How the chili got its spice:

Ecological and evolutionary interactions between fungal fruit pathogens and wild chilies

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Abstract

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Most species depend on other species for survival and reproduction, thus coevolution – reciprocal evolutionary change between species – arguably plays a dominant role in the origin and organization of biodiversity. We used a novel model system to study coevolutionary dynamics: wild chili peppers and their fruit-associated fungal pathogens. The spiciness of chilies (caused by the antimicrobial chemical capsaicin) protects chili fruits from fungi that can destroy seeds. We examined the ecological and evolutionary interactions between chilies and fungal pathogens by using a combination of field experiments, lab work, and biodiversity analyses. We demonstrate that fungal pathogen pressure varies across the landscape, and impacts fruit infection rates and alters the role of fungal insect vectors. We also show that variation in chili spiciness selects for fungal tolerance to plant defenses and drives fungal local adaptation. However, increased fungal tolerance to capsaicin comes with costs that impact fungal

competitive ability and constrain the process of coevolution. Finally, we show that the production of capsaicin by chilies impacts the biodiversity and community structure of fungal fruit inhabitants. We demonstrate that capsaicin has a positive effect on fungal diversity, and that capsaicin has differential impacts on the taxa within the fungal community. Combined, these findings provide compelling evidence that coevolutionary dynamics between plants and fungal pathogens are likely to be responsible for the major property of one of the most popular spice plants in the world: the spiciness of chilies.

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DEDICATION

To my boys, Jim and Opus.

CHAPTER 1

Title: Interactions between fruit chemistry, insects, and seed pathogens determine predispersal seed survival in wild chili peppers

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Key words: reciprocal transplant, seed predation, genotype × environment interactions, host-pathogen interactions, chemical defense, capsaicin, insect vectors

Abstract: A wide range of predators and pathogens attack fruits before dispersal, but are rarely integrated into an evolutionary framework. We examine the impact of both fungal seed pathogens and insect seed predators on the dynamics of fruit and seed infection across a geographically explicit polymorphism in fruit protective chemistry. Using a reciprocal transplant, we show that pathogen and insect predator density, plant density, and fruit chemistry all co-vary with moisture availability. Insect seed predators and fungal pathogens are mechanistically linked, as insect seed predators both directly reduce seed survival in uninfected fruits, and vector fungal pathogens that further reduce survival. Infections caused by insect-vectored fungal pathogens lead to higher seed infection severity and lower survival than fruits infected without vectors, and fungal strains isolated from insect-damaged fruits grow faster than strains isolated from fruits not exposed to insects. Insect transmission may thus select for faster growing, more virulent fungal pathogens.

Introduction:

Interactions between plants and organisms that cause seed mortality can exert strong selection on plant traits and shape the diversity and composition of plant communities. Seed predation in natural systems can occur before or after dispersal, and often involves multiple interactions among a diverse range of taxa including vertebrates, invertebrates, and seed-destroying microbes (Janzen, 1971). While a rich body of literature exists on the evolutionary (Janzen, 1969; Schupp, 1988; Hulme & Benkman, 2002) and demographic (Janzen 1970, 1971; Connell 1978; Hulme 1998; Petermann et al. 2008) consequences of post-dispersal seed predation on plants, fewer studies have assessed the impact of pre-dispersal fruit and seed predators on plant trait selection or plant population dynamics (Ehrlén, 1996; Kolb et al., 2007). Most studies of pre-dispersal

fruit and seed predation focus on a single class of antagonists such as insects (Louda, 1982; Ehrlén, 1996; Ostergård et al., 2007), birds (Galetti, 1993; Francisco et al., 2008), or mammals (Heithaus, 1981; Siepielski & Benkman, 2007), even though evidence suggests that multiple, and sometimes interacting, fruit and seed predators operate in many natural systems (Gómez, 2008; Ramírez & Traveset, 2010; Beckman & Muller-Landau, 2011).

In general, fungal pathogens are promising candidates for biotic drivers of local adaptation and trait evolution in plant populations. Interactions between fungi and plants are ubiquitous and they influence the ecological dynamics, composition, and structure of natural plant communities across multiple geographic scales (Burdon et al., 1989; Thrall et al., 1997; Gilbert, 2002; Laine, 2007; Burdon & Thrall, 2009). The outcomes of plant-fungal interactions often vary geographically between populations (Thompson, 1994, 2005; Thrall et al., 1997; Kaltz & Shykoff, 1998; Burdon et al., 2002; Laine & Laine, 2005) and reciprocal effects on the evolutionary dynamics of both species appear common (Thompson, 1994, 2005; Burdon et al., 2002) (Burdon et al. 2002; Thompson 1994; Thompson 2005).

Fungi that attack fruits and seeds may be especially promising systems in which to study pathogen driven selection on plants. The fitness impacts of seed pathogens are strong: they can impact seed development, kill seeds directly, and they can render fruits unattractive to potential dispersers (Janzen, 1977; Buchholz & Levey, 1990; Cipollini & Stiles, 1993). In addition, fruit inhabiting fungi often interact with other predators of fruits and seeds, and can depend on insect seed predators to access fruit (Mills, 1983; Hatcher, 1995; Mitchell, 2004). However, the mechanisms and extent of microbial fruit infection are poorly understood in natural systems

(Gilbert, 2002). Many microbial fruit infections are caused by fungi, and fungal dispersal can be abiotic - via wind or water - or biotic – through insect vectors (Mills, 1983; Mitchell, 2004). Biotic vectoring is also complex, because insects may simply enable fungal attack by creating entry points for fungi that are present on the skin, but unable to get inside the fruit on their own. In contrast, insects can also move fungi from fruit to fruit, by carrying it in their gut, in their proboscis, or on their bodies. The ecological and evolutionary consequences of these two types of interactions between fungal pathogens and insect predators are quite different, but are rarely explored.

Very few studies have examined the ecological or evolutionary interactions between fruits and fungal pathogens in wild populations (Herrera, 1982; Cipollini & Stiles, 1993; J. Tewksbury et al., 2008); most have focused on interactions with vertebrate dispersers (Herrera, 1982; Cipollini & Levey, 1997). We are aware of no studies that have compared the impact of biotic and abiotic pathogen transmission modes on host infection probability, infection severity, and seed survival in wild plant systems. In addition, very few studies have quantified the combined impact of insect and microbial seed predators on predispersal seed loss in natural systems (Herrera 1982).

Here we take advantage of a gradient in moisture availability that influences the relative strength of biotic and abiotic selective pressures on one of the most famously protected plant fruits – the wild chili pepper. *Capsicum chacoense* exhibits a polymorphism in fruit protective chemistry across this gradient, with all plants producing fruit with high levels of chemical defense in moist areas and, and most plants producing fruit without chemical defense in dryer areas (Tewksbury et al. 2008). We use reciprocal transplants of pungent and non-pungent chilies from wet and dry

locations to 1) quantify fungal transmission and seed infection across this gradient, 2) assess the extent to which dominant insect seed predators aide fungal transmission by acting as a vector, and compare the fitness impact of fungal infection to the potential impact of insects as seed predators, 3) assess the dynamics of local adaption of plants and two types of fungal communities: those transmitted by abiotic factors (wind and rain) and those transmitted through the foraging of an insect vector, and 4) determine the extent to which fruit pungency alone impacts fungal infection and seed mortality.

Methods:

Study system

The wild chili pepper, *Capsicum chacoense,* Hunz., is native to the Chaco region of southeast Bolivia, Argentina and Paraguay. Several characteristics make *C. chacoense* and its fruit-associated fungal pathogens a rich system for studying local adaptation and fruit-fungal interactions. The genus *Capsicum* is known for the production of a family of closely related compounds called capsaicinoids, most of which (> 90%) are in the form of capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), and dihydrocapsaicin (N-(4-Hydroxy-3-methoxybenzyl)-8-methylnonanamide). Capsaicinoids are produced exclusively in the fruits and are responsible for the fiery (pungent) taste of chili peppers. Capsaicinoids remain present in fruits throughout development and maturity (Prasad et al., 2006), and have documented antimicrobial properties (Billing & Sherman, 1998)Molina-Torres et al. 1999; Kurita et al. 2002). While most *Capsicum* species do not show intraspecific variation in pungency (Eshbaugh, 1980), *C. chacoense* is polymorphic for capsaicinoid production (Tewksbury et al. 2008; Haak et al. 2011). Two distinct, and genetically determined (Blum et al., 2002) chemical phenotypes coexist: plants with

pungent (capsaicin-producing) fruits occur in the same populations as plants with fruits that completely lack pungency. Across our 300 km study area in Bolivia, polymorphic populations of *C. chacoense* vary considerably in the proportion of plants that bear pungent fruit. This variation extends from the dry lowlands near the north-eastern part of our transect, where most chilies lack pungency, to moister, high elevation regions in the Southwestern end of our transect where all chilies are completely pungent (Tewksbury et al. 2006; Tewksbury et al. 2008). The maintenance of this polymorphism has been attributed to differences in both biotic and abiotic selective forces across the landscape. Haak et al. (2011) showed that water stress selects for non-pungency; in dry conditions, pungent plants suffer a 50% reduction in seed set relative to non-pungent plants. Selection for pungency, on the other hand, may be driven by fungal seed pathogens, which have been shown to be a significant source of seed mortality (J. Tewksbury et al., 2008). Fungal pathogens appear to gain access to seeds through the wounds created by heteropteran seed bugs, which pierce fruit to forage on seed endosperm. The frequency of insect attack on fruits is greater in moist, high elevation sites, and Tewksbury et al. (2008) argued that insect and fungal attack on seeds in these sites was a principle mechanism causing selection for pungent fruit.

Reciprocal Transplant

We took advantage of naturally existing variation in *C. chacoense* populations and established a reciprocal transplant using populations taken from two sites at either end of the polymorphism gradient. At our wet site (21.520°S, 63.781°W), where all plants are pungent, insect pressure on chili plants is high, with an average of 11 punctures per fruit, and relatively high average rainfall (about 950 mm / year). At our dry site, (20.018°S. 63.100°W) insect pest pressure is much less extreme, with < 1.5 punctures per fruit, lower annual rainfall (650 m / year), and only 38% of

plants are pungent (J. Tewksbury et al., 2008). Study sites were located about 180 kilometers apart in the Gran Chaco region, Santa Cruz, Bolivia.

We removed mature wild plants to use in this study from each field site in November 2008, prior to annual onset of flowers or fruits. We used only non-pungent plants from the dry site, as they dominated in number. We potted and transported plants to a common greenhouse environment at Vallacito Agricultural College in Santa Cruz, Bolivia where they were protected from insect attack and pruned to encourage fruit production.

In April 2009, we transported 20 plants with young fruits from each population to each field site. In each site, we created a mixed plot of potted plants; approximately 20 m^2 under a 50% black shade cloth. We kept plants in aboveground pots and randomly placed them approximately 1 m apart from each other, and watered plants daily with a watering regime that matched local conditions as closely as possible. We covered all individual fruits on each plant with a fine mesh bag to prevent insect access but still expose fruits to airborne sources of local fungal propagules. We applied treatments to bagged plants after they had been set up in the field for one week.

