TRANSMISSION OF *FUSARIUM* IN WILD CHILI PEPPER FRUITS IN THE CHACO REGION OF BOLIVIA

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2010

To my family: Carlos, Esperanza, Sara y Miguel

ACKNOWLEDGMENTS

I would like to thank my academic advisor and chair, Dr. Doug Levey, for his mentoring and guidance through this project. Additionally, I thank Dr. Joshua Tewksbury for his thoughts and help with logistics in Bolivia. I also thank Noelle Machnicki and Cat Adams of the Tewksbury lab at the University of Washington for their help and field assistance in 2008 and 2009. Special gratitude goes to Dr. Larry Winner at the University of Florida and Jhenny Salgado from CATIE, Costa Rica, for their help with statistical analyses. I am also grateful to Karen Patterson and Tangelyn Mitchel for their tireless patience providing assistance with paperwork, travel authorizations and deadline reminders. Funding was provided by grants from the National Geographic Society and National Science Foundation to Doug Levey and Josh Tewksbury. Last but not least, I thank very much my parents: Carlos y Esperanza, for their ever-present love and support in getting me to where I am today. I would like to extend my gratitude to all my friends from the Biology department at the University of Florida and fellow alumni from the Biology major at the Universidad Gabriel Rene Moreno and Museo Noel Kempff Mercado in Santa Cruz, Bolivia.

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May 2010

Chair: Doug Levey
Major: Zoology

Microbes that feed on fruits are especially detrimental for plant fitness and seed dispersal. *Fusarium* is a widespread and frequently pathogenic genus of plant fungus, important in agriculture but poorly known in non-domesticated species. Mechanisms by which wild species of *Fusarium* disperse between plants are especially poorly known. I experimentally tested two hypotheses for spread of *Fusarium* in a wild species of chili pepper, *Capsicum chacoense*. The vector hypothesis posits that insects feeding on fruits and seeds actively transmit the fungus when they travel from infected to uninfected plants. The mechanical damage hypothesis posits that fungal spores are passively dispersed through the environment and land on the surface of fruits, where they enter through wounds created by feeding insects. Additionally, I tested whether different species of insects that commonly feed on chili fruits differ in their ability to cause fungal infection and whether pungent and non-pungent fruits differ in their susceptibility to *Fusarium* infection. I carried out field experiments in southeastern Bolivia. Individual fruits were covered with small mesh bags and randomly assigned to one of five treatments. In three treatments, several individuals of one of three frugivorous insects were placed inside the bag. A fourth treatment consisted of fruits

that I poked holes in with a sterilized needle. Fruits in the fifth treatment had no holes poked (control). After seven days, fruits were removed and seeds scored for fungal infection. Overall, fungal infection scores of seeds from treatments with insects did not differ from those from the control fruits. However, fungal infection scores of seeds from fruits poked with a sterile needle were significantly lower than those of control fruits, perhaps because the poking triggered production of antimicrobial proteins (i.e., an induced defense). Collectively, these results do not support either hypothesis. Fungal infection of seeds from pungent and non-pungent fruits did not differ, a result that contradicts a previous study on the functional role of pungency in chili fruits. Long-term population studies on wild *Capsicum spp.* are required to understand factors affecting the dynamics of *Fusarium* dispersal and their effects on plant fitness. This type of research on the ecology of disease transmission in wild relatives of agricultural crops could improve our ability to predict plant-pathogen interactions in commercial settings.

CHAPTER 1
INTRODUCTION

The functional role of vertebrate-consumed fruits consumed is dispersal of the seeds within them, which is accomplished through placement of nutrients in fruit pulp to attract consumers (Edwards 2006, Lord and Westoby 2006). Those nutrients, however, attract not only frugivores that disperse seeds but also a broad range of frugivores that do not disperse seeds (Herrera 1982). Microbial frugivores are especially ubiquitous and detrimental to plant fitness; not only do they fail to disperse seeds, but they often cause rot, making fruits unattractive to seed-dispersing frugivores (Tang et al. 2005).

