BIOLOGICAL CONTROL OF COFFEE BERRY BORER: THE ROLE OF DNA-BASED GUT-CONTENT ANALYSIS IN ASSESSMENT OF PREDATION

Eric G. Chapman[1], Juliana Jaramillo[2, 3], Fernando E. Vega[4],

& James D. Harwood[1]

[1] *Department of Entomology, University of Kentucky, S225 Agricultural Science Center North, Lexington KY 40546-0091, U.S.A., eric.chapman@uky.edu; james.harwood@uky.edu;* [2] International Center of Insect Physiology and Ecology (*icipe*) P.O.Box 30772-00100 Nairobi, Kenya. [3]Institute of Plant Diseases and Plant Protection, University of Hannover, Herrenhäuser Strasse. 2, 30419 Hannover - Germany. *jjar2001@yahoo.com;* [4]*Sustainable Perennial Crops Laboratory, U. S. Department of Agriculture, Agricultural Research Service, Building 001, Beltsville MD 20705, U.S.A. Fernando.Vega@ars.usda.gov*

ABSTRACT.

The coffee berry borer, *Hypothenemus hampei*, is the most important pest of coffee worldwide, causing an estimated $500 million in damage annually. Infestation rates from 50-90% have been reported, significantly impacting coffee yields. Adult female *H. hampei* bore into the berry and lay eggs whose larvae hatch and spend their entire larval life within the berry, feeding on the coffee bean, lowering its quality and sometimes causing abscission. Biological control of *H. hampei* using parasitoids, fungi and nematodes has been reported but potential predators such as ants and predatory thrips, which have been observed in and around the coffee berries, have received little attention. This study reviews previous *H. hampei* biological control efforts and focuses on the role of predators in *H. hampei* biological control, an area in which tracking trophic associations by direct observation is not possible in part due to the cryptic nature of the biology of *H. hampei*, spending its life cycle inside the berry. The use of molecular methods to detect the presence of small amounts of prey in the digestive tracts of predators is the primary focus of this research program, and ultimately elucidating food web structure and making recommendations for biological control. We designed *H. hampei*-specific primers to demonstrate that *H. hampei* DNA can be detected in DNA extractions of a predatory thrips species, *Karnyothrips flavipes*, which preys on *H. hampei*. We demonstrate the potential of this molecular technique to unravel the trophic interactions that occur inside the coffee berry.

INTRODUCTION.

Coffee (Rubiaceae: *Coffea* spp.) is the most important agricultural commodity in over 70 countries, accounting for over US $70 billion in annual retail value (Vega *et al*. 2006). Of the more than 100 species in the genus *Coffea* (Davis *et al.* 2006), only two species, *Coffea arabica* L. and *Coffea canephora* Pierre ex A. Froehner are grown commercially. Small-scale farmers produce ~70% of the world's coffee, with over 100 million people depending on its production (Vega *et al.* 2003). Coffee is attacked by more than 850 species of insects (Le Pelley 1968, 1973). Of these, the coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae), is the only species that directly attacks the seed (Vega *et al.* 2003), and is the main threat to coffee production (Damon 2000; Jaramillo *et al.* 2006), with annual losses

exceeding US $500 million (Vega 2004). This pest was first described in 1867 in France feeding on coffee beans (Waterhouse & Norris 1989) and has subsequently spread to all coffee producing countries except Hawaii (Vega 2004) and Nepal. In this paper, we review the biology and ecology of *H. hampei*, examine various biological control efforts, and demonstrate the potential for DNA-based detection methods in unraveling trophic interactions between these cryptic pests and their natural enemies.

BIOLOGY AND ECOLOGY OF *HYPOTHENEMUS HAMPEI*.

The life cycle of *Hypothenemus hampei*.

