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ORIGINAL PAPER

Molecular diagnosis of a previously unreported predator–prey association in coffee: *Karnyothrips flavipes* Jones (Thysanoptera: Phlaeothripidae) predation on the coffee berry borer

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Abstract The coffee berry borer, *Hypothenemus hampei,* is the most important pest of coffee throughout the world, causing losses estimated at US $500 million/year. The thrips *Karnyothrips flavipes* was observed for the first time feeding on immature stages of *H. hampei* in April 2008 from samples collected in the Kisii area of Western Kenya. Since the trophic interactions between *H. hampei* and *K. flavipes* are carried out entirely within the coffee berry, and because thrips feed by liquid ingestion, we used molecular gut-content analysis to confirm the potential role of *K. flavipes* as a predator of *H. hampei* in an organic coffee production system. Species-specific COI primers designed for *H. hampei* were shown to have a high degree of specificity for *H. hampei* DNA and did not produce any PCR product from DNA templates of the other insects associated with the coffee agroecosystems. In total, 3,327 *K. flavipes* emerged from 17,792 *H. hampei*-infested berries collected from the field between April and September 2008. Throughout the season, 8.3% of *K. flavipes* tested positive for *H. hampei* DNA, although at times this figure approached 50%. Prey availability was significantly correlated with prey consumption, thus indicating the potential impact on *H. hampei* populations.

Keywords Biological control · Organic coffee · *Karnyothrips flavipes* · *Hypothenemus hampei* · Gut-content analysis · Kenya · Predator–prey interactions

J. Jaramillo and E.G. Chapman contributed equally to the experimental work in this paper and should be considered joint first authors.

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Introduction

The coffee berry borer (*Hypothenemus hampei* (Ferrari); Coleoptera: Curculionidae) is the most important pest of coffee throughout the world (Jaramillo et al. 2006). It occurs in all coffee-producing countries, with the exception of Hawaii, Nepal, and Papua New Guinea. Yearly losses are estimated at US $500 million, thus affecting the income of more than 20 million rural households in the tropics (Vega et al. 2003). Female coffee berry borers bore galleries into the endosperm of the coffee berries where they oviposit more than 200 individuals under laboratory conditions (Jaramillo 2008), resulting in both qualitative and quantitative losses through larval feeding (Le Pelley 1968;

Decazy 1990; Damon 2000). This cryptic life history inside the coffee berry makes the pest extremely difficult to control. To date, explorations for natural enemies of this insect in its area of origin in tropical Africa have mainly yielded parasitoids such as *Prorops nasuta* Waterston and *Cephalonomia stephanoderis* Betrem (Hymenoptera: Bethylidae), *Phymastichus coffea* LaSalle (Hymenoptera: Eulophidae), and *Heterospilus coffeicola* Schmiedeknecht (Hymenoptera: Braconidae) (Hargreaves 1935; Barrera 1994; Borbón-Martinez 1989).

Given the economic significance of coffee production to tropical countries and the damaging nature of the coffee berry borer, for over 100 years particular attention has been paid to identifying a predator that can be effective in biological control programs (Damon 2000). Apart from circumstantial evidence of ants occasionally preying on *H. hampei* (Bustillo et al. 2002; Infante et al. 2003; Armbrecht et al. 2005; Armbrecht and Gallego 2007) and a report by Vega et al. (1999) on *Leptophloeus* sp. near *punctatus* (Coleoptera: Laemophloeidae) as a potential predator of the pest, virtually nothing is known about the complex of predators associated with *H. hampei* and their capacity for controlling pest populations.