An especially common microbial frugivore of wild and domesticated fruits is fungus in the genus *Fusarium*. *Fusarium* is a generalist pathogen, infecting a wide variety of plants and animals and responsible for both pathogenic and non-pathogenic interactions (Nelson 2004). Although the genus includes at least 70 species, the total is uncertain because of debate about taxonomy and the advent of molecular tools now being applied to distinguish species (Leslie and Summerell 2006). Taxonomy aside, *Fusarium* is responsible for an enormous range of plant diseases, many of which have significant agricultural impacts. Thus, much of the literature on *Fusarium* is related to crop species and cultivars (Yergeau et al. 2010, Sommer et al. 2008, Goswami and Kistler 2004, Gordon and Martyn 1997, Jimenez et al. 1992) and little is known about its natural history and infection of non-domesticated plants. Depending on the host species, *Fusarium* propagules (mostly spores and conidia) can be carried by engraver beetles (Erbilgin et al. 2008), moth flies (El-Hamalawi 2008), shore flies (El-Hamalawi and Stanghellini 2005), and even hummingbirds (Lara and Ornelas 2003).

Many species of *Fusarium* are spread by animals, especially insects. Host infection can occur when animals actively transport spores internally (e.g., in their gut or mouthparts) or externally (i.e., on their body surface). Alternatively, infection can occur passively, when animals injure host tissues and thereby create an opportunity for already-present fungus to invade the host. The relative frequency of active vs. passive transmission has not been evaluated, nor has the means of transmission been tied to effects on plant reproductive fitness.

Wild fruits often contain secondary compounds that inhibit growth of pathogenic fungi (Cipollini and Levey 1997), including *Fusarium* (Agizzio et al. 2003, Tewksbury et al. 2008a). Wild chilies (*Capsicum spp.*) are a well-studied example; most species contain capsaicinoids. These compounds make chilies "hot" and protect fruits and seeds from *Fusarium* attack (Tewksbury et al. 2008a).

I studied *Fusarium* infection of a wild chili pepper, *Capsicum chacoense,* which is polymorphic for the occurrence of capsaicinoids; some individuals produce only pungent fruit, whereas other individuals produce only non-pungent fruit. Although production of capsaicin is beneficial in terms of protecting the plant from *Fusarium*, it likely entails a cost of reduced seed coat thickness, increasing the probability of seed mortality, or of reduced efficiency of water use (Tewksbury et al. 2008b).

To understand the evolutionary ecology of fruit secondary chemistry and to provide insight into potential applications to predict and prevent spread of pathogenic fungi in agricultural systems, we need to understand the basic natural history of how *Fusarium* infects wild fruits. I addressed three questions: (1) How does *Fusarium* spread among fruits in wild populations of chilies? Specifically, I tested two hypotheses: The

vector hypothesis posits that *Fusarium* is transmitted actively from infected to uninfected fruits by insects that feed on chili fruits and seeds and carry fungal propagules on their bodies. The mechanical damage hypothesis posits that *Fusarium* spores are passively dispersed (e.g., by wind) and settle on the surface of chili fruits, where they gain entrance to pulp and seeds through wounds created by insects feeding on the fruit. These hypotheses are not mutually exclusive. (2) Do different species of insects differ in their ability to cause infection of fruit and seeds? And, (3) do pungent and non-pungent fruits and seeds differ in their susceptibility to *Fusarium* infection?

CHAPTER 2
METHODS

Study Site

My study site was located 30 km from Charagua in southern Bolivia (20º01'00''S, 63º06'00''W). Typical vegetation consists of distinctive low, dense, semideciduous, subtropical trees and scrubs, many of which are thorny. The most common species of the region are Quebracho (*Schinopsis quebracho-colorado* and *Aspidosperma quebracho-blanco*), Guayacan morado (*Bulnesia sarmientoi*), Tala (*Celtis pubescens*) and Mistol (*Ziziphus rnistol*; Navarro and Maldonado 2002). The site has a wet summer season (November – February) and a pronounced dry season (April – October; Bravo et al. 2008). In terms of human impact, the area has dispersed cattle ranches and few urban settlements. Population density is approximately 0.65 humans per km², which is relatively low compared to other tropical areas of South America (Conservation International 2002).

