[a] In order of elution during gas chromatography on SPB-1000 column, compound names followed by average retention time.

[b] Values are the mean ± standard error of 3-7 replicate SDBY preparations. If standard error is absent, this compound could only be quantified in one replicate. Values followed by the same letter are not significantly different at the 0.05 level from other values in the row. Rows with no letters indicate that all values across the row are not significantly different.

[c] Dashes indicate compounds that were not quantified because the quantity fell below the level of detection

Table 3-4: Volatile components produced in significantly different quantities by beetle-associated and non-beetle-associated yeasts growing on pollen.

Component[a]	µg produced per 2 hours[b]		
	Beetle Associated Yeasts	Non-Beetle-Associated Yeasts	P value[c]
Unknown 2 (6.04)	0.94 ± 0.12	0.56 ± 0.05	0.002
2-Hydroxy-3-butanone (9.42)	3.7 ± 0.43	1.8 ± 0.34	0.001
Isobutyric acid (13.0)	1.4 ± 0.13	2.7 ± 0.36	0.008

[a] In order of elution during gas chromatography on SPB-1000 column, compound names followed by average retention time.
[b] Values are the mean ± standard error of 22-31 replicate pollen preparations.
[c] Results of one-way ANOVA.

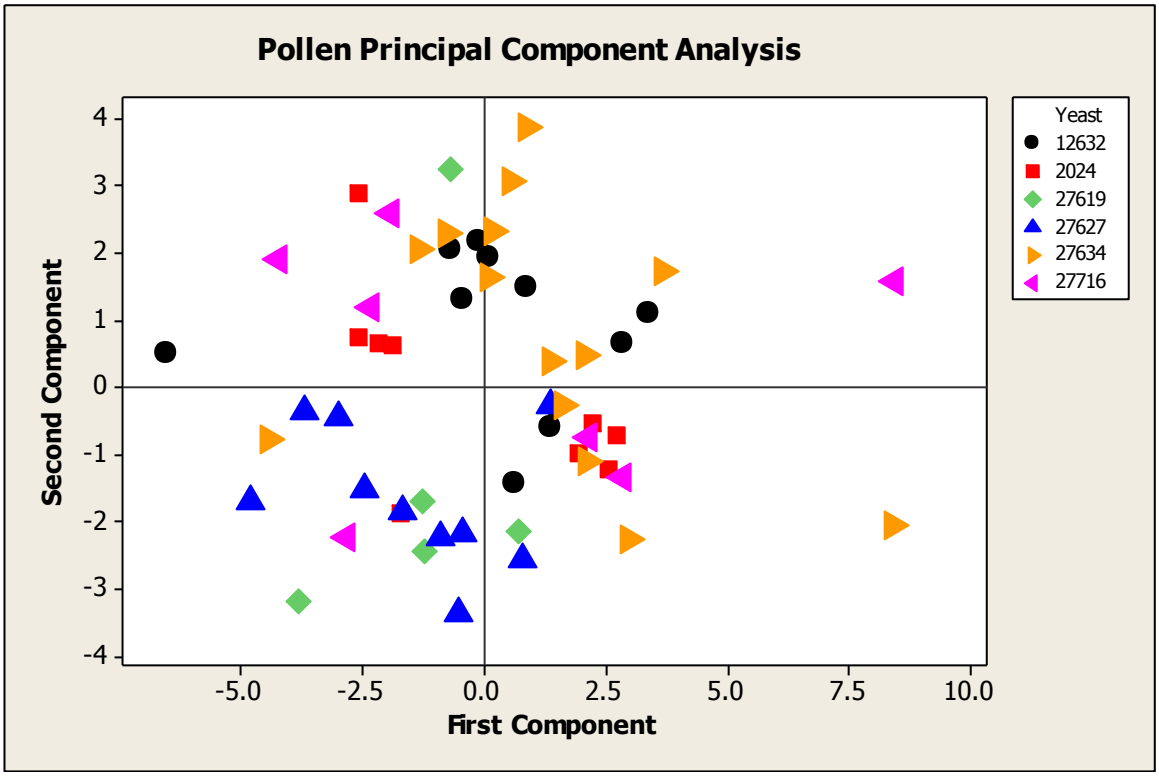

Figure 3-2: Principal component analysis of volatiles produced by yeasts growing on pollen.

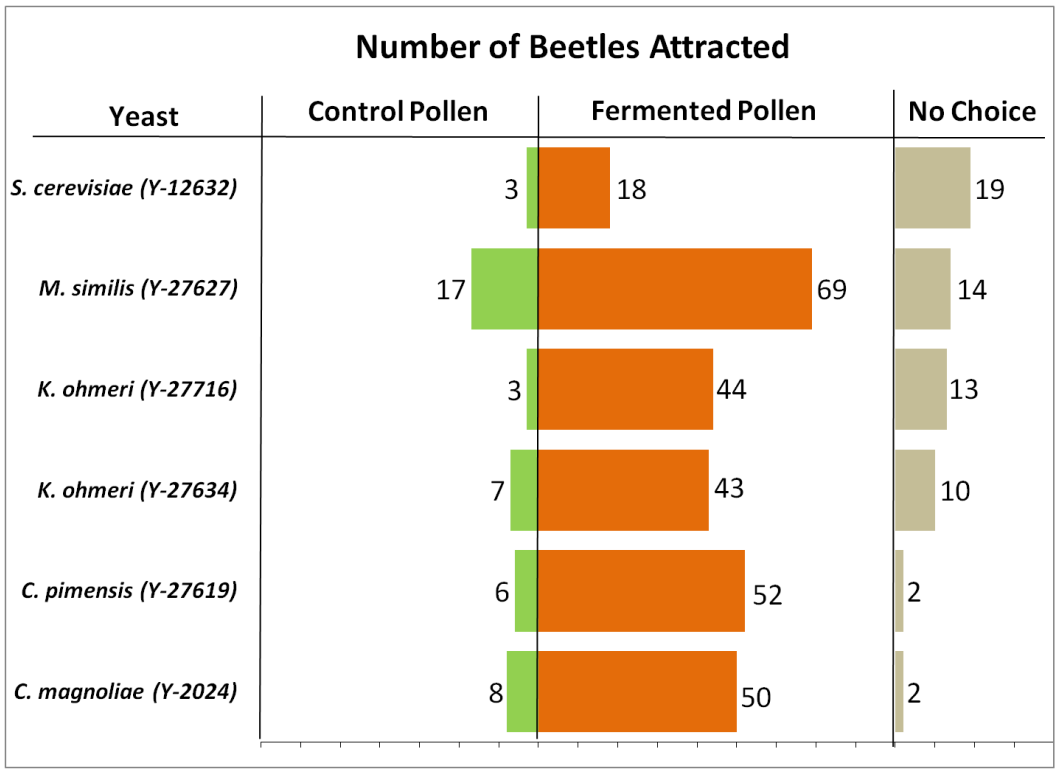

Figure 3-3: Number of adult beetles choosing control pollen or pollen fermented by one of six yeasts. All proportions significantly different from 1:1 by exact binomial test ($p \leq 0.0001$)

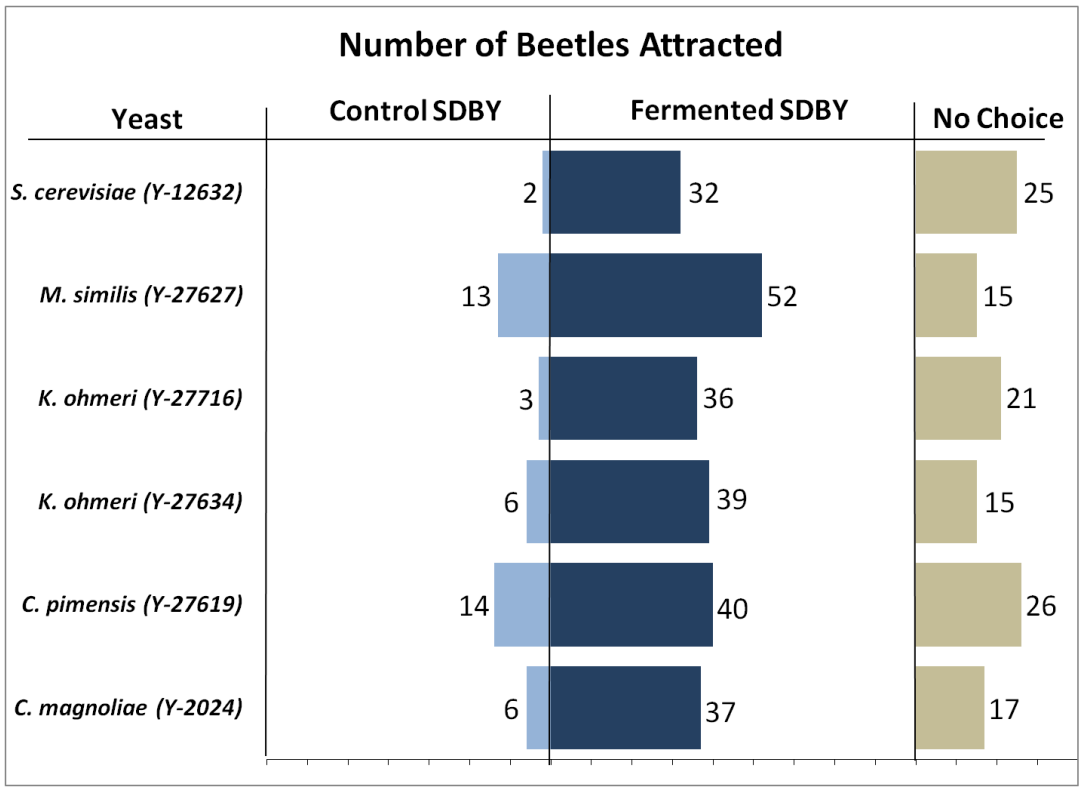

Figure 3-4: Number of adult beetles choosing control SDBY or SDBY fermented by one of six yeasts. All proportions significantly different from 1:1 by exact binomial test ($p \leq 0.0001$).