Insect surveys

Seed predators presumably reduce seed viability by inserting their stylus into the fruit and targeting the seed endosperm. Each time an insect punctures the skin of the fruit, it creates an entry point for fungal pathogens and may also act as a vector (Purcell, 2005). To determine the identity of the dominant insect seed predators at our field sites, we conducted an insect survey at each site. We used large, fine mesh insect nets to cover whole branches of or whole wild chili

plants. We shook the plants vigorously for 5 seconds and quickly removed the net, taking care to not to release caught insects. Each plant or branch was sampled once. We transferred insects to mesh holding bags so they could be sorted and counted. We categorized insects into three most common groups, (1) *Acroleucus* (2) green Pentatomids or (3) "other" and counted their occurrence at both sites.

Field experiment

The most common insect seed predator at both our sites was *Acroleucus coxalis* (Stäl) (Lygaeidae), (see table S1), thus we focused on *Acroleucus* in our experiment. Fruits on our plants were assigned to one of two infection treatments: "biotic" or "abiotic." In the biotic infection treatment, we placed a locally caught adult instar of *A. coxalis* in a mesh bag with a fruit for the duration of the experiment (7 days). For the abiotic treatment, we measured the diameter of the stylus of *Acroleucus*, and matched this with sterile acupuncture needles of equal width (Sierin, JT.12x15; 0.12mm). We used these needles to introduce 5 holes into each fruit (average number of holes left by bagged insects in a pilot study, unpublished data) before covering the fruit with a mesh bag. We applied one treatment to each fruit; each plant had a balanced set of both treatments. During setup, we wore latex gloves and surface sterilized them with 70% isopropanol to prevent cross contamination when handling new fruit. After one week, we collected and individually placed each fruit in a sealed paper envelope. We stored fruits in a cool, dry, and well-ventilated environment prior to transport to the lab.

Measurement of fungal seed infection

In the lab, we examined each insect-treated fruit under a dissecting microscope to count the number of holes present on the surface. To assess the impact of treatment on fungal fruit and seed infection, we used sterile technique remove and examine a subset of six seeds from each fruit. We assigned each seed an identification number and placed them in Petri dishes on sterile cellulose pads. We watered seeds with 1.5 ml sterile water every 3 days to keep them evenly moist. We incubated dishes in an environmental chamber on an 11 hour light/13 hour dark, 33 C/22 C° (2 hour ramp) cycle to mimic field temperature and light conditions, and rotated dishes daily. After 7 days, we used a seed-scoring standard to quantify visible fungal infection on seeds (J. Tewksbury et al., 2008). Using a dissecting microscope, we assigned each seed a value from 0 (no visible infection) to 5 (highest level of infection). All scoring was blind; observers had no knowledge of seed source, treatment, or fruit pungency. We replaced seeds in the chamber after scoring.

Seed germination and survival

To assess how fungal infection on seeds impacted seed germination and seedling survival, we checked all seeds for germination daily. Any seeds that germinated and had exposed cotyledons were transplanted into 72-well seed trays filled with Sunshine soil mix. We transferred germinated seeds with the seed coat attached. We handled all seeds with sterile forceps. We kept seedlings in the environmental chamber for 20 additional days and checked them for survival at the seedling stage. We tracked germination for 6 weeks total, seeds that had still not germinated after this point were considered non-viable.

Fungal Growth Assays

To assess if fungal growth rates differed between infection treatments, field sites, or plant types, we conducted assays of fungal growth rates in the lab. We isolated fungi from non-surviving seeds by transferring one seed per fruit to agar plates that contained dilute potato dextrose media (PDA) with a 0.2% solution of the antibiotics tetracycline, ampicillin, and streptomycin. We incubated seeds in the dark at 25° C and checked them daily for fungal growth. We subcultured isolates until axenic, transferred them onto agar slants for storage, and refrigerated them at 6° C. For growth assays, we measured fungal growth rates on two capsaicin media treatments, 0 mg/g capsaicin or 0.5 mg/g capsaicin. Our previous laboratory work has shown that even small amounts of capsaicin have a pronounced effect fungal growth (J. Tewksbury et al., 2008). For both treatments, we assayed isolates on false fruit media (FFM) that mimicked the nutritional content of *Capsicum* fruits (J. Tewksbury et al., 2008). We added capsaicin (Sigma, M2028, ≥95%) to sterilized media after it was dissolved in methanol; methanol alone was added to the 0 mg/g treatment as a control. For both treatments, we dispensed 3 ml of FFM into 60 cm petri dishes. Prior to the start of the growth assays, we grew isolates on PDA and then transferred each to the center of assay plates with the use of a 4 mm diameter cork borer to standardize the size of the initial inoculum. We inoculated each isolate onto each treatment once. Plates were kept in the dark at 25° C. We measured 2 perpendicular colony diameters (originating at a random starting point) at 24 and 48 hours after inoculation. Measurements were blind with respect to isolate origin and capsaicin treatment. We used the mean colony diameter for each isolate, and calculated rate of growth as mean mm/hr growth.

Data Analysis

We used linear mixed-effects models (LMERs) to characterize the effects of field site, plant population origin, and treatment type (and their interaction terms) on three explanatory variables: the probability of fungal infection occurring in a fruit, infection severity of 6 randomly selected seeds within each fruit, and their probability of survival. Plant was included as a random effect in all analyses except for infection severity where we included a nested random effect of fruit within plant. For fruit-level analyses, where we characterized fungal infection as present or absent, we used a binomial error distribution with a logit link function. For seed infection severity, we analyzed only fruits with infected seeds. For seed survival, we scored seeds as "alive" or "dead," created a proportional response variable (*n*/6 seeds to survive), and used a binomial error distribution in models. We analyzed both infected and uninfected seeds together in survival models but added seed infection as an additional fixed factor, which we characterized as a yes/no variable.

The number of holes per fruit varied in the insect treatment but did not vary in the needle treatment. For this reason, we could not the include number of holes as a fixed factor in our models. To assess if the number of holes had an impact on infection or survival, we created models for fruits in the insect treatment only, and included number of holes, field site, and plant population origin as fixed effects, and included plant as a random effect.

For fungal growth assays, we used LMERs to characterize the effects of treatment, field site, capsaicin level (and their interactions) on fungal growth rates, and included plant as a random effect in our models. We were unable to include plant population origin in our models because

we did not obtain any fungal isolates from local plants at the dry site, as nearly all the seeds in that group germinated and survived.

For all analyses, we identified the best subset of models using Akaike's Information Criterion (AIC_c) and reported all models with a delta AIC_c ($\Delta_{i=}$ AIC_i - AIC_{min}) of less than 2, or if fewer than 4 models had a $\Delta_i < 2$, we reported the top four models regardless of delta AIC_c. Rather than using likelihood ratio tests (LRT) with their associated *p*-values to identify only one top model with significant main effects, we performed model averaging of coefficients of our top models to acknowledge model-selection uncertainty (Burnham & Anderson, 2002). In all analyses, LRT and model averaging methodologies both revealed the same best-fit model. For models included in model averaging, we reported Akaike weights (w_i), which are the likelihood that a given model is the best of the models considered (Burnham & Anderson, 2002) as well as their regression coefficients (β) with 95% confidence intervals. We also reported the relative variable importance (w_{ip}), or the sum of w_i across all candidate models in which the variable appears (Burnham & Anderson 2002). We conducted all analyses in R (R Development Core Team, 2011) using packages lme4 (http://r-forge.r-project.org/projects/lme4/) and MuMIn (http://r-forge.r-project.org/projects/mumin/).

Results:

Fungi that gained access to fruit abiotically were twice as likely to infect fruits at our wet site as our dry site. However, this difference was not seen in fruits that *Acrolecuus coxalis* foraged on; fruit infection was the same at both sites, regardless of plant origin and pungency (Table 1, Fig. 1a, b). At the dry site, fruits exposed to *Acrolecus* were twice as likely to be infected when

compared to abiotically infected fruit (Fig. 1a). When fruits were infected, those that were exposed to *Acroleucus* had consistently higher levels of seed infection severity than fruits with abiotically transmitted fungi (Table 2; Fig. 1b, c). At the dry site, local, non-pungent plants had lower levels of seed infection severity than non-local pungent plants from the wet site (Fig. 1c). This trend was not seen at the wet site, where local and non-local plants had similar levels of infection severity. The number of foraging scars left on fruits by *Acroleucus* was not a factor in any of the top seed infection severity models; a model that included the number of scars had nearly equivalent support as the null model (null model, $\Delta_i = 2.08$, model with holes $\Delta_i = 2.07$).

Results from fungal growth assays in artificial media support our findings that pathogens transmitted by *Acroleucus* caused more severe seed infections. While all isolates grew more slowly in media with capsaicin, strains isolated from fruits infected by *Acroleucus* consistently grew faster on media with and without capsaicin (Table 2, Fig. 2). The second best-fitting model ($\Delta_i = 0.49$, Table S2) suggested that isolates vectored by *Acroleucus* were slowed more by capsaicin than isolates that entered fruits abiotically (capsaicin level × treatment interaction), but this interaction was not in the best-fit model which included only capsaicin level and treatment factors.

The probability of survival was consistently lower for seeds that came from fruits that were infected when compared to uninfected fruits (Fig. 3). Seed survival was also consistently lower for fruits exposed to *Acroleucus,* and this was true for both infected and uninfected fruits, where the insects were presumably killing seeds directly (Fig. 3). However, fungal infections associated with *Acroleucus* had larger negative impacts on seed survival at the dry site than at the

wet site (Table 3, infection × site × treatment interaction); infections transmitted by *Aroleucus* decreased seed survival 82.8% at the dry site compared to a 46.7% decrease in survival at the wet site. There were no differences in the seed survival of fruits infected abiotically across the two sites.

The number of foraging scars left by *Acroleucus* in fruits was higher at both sites than the number of needle holes (5 per fruit) we made in the abiotic fruits treatment. For uninfected fruits, the mean number of holes per fruit at the wet site was 10.08 ± 0.67 SE and 8.47 ± 0.99 SE for the dry site. In infected fruits, the mean number of holes after the insect treatment was 13.59 ± 1.21 SE at the wet site and 7.91 ± 0.73 SE at the dry site. The number of foraging scars left by *Acroleucus* impacted the probability of survival in uninfected fruits but not in infected fruits (Figure S1). When we created models of seed survival that included only the biotic treatment, adding the number of holes as a random effect in the model did not change the results.