Study Species

Capsicum chacoense is endemic to the Chaco region of southern Bolivia, western Paraguay, and northern Argentina. Adult plants are typically long-lived, range from 0.3 to 1.5 m in height and produce 1-100 fruits, each of which contains 15-20 seeds. It blooms and fruits from early February to May. Fruits take 5 to 6 weeks from the time of pollination to reach full size and a couple more weeks to become fully ripe (personal observation). Capsaicinoids are present in immature fruits, although their concentrations increase during fruit maturation (Suzuki and Iwai 1984). At the study site, individuals that produce non-pungent fruit outnumber individuals that produce pungent fruit by a 3:2 ratio (Tewksbury et al. 2006).

The most common microbial pathogen of *C. chacoense* fruits is *Fusarium semitectum* Berkley and Ravenel (hereafter "Fusarium"; Tewksbury et al. 2008a). Detecting its presence in fruits is straightforward because as the fungus proliferates it produces dark areas in fruit pulp and on the seeds. These areas increase in size and darken in color over time (Figure 2.1).

Fruits of both pungent and non-pungent varieties are fed upon primarily by three species of phytophagus insects: 1) *Acroleucus coxalis* (Lygaeidae), 2) *Edessa sp*. (Pentatomidae) and, 3) "Beetle mimic" (Pentatomidae). All of these species use their mouthparts to pierce through the skin of both ripe and unripe *C. chacoense* fruits (Figure 2.2).

Experimental Design

The study was conducted during March and April of 2008 and 2009. In 2008, only naturally-occurring fruits at the field site were used. Because I was concerned that naturally-occurring fruits might have been infected with *Fusarium* at a very early stage of development, I changed the protocol in 2009 by digging up *C. chacoense* individuals from the site before they flowered, placing them in pots with natural soil, and transporting them to a greenhouse, where they were protected from insects. They were watered once per week and fertilized. Once they had produced fruit, they were transported back to the field site. To avoid physiological stress of transplantation, I kept all of these individuals in pots and watered them as necessary in the field.

To address the three questions about the mechanism of *Fusarium* dispersal, I applied five treatments to fruits. For all treatments, fruits were individually bagged. In three treatments, I introduced one of the three common species of phytophagus insects into the bag. A fourth treatment consisted of fruits not exposed to insects in the bag

(control); its purpose was to provide an estimate of fungal infection in fruits presumably unexposed to insects. The vector hypothesis would be supported if fungal infection scores for seeds from an insect treatment were greater than those from the control. For the fifth treatment, "Holes", I mimicked attack by insects with piercing mouthparts (*Acroleucus*, *Edessa* and 'beetle mimic') by poking 5 holes with sterile acupuncture needles. The mechanical damage hypothesis would be supported if fungal infection scores for seeds from this treatment were equal to those from the insect treatments.

Originally, I planned to apply treatments in a randomized block design, with all treatments present on a given plant. Due to low abundance of fruits on each plant, however, I was unable to do so. Treatments were applied to fully grown (maximum size) fruits, both ripe (red) and unripe (green), that had no obvious scars or other damage. Treatments and controls were assigned randomly to each fruit by blindly pulling labeled mesh bags from a bucket. All plants were in the same area. To remove fungi from the fruit surface and thereby standardize the starting point of all treatments, each fruit was swiped with a 5% Hypochlorite (bleach) solution and quickly rinsed with sterile water. Immediately afterwards, a mesh bag (6 by 12 cm) was secured around the branch supporting the fruit, and insects were introduced into the bag (first three treatments only). Insects were collected from other *Capsicum* plants or from nearby vegetation. Two to five bugs of the proper species were introduced into each bag. Bags were left in place for a week, which is sufficient time for fungus to infect the fruits and seeds (personal observation). At the end of the week, all fruits were harvested and stored inside individual small paper envelopes (coin envelopes). Seeds were later scored for fungal infection. In 2008, the naturally occurring plants were left under normal

conditions, whereas in 2009 the potted plants had to be watered to avoid fruit abscission.