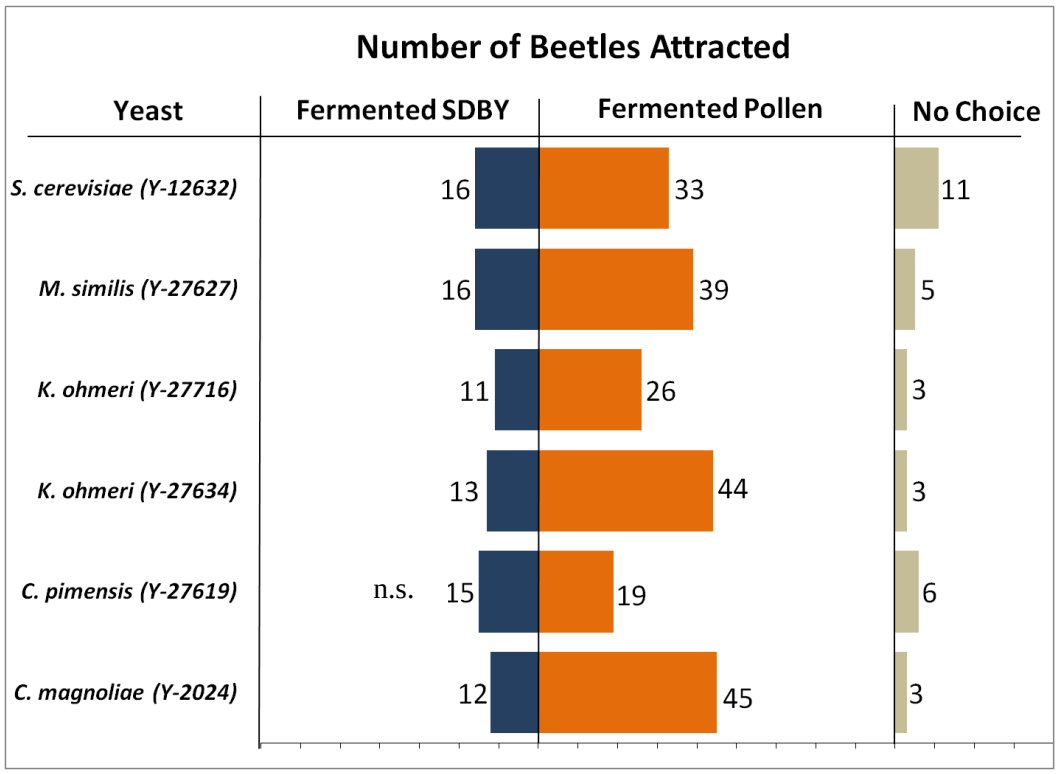

Figure 3-5: Number of adult beetles choosing SDBY or pollen fermented by one of six yeasts. All proportions significantly different from 1:1 by exact binomial test ($p \leq 0.021$), except for *C. pimensis*, (marked with n.s.) ($p = 0.608$).

Chapter 4

Ovipositional Preferences of the Small Hive Beetle and Preliminary Observations on its Mating Behavior

"A beetle may or may not be inferior to a man – the matter awaits demonstration; but if he were inferior to a man by 10,000 fathoms, the fact remains that there is probably a beetle view of things of which a man is entirely ignorant."

G. K. Chesterton, "On Humility", in "The Defendant", 1901

Abstract

The small hive beetle (*Aethina tumida*) is a parasite and scavenger of honey bee hives that has become a serious pest problem since its introduction to the US and Australia from Africa. The fecundity and rapid population growth of these beetles makes control very challenging. However, little is known about the ovipositional preferences or reproduction of the beetle. A yeast associated with the beetle, *Kodamaea ohmeri* has been shown to increase beetle reproduction, and has been hypothesized to attract small hive beetles to bee hives. The ovipositional preference of the small hive beetle for *K. ohmeri*-fermented pollen versus unfermented control pollen was examined in single-female oviposition chambers. The small hive beetles consistently chose to lay more eggs near the *K. ohmeri*-fermented pollen, but only when this pollen was fresh. After one day, the beetle preferences switched to unfermented control pollen unless new fermented pollen was added. In addition to documenting a clear preference for oviposition on fresh *K. ohmeri*-fermented pollen, initial observations on the small hive beetle mating behavior were made. These revealed that the small hive beetle has characteristic copulatory courtship behaviors including guarding, but single mating bouts were mostly infertile.

After pairs were given 48 hours to mate, fertility increased. More research is needed to understand the mating behavior of the small hive beetle, but if extended mating bouts are required for fertility, mating disruption strategies may be employed to control small hive beetle reproduction.

Introduction

The small hive beetle (*Aethina tumida* Murray) (Coleoptera: Nitidulidae) is a parasite and scavenger of western honey bee (*Apis mellifera*) hives. While in its native range of sub-Saharan Africa, it is a minor pest (Lundie 1940; Schmolke 1974), in the United States and Australia it has become a serious pest, rapidly infesting hives and causing bees to abscond (Elzen et al. 1999b; Elzen et al. 1999a; Neumann et al. 2010).

The small hive beetle belongs to the family Nitidulidae, which is a diverse group of beetles commonly associated with fungi including several species of yeasts (Benda et al. 2008; Lachance et al. 2001; Miller and Mrak 1953; Phelan and Lin 1991; Suh et al. 2005). Many Nitidulids are important pests of commodities such as stone fruits (Bartelt and Hossain 2010), oilseed rape (Ekbom and Ferdinand 2003), corn (Nout and Bartelt 1998), and stored products (Hinton 1945). They are also vectors of plant pathogens including oak mat wilt (Juzwik et al. 2004), and mycotoxin-producing fungi (Bartelt and Wicklow 1999). Despite their importance to agriculture, there is relatively little known about the oviposition or mating behavior of the group.

Oviposition behavior has been described for the small hive beetle, especially in reference to the hive environment and alternative diets. In the hive environment, early studies agreed that small hive beetles prefer to oviposit in small crevices of the hive (Lundie 1940; Schmolke 1974). Beeswax comb is preferred over crevices made of glass slides as an oviposition site (Meikle and Patt 2011), so beetles may have an odor or taste-based preference for bee-associated oviposition

sites. Small hive beetles are capable of placing eggs inside capped brood cells (Ellis et al. 2003e; Ellis et al. 2003c) and may even select certain brood cells for oviposition (Ellis et al. 2004a). Both European and African honey bees can detect these hidden eggs and remove them (Ellis et al. 2004a; Ellis et al. 2003e). Bees also remove small hive beetle eggs laid in other regions of the hive, when they can get to them (Neumann and Härtel 2004).

Diet affects small hive beetle oviposition. The highest egg production occurs on diets that include protein sources such as pollen or bee brood (Buchholz et al. 2008; Keller 2002; Meikle and Patt 2011). Bananas are also an attractive oviposition substrate (Buchholz et al. 2008). However, the small hive beetle lays a significantly smaller number of eggs on bananas and animal protein when offered bee brood or pollen as an alternative. Keller (2002) also found that females switched from a high-productivity diet (a mixture of honey and pollen) to a low-productivity diet (bananas or Manduca diet) decreased egg production within 2 days. Likewise, females switched from a low-productivity diet to high-productivity diet quickly increased egg production, indicating that oviposition rate was highly diet-dependent.

Diet also affects larval survival. Arbogast et al. (2010) noted that while lifetime egg production did not differ between females fed yeast-inoculated pollen dough and non-inoculated oranges, larval survival was only 36% on oranges compared with 79% on inoculated pollen dough. Buchholz et al. (2008) likewise found that the number of wandering larvae produced on pollen diet was higher than the number of wandering larvae produced on fruit. Yeast in the diet also affects larval production. Arbogast et al. (2009) found that larval production was significantly higher on pollen or oranges inoculated with the yeast *Kodamaea ohmeri* than un-inoculated pollen or oranges. However, whether this increase in larval production in the presence of *K. ohmeri* was the result of increased oviposition or increased larval survival is unknown. *K. ohmeri* is a yeast isolated from the small hive beetle (Benda et al. 2008) which, when growing on

pollen, attracts the small hive beetle. It has been hypothesized that this yeast attracts the small hive beetle to bee hives.

For the small hive beetle, there is trade-off in oviposition behavior, where females must chose to either lay their eggs in a location that is close to a rich food source or safe from detection by honey bees in the hive. The presence of the yeast *K. ohmeri* or other yeasts may tilt the balance of this trade-off by providing a rich larval food source or enabling females to produce more eggs (Arbogast et al. 2009; Keller 2002). As of yet, however, there have been no choice tests reported to assess the ovipositional preference of small hive beetles for yeast-fermented substrates. *K. ohmeri* produces beetle-attractive volatiles which may influence ovipositional choices, and beetles may increase ovary activation in response to increased nutrition of yeast-inoculated pollen. Ovary development can also be influenced by other factors such as mating (Perez-Mendoza et al. 2004).

In preliminary studies of small hive beetle oviposition behavior, the author noted a high proportion of infertile eggs produced by young small hive beetle females (less than 1 week old). Porter (1986) reported that *C. dimidatus* would lay "flat," infertile eggs in various numbers under all conditions. However, when small hive beetles producing infertile eggs were dissected, their spermathecae were found to be empty. This finding led the author to question when and how small hive beetle females are inseminated.

As far as the author is aware, nitidulid mating behavior has not been described anywhere in the literature. Competition between males for oviposition sites and access to females has been described in one nitidulid, *Librodor japonicas* (Okada and Miyatake 2006; Okada and Miyatake 2004), but this species is unusual in that males of this species possess enlarged mandibles for fighting, which are absent from most of the Nitidulidae. Nitidulids have been noted to mate multiply (Weber 1975), though beyond this observation little is known. Mating behavior throughout the superfamily is also relatively undescribed. Beetles in a sister taxon to the

Nitidulidae, Erotylidae, have been described using chirping to communicate with females and rival males during courtship and mating (Ohya 2001). It is surprising that the mating behavior of a group as widespread as the Nitidulidae has been overlooked, since understanding mating behavior is critical to predicting population growth and devising control methods. Mating behavior can also tell us much about the evolution of a species (Thornhill and Alcock 1983).

The purpose of this study is to fill in several gaps in our understanding of the small hive beetle's reproductive cycle. The first goal is to determine if the small hive beetle exhibits true ovipositional preference for yeast-fermented food and to examine the influence of diet and presence of males on the rate of ovary development. The second goal is to describe the mating behavior and reproductive anatomy of the small hive beetle including structure of the endophallus and spermatheca as groundwork for future studies on mating biology of this important pest insect.

Methods

Insects

A small hive beetle colony was established from beetles collected from beehives in Florida and Pennsylvania and maintained for 2 years on moistened bee-collected pollen (Brushy Mountain, Moravian Falls, NC) at 30 ± 3°C. Adults were maintained in 2-quart plastic canisters (Rubbermaid® 12 × 18.5 cm) at an average density of 50-200 beetles per canister. Canisters were filled by date, so beetles in a single canister emerged within 0-5 days of each other. Beetles laid eggs in a crevice between two plastic diet cup lids snapped together. Eggs and larvae were transferred to moistened bee-collected pollen and fed until they reached the wandering phase. Wandering larvae were then transferred to moistened sand placed in plastic, rectangular containers (Glad® 3.07L, 24 × 24× 10cm) or pupation. After emergence, beetles were collected

with an aspirator and introduced into new plastic canisters. For oviposition experiments, beetles at least 1 week old were used. For ovary development experiments and mating observations beetles were sexed within 24 hours of emergence and males and females held separately.