In April 2008, during routine dissections of coffee berries (*Coffea arabica* L. var. Ruiru 11) as part of a study aimed at discovering unknown natural enemies of *H. hampei* in western Kenya (Jaramillo et al. 2009a), adult predatory thrips *Karnyothrips flavipes* Jones (Thysanoptera: Phlaeothripidae) were observed for the first time feeding on eggs of the coffee berry borer. Subsequent observations in the laboratory revealed that *K. flavipes* adults were also capable of preying on larval stages of *H. hampei.* Only *K. flavipes* adults were observed preying on *H. hampei* immature stages. *K. flavipes* enters the coffee berry through the tiny (ca. 1-mm diameter) hole bored by *H. hampei* and oviposits inside the coffee berries between the pulp and the parchment of the beans. Newly hatched thrips complete their development inside the berry, while adults spend most of their life inside the galleries bored by *H. hampei* (Jaramillo 2008). Interestingly, out of >7,400 described species of thrips (Mound 2007; Mound and Morris 2007), fewer than 50 exhibit a capacity for predation (Ananthakrishnan 1979). *K. flavipes* is a generalist predator of cosmopolitan distribution (Zimmerman 1948; Priesner 1960, 1964; Pitkin 1976; Ananthakrishnan 1979; Mound and Marullo 1996) that feeds mainly on scales, mites, whiteflies, and other thrips, and is frequently associated with bamboo and other Poaceae (Priesner 1960, 1964). However, it can also be found in the canopy of fruit trees preying on scales, mites, and herbivorous thrips (Collins and Whitcomb 1975; Hoddle et al. 2002; Childers and Nakahara 2006).

Sustainable certification schemes of coffee, e.g., organic coffee (Giovannucci and Koekoek 2003), have stimulated the search for integrated control strategies against insect pests and thus an increase in area of low-input coffee agroecosystems. With the reduction of chemical inputs, community complexity typically increases and typically includes a diverse assemblage of generalist predators that have the capacity to provide valuable levels of pest control (Sunderland et al. 1997; Symondson et al. 2002).

Tracking trophic interactions can sometimes be undertaken through direct observation (Nyffeler 1999; Pfannenstiel 2008) or dissection of midgut contents (Harwood and Obrycki 2005; Weber and Lundgren 2009). However, because >79% of predacious terrestrial arthropods utilize extra-oral digestion (Cohen 1995), gut content identification via visual examination is impossible for the vast majority of predators as feeding by liquid ingestion leaves no morphological clues to prey identity. By contrast, the use of molecular methods to detect the presence of small amounts of prey DNA in the digestive tracts of predators both elucidates the linkages between generalist predators and their prey in the field (Symondson 2002; Sheppard and Harwood 2005; Weber and Lundgren 2009) and removes the subjective nature of visual identification of indigestible fragments of prey exoskeleton in the gut. More importantly, understanding mechanisms of predation by generalist predators using such approaches has enabled accurate determination of food-web processes in terrestrial agroecosystems and has implicated many predators and parasitoids in the biological control of economically important pest species throughout the world (e.g., Harwood et al. 2007; Juen and Traugott 2007; Fournier et al. 2008; Traugott et al. 2008). Utilizing molecular gut content analysis, we confirmed the role of *K. flavipes* as a predator of *H. hampei*, a system that could have important implications for the future management of coffee pests throughout the world. Our principal research objectives were to develop a molecular detection system and to utilize this approach to corroborate the trophic connectedness between *K. flavipes* and *H. hampei* in Kenya.

Materials and methods

Identification of thrips specimens and laboratory observations

In April 2008, upon discovery that thrips were emerging from field collected coffee berries, they were sent for identification to Dr. Steve Nakahara (Systematic Entomology Laboratory, US Department of Agriculture, Agricultural Research Service, Beltsville, Maryland, USA) and identified as *K. flavipes*. Subsequently, all thrips emerging from field collected *H. hampei*-infested berries were transferred into 0.5-mL microcentrifuge tubes, preserved

in 99% ethanol and stored at −20°C for gut-content analysis.