Discussion:

Fungal pathogens of *C. chacoense* are not distributed evenly in the environment, creating different fruit infection probabilities across the landscape. In previous work, we (Tewksbury et al. 2008) showed that capsaicin production in polymorphic *C. chacoense* populations increased with increased risk of microbial attack on fruits across a geographic gradient in Southeast Bolivia. In that study, however, microbial pressure was not measured directly; rather it relied on the positive relationship between fungal seed infection and the number of insect scars per fruit as evidence of microbial attack. Populations with the highest number of insect scars per fruit were located on the wetter, high elevation parts of the gradient. Our models of the probability of fruit

infection indicated that a site × treatment interaction was important (Table 1), suggesting a difference in fungal infection probability each site. Our data show that microbial pressure was higher at the wet site, and thus may contribute to the selective advantage of pungency in populations of the geographic gradient that have relatively high annual precipitation. Biotic factors such as and host density, phenology, and vector relationships, as well as abiotic factors such as moisture and temperature have all been associated with patchy or uneven fungal pathogen distributions (Burdon et al. 1989).

Wild *Capsicum* fruits in this system face two different and interacting pre-dispersal seed predators: insects and fungi. *Acroleucus*, the dominant Lygaeid insect in chili populations, directly predates on seeds by inserting its stylus into the seed, injecting digestive enzymes into the endosperm, and sucking up the partially-digested result (Beck et al., 1958). Estimates of the direct impact of insect seed predation on seed survival in non-agricultural settings are few and vary dramatically (Janzen 1971; (Crawley, 1989). In uninfected *C. chacoense* seeds, direct seed predation reduced seed survival by 47-53%, depending on field site, which is consistent with studies on other Lygaeids that showed seed losses of greater than 50% in several plant species (Flemion, 1958). In addition, the number of holes left by insects in uninfected fruit had an impact on seed survival, thus our results clearly link external fruit damage to seed predation.

In addition to these direct impacts, insects with piercing-sucking mouthparts are also able to vector fungal pathogens between infected fruits (Mitchell, 2004), thus both the insects and the fungi are considered primary seed consumers (Janzen, 1970). In our study, over 50% of fruits in the insect treatment were infected at both sites. Vector impact was even greater at the dry site,

where fruits in the needle treatment had a comparatively low probability of infection (Fig 2a). This suggests that vectors can potentially temper the magnitude of environmental difference between sites. In Bolivia, vectors are less abundant at dry sites (J. Tewksbury et al., 2008), but our results suggest that they may have a larger per capita impact than more abundant insects at wetter sites. Fungal vectoring compounds the detrimental impact of direct seed predation by insects because the introduction of fungal pathogens into fruit can infect seeds left undamaged by insects. In *C. chacoense*, fruits contain an average of 17 seeds (J.J. Tewksbury et al., 2008) and the likelihood that insect seed predators will damage every seed is low. However, if fungal propagules are introduced to a single seed, all seeds within the fruit are vulnerable to the spread of fungal infection due to their close proximity. Indeed, in infected fruits, the number of holes left by insects was not a predictor for seed survival, suggesting that, once infected, damage from fungal pathogens was the primary determinant of survival, not direct insect predation. In addition, fungal pathogens on insect mouthparts are most likely to come from other infected fruits visited; thus they represent a non-random subset of the fungal community that is likely well-adapted to exploit seed resources. Theory on the evolution of virulence (the degree of damage a pathogen causes to a host) predicts that vector-borne pathogens should be more virulent than non-vectored pathogens (Ewald, 1994). We present evidence that isolates transmitted by insects grow faster than those from the needle treatment fruits, both in terms of the amount of damage seen on seeds (Fig 1; c and d) and in growth assays (Fig 3). In the needle treatment, we used sterile needles to puncture fruits, so the most likely source of these infections was wind- or water-dispersed propagules. Increased virulence is also a predicted outcome of models of multiple infection in hosts. Coexistence of multiple genotypes within a host favors those that can grow fastest when growth rate is correlated to transmission rate (May & Nowak,

1995). In chilies, multiple infection may be common under natural field conditions, because insects visit and predate upon many different fruits in a population. Faster growth rates in our insect-treated isolates may be related to an increased probability of transmission by vectors.

We found some evidence for plant local adaptation. In the needle treatment, local, non-pungent fruits at the dry site had a lower probability of fungal infection (Fig. 1a) and lower seed infection severity (Fig. 1c). At the wet site, fewer local, pungent fruits were infected than non-local non-pungent fruits, but there were no population-level differences in seed infection severity (Fig. 1c). In the insect treatment, however, the probability of fruit infection was uniformly high with almost no population differences among sites, (Fig.1b) and the only difference in seed infection severity was at the dry site, where non-local plants had a higher mean seed infection score (Fig. 1d). There was no evidence of local adaptation to the effects of direct seed predation and little evidence that plants are able to adapt to the more virulent fungal pathogens carried by insects; fruits and seeds in the insect treatment generally faired worse overall. The relative short generation time of microbes, combined with multiple selective events caused by vectoring pathogens from fruit to fruit may result in rapid adaptation on the part of vectored pathogens, when compared to wind- and water- dispersed pathogens likely represented in the needle treatment.

The more pronounced population-level differences in infections seen at the dry site may be driven, in part, by a higher water stress response in pungent plants. Haak et al. (2011) demonstrated that pungent plants have reduced water use efficiency (and greater stomatal density) when compared to non-pungent plants, leading to reproductive tradeoffs in drought

conditions. Under water stress, pungent plants produced 50% fewer seeds than non-pungent plants. While the plants used in this experiment were watered daily, they were kept in above ground pots; thus they were difficult to keep consistently and evenly moist under field conditions. At the dry site, where daily temperatures are higher, it is likely that plants experienced some water stress and the non-local (pungent) plants would have been more vulnerable the non-pungent local plants. Water stress has long been known to be a contributing factor to disease susceptibility in plants (Schoeneweiss, 1975) and our results suggest that in addition to reductions in seed production, water stress may impact the ability of *C. chacoense* to resist pathogen infection.

In our experiment, population origin and pungency are linked (all plants in the wet site are pungent; all plants in the dry site are non-pungent), thus we are able to assess the relative importance of local adaptation vs. defensive chemistry in the susceptibility of fruits to microbial attack. Our results suggest that the benefits of pungency do not outweigh the benefits of being local. Non-local, pungent plants fared worse at the dry site despite having chemically defended fruit. Our assay results confirmed that even the addition of a small amount of capsaicin (0.5 mg/g) had a large impact on fungal growth rates (Fig. 3). Thus, all else being equal, seed infection frequency and severity in non-pungent plants should be greater than in pungent plants. Our results suggest that differences in a single trait, like the production of capsaicin, are unlikely to predict the complex outcomes of interactions between pathogens and plants. Local adaptation to pathogens is likely governed by many interacting traits and selective pressures, some of which may be poorly understood. Our results underscore the importance of reciprocal transplant

experiments; the movement of whole phenotypes is often necessary to uncover which traits and selective pressures govern local adaptation in natural settings.

Acknowledgements:

We would like to thank David Haak, Marie Clifford, and Diana Rocabado for helping with plant care both at UW and abroad. Museo Noel Kampff and Vallecito Agricultural College in Santa Cruz, Bolivia both provided critical logistical support. Alexandra Hart provided thoughtful comments that greatly improved the manuscript. This research was supported with funding from the National Science Foundation Doctoral Dissertation Improvement Grant (DEB-0808582) to N. Machnicki and J. Tewksbury.

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Figures:

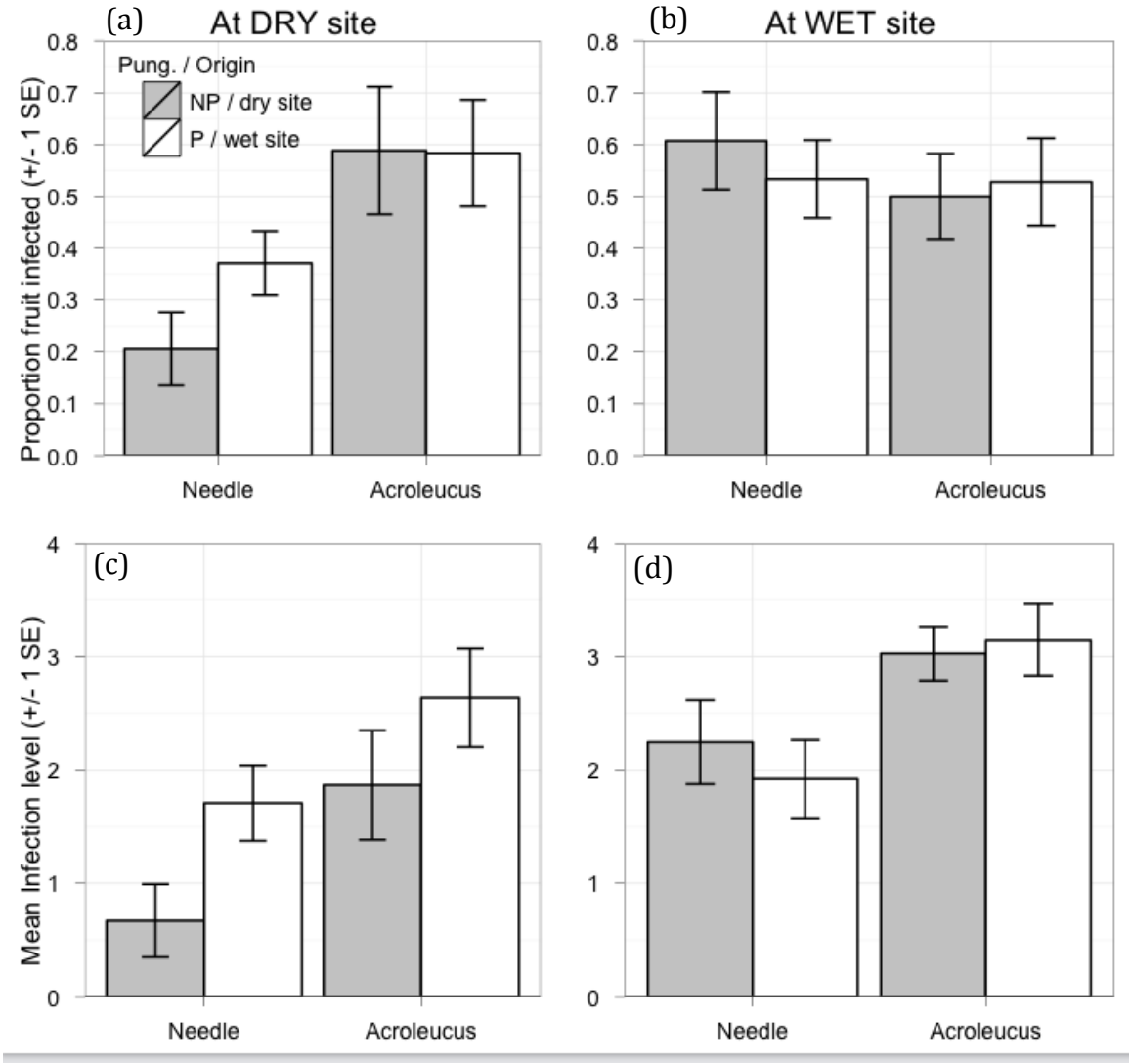


Figure 1. (a) The mean proportion of fungal seed infection in fruits damaged with sterile needles or foraged on by the insect *Acroleucus* at the dry (b) and wet field site. The mean seed infection score of infected fruits damaged with sterile needles or foraged on by *Acroleucus* at the dry (c) and wet (d) field site. Means were first calculated within plant to account for differences between plants; error bars are ± 1 SE.