Ovipositional preferences day 1 and day 2

Oviposition tests were carried out in pairs of diet cups to determine if small hive beetle females preferred to oviposit on yeast-fermented pollen or control pollen. Diet cup pairs were constructed in the following manner. A single-hole punch was used to create a uniform hole approximately 1 cm below the rim of each cup. Two diet cups were joined by placing a 3 cm piece of drinking straw between the two holes such that a beetle could travel between the two cups through the straw. A piece of pantyhose cut into a 5 cm by 5 cm square was lightly stretched over the top of the diet cup before the lid was snapped on to provide a space for the beetles to oviposit between the lid and the fabric.

K. ohmeri-fermented pollen diet was prepared by the following method. *K. ohmeri* isolated from the small hive beetle (NRRL Y-27634), obtained from the ARS culture collection, was transferred from glycerol stocks into sterile sabouraud dextrose broth (Atlas 2004) with 1% yeast extract (SDBY) and incubated in a shaker-incubator at 28°C to a high cell density corresponding to a colony count of approximately 1×10^5 cfu/µl. Sterile pollen was prepared by gamma irradiating (10kGy) commercially available bee-collected pollen (Brushy Mountain, Moravian Falls, NC) at the Penn State Radiation Science and Engineering Center. To obtain fermented pollen, 16 g of sterile pollen was placed in a petri dish and 10 ml of yeast culture was added. Pollen dishes with yeast were incubated in a humid container at 28°C for 3 days before use, when preliminary experiments showed that volatile production was high and stable. For control pollen diet, sterile pollen mixed with 10 ml of sterile SDBY was used. A 0.5 ml aliquot of

K. ohmeri-fermented pollen or control pollen was placed in each diet cup with a sterile plastic syringe prior to introduction of a single female small hive beetle. No-choice controls were carried out by allowing beetles to oviposit in single, intact diet cups containing either *K. ohmeri*-fermented or control pollen.

Beetles were allowed to oviposit for up to 2 days without changing the pollen or pantyhose. Under the rearing conditions used in these experiments, nearly 100% of fertile eggs hatched within 36 hours of being laid, so it was possible to count eggs laid on day 2 separately from eggs laid on day 1.

For choice experiments, a total of 98 females were assayed in 3 replicate experiments for day 1 choices. Day 2 choices were recorded for a total of 68 females in 3 replicate experiments. For no-choice experiments, 31 females were assayed on each diet in 3 replicate experiments. These sample sizes exclude females that failed to lay any eggs during the experiment (29% for day 1 and 16% for day 2, 11% for no-choice). Ovipositional preference of females for *K. ohmeri*-fermented or control pollen was analyzed with a one-tailed paired t-test. Day 1 egg counts and day 2 egg counts were analyzed separately. Counts of eggs laid by females given either *K. ohmeri*-fermented or control pollen in no-choice experiments were analyzed by t-test.

Effect of new substrate on day 2 ovipositional preferences

Effect of adding fresh control pollen or *K. ohmeri*-fermented pollen on ovipositional preferences after 24 hours was tested by the following method. Single females were allowed to oviposit in diet cup pairs as above. One side contained control pollen and the other side contained *K. ohmeri*-fermented pollen as described above. After 24 hours, eggs were counted on pantyhose above each substrate. Lids and pantyhose were then gently removed, preserving eggs in place. Females were randomly assigned to one of three treatments: new control pollen, new *K. ohmeri*-

fermented pollen, or no change (lids removed, then replaced, but no exchange of food). There were 7 females in each treatment. Lids and pantyhose with eggs were replaced and females were then allowed to oviposit for another 24 hours before counting day 2 eggs. Females which failed to lay eggs on either day 1 or day 2 were removed from the analysis. Statistical analysis of day 2 ovipositional preference was restricted to females which chose to oviposit on *K. ohmeri*-fermented pollen on day 1. Day 2 ovipositional preferences were analyzed using a paired t-test.

Effect of egg removal on day 2 ovipositional preference

The effect of removing eggs on day 2 ovipositional preference was tested by the following method. Females were allowed to oviposit in diet cups pairs prepared as above. One side contained control pollen and the other side contained *K. ohmeri*-fermented pollen as described above. After 24 hours, eggs were counted on pantyhose above each substrate. For 15 females, all eggs were removed from the diet cup lids by removing the panty hose and wiping any remaining eggs from the diet cup lid with a clean Kimwipe®. Egg removal ensured very few larvae would be present in the diet cups on day 2. However, since females attempted to lay eggs elsewhere in the diet cup, a small number of larvae (< 10 per diet cup) were sometimes found on day 2. After removing eggs, clean pantyhose was placed on the diet cups and the lid replaced. As a control, for 16 females, diet cup lids were removed then replaced, preserving any eggs that had been laid. Females were then allowed to oviposit for another 24 hours before counting day 2 eggs. Females which failed to lay eggs on either day 1 or day 2 were removed from the analysis. Statistical analysis of day 2 ovipositional preference was restricted to females which chose to oviposit on *K. ohmeri*-fermented pollen on day 1. Day 2 ovipositional preferences were analyzed using a paired t-test.

Ovary development

In addition to ovipositional preference, ovary development was assessed in response to diet and male presence. Ovary development of newly emerged females was assessed in response to five different diets: pollen fermented by *K. ohmeri* prepared as described above, pollen fermented by *Saccharomyces cerevisiae* (NRRL Y-12632), also obtained from the ARS culture collection, prepared in the same manner as *K. ohmeri*-fermented pollen, control pollen, honey, and water.

For diet experiments, females emerged within 24 hours were separated from males and placed in groups of 5 females in diet cups. To assess the effect of males on ovary development, 3 females were caged with 2 males, each at least 24 hours old. Two males were necessary in case one of them expired during the experiment. Each diet cup was provided with 0.5 ml of food, which was replaced daily by transferring females to a new diet cup with fresh food. Diet cups in the male-effects experiment were all fed the same moistened bee-collected pollen diet fed to the colony. At each time point, ovaries were harvested from 5-6 females for each food treatment. These experiments were repeated at least 3 times for a total of at least 15 females per treatment per time point.

Dissections of ovaries were accomplished by the following method. Beetles were anesthetized on ice before dissection. One at a time, beetles were pinned ventral side up to a Slygard® silicon elastomer dish by placing two size zero insect pins through the area of the mesepisternum and mesepimeron, lateral to either mesocoxae. After removing legs, a shallow incision was made with spring scissors (straight, 2.5 mm cutting edge, Vannas®) from the tip of the abdomen to the first ventrite on either side of the abdomen. Lifting the last abdominal ventrite, all the abdominal ventrites together were separated from the organs below by severing remaining exoskeletal connections at the tip of the abdomen and around the posterior margin of

the metacoxae. Saline (0.09% NaCl) was used to float the internal organs. The spermatheca was located by cutting the ovipositor apparatus free of its connection to the last abdominal tergite and pulling it down. Moving the ovipositor down and side to side enabled visualization of the bean-shaped spermatheca, usually attached to the dorsal side of the common oviduct, in close approximation to the posterior midgut. Pulling down on the ovipositor and attached common oviduct also enabled visualization of undeveloped ovaries in the anterior body cavity. More developed ovaries filled the abdomen and were easily located after removing the abdominal ventrites. Ovaries of ovipositing females were greatly increased in size and delicate such that at the slightest pressure they would rupture and release fully developed eggs. Ovaries were removed for assessment of ovary development and each pair placed in a well of a 96-well plate with 100 µl of saline. Full and empty spermathecal structure was also observed by dissecting spermathecae from mated and unmated females and creating a wet-mount slide with saline.

Ovary development was assessed on a 5-point scale as follows. Level 1: Ovaries undeveloped. Level 2: Ovarioles enlarged, still translucent. Level 3: Ovarioles enlarged, distinct opaque sections where eggs are undergoing development present. Level 4: Near-to-fully developed eggs visible in proximal area of the ovarioles, but no eggs being released. Level 5 (egg-laying female): Fully developed eggs released in oviducts. See Figure 4-4 for photographic comparison of the 5 stages of ovary development.

Mean ovary development was analyzed using a general linear model with factors female food or male presence and days fed for days 1-3 of ovary development.

Single mating observations

In order to document the mating and courtship behavior of the small hive beetle, mating observations were made in a walk-in incubator at 33°C under red light. Groups of 5 virgin

females were introduced into a mating arena consisting of an inverted plastic petri dish. The mating arena also contained a droplet of *K. ohmeri*-fermented pollen dough on a glass coverslip (see below for preparation of fermented pollen dough), which the beetles fed upon as needed. One or two males at a time were marked with a small dot of white nail polish on the elytra and introduced into the mating arena. Using a laptop equipped with The Observer® software (Noldus) attempted matings, mountings, and copulations were recorded for interval and duration. Mating observations were ceased if no copulations had occurred within 5 minutes of introduction. If copulation occurred, mating was allowed to continue until the male dismounted and moved away from the female. Mated males and females were then collected and females were caged singly in a diet cup to confirm fertility. A piece of pantyhose cut into a 5cm by 5cm square was lightly stretched over the top of the diet cup before the lid was snapped on to provide a space for the beetles to oviposit between the lid and the fabric. Matings were considered fertile if any eggs laid by the female hatched within 4 days of mating.

Effect of 48 hour mating opportunity and previous mating experience on fertility

In addition to direct observations of mating behavior during a single bout of mating, the effects of lengthier mating opportunity and previous mating experience on pair fertility was tested. In the following experiments, males and females were given 48 hours together to accomplish mating. No effort was made to determine how many mating bouts would occur over this time period, but presumably more than one, given that preliminary observations documented that multiple matings occurred frequently. In order to obtain males with and without previous mating experience, newly emerged males were placed into one of three caging treatments: singly caged, single-sex group, or mixed-sex group. Singly-caged males were each given an individual diet cup. Size of mixed-sex and single-sex groups varied from 5-50 individuals, depending on the

number of beetles available on a given day. Small groups of beetles (5-7 beetles) were housed in a single diet cup. Larger groups of beetles were housed in plastic 50-ml centrifuge tubes with perforated lids. All caging treatments were fed the same moistened bee-collected pollen diet ad libitum. The sex ratio of the mixed-sex groups was 1:1. Males in mixed-sex cages were assumed to have had the opportunity to gain mating experience before the beginning of the next part of the experiment, while singly caged males and males in single-sex cages were assumed to have no mating experience. Females were held together in diet cups or 50-ml tubes. Beetles were allowed to remain in these groups for 2-5 days post-emergence.