DNA extraction and PCR analysis

Due to the small size of the thrips (<2 mm), total DNA was extracted from crushed whole specimens (after Harwood et al. 2007, 2009) using QIAGEN DNeasy Tissue Kits (QIAGEN Inc., Chatsworth, California, USA) following the manufacturer's animal tissue protocol with the following exception: after incubating at 56°C for 1 h in buffer ATL and proteinase K, the thrips were broken into pieces in the buffer solution with sterile pipette tips and returned to the incubator to complete tissue lysis. Polymerase chain reaction (PCR) was performed to amplify cytochrome *c* oxidase I (COI) from all insect species associated with the coffee berry using the primers LCO-1490 and HCO-2198 (Folmer et al. 1994). PCR reactions (50 μL) consisted of 1X QIAGEN PCR buffer, $MgCl_2$ (1.5 mM for *H. hampei*; 5 mM for all other species associated with the coffee berry—(Electronic supplementary material, Table 1)), 0.2 mM of each dNTP, 0.5 mM of each primer, 1U QIAGEN *Taq* and template DNA (5 μL of total DNA). PCR reactions were carried out in a Bio-Rad PTC-200 thermal cycler (Bio-Rad Laboratories, Hercules, California, USA). The PCR cycling protocols were 94°C for 1 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 (Lonza, Rockland, Maine, USA) stained with ethidium bromide (0.1 mg/μL) was done to determine reaction success. PCR reactions that yielded significant product were purified with QIAGEN MinElute PCR purification kit according to the manufacturer's guidelines. Cycle sequencing reactions were carried out in both the forward and reverse directions in an ABI 9700 thermal cycler using the ABI Big-Dye Terminator mix (v. 3.0; Applied Biosystems, Foster City, California, USA).

Forward and reverse COI sequences from the same individual were aligned using AlignIR (v. 2.0, LI-COR Biosciences Inc., Lincoln, Nebraska, USA). BLASTN searches (Karlin and Altschul 1990, 1993) of the GenBank database were performed to determine whether the sequences significantly matched those of the same or related species and to rule out the possibility that they were from an organism infecting the insect being extracted. Multiple sequence alignments were done using CLUSTAL X (Larkin et al. 2007). The multiple alignment was used to design two pairs of species-specific COI primers (Table 1) for *K. flavipes* and *H. hampei*. One pair (Kflav-COI-F and Kflav-COI-R) was designed to amplify a 604 bp fragment of *K. flavipes* COI to confirm successful extraction of DNA from these predators. A second pair (CBB-COI-F and CBB-COI-R) was designed to amplify a 185 bp fragment of *H. hampei* COI, and was screened for cross-reactivity against 76 insect, mollusc and nematode species, including all insects concealed within coffee berries in Kenyan agroecosystems (i.e., undetermined aleyrodid and tephritid species from Kenya, *Prorops nasuta* Waterston, *Heterospilus* sp., *Aphanogmus dyctinna*, *Tapinoma* sp., *Karnyothrips flavipes*) (Jaramillo et al. 2009a) (Electronic supplementary material, Table 1). The non-target organisms were randomly selected representatives of 41 invertebrate families (in addition to the seven families occurring within the berries) and were collected either in pitfall traps, with sweep nets or by hand and preserved immediately after collection in >95% ethanol. CBB-COI-F and CBB-COI-R were also used to detect the presence of *H. hampei* DNA in *K. flavipes* DNA extractions. The PCR cycling protocol for these primers was the same as those listed above except that QIAGEN HotStarTaq *Plus* was used (with an initial 5 min denaturing step) and an annealing temperature of 56°C. To determine PCR reaction success utilizing *H. hampei*-specific primers, electrophoresis of 10 μL of PCR product in 3% SeaKem agarose was performed 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.

Table 1 Species-specific primer sequences and GenBank Accession numbers

Species	Primer name	Primer sequence (5′-3′)	Size (bp)	Tm (°C)[a]	GenBank Accession No.
Karnyothrips flavipes	Kflav-COI-F	CTGATCAGGAATCTGTGGCTTA	562	54	FJ824171
	Kflav-COI-R	GTAGGGTCACCTCCTCCTGT		57	
Hypothenemus hampei	CBB-COI-F	TTGACAAAGGAGCAGGAACA	145	54	FJ824170
	CBB-COI-R	TTCTGGCTGTATCCCAGGAG		55	

Size refers to the length of the amplified fragment not including the primer sequences

[a] Melting temperature was calculated using Promega's base-stacking method: http://www.promega.com/biomath/calc11.htm. Note: a 1.5 mM Mg^{2+} solution (the minimum that we used) will raise the T_m 5–8°C above what most T_m calculators estimate (von Ahsen et al. 1999; Nakano et al. 1999).