Measurement of Level of Fungal Infection

Fusarium infection of chili seeds can be quantified by the extent and darkness of discoloration caused by the fungus. To do so, I used the visual scale described in Tewksbury et al. (2008a: Figure 2.1). This scale ranges from 0 for uninfected seeds (seeds uniformly pale yellow, no discoloration) to 5 for heavily infected seeds (seeds uniformly black or dark gray). I scored all seeds from fruits in the 2008 experiment but only six randomly-selected seeds per fruit for the 2009 experiment. In all cases, I scored seeds blindly (i.e., I did not know which treatment seeds had experienced) and calculated the mean seed infection score per fruit by adding the infection scores and dividing the result by the number of seeds examined.

Data Analysis

Fungal scores of seeds from 2008 and 2009 control fruits were compared with a t-test and found to not differ ($p=0.179$, two-sample unequal variances; seeds from control fruits were used because they constituted the group with the largest sample size). Thus, I combined data from the two years in further analyses. Likewise, to determine the extent to which my inability to replicate all treatments on each plant may have increased the observed variation among treatments, I compared fungal infection scores of seeds from the two treatments that most commonly co-occurred on a given plant (*Acroleucus* and Control), blocking by plant. The result did not differ from the model I ultimately used for analysis, which did not include plant as a blocking variable (see Results).

I used a one-way ANOVA to assess difference among treatments in mean fungal score of seeds per fruit. To correct for heteroscedasticity, I applied Welch's method for unequal variances. To perform pair-wise comparisons between treatments, a two sample t-test for unequal variances was used with Satterthwaite's approximation for degrees of freedom. The alpha value was reduced (Bonferroni method) to control for multiple comparisons.

Additionally, I compared the total number of uninfected seeds (infection score of zero) to the total number of infected seeds (all non-zero scores; see table 2.2), using a chi-square test to detect potential differences among treatments.

Table 2-1. Description of treatments and sample size.

Treatment	Description	Sample size (# of Fruits)
Acroleucus (Lygadae)	2 adult insects or 5 nymphs	22
Edessa sp. (Pentatomidae)	2 adults	10
Beetle mimic (Pentatomidae)	2 adults	6
Holes	5 holes poked with an acupuncture needle	36
Control	None of the above	29

Table 2-2. Number of uninfected and infected seeds per treatment.

	Control	Acroleucus	Beetle mimic	Pentatomidae	Holes
Uninfected	83	84	19	39	95
Infected	69	48	7	21	42
Total seeds	152	132	26	60	137

Figure 2-1. Capsicum chacoense seeds infected by *Fusarium*. Numbers represent fungal infection scores (1 = low; 5 = high). Images taken from Tewksbury et al. (2008a).