Developing coffee berries are typically attacked by single mated female *H. hampei* from between eight weeks after flowering until harvest (>32 weeks) (Baker 1999). It takes up to eight hours for adult female *H. hampei* to bore through a coffee berry to reach the endosperm (Sponagel 1994; Fig. 1). A female *H. hampei* lays 200-300 eggs over a period of 60 days (Jaramillo 2008). At 27°C, the egg stage averages 4.3 days, the three larval stages average a total of 12.0 days, and the pupal stage 5.2 days (Jaramillo 2008). Due to the 60-day oviposition period, all life stages co-occur in the berry. Mating occurs between siblings within the berry (Bustillo *et al.* 1998), after which the female either remains in the berry to commence oviposition, or exits in search of another berry to colonize (Baker *et al.* 1992). High temperature and relative humidity triggers emergence of mated females (Baker *et al.* 1992) but males spend their entire life cycle inside the berry (Ticheler 1961). During the interharvest period, a single berry may contain as many as 150 adult *H. hampei*, and reproduction only ceases once all resources have been consumed (CENICAFÉ 1993).

Fig. 1. (a) Female *Hypothenemus hampei* penetrating a coffee berry (b) *Hypothenemus hampei* life stages inside a coffee berry. (Photographic acknowledgment: G. Hoyos CENICAFÉ, Chinchiná, Colombia).

Damage caused by *Hypothenemus hampei*.

Hypothenemus hampei causes three types of damage. First, because they feed on the endosperm, there are both losses in overall yield and in quality of the beans (Le Pelley 1968; Moore & Prior 1988). Secondly, mature berries become vulnerable to

further insect attack and infection by fungi due to the physical damage caused by the boring and feeding activities of *H. hampei* (Waterhouse & Norris 1989). Thirdly, *H. hampei* infestations can result in arrested development, decay or premature fall of the berry (Le Pelley 1968).

BIOLOGICAL CONTROL OF *HYPOTHENEMUS HAMPEI*.

Species of parasitic and predatory Hymenoptera and entomopathogenic nematodes and fungi, have all been examined to various degrees for their potential role as biological control agents of *H. hampei*.

Parasitoids.

Three species of bethylid wasps are larval-pupal ectoparasitoids of *H. hampei*: *Cephalonomia stephanoderis* Bertrem, *C. hyalinipennis* Ashmead, and *Prorops nasuta* Waterson. *Cephalonomia stephanoderis* and *P. nasuta* are of African origin and have been introduced into the Americas where populations have become established. In contrast, *C. hyalinipennis* was first reported as a parasitoid of *H. hampei* in Mexico (Pérez-Lachaud 1998), but was previously documented in Europe, the United States, and Canada (Pérez-Lachaud *et al.* 1999). These three species also fed on *H. hampei* eggs (Jaramillo *et al.* 2006). However, the impact of *C. stephanoderis* and *P. nasuta* has been minimal (Damon 2000; Baker *et al.* 2002). Interspecific competition among these three bethylid species has also been reported in laboratory studies with evidence for aggressive host and brood guarding behavior (Batchelor *et al.* 2006). Furthermore, *C. hyalinipennis* exhibited hyperparisitoid behavior when provided with *C. stephanoderis and P. nasuta* larvae, leading Pérez-Lachaud *et al.* (2002, 2004) to conclude that *C. hyalinipennis* is a facultative hyperparisitoid. Because repeated augmentative releases of a single bethylid species yields parasitism levels below 5% (Baker 1999), the potential reduction in efficiency of these parasitoids, if released together, may be negligible. Therefore, a positive economic impact may be derived from the presence of multiple bethylid species, especially if examined on a regional basis.

Phymastichus coffea LaSalle (Hymenoptera: Eulophidae), native to Africa, is a gregarious endoparasitoid of adult female *H. hampei* (Borbón-Martinez 1989) and can parasitize *H. hampei* females within hours of emerging (Jaramillo *et al.* 2006). Once parasitized, female *H. hampei* cease oviposition and usually die after 12 days (Feldhege 1992). Additionally, the highest levels of parasitism were recorded by Jaramillo *et al.* (2005) in berries less than 160 days old, before female *H. hampei* reached the endosperm of the berry, thus preventing damage to the coffee bean. Mass release of *P. coffea* should therefore be timed such that the majority of *H. hampei* have not completely bored into the berries (Jaramillo *et al.* 2005). First introduced in Colombia in 1996, *P. coffea* has become established in North, Central and South America (Baker *et al.* 2002).