Feeding trials

Feeding trials were conducted to determine the detection period of *H. hampei* DNA in *K. flavipes* following consumption. Two hundred *K. flavipes* adults that emerged from field-collected coffee berries were placed individually into 0.5 mL microcentrifuge tubes containing a moistened cotton ball to provide water and humidity for survival. The thrips were maintained in an incubator at 25±1°C, 70±5% relative humidity (rh) and L12:D12 photoperiod, to resemble the prevalent climatic conditions of the coffee plantation. The thrips were starved for a period of 48 h (water provided) and subsequently allowed to feed on a single target prey (one *H. hampei* egg). All thrips were observed to feed for 2 h whilst predators that did not feed were discarded. At the end of this feeding period (t=0 h), 15 individuals were placed in 99% ethanol and stored at −20°C. After feeding, thrips were transferred into clean containers and provided an ad libitum supply of alternative prey (the *H. hampei* parasitoid *Prorops nasuta*) to mitigate the effects of starvation on digestion rates and DNA detectability (Greenstone and Hunt 1993; Chen et al. 2000). Additional samples of 15 thrips were transferred to 99% ethanol and stored at −20°C after 2, 4, 6, 8, 12, 16, 20, 24, 30, 36, and 48 h.

DNA was extracted from each of the feeding trial specimens as previously described. Extraction success was confirmed using the *Karnyothrips* primers and presence of prey DNA determined with the *H. hampei*-specific primers (Table 1).

Field sampling

A total of 17,792 *H. hampei*-infested coffee berries that had fallen to the ground from 100–150 trees were collected on a weekly basis from January to September 2008 in an organic non-shaded *Coffea arabica* L. (var. Ruiru 11) plantation (ca. 2,000 trees, ca.1 ha) in the Kisii area of Western Kenya (00° 25′ S, 34° 28′ E; 1,510 m above sea level). This encompassed the two main harvest periods for coffee in Kisii (May/June and September/October). To avoid fungal contamination, the berries were surface-sterilized in the laboratory following the protocol of Pérez et al. (2005), allowed to dry at room temperature and placed in plastic containers (40×40×20 cm) layered with a mixture of plaster of Paris and activated charcoal to prevent the desiccation of the berries. Containers were closed with perforated lids (55-mm diameter) covered with a thrips-proof net (64 μm mesh) and maintained under controlled conditions (25±1°C; 70%±5% rh; L12:D12 photoperiod). This methodology allows keeping the insects and berries in viable condition for extended periods of time (see Jaramillo et al. 2009b).

For molecular gut-content analysis, emerging *K. flavipes* adults were immediately collected from coffee berries and preserved as previously described. In parallel with the collection of *K. flavipes* for molecular gut-content analysis, beetle-infested coffee berries were sampled weekly to enable prey availability to be correlated with predation rates; emergence frequencies of *K. flavipes* (after Jaramillo et al. 2009b) were recorded daily. Dissections of berries were not carried out to avoid desiccation of *K. flavipes* immature stages.

Statistical analysis

The feeding trial data were analyzed by examining the percent positive for each time period using a probit (dose-response) model (v. 10; Systat Software Inc., San Jose, California, USA) in order to determine the decay rate of *H. hampei* DNA in the digestive tract of the thrips and to determine the DNA half-life (after Payton et al. 2003; Greenstone et al. 2007). SigmaPlot also calculated upper and lower 95% confidence interval bands for the resulting curve.