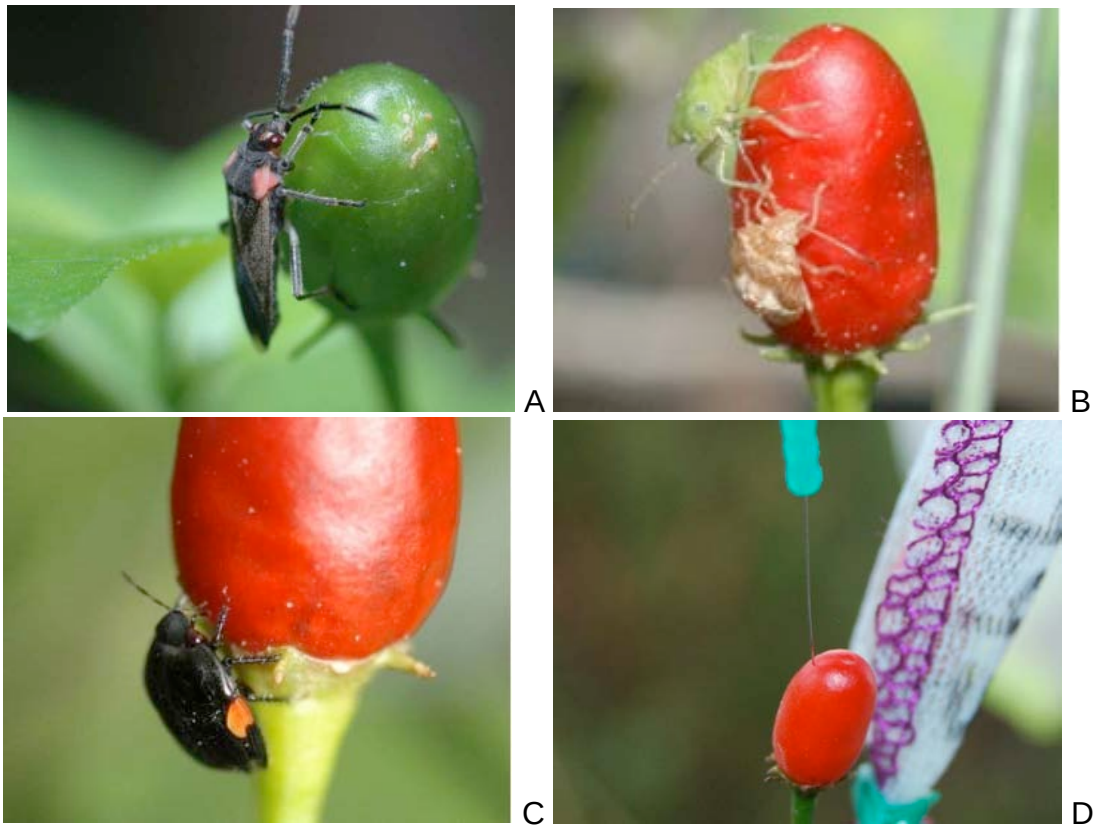

Figure 2-2. Hemipteran insects feeding on *Capsicum chacoense* fruits: A) *Acroleucus*, B) *Edessa sp.* (Pentatomidae) nymphs, C) "Beetle mimic" (Pentatomidae). D) Hole treatment, using an acupuncture needle. (Pictures A to C taken by Tomas Carlo)

CHAPTER 3
RESULTS

Seeds from 103 fruits (59 from 2008, 44 from 2009; 34 non-pungent, 69 pungent) were scored for fungal infection. Effect of plant was not significant; fungal scores of seeds from *Acroleucus* and Control fruits did not differ when controlling for mother plant ($F_{1,17} = 0.54$, $p = 0.91$). Fungal infection scores were significantly different among treatments (P=0.034, one-way ANOVA).

Most of the variation in fungal infection score occurred between Holes versus Control and *Acroleucus* fruits (Figure 3.1). Post-hoc tests for fungal infection scores from the best-replicated insect treatment, *Acroleucus*, did not differ from fungal infection of Control fruits ($p = 0.69$). In contrast, fungal infection scores of seeds from Holes fruits averaged 69.7% and 73.9% less than of those from Control and *Acroleucus* treatments, respectively ($p = 0.011$, $p = 0.017$, respectively). Pair-wise comparisons were not performed with Pentatomids (*Edessa sp.* and Beetle mimic) due to small sample sizes.

Fungal infection scores were not significantly different among seeds from pungent and non-pungent fruits (P=0.46; figure 3.2; the Beetle mimic treatment was excluded due to small sample size).

The total number of uninfected seeds did not differ among treatments (P=0.55; table 2.2).