Predators.

Heterospilus coffeicola Schmiedeknecht (Hymenoptera: Braconidae) is a wasp native to Africa that preys on *H. hampei*. A female *H. coffeicola* lays one egg per berry and its larva consumes *H. hampei* eggs and larvae (Murphy *et al.* 2001). Due to

difficulties encountered in rearing, *H. coffeicola* has not been used in biological control programs. An unidentified African species of *Leptophloeus* (Coleoptera: Laemophloeidae) has also been observed as a predator of *H. hampei* larvae (Vega *et al.* 1999), and nine ant genera (Hymenoptera: Formicidae) prey on *H. hampei* in Colombia (*Brachymyrmex*, *Crematogaster*, *Paratrechina*, *Pheidole*, *Solenopsis*, and *Wasmannia*; Bustillo *et al.* 2002; Armbrecht *et al.* 2005) and Mexico (*Azteca*, *Pseudomyrmex* and *Tapinoma*; Infante *et al.* 2003). *Solenopsis picea* Emery is considered to be the most efficient *H. hampei* predator; it enters the berry, removes immature *H. hampei* and carries them back to their nests (Armbrecht & Gallego 2007). Ultimately the value of generalist predators lies in their role in conservation biological control, thus impacting pest species throughout the season and potenitally regulating pest populations when specialist natural enemies are absent.

Recently, a predatory thrips, *Karnyothrips flavipes* (Jones) (Phlaeothripidae), has been observed consuming *H. hampei* eggs and larvae inside coffee berries (Jaramillo 2008). *Karnyothrips flavipes* has a wide distribution, including North, Central and South America, the Pacific region, India, the Mediterranean, Palestine, Egypt, Europe, and South and Central Africa. It is a generalist predator that feeds mainly on scales, mites, whiteflies and other thrips, and is frequently associated with bamboo and other species of Gramineae (Priesner, 1960, 1964). This is the first time *K. flavipes* has been reported as a predator of Coleoptera and associated with non-graminaceous plants.

Entomopathogenic Nematodes and Entomopathogenic Fungi.

In laboratory studies, *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) and *Steinernema feltiae* (Filipjev) (Rhabditida: Steinernematidae), were able to locate and penetrate *H. hampei*-infested berries, resulting in high *H. hampei* mortality (Molina & López 2002). Furthermore, these species are able to reproduce inside immature and adult *H. hampei* (Lara *et al.* 2004). In Mexico, *Metaparasitylenchus hypothenemi* (Tylenchida: Allantonematidae) was observed parasitizing *H. hampei* (Castillo *et al.* 2002). While it does not cause high mortality levels, it significantly impacts female fecundity (Poinar *et al*. 2004).

Beauveria bassiana (Balsamo) Vuillemin (Ascomycota: Hypocreales) is known to infect *H. hampei* adults, and has been reported in many countries (Damon 2000). Application of extremely high doses (>10^{10} spores/tree) is effective in controlling *H. hampei*, but such concentrations are not economically feasible (Posada 1998). Studies aimed at reducing the necessary dosage by increasing the virulence of *B. bassiana* and *Metarhizium anisopliae* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) through genetic modifications are in their infancy (Góngora 2005; Pava Ripoll *et al.* 2008). Recent research has attempted to introduce *B. bassiana* as a fungal endophyte in coffee plants, in an attempt to have it become established systemically throughout the plant (Vega *et al.* 2008).

ROLE OF DNA-BASED DETECTION OF PREDATOR-PREY INTERACTIONS.