Differences in *Karnyothrips flavipes* emergence across months (April to September) were analyzed by analysis of variance, using the PROC MIXED procedure of SAS (SAS Institute 1999). Orthogonal contrasts were used to test the significance of mean differences between months. The significance level was set at P=0.05.

Results

Coffee berry borer primer specificity

The *H. hampei*-specific primers (Table 1) did not elicit amplification of DNA from any other species screened, i.e., species commonly found inside coffee berries (in the microhabitat within which *K. flavipes* occurs) and 69 other

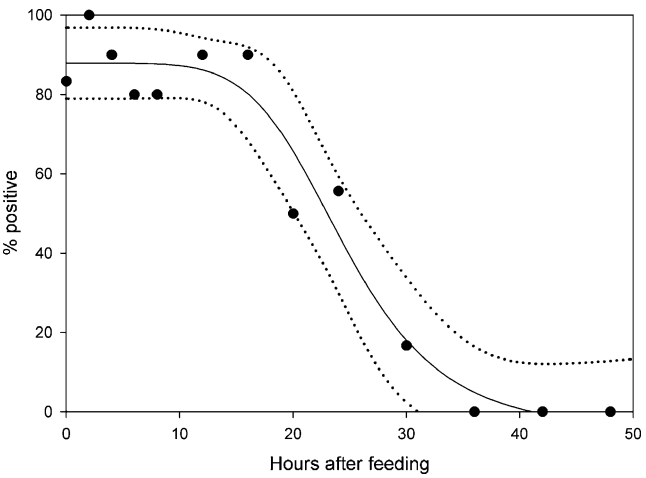

Fig. 1 Time course of detection of DNA of *Hypothenemus hampei* following consumption by *Karnyothrips flavipes*. The *dotted lines above and below* the curve are 95% confidence interval bands

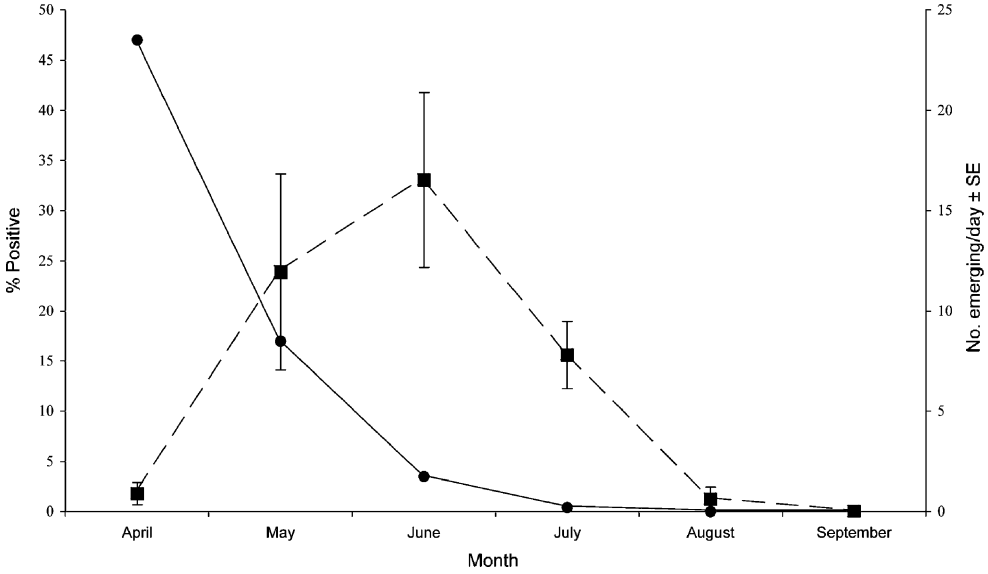

Fig. 2 Temporal relationship between the emergence of predators and feeding activity upon *Hypothenemus hampei* (data pooled by month). *Solid line* percent of predators positive for *H. hampei* DNA; *dashed line* mean number of thrips (± SE)

non-target organisms representing 41 families in 11 other invertebrate orders (Electronic supplementary material, Table 1). This extensive cross-reactivity screening confirms the specificity of the primers to *H. hampei*, and thus reliability for use in field-based food web studies.