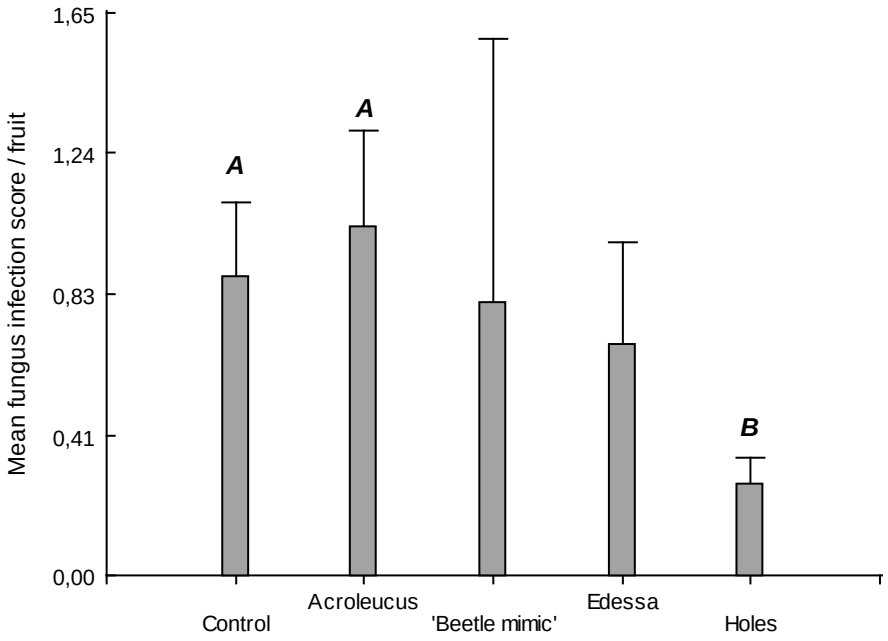

Figure 3-1. Fungal infection scores of seeds from five treatments. Error bars show standard error of the mean. Treatments with the same letter are not significantly different, based on post-hoc tests. Treatments with different letters are different at $P < 0.05$. Lack of letters indicates that post-hoc tests were not conducted (see text).

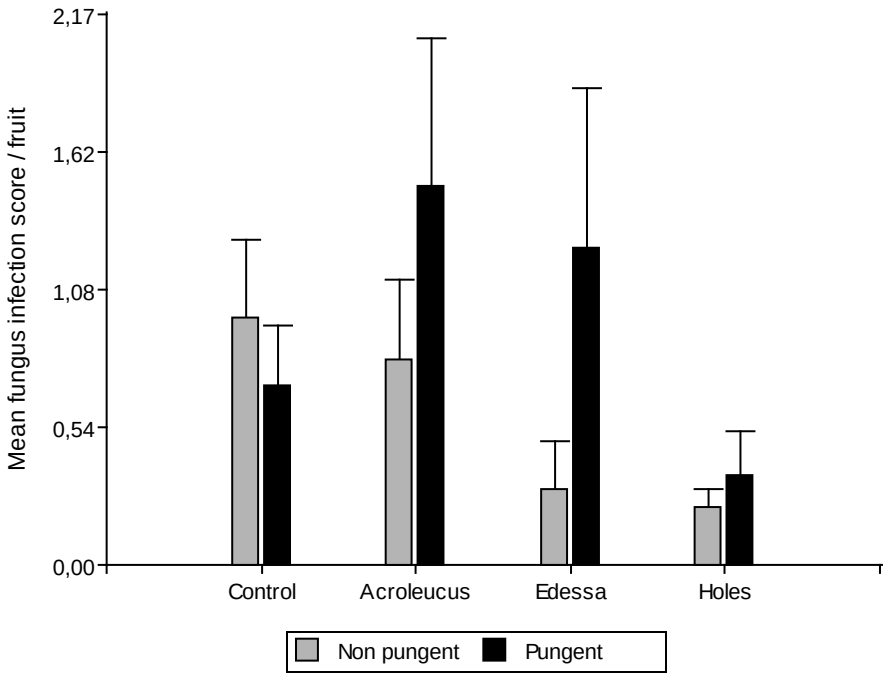

Figure 3-2. Effect of pungency on *Fusarium* infection; grouped by treatment. Error bars show standard errors.

CHAPTER 4
DISCUSSION

Under the vector hypothesis I expected seeds from fruits in the three treatments that included insects to have higher infection scores than seeds from Control fruits. However, they did not. The relatively high fungal infection score of seeds from Control fruits suggests an undetected route of entry of the fungus into fruits – a route that I obviously did not control or manipulate. Under the mechanical damage hypothesis, seeds from the Holes treatment should have had similar infection scores as seeds from the three treatments with insects. On the contrary, seeds from pin pricked fruits showed significantly lower fungal infection scores than seeds from the *Acroleucus* treatment. The same pattern was apparent between seeds from Holes versus the other two treatments with insects, although post-hoc tests were not attempted due to low sample sizes.

The comparisons among treatments discussed in the previous paragraph are based on average scores of fungal infection. An alternative approach is to treat fungal infection as being either present or absent on a seed and to test whether seeds from some treatments are more or less likely to be infected than seeds from other treatments. When I analyzed the data this way I found that the total number of uninfected seeds did not differ among treatments -- exposure to foraging insects and pin pricks did not affect the likelihood that fungus would be detected on seeds.