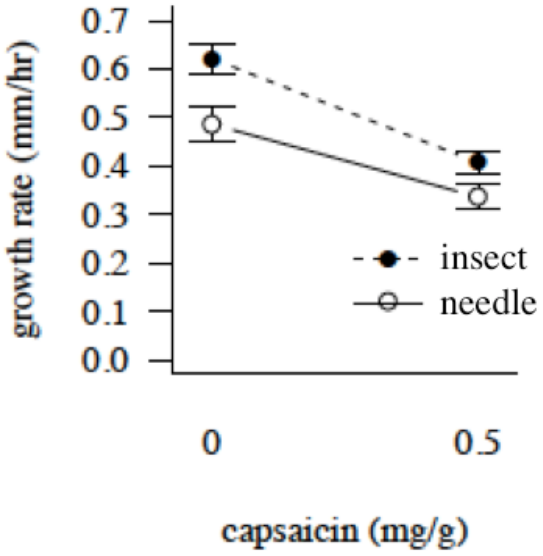


Figure 2. Mean growth rate (mm/hr) of fungal isolates obtained from fruits exposed to insect (A; closed symbols) or needle (H; open symbols) treatments at 0 mg/g or 0.5 mg/g capsaicin. Error bars are ± 1 SE

Figure 3.

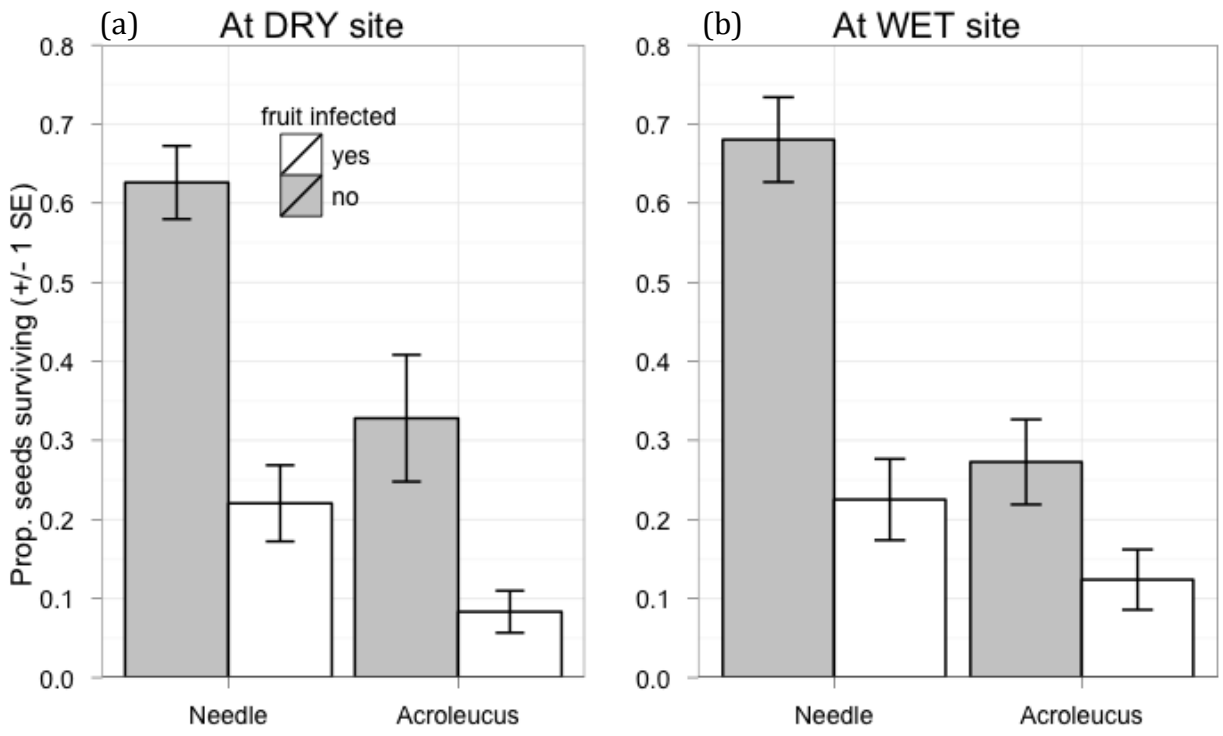


Figure 3. The mean proportion of seeds taken from needle and *Acroleucus* treatments that survived at the dry (a) and wet (b) field sites. Dark bars represent seeds from uninfected fruit and white bars represent seeds from infected fruit. Error bars are ± 1 SE.

Tables:

Table 1. Results of model selection and model averaging for general linear mixed models (GLMM) of the probability of fungal infection occurring in fruits. In all cases, we examined the impact of field site, treatment, population origin, and all interactions on fruit infection. Tables include all models where the AIC_c of the best model (Δ_i) is ≤ 2 or the top 4 models if fewer than 4 have a $\Delta_i \leq 2$. Variables included in each model are indicated with a • symbol. Model rank order is determined by Δ_i. Akaike weights (w_i) are the likelihood that a given model is the best of the models considered (Burnham & Anderson, 2002). Regression coefficients (β) represent averages of β_i values across considered models, weighted by the w_i of each model. 95% confidence intervals of β that do not cross zero are indicated in bold. Relative variable importance (w_{ip}) is the sum of w_i across all candidate models in which the variable appears (Burnham & Anderson 2002). Only variables included in the considered models are listed, they are shown in decreasing order of w_{ip}.

Fruit infection	**Model rank**				**Model average**			
Variables	**1**	**2**	**3**	**4**	**β**	**95% CI**		**w_{ip}**
treatment	•	•	•	•	0.171	-0.499	0.841	1.00
site	•	•	•	•	0.213	-0.679	1.110	1.00
site × treatment	•	•	•	•	**-1.250**	**-2.210**	**-0.291**	1.00
population origin		•	•	•	0.022	-0.466	0.509	0.49
site × population			•		0.120	-0.542	0.781	0.18
population × treatment				•	0.020	-0.296	0.336	0.09
Δ_i	0	1.64	2.11	3.53				
w_i	0.51	0.22	0.18	0.09				

Table 2. Results of model selection and model averaging for general linear mixed models (GLMM) of the mean seed infection score of infected fruits. In all cases, we examined the effect of field site, treatment, population origin and all interactions on infection. Table organization as explained in Table 1. 95% confidence intervals of β that do not cross zero are indicated in bold.

Seed infection	**Model rank**				**Model average**			
Variables	**1**	**2**	**3**	**4**	**β**	**95% CI**		**w_{ip}**
treatment	•	•	•	•	**-0.962**	**-1.152**	**-0.407**	1.00
site	•	•	•	•	**-1.030**	**-1.990**	**-0.064**	1.00
population origin	•		•	•	0.007	-0.591	0.620	0.71
pop. origin × site	•			•	0.563	-0.711	1.840	0.54
pop. origin treat.				•	-0.023	-0.428	0.382	0.15
Δ_i	0	0.64	1.67	1.96				
w_i	0.39	0.29	0.17	0.15				

Table 3. Results of model selection and model averaging for general linear mixed models (GLMM) of seed survival in fruits. In all cases, we examined the effect of infection, treatment, field site, and population origin (and all their interactions) on survival. Table organization as explained in Table 1. 95% confidence intervals of β that do not cross zero are indicated in bold.

Survival	**Model rank**				**Model average**			
Variables	**1**	**2**	**3**	**4**	**β**	**95% CI**		**w_{ip}**
infection	•	•	•	•	**-1.030**	**-1.640**	**-0.416**	1.00
treatment	•	•	•	•	**2.310**	**1.870**	**2.840**	1.00
site	•	•	•	•	0.478	-0.589	1.540	1.00
site × treatment	•	•	•	•	**-1.350**	**-2.150**	**-0.544**	1.00
infection × treatment	•	•	•	•	**-1.040**	**-1.890**	**-0.182**	1.00
infection × site	•	•	•		-0.820	-1.920	0.277	0.85
infection × site × treatment	•	•	•		1.260	-0.217	2.730	0.85
population		•	•		0.165	-0.566	0.895	0.37
population × site					-0.166	-1.100	0.768	0.17
Δ_i	0	1.76	2.11	2.36				
w_i	0.48	0.20	0.17	0.15				

Supplemental Information

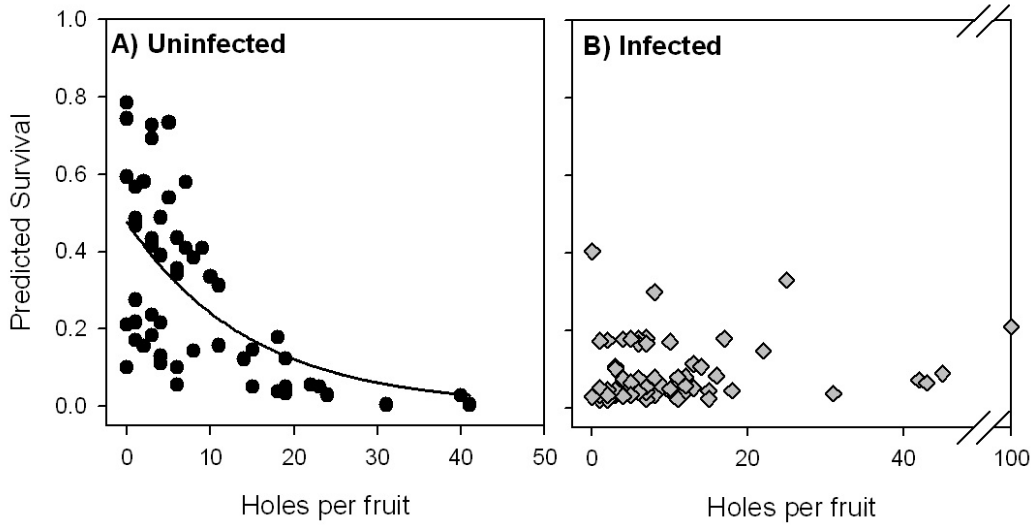
Table S1. Number of insects of each type surveyed at both field sites. Numbers in parenthesis indicate the percentage of total insects surveyed at a single site.

	Acroleucus coxalis	**Green pentatomid**	**Other**	**Total insects per site**
Wet site	96 (80.1%)	20 (16.8%)	3 (2.5%)	119
Dry site	47 (55.3%)	17 (20.0%)	21 (24.7%)	85
Total	70.1%	18.1%	11.8%	204

Table S2. Results of model selection and model averaging for general linear mixed models (GLMM) of the mean growth rates of fungal strains isolated from needle and insect treated fruits on media with and without capsaicin. We examined the effect of field site, treatment, and media capsaicin level (and all their interactions) on fungal growth rate (mm/h). Table organization as explained in Table 1. 95% confidence intervals of β that do not cross zero are indicated in bold.