Detection of coffee berry borer DNA in *Karnyothrips flavipes*

The *H. hampei*-specific primers (Table 1) were used to screen for the presence of *H. hampei* DNA in extractions of *K. flavipes* from the laboratory feeding trials. The binary regression using a probit model was fitted to the data with an r^2=0.96 (goodness-of-fit test; F=65.4, df=3.9, P< 0.0001) and estimated a DNA detectability half-life of 24.6 h. The reaction success remained high (≥80%) from 0 to 16 h after feeding and dropped to 0% by 36 h, following a non-linear model (Fig. 1). This demonstrated that a 185 bp fragment of *H. hampei* COI DNA could remain intact for up to 30 h in *K. flavipes* digestive tract (see Table 1 for the GenBank accession number for this 145 bp sequence).

Analysis of field-collected *Karnyothrips flavipes*

In total, 3,327 *K. flavipes* emerged from 17,792 *H. hampei*-infested berries collected from the field between April and September 2008.

Hypothenemus hampei DNA was detectable in 8.3% of DNA extractions of field-collected *K. flavipes* over the study period (range, 47% (April); 0% (August)). The percentage of *K. flavipes* testing positive for *H. hampei* DNA and the mean number of thrips emerging from the coffee berries (± SE) over the same time period are presented in Figs. 2 and 3. The highest percentage of positive results occurred in April, the first 17 days of the emergence period, where 47% of emerging *K. flavipes* tested positive for *H. hampei* DNA. As the number of emerging *K. flavipes* peaked (June), the percent testing positive declined to 3.5%, with only 0.4% of *K. flavipes* that emerged after June screening positive for target DNA.

The numbers of emerging *K. flavipes* from field samples showed highly significant temporal variability from April to September ($F_{5,\ 180}$=11.18, P<0.0001) (Fig. 3). The trend of the *K. flavipes* population was found to be quadratic ($F_{5,\ 180}$= 31.55, P<0.0001), thus average *K. flavipes* population density increased from April, reached its highest numbers in July and decreased thereafter (Fig. 3).

Discussion

Even though the coffee berry borer has been studied for over 100 years, there have been no confirmed reports of a

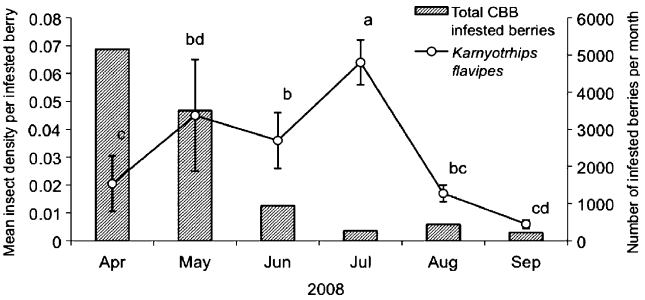

Fig. 3 Mean density of *Karnyothrips flavipes* (± SE) and the total number of infested coffee berries collected per month from the ground strata (n=17,792) in a coffee agroecosystem in western Kenya. *Letters on each date point* (months), indicate significant differences of the means (P>0.05)

coffee berry borer predator. Based on observations in the field and laboratory, we confirmed that the predatory thrips, *K. flavipes*, was preying on *H. hampei*, and exhibited high levels of predation throughout the season, overall 8.3% of *K. flavipes* tested positive for *H. hampei* DNA, with this figure approaching 50% early in the season (April). Our results have confirmed for the first time the presence of a coffee berry borer predator, based on molecular gut-content analysis.