Males 2-5 days old from each caging treatment were separated into individual diet cups and provided with two virgin females 2-5 days old and moistened bee-collected pollen diet. A piece of pantyhose cut into a 5 cm by 5 cm square was lightly stretched over the top of the diet cup before the lid was snapped on to provide a space for the beetles to oviposit between the lid and the fabric. After 48 hours, males were removed and females were transferred to a new diet cup with fresh diet and a fresh piece of pantyhose. If any eggs had been laid in the first 48 hours, the previous diet cup and pantyhose was kept to monitor for egg hatching. Diet cups were monitored for production of fertile eggs for two more days after the male was removed. A mating was considered fertile if any eggs had hatched in 4 days of observing oviposition. This experiment was repeated on three separate dates with a total of 40 singly-caged males, 35 mixed-sex caged males, and 24 single-sex caged males. A chi-squared test of independence was used to determine if the proportion of fertile matings was significantly different between male caging treatments and previously observed matings.

Structure of male genitalia and female spermatheca

Structure of genitalia and spermathecae are vital to reproductive success and thus are under strong selective pressure. The morphology of these and mating-associated structures such as spermatophores can shed light on reproductive biology of a species, particular in relation to sexual selection, male-male competition, and male-female conflict in mating (Arnqvist and Thornhill 1998; Eberhard 1985; Flowers and Eberhard 2006; Thornhill and Alcock 1983). Structure of the male genitalia of the small hive beetle was observed by squeezing male beetles firmly until the endophallus was fully everted. A small binder clip placed on the body of the beetle was used to maintain the pressure of fluid necessary to keep the endophallus fully inflated while it was measured and photographed. The structure of the spermatheca was examined by dissecting out the spermathecae of several mated and unmated females as described above, placing them on slides with a droplet of saline (0.85% NaCl), and viewing under the microscope. Spermatophores were not observed in the reproductive tracts of any mated females and thus spermatophore structure was not described.

Results

Ovipositional preferences day 1 and day 2

In choice chambers, beetles laid an average of 78 ± 4 (SE) eggs per female on day 1 and 102 ± 8 eggs per female on day 2. In no-choice diet cups, beetles laid an average of 103 ± 8 eggs per female. When offered a choice between *K. ohmeri*-fermented pollen and control pollen, female small hive beetles laid more eggs over the *K.ohmeri*-fermented pollen for the first day of oviposition ($p < 0.0005$). However, after the second day, females reversed their preference, and

laid more eggs over the control pollen ($p = 0.001$) (Figure 4-1). There was no significant difference in the average number of eggs laid by females in no-choice diet cups fed either *K. ohmeri*-fermented pollen or control pollen (*K. ohmeri*-fermented pollen mean ± SE: 97 ± 12, control pollen: 110 ± 12, $p = 0.458$).

Effect of new substrate on day 2 ovipositional preferences

When substrates were not changed, female oviposition on day 2 followed the same pattern as previous choice tests, with females preferring to lay eggs on the control pollen. However, this difference was not statistically significant ($p = 0.101$) in this experiment. When females were offered new *K. ohmeri*-fermented pollen, they preferred to lay eggs on this new substrate instead of control pollen on day 2 ($p = 0.002$). When offered new control pollen, however, there was no difference between the number of eggs laid on new control pollen or the old *K. ohmeri*-fermented pollen ($p = 0.236$) (Figure 4-2).

Effect of egg removal on day 2 ovipositional preference

Removing eggs did not affect day 2 ovipositional preferences. Both groups of females, either those whose previously laid eggs were removed or those who oviposited in the presence of their previously laid eggs, preferred to lay eggs on the control pollen on day 2 (Figure 4-3). However, the preference was not statistically significant for the egg-removed treatment ($p = 0.076$), but was statistically significant for the controls ($p = 0.001$).

Ovary development

Level of ovary development increased with number of days fed ($p < 0.0005$). The interaction between days fed and male presence was not significant, indicating that male presence did not significantly affect ovary development ($p = 0.064$). Females fed honey or water failed to activate ovaries above level 2, and were thus eliminated from the analysis. The interaction between the remaining diets and days fed was not significant ($p = 0.147$), indicating that pollen fermentation with neither *K. ohmeri* nor *S. cerevisiae* affected ovary activation (Figure 4-5). Virgin females fed pollen-containing diets frequently reached full ovary activation after 3 days and laid infertile eggs, indistinguishable from fertile eggs except that these infertile eggs all shriveled and dried up within 24 hours of being laid.

Single mating bout observations

General pattern of mating behavior

Initialization of beetle mating behavior followed a consistent pattern with no apparent courtship. A male would encounter a female, then rapidly mount, assume the mating position, and attempt mating (Figure 4-6). Among the Nitidulidae, the male genitalia is borne on an 8th abdominal segment, in the middle of which, the 9th tergite and sternite form a highly sclerotized tubular structure similar to a duck's bill (Figure 4-7), which guides the ejaculatory duct into the genital opening (Parsons 1943). The 9^{th} tergite is also known as the tegmen, while the 9^{th} sternite is also known as the median lobe (Düngelhoef and Schmitt 2010, Cline 2005). Attempted matings began with the male making a swift side-to-side motion, during which the tegmen could be seen extended over the edge of the female's abdomen, apparently searching for the genital opening. After locating the genital opening, the tegmen locked under the female's abdomen and stayed in

place for the duration of copulation. The male then grasped the tip of the female abdomen with his metatarsi (Figure 4-6). Initiation of copulation was accompanied by repeated stroking of the female's elytra with pro- and meso-tarsi, which ceased 2-3 seconds into the copulation.

Males attempted mating as early as 24 hours old, but fertile matings were only observed when males were at least 48 hours old. Females were capable of fertile matings at 24 hours old. When allowed, males would attempt mating with multiple females with no interval between matings. When allowed, females would accept mating from multiple males with no interval between matings. Such matings occasionally occurred, despite the experimenter's attempts to catch the beetles before they began copulating again. These multiple matings were not included in the analysis.

Single mating bout success and fertility

In 41% of the trials, no mating attempts were observed. The percent of males attempting copulation that ultimately achieved copulation was 70%. A large number of apparently successful copulations resulted in no production of fertile eggs (73% of all observed matings). The number of matings observed that resulted in the subsequent production of fertile eggs was 28 of 103. The following details are reported from these fertile matings. Males remained mounted on females an average of 321 ± 65 (SE) seconds, copulating an average of 3.3 ± 0.46 times per mounting for an average of 53 ± 8 seconds per copulation. While many matings were apparently unsuccessful, the values for average copulation number and average copulation duration did not differ significantly between infertile and fertile matings (copulation number: $p = 0.379$, duration per copulation: $p = 0.277$), as evaluated by a t-test. The only factor that differed significantly between fertile and infertile matings was the average time spent mounted on the female ($p = 0.012$). During fertile matings, males remained mounted on females an average of 321 ± 65 (SE) seconds, while during

infertile matings, males remained mounted on females an average of only 142 ± 17 seconds. Mated females that failed to produce fertile eggs produced instead infertile eggs.

Male-male fighting

Of the over 100 pairs observed in arenas where multiple males were present, males were observed fighting on 3 occasions. Attacking males were identified as they attempted to dislodge already mounted males. This the attacking male accomplished by flipping the pair over, using his head to flick them upward. Attacking males also butted heads with rival males or with the desired female. If challenged while mounted but not copulating, the mounted male would quickly move backward and assume the mating position apparently in an effort to block efforts of the rival male. One fight between two males escalated to include chasing each other and climbing on top of each other. After climbing on top of his rival, the male appeared to attempt mating with his rival. One of the three fights resulted in a turnover, where the attacking male managed to achieve copulation with the desired female. The other two fights resulted in either no success in separating the mating pair or a prolonged fight between the rival males in which it was impossible to determine if the victor was the original copulating male or the challenger.

Interesting male-male interactions were also observed on two occasions in arenas with 5 to 7 males awaiting the addition of females. These interactions appeared to be attempted matings, similar to the behavior observed in one of the fights between rival males.

Effect of 48 hour mating opportunity and previous mating experience on fertility

When males were given 48 hours to inseminate females, the fertility rate was much higher than when only one mating bout was allowed ($\chi^2 = 31.6$, df = 1, $p < 0.0001$). Cage

treatment – singly-caged, single-sex caging, and mixed-sex caging – did not affect the proportion of matings that were fertile ($\chi^2 = 0.027$, df = 2, p = 0.987) (Figure 4-8).

Structure of male genitalia and female spermatheca

The structure of the small hive beetle male genitalia was surprisingly complex. The endophallus is approximately 2.5 mm long by 0.5 mm wide, uniquely decorated by lobes and sclerites, and appears to bear a patch of denticles or sensillae on the basal portion (Figure 4-8).

The spermatheca of the small hive beetle is a balloon-like structure accompanied by a large accessory gland which joins the spermatheca near the opening of the spermathecal duct. The spermathecal duct is very long (Figure 4-10A). When fully mated, the spermatheca is noticeably opaque under the dissecting microscope. When the spermatheca of a mated female is placed under a coverslip, it readily ruptures and releases a tightly tangled ball of sperm (Figure 4-10B).

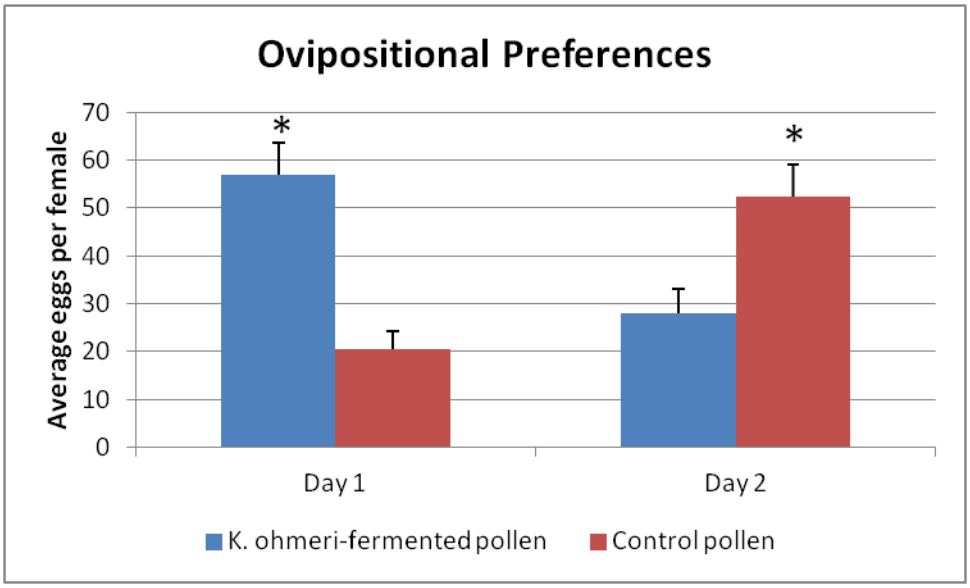

Figure 4-1: Oviposition preference of small hive beetle females for *K. ohmeri*-fermented pollen versus control pollen after one day of oviposition and two days of oviposition. Significant differences (one-tailed t-test $p < 0.05$) are indicated by an asterisk.