Seed infection	**Model rank**				**Model average**			
Variables	**1**	**2**	**3**	**4**	**β**	**95% CI**		**w_{ip}**
capsaicin level	•	•	•	•	**-0.198**	**-0.263**	**-0.132**	1.00
treatment	•	•	•	•	**-0.082**	**-1.156**	**-0.007**	1.00
cap. level × treat.		•		•	0.029	-0.063	0.122	0.44
site			•	•	0.010	-0.054	0.075	0.29
Δ_i	0	0.49	1.75	2.24				
w_i	0.40	0.31	0.17	0.13				

Figure S1. Predicted survival of uninfected (A) and infected (B) seeds from the insect treatment based on the number of holes per fruit. We used a GLMR with plant included as a random effect and a binomial error distribution to find predicted seed survival values based on observed number of holes per fruit.



CHAPTER 2

Title: Selection by plant defensive chemistry drives adaptation and tradeoffs in a community of fungal pathogens

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Key words: Diffuse coevolution; capsaicin; *Capsicum;* seed pathogens; host-pathogen interactions; coevolutionary arms race

Abstract: Coevolution, or reciprocal evolutionary change between interacting species, is central to our most important evolutionary stories. While we have long recognized the importance of biotic interactions in the evolutionary process, only recently has the field of coevolution begun to explore testable hypotheses on the importance of complex landscapes, multiple selection pressures, and the constraints on evolutionary and ecological outcomes. We used the fruits of wild chilies that vary geographically in the frequency and intensity of capsaicin production and

their associated fungal pathogens to ask 1) if pathogens can develop tolerance to capsaicin and 2) and if tolerance to capsaicin is associated with metabolic costs or tradeoffs. We show that while capsaicin clearly inhibits the growth of all fungal strains tested, isolates that originate from hotter environments (those from capsaicin-producing fruits and those from populations dominated by capsaicin-producing plants) were less impacted by capsaicin. In addition, fungal tolerance to capsaicin appears to come with costs; in the absence of capsaicin, strains originating from less pungent populations grew faster than those originating from pungent fruits. This pattern was also apparent across isolates taken from three different *Capsicum* species that vary considerably in amount of capsaicin they produce. Isolates from the hottest fruits grew slowest in the absence of capsaicin. Our results suggest that fruit chemistry selects for fungal tolerance to capsaicin, but tradeoffs exist that likely limit the competitive ability of tolerant isolates in low-capsaicin environments. Fungal tolerance for capsaicin also likely drives selection for increased capsaicin production by plants, creating the potential for a coevolutionary arms race.

Introduction:

Coevolution, or reciprocal evolutionary change between interacting organisms, is widely believed to a major driver of the Earth's biodiversity (Thompson, 1994, 2005). Studies have pointed to the radiation of angiosperms (Tiffney, 2004), the diversification of insect pollinators (Ehrlich & Raven, 1964) and the evolution of fruit-frugivore relationships (McKey, 1975) as products of the coevolutionary process. Historically, the term coevolution has described a wide spectrum of coevolutionary interactions (Thompson, 1989). These have ranged from relationships between tightly linked species pairs (Zangerl & Berenbaum, 2003) or gene-for-gene interactions (Flor, 1971; Thompson & Burdon, 1992), to more diffuse patterns of coadaptation seen among taxonomically related groups- such as those seen in butterflies and their host plants

(Ehrlich & Raven, 1964). In the early 1980s, overly broad definitions of coevolution came under criticism (Janzen, 1980; Mitter & Brooks, 1983), and stricter criteria for identifying coevolution (closely matched, coevolved traits between pairs of species and matched phylogenies indicating cospeciation) were widely adopted. As an unfortunate result, diffuse coevolution was often dismissed as less than "true" coevolution (Janz, 2011), and studies of reciprocal adaptation between interacting groups of organisms became less common. However, more recently the case has been made that most coevolution likely takes place somewhere between these two extremes (Thompson, 1994, 2005). We now have evidence that coevolutionary interactions are frequently contextual, forming interaction mosaics in which conditionality and constraints on evolutionary and ecological outcomes shift on both spatial and temporal scales (Thompson 2005; Bronstein 1994; Berenbaum et al. 1986; Thompson and Cunningham 2002).

This has led to a renewed focus on the community context of coevolution and its role in shaping adaptive traits among assemblages of interacting species (Strauss & Irwin, 2004; Haloin & Strauss, 2008). Particularly strong evidence supporting this idea has come from a series of studies that documented conditional shifts in pine cone morphology under contrasting selection imposed by crossbills, insects and squirrels (Benkman, 1999; Benkman et al., 2001, 2003). Outside of these experiments, most studies of evolution within a community context have demonstrated adaptive responses of a single host species to selection by a community of consumers (Strauss & Irwin, 2004), but have not looked at the reciprocal adaptive responses of the consumers. Given the likely importance of community context in coevolutionary outcomes, there is a need for more experimental approaches that consider the responses of both communities of consumers and their hosts (Inouye & Stinchcombe, 2001).

Strong interactions are likely to occur between organisms and their enemies. For this reason, studies of antagonistic interactions, especially in plant systems, have contributed to the development of such ideas as the 'evolutionary arms race,' (Ehrlich & Raven, 1964; Dawkins & Krebs, 1979) and the Red Queen Hypothesis (Van Valen, 1973), which predict that coevolution will result in continuous or cyclic host adaptation in response to escalating counter-adaptation of their enemies. While support exists for arms race coevolution within gene-for-gene systems (Thompson & Burdon, 1992; Burdon & Thrall, 2009)), and in those with a high degree of plant/antagonist specificity (Zangerl & Berenbaum, 2003), studies that take a more diffuse or community approach to arms race coevolution are rare (Strauss et al., 2004). This has likely contributed to the view that arms race coevolution is only possible when plants interact with highly specialized enemies (Agrawal & Heil, 2012). In contrast, others have suggested that many broad-spectrum adaptations used by plants against suites of attackers are more common than those that evolved as a result of one-on-one coevolutionary arms races (Fox, 1981; Rausher, 1996, 2001). If the latter assertion is true, under what conditions did broad-spectrum defenses evolve? It is widely accepted that the morphological and chemical diversity of broad-spectrum plant defenses are a product of plant-enemy coevolution, but we still currently lack strong empirical evidence of diffuse or community selection in shaping those defenses.

Part of the challenge lies in selecting tractable model systems in which the plant and antagonist have clear, quantifiable reciprocal fitness impacts and whose traits can either be experimentally manipulated or exhibit natural variation across the landscape. The system we use in this study- chili peppers and their fruit-associated fungal pathogens- meet these criteria and provide an

exceptionally clear window on the coevolutionary dynamics leading to the major property of the most popular spice plant in the world: the spiciness of chilies. Previous work in this system has shown that fungal pathogens place strong selection on chilies by infecting seeds and lowering seed survival, providing a direct link to plant fitness. Fungal infection selects for the production of capsaicinoids, a constitutive, broadly-antimicrobial group of defensive chemicals produced only by plants in the genus *Capsicum* (Eshbaugh, 1980). Capsaicinoids are produced exclusively in the fruit and have been shown to inhibit fungal growth (Tewksbury et al., 2008). While most *Capsicum* species do not show intraspecific variation in pungency (Eshbaugh, 1980), several ancestral wild species exhibit a well-documented and geographically structured polymorphism in capsaicin production (Tewksbury et al., 2006). In *C. chacoense,* this variation corresponds to the strength of microbial selection pressure on *C. chacoense,* suggesting that capsaicin production has evolved to mediate microbial seed attack (Tewksbury et al., 2008). However, coevolution requires reciprocal adaptation, and to date most of the work in this system has focused on the evolutionary responses of the plant.

In this study, we take advantage of variation in capsaicin production to look for variation in the ability of the fungal pathogens to tolerate capsaicin. To do this, we isolated fungal pathogens from the fruits of wild chilies taken from populations that vary considerably in their production of capsaicin. We used all isolates obtained and did not attempt to limit our investigation to one fungal species; thus the fungi in our study represent a guild of non-specialized seed pathogens. This group of pathogens allows us to ask a largely unaddressed question in studies of coevolution: are community-level responses to an escalation in host defense possible? Specifically, we use fungal assays to quantify the growth rates of fungal isolates to address the

following 3 questions: 1) Does capsaicin inhibit the growth of all fungal fruit isolates? 2) Do isolates that originate from "hotter" environments grow faster in the presence of capsaicin than those from less "hot" environments? and 3) Is there a evidence that increased capsaicin tolerance comes with metabolic costs or trade offs? Answers to these questions shed light on the reciprocal evolutionary dynamics between fungal pathogens and their chili pepper hosts and the potential for a coevolutionary arms race.

Methods:

Study system

The fiery (pungent) flavor of chili peppers is caused by a group of phenolic amine compounds known collectively as capsaicinoids. These compounds are produced exclusively by plants in the genus *Capsicum* (Solanaceae), and are only found in fruits (Cordell & Araujo, 1993; Levey et al., 2007) where they remain present through development and maturity. Capsaicinoid production is hypothesized to be a monophyletic derived trait, and while most derived species of *Capsicum* exclusively produce pungent fruit (Eshbaugh, 1980), several basal species have been described as polymorphic for pungency- pungent and non-pungent plants coexist (Tewksbury et al., 2006, 2008; Haak et al., 2011). *Capsicum chacoense, C. eximium* and *C. baccatum*, are all polymorphic species native to the Chaco region of southeast Bolivia, Argentina and Paraguay. Of these species, *C. chacoense* has been studied most extensively (Tewksbury et al. 2008; Haak et al. 2011). Throughout their range in Bolivia, polymorphic populations of *C. chacoense* vary considerably in the proportion of plants that bear pungent fruit. This variation extends from the dry lowlands, where most chilies lack pungency (35% of plants are pungent), to moister, high elevation regions where all chilies are completely pungent (Tewksbury et al. 2006; Tewksbury et

al. 2008). In addition to this variation, the amount of capsaicin produced by pungent fruits positively co-varies with the proportion of fruits that are pungent (Fig 1.) The maintenance of this polymorphism has been attributed to differences in both biotic and abiotic selective forces across the landscape. Haak et al. (2011) showed that water stress selects for non-pungency; in drought conditions, pungent plants suffer a 50% reduction in seed set relative to non-pungent plants. Selection for pungency, on the other hand, is likely driven by fungal seed pathogens, which have been shown to be a significant source of seed mortality (Tewksbury et al., 2008). Capsaicinoids have documented antimicrobial properties (Billing & Sherman 1998; Molina-Torres et al. 1999; Kurita et al. 2002) and capsaicin production in wild plants has been connected to decreased attack of fruits and seeds by fungal pathogens (Tewksbury et al. 2008; Machnicki et al, in prep.).

Survey of wild C. chacoense populations

We surveyed 6 chili populations, distributed across a 1,600 km^2 area in southeastern Bolivia. To determine the proportion of pungent plants at each population, we classified each plant as pungent or non-pungent by tasting at least one ripe fruit per plant. Plants either produce capsaicin, resulting in pungent fruits, or they produce no capsaicin, resulting in fruits completely devoid of pungency, making the determination of pungency feasible in the field. Plant population pungency in each location was determined by finding the proportion of pungent plants observed over the total number of plants surveyed over multiple years between 2003-2008.