Previous studies have shown that adult shore flies, fungus gnats and moth flies effectively serve as vectors of *Fusarium* macroconidia in greenhouse plants (El-Hamalawi and Stanghellini 2005) and that engraver beetles carry *Fusarium* on their exoskeletons and transfer it among pine plantations (Erbilgin et al. 2008). Likewise, it

has been confirmed that vertebrate pollinators actively transmit *Fusarium* spores into the flowers that they pollinate (Lara and Ornelas, 2003). In the only study I am aware of relating to *Fusarium* transfer in wild fruits, Tewksbury et al. (2008a) found that *Fusarium* infection scores of *Capsicum chacoense* seeds increased with the number of foraging scars left by insects on the surface of the fruit containing those seeds.

It is perplexing that the use of sterile needles to poke holes in chili fruits reduced the level of fungal infection. A potential explanation is induced defense. There is the possibility that the damage produced by needles affected the fruit in such a way that triggered production or release of defense-related proteins. Such proteins have been described in many plant species upon senescence or wounding (Van Loon et al. 2006). Molecular work has shown that *Capsicum annuum*, another species of chili, produces antimicrobial proteins in response to microbial attack and that those proteins are highly effective in reducing bacterial and fungi infection (Lee et al. 2008). Recent work has also revealed an effective antimicrobial peptide lipid transfer protein in *Capsicum annuum*, particularly effective in inhibiting growth of *Fusarium oxysporum* (Cruz et al. 2010). It remains unclear, however, whether any of these compounds are released or produced in response to physical damage to fruits. It is also unclear why a defensive response would be triggered by an artificial stimulus (needle pokes) and not by natural stimuli (insect feeding).

With respect to effects of pungency on fungal infection, I found no effect of pungency on *Fusarium* infection of seeds. In contrast, a previous study concluded that pungent fruits are significantly less prone to microbial attack than non-pungent fruits (Tewksbury et al. 2008a). This discrepancy may have arisen because Tewksbury et al.

(2008a) treated seeds differently before assessing levels of fungal infection. In particular, they allowed fungal growth for six months and scored seeds on the first day of germination when seeds had been placed on moist filter paper on top of natural soil to simulate post-dispersal conditions. Perhaps pungency plays a larger role in protection of seeds from *Fusarium* after dispersal, rather than before dispersal – the stage considered in my study.

I propose further studies using same design with longer treatment times and scoring of seeds *in-situ*.

CHAPTER 5
CONCLUSION

Long-term population studies on wild *Capsicum spp.* are required to understand the ultimate factors affecting dynamics of *Fusarium* dispersal, which affects fruit and seed infection. The lack of significant difference in fungal scores of seeds from fruits of insect treatments relative to controls does not necessarily allow one to rule out *Fusarium* transmission by insects. It is possible that *Fusarium* is actively dispersed by insects earlier in the plant's life cycle. It may additionally be dispersed by wind. The mechanisms of *Fusarium* dispersal in the wild may be affected by density of plants as well – a factor I did not examine but suggest be manipulated in future experiments. Research in ecology of diseases and their vectors should be encouraged in wild relatives of crop plants to improve our understanding of plant-pathogen interactions. Doing so may reveal ecological alternatives to chemical or genetic protection of agricultural crops, thereby avoiding potentially negative side effects of traditional means of combating plant pathogens.

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BIOGRAPHICAL SKETCH

Carlos E. Manchego was born in Santa Cruz, Bolivia. In May 2006 he received a Bachelor's degree in biology at the Universidad Autónoma Gabriel René Moreno. He was an *ad honorem* collaborator in the GIS department at the Natural History Museum Noel Kempff Mercado, as well as a Teaching Assistant in cellular biology. In August 2007, Carlos began graduate studies in the Department of Zoology at the University of Florida, working with Dr. Doug Levey and in close collaboration with the Tewksbury lab at the University of Washington. During his time in graduate school Carlos has taught 4 semesters of Introductory Biology and 2 semesters of Molecular Physiology courses. He performed research in Bolivia and spent a semester in Costa Rica, studying tropical ecology and conservation with the Organization of Tropical Studies.