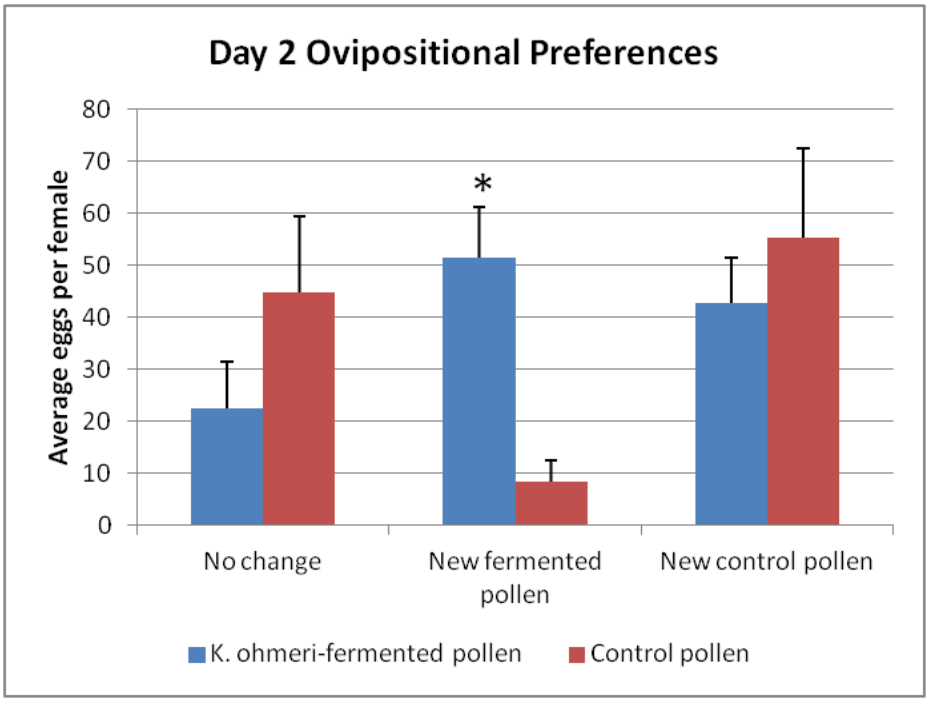

Figure 4-2: Day 2 ovipositional preferences of females given new *K. ohmeri*-fermented pollen, new control pollen, or no new substrate after 24 hours. Significant differences (one-tailed t-test $p < 0.05$) are indicated by an asterisk.

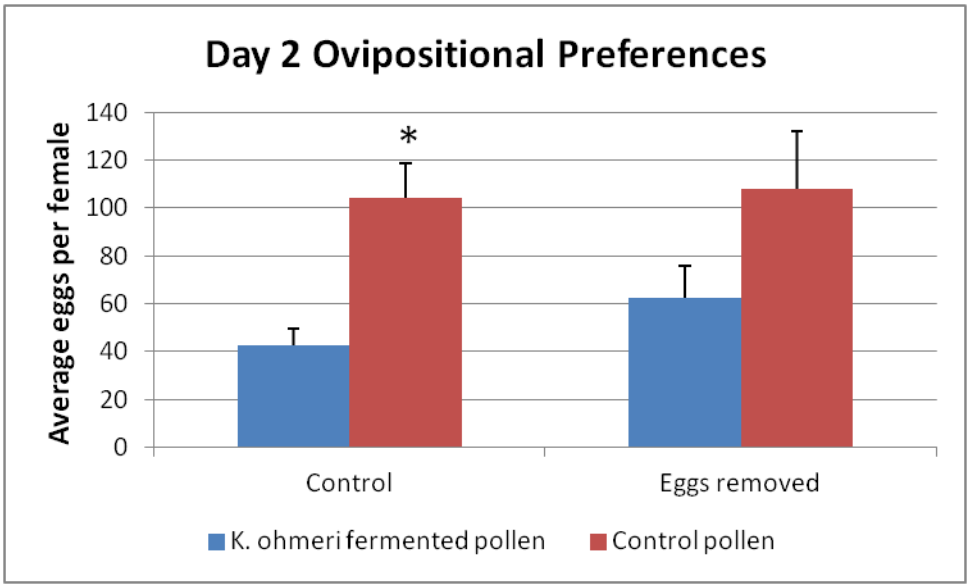

Figure 4-3: Day 2 ovipositional preferences of females after previous day's eggs were removed or left behind to hatch (control). Significant differences (one-tailed t-test $p < 0.05$) are indicated by an asterisk.

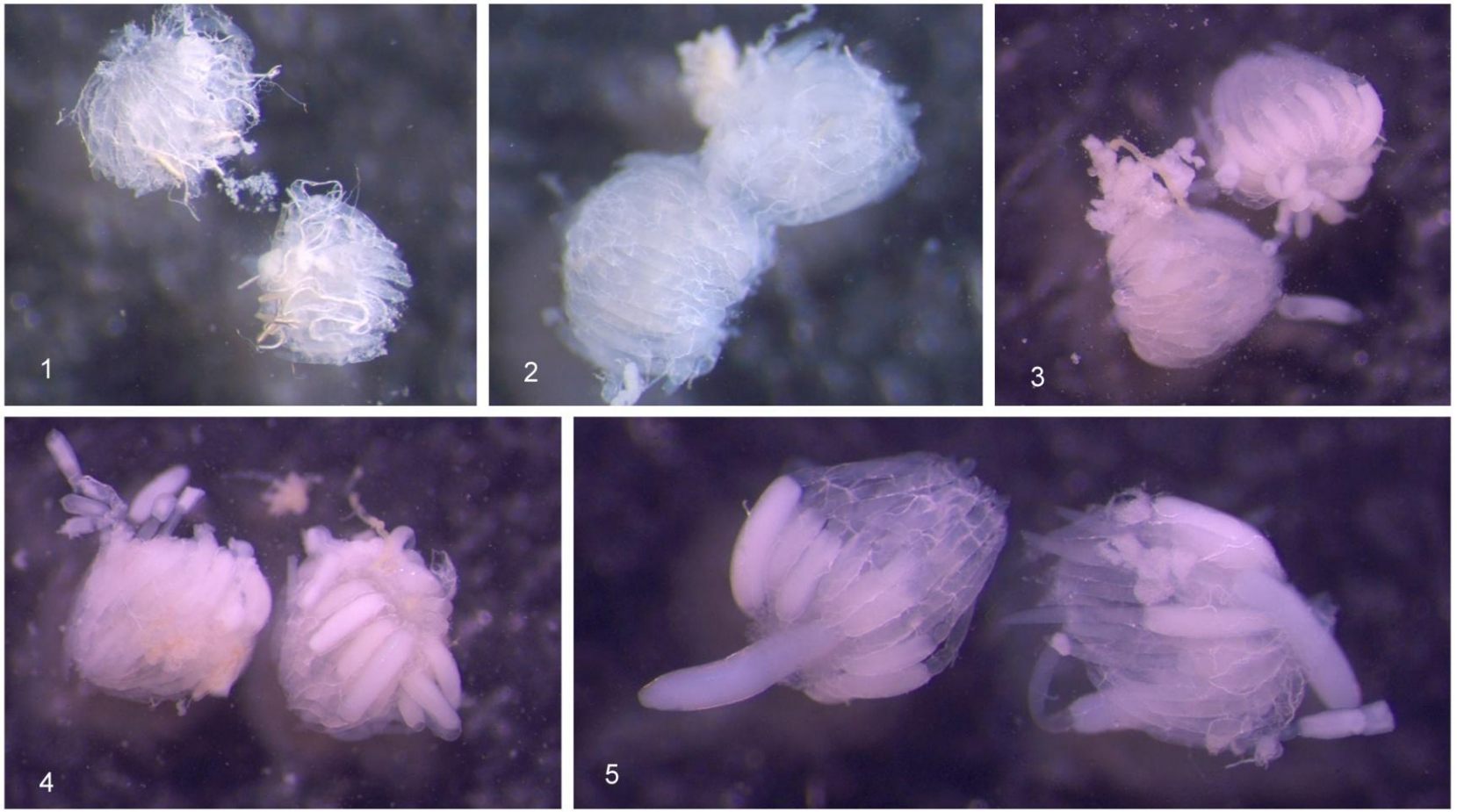

Figure 4-4: Stages of ovary development in the small hive beetle. 1: Ovaries undeveloped. 2: Ovarioles enlarged, still translucent. 3: Ovarioles enlarged, distinct opaque sections where eggs are undergoing development present. 4: Near-to-fully developed eggs visible in proximal area of the ovarioles, but no eggs being released. 5 (egg-laying female): Fully developed eggs released in oviducts.

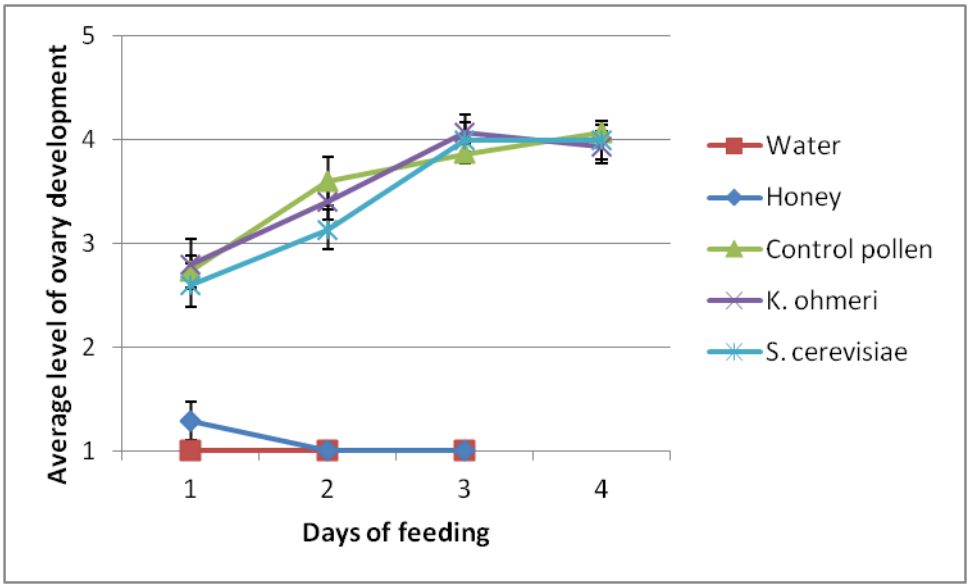

Figure 4-5: Average ovary development level (with SE) of newly emerged females for 5 diets over 4 days. See Figure 4-4 for photographic comparison of ovary development levels.