Fruit Collection

We collected fruits from 6 populations of wild *C. chacoense* in Southeastern Bolivia from 2006-2008 that varied in pungency from 34-100% plant pungency. We haphazardly collected mature fruits from plants, collecting from up to 10 pungent and 10 non-pungent plants per population when possible. Fruits were placed in labeled paper envelopes and stored in a cool, dry and well-ventilated environment prior to transport to the lab. Fruits dried in their envelopes during field storage due to the naturally low ambient moisture content of *C. chacoense* fruits. In addition, we collected fruits of two other *Capsicum* species that produce much higher quantities of capsaicin in pungent fruits, *C. eximium* (21.42 mg/g dry weight) and *C. baccatum* (37.52 mg/g dry weight).

Fungal isolation

In the lab, we stored dry fruits in paper envelopes at 6° C prior to isolation. We isolated fungi from fruits by surface sterilizing and placing them on agar plates containing half-strength potato dextrose agar (PDA) with a 0.2% solution of the antibiotics tetracycline, ampicillin, and streptomycin. Since fruits were dry at the time of isolation, they quickly absorbed any solutions we placed them in for purposes of surface sterilization. To prevent total saturation of the fruits, we used sterile forceps to dip whole fruits into a 70% ethanol solution for 2 seconds, followed by a 2 second dip into a 30% bleach solution. After wetting the fruits with the bleach solution, we allowed the bleach-covered fruits to sit for 2 minutes in an empty sterile Petri dish, followed by a triple rinse with sterile DI water. We used sterile scalpels to cut the fruits in half prior to transferring them to PDA plates. We incubated fruits in the dark at 25° C and checked them daily for fungal growth for up to two months. We subcultured all isolates from fruits onto new PDA plates. We stored pure cultures on slants and refrigerated them at 6° C until we performed

growth rate assays. For *C. chacoense,* we isolated and assayed growth rates for 120 isolates; 65 from pungent plants and 55 from non-pungent plants.

For between species comparisons, we obtained isolates from *C. eximium* and *C. baccatum,* using fruits from a single population for each species. *C. chacoense* isolates used in between species comparisons came from the San Julian population, where 39% of the plants are pungent and the average fruit pungency is 2.85 mg/g dry weight capsaicin. We obtained 6 isolates per species, 3 pungent and 3 non-pungent. These were isolated and stored using the same methods described above.

Fungal growth assays

We assayed fungal growth rates of isolates obtained from *C. chacoense* populations on two treatments: media with capsaicin (0.5 mg/g dry weight) and a capsaicin-free control. Our previous laboratory work has shown that even small amounts of capsaicin have pronounced effects on fungal growth and 0.5 mg/g capsaicin is an ecologically-relevant level of pungency for *C. chacoense* (Tewksbury et al 2008). We used an agar-based false fruit media (FFM) that mimicked the nutritional content of *Capsicum* fruits (Tewksbury et al., 2008). We added capsaicin (Sigma, M2028, ≥95%) to sterilized media by dissolving it in 1 ml methanol; 1 ml methanol was added to the 0 mg/g treatment as a control. After media was thoroughly homogenized, we dispensed 5 ml of FFM into individual 60 cm petri dishes. Before assays began, we grew each isolate on PDA for approximately 1 week. To inoculate assay plates, we used a sterilized 4 mm diameter cork borer to cut plugs of standard size from the growing edge of each isolate and transferred each to the center of an assay plate. Isolates were replicated

between 1-3 times on each treatment. After inoculation, plates were kept in the dark, incubated at 25° C, and rotated daily within the incubator. With the use of dissecting microscopes, we measured 2 perpendicular colony diameters (originating at a random starting point) 48 hours after inoculation. Measurements were blind with respect to isolate origin and capsaicin treatment. We found growth rates by using the mean of the two colony diameters for each isolate, and calculated rate of growth as mean mm h^{-1} growth.

To further test the hypothesis that isolates originating from more pungent environments incur metabolic costs, we performed an additional set of growth assays on capsaicin-free media with an independent set of fungal strains isolated from the fruits of *C. chacoense, C. eximium, and C. baccatum.* For each species, we assayed 6 isolates, 3 from pungent fruits and 3 from non-pungent fruits. Each isolate was replicated on PDA 6 times and measured as described in the methods above.

Data analyses

We used linear mixed-effects models (LMERs) to characterize the effects of treatment (control or capsaicin media), population pungency (the proportion of pungent plants in a population) and individual fruit pungency on fungal growth rates. We used a normal error distribution and included isolate ID as a random effect in all analyses to account for repeated measures (replicates) of the same isolate within each treatment. To clarify any overall differences between isolates from different fruit types, we also modeled growth rates for isolates derived from pungent or non-pungent fruits separately and examined only the effects of treatment and

population pungency (and their interactions) on these groups. For between species analyses, we used the same analyses as described above, but excluded treatment as a factor in our models because assays were performed only on capsaicin-free media.

For all analyses, we identified the best subset of models using Akaike's Information Criterion (AIC_c) and either reported all models (for analyses with fewer than 4 total models) or reported top models with a delta AIC_c ($\Delta_{i=}$ AIC_i - AIC_{min}) of less than 3. Rather than using likelihood ratio tests (LRT) with their associated *p*-values to identify only one top model with significant main effects, we performed model averaging of coefficients of our top models to acknowledge model-selection uncertainty (Burnham & Anderson, 2002). In all analyses, LRT and model averaging methodologies both revealed the same best-fit model. For models included in model averaging, we reported Akaike weights (w_i), which represent the likelihood that a given model is the best of the models considered (Burnham & Anderson, 2002) as well as their regression coefficients (β) with 95% confidence intervals. We also reported the relative variable importance (w_{ip}), or the sum of w_i across all candidate models in which the variable appears (Burnham & Anderson 2002). We conducted all analyses in R (R Development Core Team, 2011) using packages lme4 (http://r-forge.r-project.org/projects/lme4/) and MuMIn (http://r-forge.r-project.org/projects/mumin/).

Results:

The addition of capsaicin to the media slowed growth rates for all fungal isolates considerably (Fig. 2; Table 2, treatment main effect), regardless of their plant or population origin. All top-ranked models included treatment as an important factor (Table 2). However, the top-ranked

models also included population pungency and the interaction between treatment and population (Table 2). While the population origin of fungal isolates did not have an impact on growth rates in the capsaicin treatment, on capsaicin-free media, isolates that came from populations with a higher proportion of pungent plants grew slower than those from low pungency populations (Fig. 2). There was weaker support for including plant pungency (whether isolates originated in pungent or non-pungent fruits) in our models. Fruit pungency was included all but the top ranked model; those that included fruit pungency had about half the support of the best model (Table 2; $w_i = 0.42$, $w_i \leq 0.25$, respectively).

To clarify the importance of fruit pungency, we analyzed the growth rates of isolates from pungent and non-pungent fruits independently. We found that isolates from pungent and non-pungent fruits behaved differently in the presence of capsaicin. Isolates from non-pungent plants grew fastest when they came from populations with a low proportion of plant pungency, while the reverse was true for isolates that originated in pungent fruit. Isolates from pungent fruits grew fastest when they came from populations where most of the plants were pungent (Fig. 3). Our models supported these results; the addition of population pungency strongly improved model fit in isolates originating in pungent fruit, but not in non-pungent fruit (Table 3). In addition, the model that included a treatment by pungency interaction term had much higher support (Table 3, $w_i = 0.99$) than any other model (Table 3; treatment x population pungency; LRT $p = 0.0001$).

On capsaicin-free media, fungal growth rates were slowest when isolates came from populations with the highest proportions of pungent plants (Fig. 3, Fig. 4a). This suggests that originating in high pungency environments comes with metabolic costs: isolates are less competitive in a

capsaicin-free environment. We tested this idea with an independent set of isolates originating from three different *Capsicum* species that vary considerably in the intensity of capsaicin production (2.85-37.50 mg/g capsaicin). We found the same relationship; isolates that came from less-pungent species grew faster on capsaicin-free media then isolates from more pungent species (Fig. 4b).

Discussion:

Impacts of capsaicin on fungal growth

Capsaicin had clear negative impact on fungal fruit inhabitants, and slowed the growth of isolates originating from all *Capsicum* populations and fruit types (Fig. 2). This finding is consistent with previous work on bacteria and *Saccharomyces cerevisiae* that demonstrated broad antimicrobial properties of capsaicin (Cichewicz & Thorpe, 1996; Molina-Torres et al., 1999; Dorantes L. et al., 2000). The effect of capsaicin on filamentous fungi has received comparatively little attention, although one study (Xing et al., 2006) found that capsaicin effectively slowed (but did not stop) the growth of two fungal species associated with fruit storage rot.

Tewksbury et al. (2008) were the first to study the effect of capsaicin on fungi in an ecologically relevant context. They showed that pungent fruits had lower visible fungal seed damage than non-pungent fruits, and assays of fungal growth in capsaicin media verified that capsaicin slowed the growth of *Fusarium* isolated from a limited number of seeds. However in natural systems, *Capsicums,* like most plants, interact with a diverse community of fungal antagonists (see Chapter 3) which likely exhibit variation in their sensitivity to capsaicin. Studies that test the

effect of plant chemical defenses against multiple enemies are rare, even though this is the most likely scenario for the evolution of broadly effective plant chemistry. The present study addresses this gap and is the first to test the effect of defensive host chemistry on fungal diversity representative of the diversity found under natural conditions.

While the exact mechanisms that underlie capsaicin's inhibitory effects on filamentous fungi are still uncertain, previous studies indicate that it may potentially impact multiple cellular processes. Capsaicin was shown to induce expression in 39 of approximately 6,000 genes of *Saccharomyces cerevisiae* that varied considerably in function (Kurita et al., 2002). Capsaicin has also been shown to inhibit two key enzymes in the mitochondrial respiratory chain of several bacterial species, and partially inhibit those of *Saccharomyces cerevisiae,* which lack one of the enzymes (Xing et al., 2006). In addition, capsaicin has been shown to change the fluidity of bacterial cellular membranes and interfere with membrane protein function (Tsuchiya, 2001).

Fungal tolerance of capsaicin

In the present study, we show that fungal isolates that originated in populations with more pungent plants were less slowed by capsaicin, but only when they came from pungent fruits (Fig. 3). Growth rates in fungi are directly related to their competitive ability within a fruit; those that grow fastest are most likely to establish themselves on a limited resource (seeds within a fruit), as well as increase their probability of transmission to a new fruit via wind or insect-dispersed propagules. Our results are notable for several reasons. First, we demonstrate for the first time that variation in the ability of fungi to grow in the presence of capsaicin exists, and that this variation appears to be driven by capsaicin production in their "home" environment. Capsaicin

production in a "home" environment can be characterized in two ways: 1) by the frequency of capsaicin-producing plants in a population and 2) by the pungency of an individual fruit. In addition, these factors also co-vary; as the frequency of pungency increases in a population, the amount of capsaicin produced by pungent fruits also increases. The differences in capsaicin tolerance we observed between fruit types suggest that selection at the fruit level may be the primary factor driving fungal adaptation to capsaicin in this system- arguably more important than population pungency. If by plant population pungency drives selection, we would expect to find very similar fungal growth rates in pungent and non-pungent fruits from the same populations. However, fruit pungency alone is unlikely to account for increased capsaicin tolerance as population pungencies increase, suggesting that both factors play a role in shaping fungal adaptation to capsaicin. The frequency of pungent plants in a population is relevant because as it increases, the probability that a pathogen will encounter a pungent fruit increases.