Tracking trophic interactions by direct observation is not always possible. This is especially true for small, elusive insects such as those that spend nearly all of their life cycles within a host plant. Visual inspection of gut content is only possible when a

portion of the ingested prey is resistant to digestion. By contrast, molecular methods can detect the presence of small amounts of prey in the digestive tracts of predators (Agusti *et al.* 2003; Harper *et al.* 2005; Read *et al.* 2006; Harwood *et al.* 2007; Juen & Traugott 2007; Fournier *et al.* 2008). In these studies, DNA is extracted from the predator and prey species under study and sequenced using universal primers. Mitochondrial genes have been favored because they have a large copy number relative to most nuclear genes and there are less conserved (e.g., COI) and more conserved (e.g., 12S rDNA) areas offering both species- and group-specific primer possibilities. Once sequences are obtained for a group of focal taxa, primer pairs can be designed to exclusively amplify a small region of DNA (usually <300 base pairs) of a prey species. Because the DNA of prey species remains variously intact in the digestive tract of predators for a period of time after predation occurs, prey DNA can be extracted and detected with species-specific primers. Field collected predators can then be screened for predation, enabling an accurate assessment of biological control and examination of trophic interactions that would be extremely difficult to observe directly. These molecular approaches are becoming an essential part of biological control assessment and complement existing antibody-based studies (Hagler & Naranjo 2005) that continue to prove invaluable for examination of large-scale predator-prey interactions in the field. Here, we present preliminary results of the use of *H. hampei*-specific primers to detect *H. hampei* predation by *K. flavipes*.

MATERIALS & METHODS.

Coffee berries were collected from a plantation in western Kenya and dissected to determine the insect fauna of the berries. Additional coffee berries were put into containers from which the emerging insect fauna could be collected. Insects were preserved in >95% ethanol and stored at -20°C. Total DNA was extracted from whole specimens using QIAGEN DNeasy Tissue Kits following the animal tissue protocol. Polymerase chain reaction (PCR) was performed to amplify cytochrome *c* oxidase I (COI) from the insect species that have been found associated with the coffee berry [*Aphanogmus goniozi* Dessart, *H. hampei*, *K. flavipes*, *P. nasuta*, *Tapinoma* sp., Aleyrodidae (Hemiptera) and Drosophilidae (Diptera)] using the primers LCO-1490 and HCO-2198 (Folmer *et al.* 1994). PCR reactions (50µL) consisted of 1X QIAGEN PCR buffer (1.5 mM $MgCl_2$), 0.2 mM each dNTP, 0.5 mM each primer, 1U QIAGEN *HotStarTaq® Plus* and 5µL template DNA. PCR reactions were carried out in a Bio-Rad thermal cycler. PCR cycling protocols were 94°C for 5 min followed by 50 cycles of 94°C for 45 s, 40°C for 45 s, 72°C for 45 s and a final extension of 72°C for 10 min. Electrophoresis of 10 µL of PCR product in 1.5% SeaKem agarose stained with ethidium bromide was done to determine reaction success. PCR reactions that yielded significant product were purified with QIAGEN MinElute PCR purification kit. Cycle sequencing was carried out in both the forward and reverse directions using the ABI Big-Dye Terminator mix (v. 3.0) in an ABI 9700 thermal cycler, and run out in an ABI 3730xl sequencer.

Forward and reverse COI sequences from the same individual were assembled using AlignIR (v. 2.0). Multiple sequence alignments were done using CLUSTAL_X (Larkin *et al.* 2007). This alignment was used to design two pairs of COI primers (Chapman, Jaramillo, Vega & Harwood, unpublished). One pair was designed to amplify a 646 bp fragment of *K. flavipes* COI to check whether the *K. flavipes* DNA extractions were successful. A second pair was designed to amplify a 185 bp

fragment of *H. hampei* COI, and was screened for cross-reactivity against all other insects that were found associated with the coffee berry including starved *K. flavipes*. This primer pair was used to detect the presence of *H. hampei* DNA in *K. flavipes* DNA extractions. To determine PCR reaction success utilizing the *H. hampei*-specific primers, electrophoresis of 10µL of PCR product in 3% SeaKem agarose was done to separate the 185 bp PCR product from the glycerol-bromphenol blue-based loading dye. Positive controls containing *H. hampei* DNA and negative controls were included in each PCR.