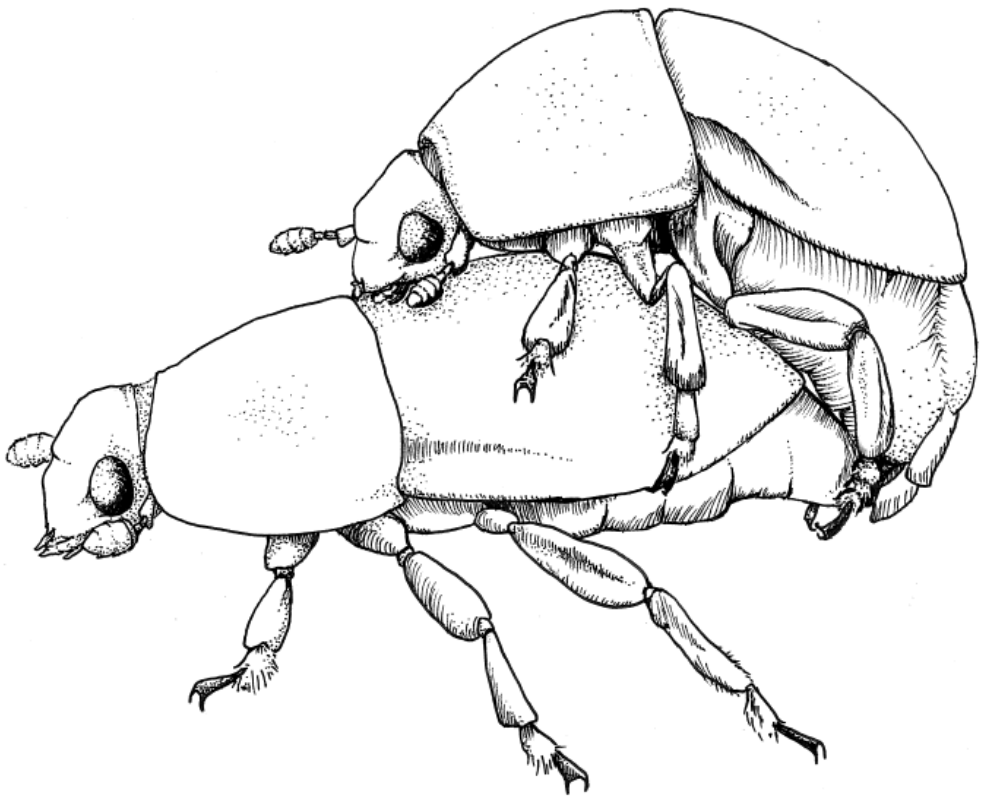

Figure 4-6: Copulating small hive beetles, male above. Drawing by Kerry Mauck.

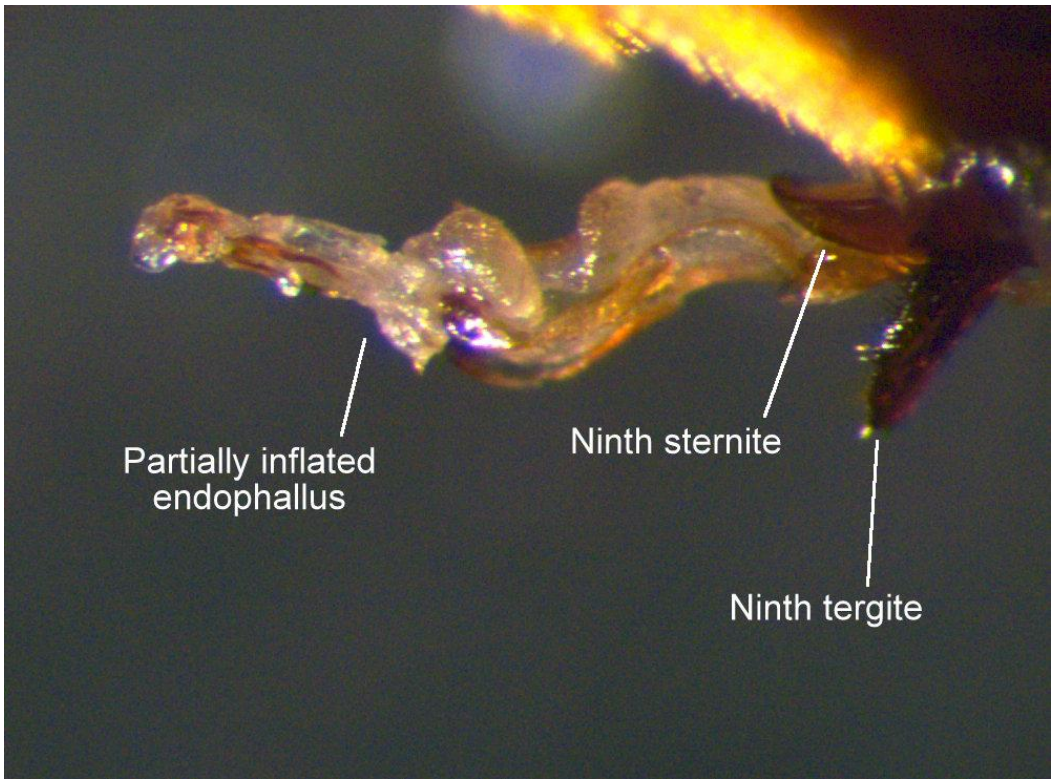

Figure 4-7: Small hive beetle adeagus, showing 9th sternite (median lobe) and tergite (tegmen).

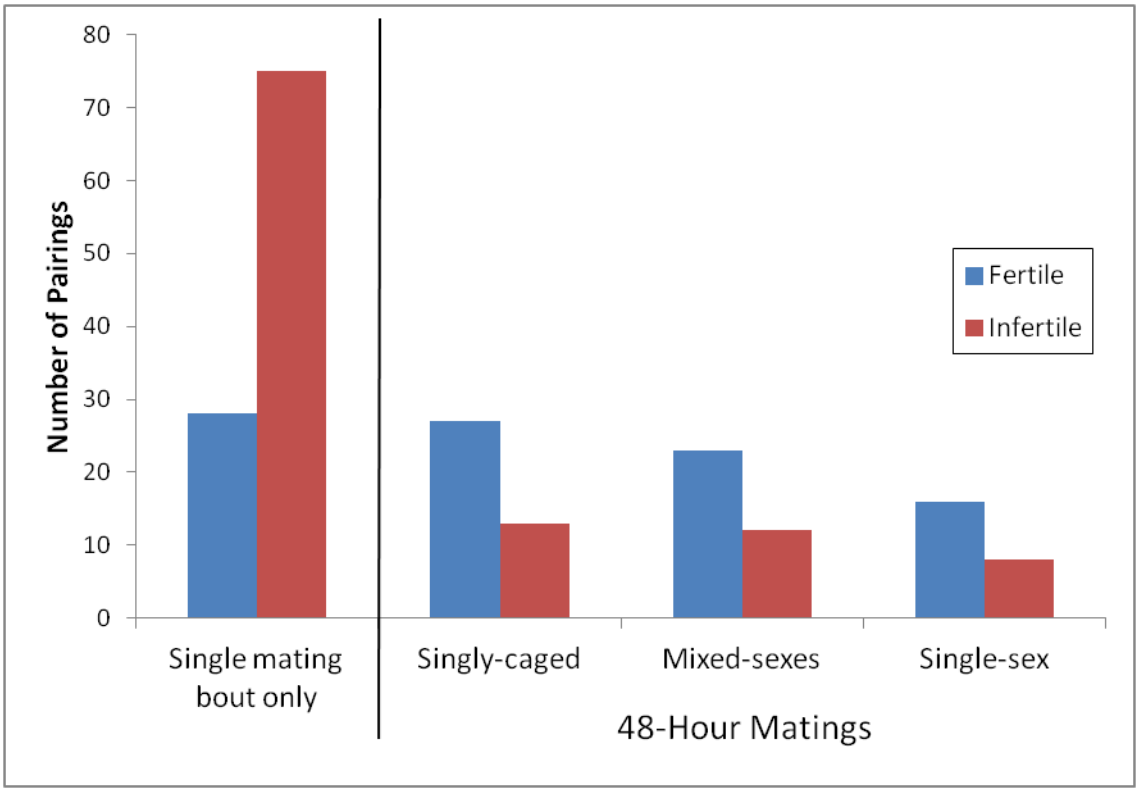

Figure 4-8: Fertility of 48-hour mating periods with males caged singly, with females, or with other males, as compared with fertility of single mating bouts.