Second, we show that it is possible to detect evidence of fungal adaptation to plant defensive chemistry even in a multispecies assemblage of pathogens. In this study, we did not focus on variation in one or two specific fungal species and instead took a functional, community-based approach by using all fungi that we were able to isolate from fruit. While this approach has the advantage of more closely reflecting the actual diversity of fungal players in the field, the occurrence of high interspecies variation could potentially obscure patterns of variation in fungal growth among fruits or populations. Remarkably, patterns of tolerance were apparent even among presumably unrelated fungal isolates, suggesting that while capsaicin places strong selective pressures on multispecies assemblages of fungal antagonists, this functional guild of

fungi has the potential to reciprocally adapt in what can be best described as "diffuse" co-evolution.

How might selection above the species level operate? One possibility is that the composition of fungal community in the environment is relatively consistent across the landscape, and selection at the level of the fruit "filters" a subset of the community that is able to tolerate capsaicin. Essentially, capsaicin may narrow the niche space available to most pathogens, resulting in a smaller assemblage of species that might be considered capsaicin specialists. If this were true, we would expect the number of species found in pungent fruits to be lower, and potentially different than those found in non-pungent fruits. In addition, we would also expect that as the proportion of pungent plants increased across populations, the community of fungi present in all fruits would decrease to reflect more capsaicin specialists. Alternatively, fungal community composition in fruits may mirror the community in the environment, but variation in fungal tolerance to capsaicin is large enough within fungal species that only the most tolerant genotypes survive in pungent fruit. This would result in a similar species composition in pungent and non-pungent fruit, and across populations, but within a single fungal species, those from pungent fruit or more pungent locations would be predicted to have higher capsaicin tolerance. Of course, these scenarios are not mutually exclusive, and future work is needed to distinguish between these possibilities. We are currently working to identify isolates and characterize fungal community structure in an effort to better understand the selection dynamics at play.

The cost of capsaicin tolerance: between populations and between species

If tolerance to capsaicin had no metabolic costs, we would expect that its effectiveness would diminish (and perhaps cease) over time as capsaicin resistance spread through fungal populations. While we confirmed in the present study that capsaicin has indeed not lost its ability to slow fungal growth (Fig. 2), we also uncovered evidence of metabolic costs in isolates from populations where pungency is more common (Fig. 4a) and where fruits produce more capsaicin (Fig. 4b). Within *C. chacoense,* isolates from populations with the most pungent fruits grew slowest (Fig. 4a). While these isolates have a competitive advantage when capsaicin is present, their comparatively slow growth in the absence of capsaicin may result in their being outcompeted by less tolerant, but faster growing isolates and counteracting selection for pungency. The proportion of plants that are pungent in a population represents the probability that fungi will end up in pungent fruit. In populations where most of the plants lack capsaicin, capsaicin tolerance could be a net disadvantage if competition within fruits is limiting.

While the amount of capsaicin produced by pungent plants across *C. chacoense* populations varies, the degree of variation is small when compared to closely related *Capsicum* species. To see if the amount of capsaicin in a fruit had an impact on fungal isolates, we compared the growth rates of isolates taken from three species of *Capsicum* that varied considerably in capsaicin production. We show that even across *Capsicum* species, isolates from the most intensely pungent fruits grow the slowest in the absence of capsaicin (Fig 4b). Overall, while further experimentation (within-fruit isolate competition) is necessary to test some of our assumptions about competition empirically, the apparent existence of functional trade-off for capsaicin tolerance may explain why the effectiveness of capsaicin remains relatively high.

Conclusions

We demonstrated, in a multispecies assemblage of fruit-infecting fungi, that capsaicin tolerance was highest in isolates that originated in populations that were dominated by pungent plants. In addition, we showed that there were constraints associated with tolerance to capsaicin; isolates that were the most tolerant grew the slowest in the absence of capsaicin. This pattern was also apparent in isolates taken from three different *Capsicum* species that vary considerably in amount of capsaicin they produce. Our results suggest that fruit chemistry selects for capsaicin tolerance in a diffuse guild of fungi, but tradeoffs exist that likely limit the competitive ability of tolerant isolates in low-capsaicin environments. Fungal tolerance for capsaicin also likely drives selection for increased capsaicin production by plants, creating the potential for a coevolutionary arms race.

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Figures:

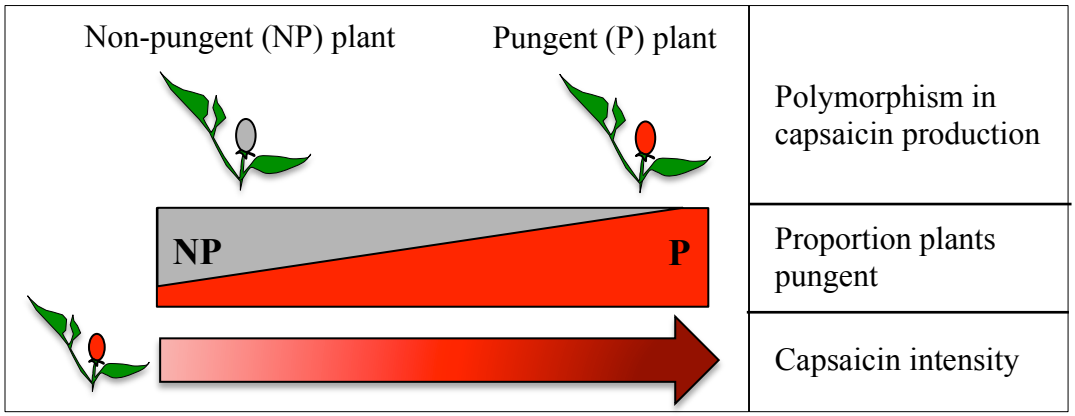

Figure 1. Schematic of the polymorphism in capsaicin production and the positive relationship between the proportion of pungent plants in a population and the capsaicin intensity (production of capsacin in mg/g) of pungent plants.

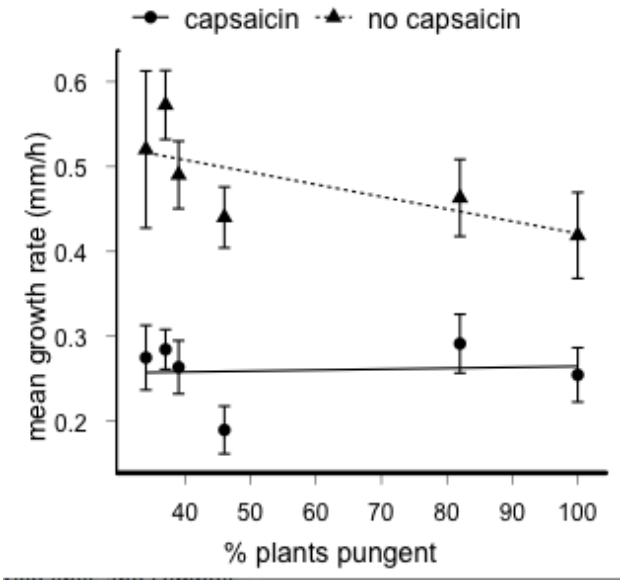

Figure 2. The impact of capsaicin on fungal growth rates. Mean growth rates (mm/h) of isolates originating from *C. chacoense* populations that vary in the proportion of plants that are pungent. Circles and the solid line represent growth rates in media with no capsaicin; triangles and the dotted line represent growth in the presence of capsaicin. Error bars are ±1 SE . LMER, treatment × population pungency interaction.

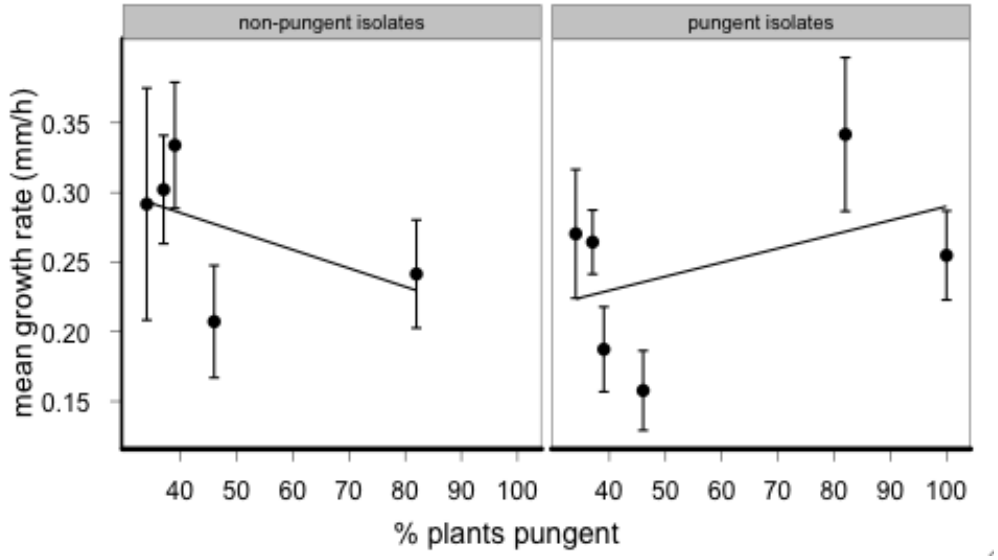

Figure 3. Mean growth rate of isolates originating from pungent and non-pungent *C. chacoense* plants in populations that vary in the proportion of plants that are pungent. Fungal assays were performed on media containing capsaicin. Error bars are ±1 SE .