RESULTS.

PCR using the *H. hampei* specific primers was done to examine the feeding relations of *K. flavipes* and confirm specificity of the primers. Figure 2 shows an agarose gel loaded with PCR reactions with DNA from all of the species associated with the coffee berry (listed above). The *H. hampei*-specific primers produced amplicons of expected size from PCRs with extractions of *H. hampei* DNA (Fig. 2: top and bottom: lane 2), *K. flavipes* fed *H. hampei* (Fig. 2: top: lanes 3-7), and *K. flavipes* that emerged from coffee berries (Fig. 2: bottom: lanes 3-5). PCR reactions did not produce products for starved *K. flavipes* (Fig. 2: bottom: lanes 6-8) or any other insect found to be associated with the coffee berry (Fig. 2: top: lanes 8-18).

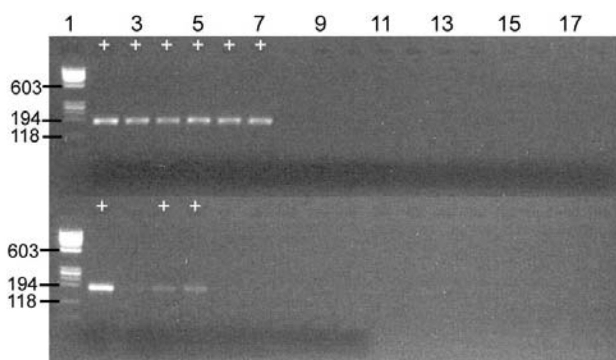

Fig. 2. Agarose gel loaded with PCR products using *H. hampei* specific primers. Numbers across the top indicate lane number, positive results are indicated by "+" and numbers along the side indicate the size (bp) of three bands in the PhiX174/Hae III size standard. Top row: lane 1: size standard; lane 2: *H. hampei* (positive control); lanes 3-7: *K. flavipes* fed with *H. hampei*; lanes 8-9: *Prorops nasuta*; lanes 10-11: *Tapinoma* sp.; lanes 12-13: undetermined Drosophilidae species; lanes 14-15: undetermined Aleyrodidae species; lanes 16-17: *Aphanogmus goniozi*; lane 18: *Heterospilus* sp.; Bottom row: lane 1: size standard; lane 2: *H. hampei* (positive control); lanes 3-5: *K. flavipes* that emerged from coffee berries; lanes 6-8: starved *K. flavipes*; lanes 9-10: negative controls.

DISCUSSION & FUTURE DIRECTIONS.

Our results show that it is possible to detect *H. hampei* DNA from extractions of a tiny (<2mm long) predatory thrips species, *K. flavipes*. We used this technique to demonstrate that *K. flavipes* emerging from coffee berries feed upon *H. hampei*. We are currently in the process of completing a year-long study to decipher the level of

trophic connectedness between this abundant generalist predator and *H. hampei*, an economically damaging pest of coffee. Because *K. flavipes* has a geographic distribution that encompasses *H. hampei's* range*,* the results of our continuing study could have important implications for biological control in coffee worldwide. Because the *H. hampei*-specific primers do not amplify the DNA of any of the other insect species associated with these systems, the primers have potential to be used to determine the connectedness of other predators (e.g., ants) to *H. hampei*. Furthermore, because we are compiling DNA sequences from as many of the insects associated with the coffee berry as possible, we have the potential to design species-specific primers for each coffee berry-frequenting insect species. This would enable us to unravel the trophic structure of the coffee berry insect fauna, which could lead to a much improved understanding of the role of natural enemies in control of *H. hampei*.

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Forest Health Technology Enterprise Team
Morgantown, West Virginia

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Forest Service