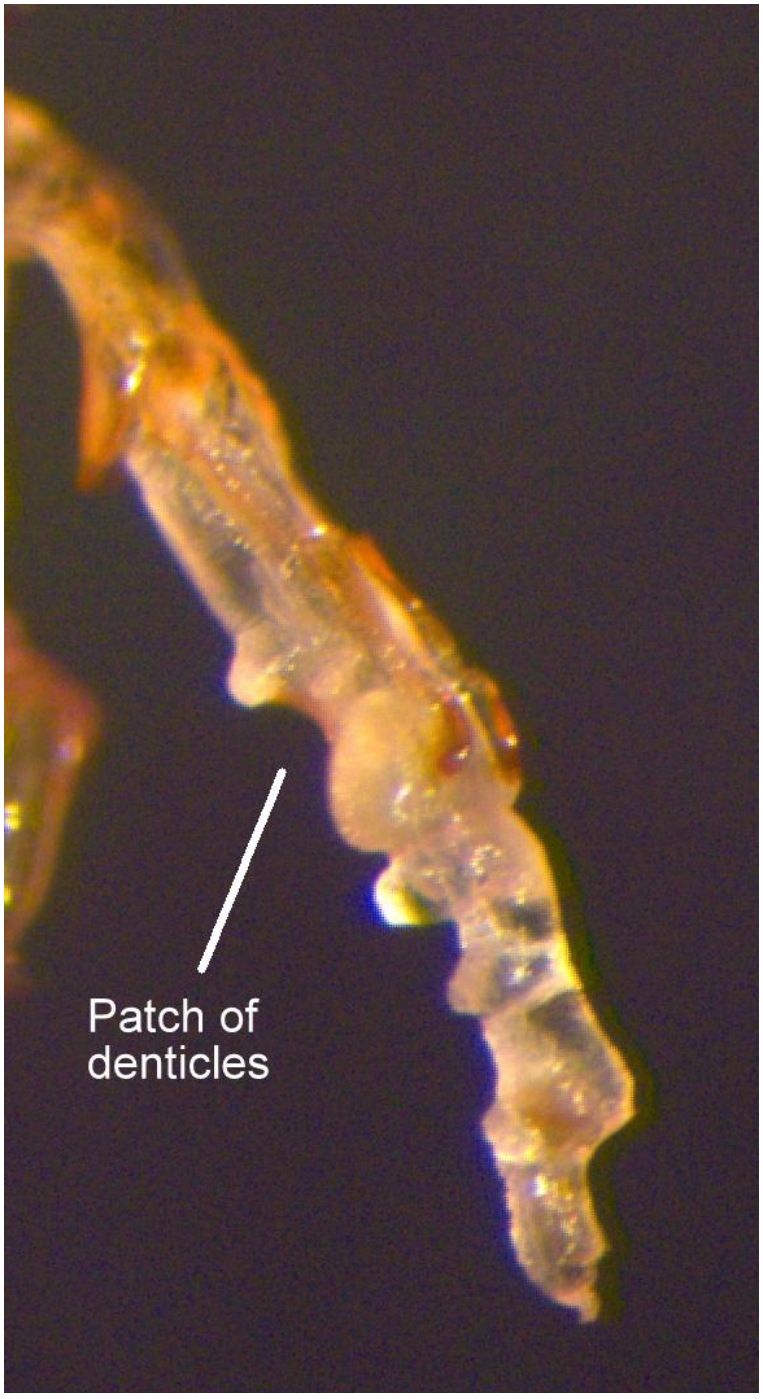

Figure 4-9: Inflated endophallus of the small hive beetle

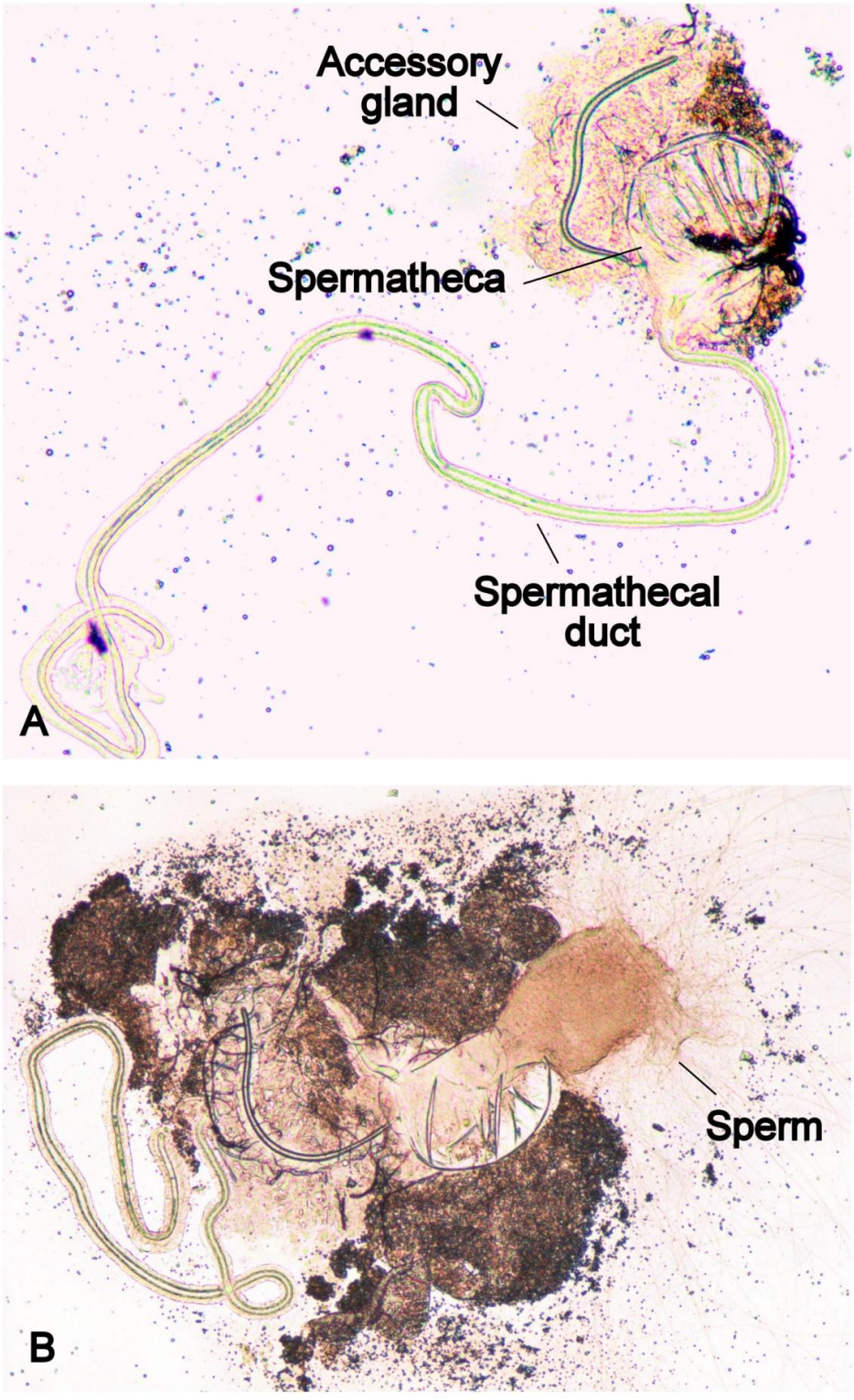

Figure 4-10: Small hive beetle spermatheca of unmated female (A) and mated female (B). Magnification 40X.

Discussion

Oviposition behavior and ovary development

While beetles did prefer to lay eggs on *K. ohmeri*-fermented pollen, no choice experiments and ovary development experiments indicated that this increased oviposition was not due to increased egg-laying or increased ovary activation. *K. ohmeri*-fermented pollen has been shown to be extremely attractive to both male and female small hive beetles (Torto et al. 2010a; Torto et al. 2007c; Torto et al. 2007a).

Follow up experiments on the effects of new substrate and egg removal on day 2 oviposition confirm that ovipositional preference is strongly influenced by volatiles produced by *K. ohmeri*-fermented pollen. The only change that caused females to continue to prefer to lay eggs on *K. ohmeri*-fermented pollen after 24 hours was adding new *K. ohmeri*-fermented pollen. Pollen fermented by *K. ohmeri* for an extended period of time has been shown to be far less attractive than freshly fermented pollen (Torto et al. 2007a). During the first day, females may also have inoculated the unfermented, control pollen with yeast by walking around, such that by the second day, this control pollen had begun to produce attractive volatiles, accounting for the increase in oviposition on the control pollen after two days. Presence of beetle larvae has been shown to inhibit oviposition in small hive beetles (Keller 2002). However, removing the eggs laid during day 1 did not alter the preference for control pollen on day 2.

Neither fermentation by *K. ohmeri* nor *S. cerevisiae* increased ovary development in the small hive beetle. These results conflict somewhat with Keller's (2002) observations of the small hive beetle and Hopkins and Ekbom's (1999) observations with the nitidulid *Meligethes aeneus* that beetles switched from one food to another changed their oviposition rate in response to food. However, the alternative diets used by both of these studies were extremely low quality compared

with the preferred diet. It is possible that *K. ohmeri*-fermented, *S. cerevisiae*-fermented, and control pollen are all high-quality diets, such that differences in oviposition were not detectable. Meikle and Patt (2011) found diet only affected oviposition at 35°C. Arbogast et al. (2010) found that lifetime egg production did not differ between diets of pollen dough and oranges, though larval survival was greatly reduced on oranges. It seems, then, that diet has a more profound effect on larvae than adults of the small hive beetle. Further studies on the effect of yeast on reproduction of the small hive beetle should focus, therefore, on larval development. Nevertheless, the small hive beetle clearly prefers to lay eggs near *K. ohmeri*-fermented food and as such the presence of this yeast in the hive may increase beetle attraction and increase the chances that beetles will reproduce in the hive.

Mating behavior

Guarding behavior

Small hive beetle mating behavior proved complex. Small hive beetle males formed prolonged associations with females, alternating between copulation and a passive phase, during which the male appeared to guard the female. Guarding behavior is typically associated with male-male competition (Thornhill and Alcock 1983). Though fighting was observed rarely, it is possible that guarding behavior may help ensure paternity in a situation where multiple males are present. However, in single mating bouts, the guarding period was relatively short, and ended well before females laid any eggs, allowing for other males to inseminate the female. Also, the functional sex ratio of the small hive beetle is skewed toward females, so there is probably no shortage of females to mate with in any given location (Ellis et al. 2002b; Neumann et al. 2001c; Spiewok and Neumann 2012). Cryptic female choice may provide alternative explanation for

guarding behavior observed in the small hive beetle. To consider this alternative, we must examine the mating biology of the Chrysomeloidea, a large, diverse sister taxon to the Cucujoidea which contains the Nitidulidae (Hunt et al. 2007).

Cryptic female choice?

The mating behavior of the small hive beetle followed a similar pattern to that of many Chrysomelid beetles. Chrysomelids often alternate between active and passive phases of copulation while remaining in contact with the female (Dickinson 1997). However, this "guarding" behavior is not to ensure paternity as in other insects. The passive phase of mating in the Chrysomelidae is thought to be a form of copulatory courtship (Dickinson 1997; Rodriguez 1994; Rodriguez 1993). Copulatory courtship as defined by Eberhard (1997) is courtship behavior that occurs after intromission has begun. Cryptic female choice is thought to have selected for these behaviors in the Chrysomelidae and other insects (Dickinson 1997; Eberhard 1997). In species with cryptic female choice – where females can control sperm use post-copulation – males must seek to influence the female's choice to accept and utilize his sperm. Copulatory courtship behavior such as stroking and guarding are thought to influence female choice. Therefore, prolonged associations offer more opportunity for the male to "convince" the female to accept his sperm.

It is possible that cryptic female choice is at least partially to blame for the high rate of infertile matings observed in the small hive beetle. Fertility of small hive beetle matings was related to the length of the passive phase, or time spent on the female regardless of number or duration of copulations completed during their association. The results from the 48-hour mating experiments tend to support this hypothesis that extended association improved fertility of

matings. The structure of the small hive beetle endophallus, which proved to be complex, may also play a role in copulatory courtship to influence cryptic female choice (Eberhard 1985).