The pest-specific primers reported here were shown to have a high degree of specificity for *H. hampei* DNA, as they did not produce any PCR product from DNA templates of other insects associated with the coffee berry or a diverse array of non-target taxa from 11 orders. This demonstrates the high degree of specificity of the primers for *H. hampei* within the coffee system and that in rare instances when other insects penetrate the coffee berries, these primers are not likely to produce false positive results. The primers were also sensitive enough to produce PCR product from DNA extractions from this extremely small predator and, most importantly, *H. hampei* DNA remained detectable in the predator such that a high percentage (80–90%) of positive PCR results were obtained from feeding trials up to 16 h after feeding. Furthermore, with a detectability half-life of ~24 h and ~17% positive results at 30 h (Fig. 1), the implications are that the *K. flavipes* specimens that tested positive very likely fed on *H. hampei* within 24–30 h of exiting the coffee berry.

Population density of *K. flavipes* emerging from field collected berries increased from April, reached its highest numbers in July and decreased thereafter. Most likely, most of the *K. flavipes* adults penetrated *H. hampei*-infested coffee berries during the dry season, in December and January. Our results demonstrate the trophic connections between *K. flavipes* and *H. hampei*, especially during April and May, when nearly 50% of thrips emerging from coffee berries tested positive for *H. hampei* predation.

It is currently unknown whether *K. flavipes* is preying on *H. hampei* in other coffee producing countries. However, based on (1) our observations of *K. flavipes* preying on *H. hampei* in Kenya, (2) that *K. flavipes* also occurs throughout the coffee-growing areas of the world, and (3) that other Phlaeothripidae have been shown to be important predators (Bailey and Caon 1986; Kakimoto et al. 2006), the potential is high for *K. flavipes* to have a significant impact on *H. hampei* populations in other regions. Most predatory thrips are generalist predators (Lewis 1973), and some such as *Franklinothrips orizabensis* Johansen, *F. vespiformis* (D. L. Crawford; Aeolothripidae) and *Scolothrips takahashii* Priesner (Thripidae) are used as biological control agents (Hoddle et al. 2001; Larentzaki et al. 2007; Ding-Xu et al. 2007). Although the importance of *K. flavipes* as a predator of *H. hampei* remains to be fully quantified, the fact that *K. flavipes* occurs in many other coffee producing nations (Zimmerman 1948; Priesner 1960, 1964; Pitkin 1976; Ananthakrishnan 1979; Mound and Marullo 1996) means that the introduction of borer-feeding *K. flavipes* populations into coffee-producing areas should be relatively easy and will not pose problems associated with the introduction of an organism not present in the country, which might require environmental impact assessment studies and regulatory permits, among others.

The introduction of generalist predators is rarely successful in classical biological control (Howarth 1991), as they are frequently associated with negative effects on the indigenous fauna (Elliott et al. 1996). However, most of these introductions happened > 20 years ago, and lately Symondson et al. (2002) reviewed more recent cases on native generalist predators such as mirids, anthocorid bugs, spiders, and carabids that effectively contribute to pest population regulation in various ecosystems. Therefore, if future studies in other regions confirm the findings presented here, then efforts should be targeted towards conservation biological control using a range of habitat management and diversification techniques (e.g., Tscharntke et al. 2005, 2007; Jonsson et al. 2008). For instance, several studies have shown the enhancing effect of shade trees in coffee plantations on the diversity and efficacy of natural enemies of *H. hampei* (Perfecto et al. 1996, 1997; Richter et al. 2007; Teodoro et al. 2008). Furthermore, because *K. flavipes* is a generalist predator that also feeds on scales, mites, whiteflies, and other thrips (Priesner 1960, 1964), alternative habitat that harbours these potential prey species could be provided in and around coffee plantations so that *K. flavipes* is available to feed on *H. hampei* when borer populations are in the early stages of attacking coffee plantations.

Hypothenemus hampei remains the most devastating pest of coffee throughout the world, and the findings presented in this paper, which combine ecological and molecular studies, provide coffee growers and coffee scientists with new insights into a biological control agent that could be conserved and augmented in coffee growing regions where it occurs. Further deciphering the trophic linkages of this newly discovered predator-prey association in other coffee producing countries could make a significant contribution to integrated pest management of *H. hampei*, in particular through enhanced biological control.

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