The 30% infertility of extended matings is still puzzling. Temperature is known to affect fertility of the small hive beetle. At 35°C, beetles were 30-76% infertile (Meikle and Patt 2011). At more moderate temperatures (28-32°C), infertility has been reported to vary from 8% to 20% (Arbogast et al. 2010; Meikle and Patt 2011). It is possible that there is a low level of natural infertility in some populations of the small hive beetle or that lab rearing conditions are not optimal. Future work should assess the mating status of field-caught females to determine if mating is indeed a barrier to small hive beetle growth.

Mating disruption for control of the small hive beetle

If our findings are applicable to field populations, small hive beetles may need an extended period of mating in order to begin laying fertile eggs. Mating disruption strategies could help control the small hive beetle. These strategies might include removing alternative food sources such as hive refuse piles where beetles can mate freely, or eliminating crevices in hives big enough to hide a mating pair of small hive beetles, forcing them to mate in open areas where bees can harass them. Likewise, maintaining colony strength would ensure harassment by bees would keep beetles on the move, preventing extended matings.

Conclusions

The results of these studies offer an intriguing window into the reproductive behavior of the small hive beetle. We can conclude that control of small hive beetle reproduction at the oviposition stage may be difficult, given their ability to lay eggs on a wide range of diets.

However, control of the larval population may be more feasible, sanitation practices to reduce the availability of larval food sources in the hive and mating disruption strategies to limit beetle fertility. More research is needed to understand the intricacies of small hive beetle mating behavior.

Chapter 5

Conclusions

The data gathered in this dissertation has answered some questions about small hive beetle-yeast interactions and basic biology of these two organisms, but has raised still more questions which demand further research. I will consider a few of the outstanding issues in this section.

In the first part of this dissertation, the research question was "What factors influence the ability of *K. ohmeri* to grow in bee hives?" I gained a partial answer by determining that high water activity (and therefore high ambient humidity) is necessary for the growth of *K. ohmeri* on pollen. This result clearly indicates that if yeasts such as *K. ohmeri* are important in the attraction of small hive beetle to bee hives, they may only be active when humidity is high in the hive. The influence of humidity may explain the severity of small hive beetle infestations in sub-tropical regions such as southern Florida and Queensland, Australia as opposed to the relative insignificance of small hive beetle to beekeepers in drier climates. Perhaps year-round high humidity is necessary for growth of *K. ohmeri* and production of beetle-attractive volatiles which may act as an aggregation signal. Experiments confirmed that yeast was capable of growing on bee bread even when the small hive beetle was not present, but beetle larvae and beetle frass were potent vectors of *K. ohmeri*. Even newly emerged adult small hive beetles carried *K. ohmeri*, and could potentially vector this yeast. These findings indicate that the highest likelihood scenario for *K. ohmeri* causing small hive beetle aggregations is where some small hive beetles are already present in the hive. However, since the second part of this dissertation found evidence that many other yeasts produce beetle attractants and grow on pollen, it is possible that yeasts already present in the hive could attract and cause aggregations of small hive beetles in bee hives. The next question to be answered more fully is if yeasts in the hive can cause field aggregations of

small hive beetle. These future field experiments should control for hive humidity either by artificially increasing hive humidity or experimenting in regions and seasons where humidity is consistently high, and, of course, any efforts should include in-hive monitoring of humidity throughout the experiment. A systematic hive yeast sampling method such as I used in my field experiments should be employed to verify the presence and growth of yeasts under humid conditions.

The question of the existence of a specific relationship between small hive beetle and the yeast *K. ohmeri* was answered with a resounding "no" in this dissertation. Many yeasts, including those present in the bee hive already, were found to attract the small hive beetle and could potentially cause beetle aggregations. The small hive beetle's catholic response to yeast odors tends to confirm the previous hypothesis, that nitidulids are chemical generalists (Blackmer and Phelan 1992). The similar odors produced by yeasts and bee hives may have initially attracted the small hive beetle's ancestors to bee hives, but cannot explain why they are not frequently found on other substrates. We must look elsewhere, then, to answer the question of why the small hive beetle specializes on bee hives when myriad other hosts are available. In fact, new information on the small hive beetle has revealed that these bee hive specialists retain a high degree of attraction to fruit-specific volatiles (P. Teal, personal communication), even though the small hive beetle is only infrequently found associated with fruits. Such attractive odors can be used to trap and control the small hive beetle, but do not provide useful information about the evolution of specialization of the small hive beetle. We must now ask ourselves not what chemical adaptations, but what behavioral adaptations have led to the evolution of the small hive beetle's specialization on bee hives. The most obvious behavioral adaptation of the small hive beetle to bee hives is its ability to solicit food from honey bee workers while imprisoned in the bee hive. A provocative hypothesis has recently been proposed to explain how small hive beetles actually seek out confinement locations, perhaps to take advantage of the gullible guard bees which can be

tricked into feeding the confined beetles (Atkinson and Ellis 2011a, b). Future research should focus on what, if any, benefits, including fitness advantages, small hive beetles reap from their imprisonment in the hive. Perhaps this unique behavioral adaptation is the key to understanding the evolution of the small hive beetle.

In the third part of this dissertation, I further explored the association between the small hive beetle and *K. ohmeri* by examining its effect on oviposition. Small hive beetle females preferred to lay their eggs near *K. ohmeri*-fermented pollen, but only when this fermented pollen was fresh. This provides a very interesting path for future research. It is possible that fermented pollen is only attractive for oviposition for a short window of time, indicating that the nutritional benefit of yeast fermentation may only exist for a short period. This result is supported by the speed at which small hive beetle eggs hatch at 30°C, as if the beetle larvae must quickly take advantage of the substrate. It has been proposed that *K. ohmeri*-fermented pollen has an "expiration date" between 7 and 14 days, after which beetles were no longer attracted (Torto et al. 2007). Why this loss of ovipositional preference occurred should be more fully explored. Was it due to the loss of attractive volatiles or the presence of repellants? If ovipositional repellants exist, these could be developed into an effective small hive beetle control method. Future research should also attempt to quantify the benefits of yeast to the small hive beetle. Since no benefit was detected in egg output or ovary development, it is possible that the small hive beetle larvae benefit most from yeast. The difficult part of these experiments will be to eliminate the influence of yeast already present on the small hive beetle larvae. Small hive beetle eggs and larvae carry yeast and yeast-free larvae are very difficult to obtain (N. Benda, personal communication). Possible strategies for eliminating the yeast already on the beetle may be to treat eggs or small larvae with a low dose of fungicide initially, then transfer larvae to sterile or yeast-inoculated diet, since fungicide will doubtless affect the growth of larvae and will be difficult to control for

otherwise. If yeast presence has a strong effect on larval growth, it may be worthwhile to investigate methods for controlling yeast in bee hives and on small hive beetles.

A final intriguing result from this dissertation was the first description of the mating behavior of the small hive beetle. Single mating bouts were exceptionally infertile, while 48-hour mating periods allowed for a much higher rate of fertility. Mating experience did not appear to influence the fertility of matings. Since females producing infertile eggs were consistently found without any sperm in their spermathecae, there are two possible explanations for why this infertility existed which must be investigated further. One is that small hive beetle females exert powerful cryptic female choice, rejecting sperm from males unless the male spends an extended time engaging in copulatory courtship behaviors such as guarding. Very little is known about the natural mating habits of the small hive beetle. Sometimes they have been observed mating in the hive, but it is possible that small hive beetles also mate before entering hives, avoiding the harassment of bees and allowing for extended matings. Collecting small hive beetle females in hives and outside of hives and examining their spermathecae for sperm could possibly answer this question. The other possible explanation for the high rate of infertility is that male small hive beetles in this experiment were unable to transfer sperm due to some unknown factor. Infertility of up to 20% has previously been reported for small hive beetle pairs in the lab. Diet and rearing conditions may have a profound effect on small hive beetle male fertility. If diet is responsible for the infertility seen in these experiments, it would be well worth discovering what factors in the diet effect beetle fertility for their potential as small hive beetle "birth control." Use of live/dead staining techniques could shed light on the proportion of live sperm which small hive beetle males carry and the influence of diet on this proportion.

In conclusion, there is much that it is still mysterious about the pest of honey bees known as the small hive beetle. As a nitidulid with the ability to feed on many substrates, it has chosen a difficult path by associating itself with honey bees. It has retained its ancestral preferences for

fermentation volatiles and symbiotic yeasts. However, relationships between nitidulids and fungi are remarkably flexible. Small hive beetle researchers must be wary of becoming too focused on a single symbionts or a single attractant. The small hive beetle, like other nitidulids, is successful, not due to its ability to specialize, but its adaptability. Small hive beetle researchers must, likewise, embrace the multiplicity of hypotheses and remain open minded to the many possibilities of this system if research is to continue forward.

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Master of Science in Entomology, 2006. University of Florida, Gainesville, FL.
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TEACHING EXPERIENCE

NSF GK-12 Teaching Fellow, North Lincoln Hill Middle School, Phillipsburg PA, 2011-2012
Teaching Assistant:

- Introduction to Entomology (Penn State University ENT 313).
- Insect Connections (Penn State University ENT 202).
- Principles of Entomology (University of Florida ENY 3005).

Invited lectures

- Delaware Valley College apiculture class, 2012 – "The Small Hive Beetle."
- Foundations of Linguistics (Penn State University LING 100), 2011 – "Animal communication."
- High school homeschool group, 2011 – "Introduction to Chemical Ecology."

WISE workshop coordinator, 2008

SKILLS

Experimental techniques: Microscopy, analytical chemistry, microbiology, molecular biology, insect rearing, aquatic sampling, animal behavior, beekeeping, gardening, statistical analysis.
Classroom technology: Smartboard, Power-point, Elmo opaque projector.
Online assessment tools: ANGEL course management.
Web-authorship: HTML, CSS, JAVA, Javascript, Blogging using Blogger and Wordpress.

PERSONAL INTERESTS

Social: Service and bible study through church and Cru-affiliated graduate student ministry, discussing issues of faith and academic integration.
Travel: Avid traveler to 19 countries on 5 continents, including: research trip to Kenya, missions trips, teaching English as a second language at summer camps in Czech Republic and Hungary, backpacking in Argentina, and beekeeping tour of New Zealand.
Hobbies: Knitting, writing, reading, cooking, insect collecting, maintaining community garden plot, running (completed first half-marathon in February 2012), hiking, backpacking, camping, canoeing.

PUBLICATIONS

Frazier, M., E. Muli, T. Conklin, D. Schmehl, B. Torto, J. Frazier, J. Tumlinson, J. D. Evans, S. Raina. 2010. A Scientific Note on *Varroa destructor* found in East Africa; threat or opportunity? *Apidologie*. 41: 463-465.
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