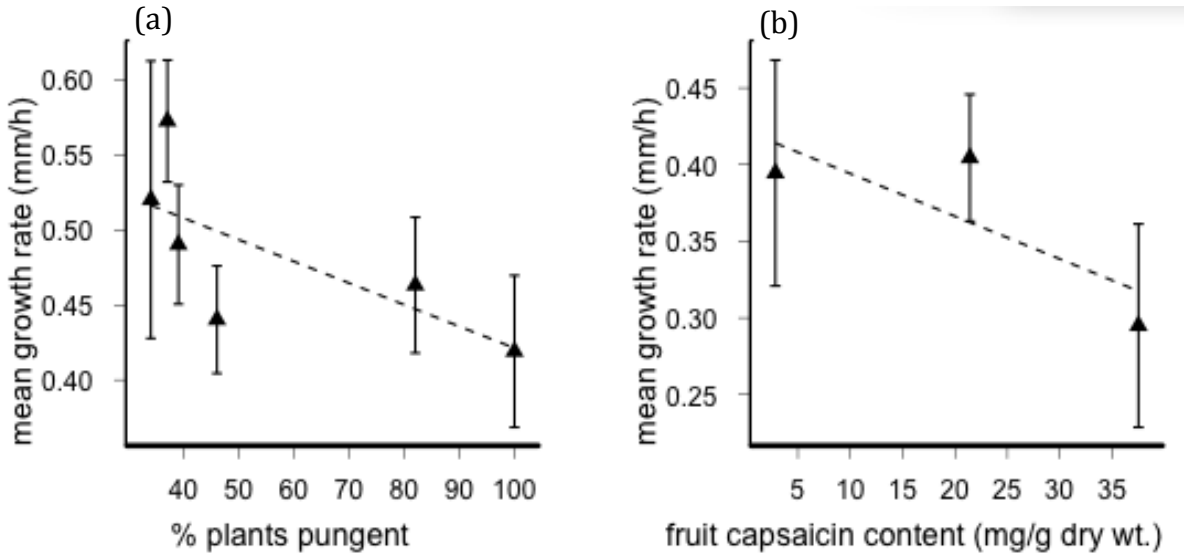

Figure 4. Mean growth rates (mm/h) on capsaicin-free media of isolates from (a) *C. chacoense* populations that vary in the proportion of plants that are pungent and, (b) from three species of *Capsicum* (*C. chacoense, C. eximium, and C. baccatum,* respectively*)* that vary in the intensity of capsaicin production. Mean capsaicin content of fruits (mg/g dry weight) was determined by HPLC.

Tables:

Table 1. The proportion of pungent (capsaicin-producing) plants in each population and the number of independent isolates used in experimental assays from each plant type.

Population name	Proportion of plants pungent	# of isolates used from P plants	# of isolates used from NP plants
Pipeline	34%	7	2
Ibasiriri	37%	13	11
San Julian	39%	11	12
Yuquiti	46%	7	13
River ranch	82%	12	12
Tres Aguadas	100%	12	0

Table 2. Results of model selection and model averaging for linear mixed-effects models (LMER) of mean fungal growth (mm/hr). We included treatment (capsaicin or control media), the population pungency, the pungency of individual fruits and their interactions as fixed factors. We included isolate in all models as a random effect to account for repeated measures (replicates) within isolate. Tables include all models where the AIC_c of the best model (Δ_i) is ≤ 3. Variables included in each model are indicated with a • symbol. Model rank order is determined by Δ_i. Akaike weights (w_i) are the likelihood that a given model is the best of the models considered. Regression coefficients (β) represent averages of β_i values across considered models, weighted by the w_i of each model. Relative variable importance (w_{ip}) is the sum of w_i across all candidate models in which the variable appears (Burnham & Anderson 2002). Only variables included in the considered models are listed, they are shown in decreasing order of w_{ip}.

Growth rate	**Model rank**				**Model average**			
Variables	**1**	**2**	**3**	**4**	**β**	**95% CI**		w_{ip}
treatment	•	•	•	•	**-0.256**	**-0.292**	**-0.219**	1.00
population pungency	•	•	•	•	-0.002	-0.004	0.001	1.00
treatment × pop pung.	•	•	•	•	**0.001**	**0.001**	**0.002**	1.00
plant pungency		•	•	•	-0.055	-0.214	0.103	0.58
pop pung. × plant pung.		•		•	0.001	-0.002	0.003	0.35
treatment × plant pung.				•	-0.001	-0.012	0.010	0.10
Δ_i	0	1.07	1.25	2.83				
w_i	0.42	0.25	0.23	0.10				

Table 3. Results of model selection and model averaging for linear mixed-effects models (LMER) of mean fungal growth rates (mm/hr) in pungent and non-pungent fruits. We included treatment (capsaicin or control media), the population pungency and their interactions as fixed factors. We included isolate in all models as a random effect to account for repeated measures (replicates) within isolate. Table organization as explained in Table 1. 95% confidence intervals of β that do not cross zero are indicated in bold.

Growth rate: pungent	**Model rank**			**Model average**			
Variables	**1**	**2**	**3**	**β**	**95% CI**		w_{ip}
treatment	•	•	•	**-0.275**	**-0.328**	**-0.222**	1.00
population pungency	•		•	-0.001	-0.002	0.001	0.99
treatment × population pungency	•			**0.001**	**0.001**	**0.002**	0.99
Δ_i	0	10.09	12.15				
w_i	0.99	0.01	0				
Growth rate: non-pungent	**Model rank**			**Model average**			
Variables	**1**	**2**	**3**	**β**	**95% CI**		w_{ip}
treatment	•	•	•	**-0.207**	**-0.259**	**-0.155**	1.00
population pungency	•	•		-0.002	-0.004	0.001	0.70
treatment × population pungency		•		0.001	-0.001	0.001	0.31
Δ_i	0	0.48	0.58				
w_i	0.39	0.31	0.30				

CHAPTER 3

Title: Fruit defensive chemistry shapes the diversity and community structure of fungi associated with the seeds of wild chili peppers

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Key words: Fungal diversity; Ascomycota; ITS; *Phomopsis; Fusarium;* capsaicin; defensive chemistry; *Capsicum;* seed pathogens

Abstract: Fungal fruit and seed infections are an important cause of seed mortality, contributing to recruitment limitation that shapes the diversity and composition of plant communities. It has been proposed that noxious or toxic compounds present in fruits function to protect seeds against microbial seed predators. However, the impact of defensive chemistry on the incidence, diversity and community structure of seed-infecting fungi has rarely been investigated. We used a polymorphism for capsaicin production in a wild chili species (*Capsicum chacoense*) to study the impact of fruit chemistry on fungal seed infection, seed survival, and fungal diversity. We combine a broad geographic survey of wild chili populations, a fruit infection experiment, and the use molecular tools to characterize the fungal community of wild chili seeds. We found that seed infection had significant negative effects on seed survival and fungal infection rates increased when hemipteran insects damaged fruit. Capsaicin production in fruits did not decrease the probability of infection, but once infected, seeds from some pungent fruits were more likely to survive. Fruit and seed fungi comprise a diverse community at the genus level, but are dominated by isolates identified as *Phomposis* and *Fusarium*. We report that multiple factors-field site, the activity of insect vectors, and the production of capsaicin, all contribute significantly to shaping the diversity and community structure of fungal fruit and seed inhabitants. We show that the presence of defensive chemistry in fruits has a positive effect on fungal diversity, and suggest that plant secondary chemistry may play a central role in determining the diversity and composition of fungal communities that inhabit plants.

Introduction:

Selection by microbial seed predators has been frequently cited as an explanation for the presence of toxic or noxious compounds in fruit (Janzen, 1977; Cipolini, Martin L. & Stiles, Edmund W., 1993; Cazetta et al., 2008). The nutritional qualities that attract dispersers to ripe fruits can also attract consumers that consume seeds and do not contribute to seed dispersal (Janzen, 1971; Herrera, 1982). While many studies have focused the role of fruit chemistry in mediating interactions with vertebrate and insect fruit and seed consumers (Herrera, 1982; Zangerl & Berenbaum, 1997; Eriksson & Ehrlén, 1998; Tewksbury & Nabhan, 2001; Levey et al., 2006), few studies have included fungal fruit consumers in their consideration of fruit-frugivore interactions (Cipolini, Martin L. & Stiles, Edmund W., 1993; Biere et al., 2004). Microbial consumers that attack fruits and seeds have a direct negative impact on plant fitness, and create a selective advantage for plants that develop strategies to resist fruit and seed rot without deterring legitimate dispersers (Cipollini & Levey, 1997). Plant investment in defensive chemistry is costly and often results in tradeoffs (Zangerl & Berenbaum, 1997; Haak et al., 2011), thus their production suggests that potential costs are outweighed by the benefit of seed protection. Fungal seed consumers are a ubiquitous and understudied source of seed loss that may drive the production of defensive chemistry in fruits and seeds.

The ecological implications of seed predation by mammals and insects has been studied extensively (Janzen, 1971; Louda, 1982; Schupp, 1988; Silman et al., 2003), however, even basic information about the contribution of fungi to seed losses in natural systems is sparse (Gilbert, 2002). The diversity and impact of seed fungi in soil seed banks has been studied in a few

species (Gallery et al., 2007; U'ren et al., 2009) and suggests that fungal communities are diverse and often have significant, but variable impacts on seed losses in plants. While the impacts of fruit defensive chemistry on fungal seed predators have been the subject of a few studies (Cipollini & Stiles, 1993; J. Tewksbury et al., 2008), to our knowledge no studies have examined the diversity of fungal communities that attack seeds prior to dispersal or contact with the soil. In addition, we lack basic information on how host plant defensive chemistry impacts the diversity and community structure of fungal inhabitants. A few studies of foliar endophytic fungi have shown that fungal sensitivity to plant chemistry varies and may determine the host specificity and diversity of endophytic fungal communities (Arnold et al., 2000; Saunders & Kohn, 2009). Variation in tolerance to plant chemistry among fungal taxa is likely to impact the competitive dynamics and diversity of fruit and seed fungi, yet we currently lack an explicit framework for predicting how shifts in host chemistry impact host fungal diversity. Host chemistry imposes stress on fungal inhabitants, which may decrease diversity and limit fungal colonization to a reduced subset of tolerant taxa. On the other hand, the roles that environmental stress, disturbance, and heterogeneity play in decreasing competitive exclusion and increasing diversity are well-documented and central ideas in ecology (Simpson, 1949; MacArthur & Wilson, 1967; Ricklefs, 1977). Thus, increased stress imposed by plant chemistry may have positive effects on fungal diversity. Are general ecological principles that operate on landscape or habitat scales applicable to the fungi that inhabit plants? To address this question, basic information on the identity and diversity of fungal inhabitants is needed.

In this study, we used molecular methods to characterize the fungal community of fruits and seeds in a plant famous for the production of fruit chemistry: the wild chili pepper. We used the

nuclear ribosomal internal transcribed spacer region (ITS) to identify fungal isolates. ITS is frequently used as a "barcoding" locus in fungi due to the ease of its amplification and its high representation in large databases such as GenBank (Seifert, 2009). However, there are difficulties associated with the use of ITS; sequences can be difficult to align across diverse taxa, especially for those that currently lack strong phylogentic species concepts (Vandenkoornhuyse et al., 2002; Arnold, 2007) and species identification with ITS can be problematic for some groups, especially those that are underrepresented in the GenBank database. To avoid misapplying species names, some studies of fungal diversity have instead grouped species into operational taxonomic units (OTUs) based on shared sequence similarity (Arnold et al., 2000; Gallery et al., 2007). While this methodology has provided good estimates of overall diversity, the phylogenetic placement of fungi is often underemphasized. In this study, we used conservative estimates of diversity and identified seed fungi to genus, thus avoiding some of the problems associated with ITS species identification while still gaining information about the taxonomic placement and relative diversity of isolates.

We combine a broad geographic survey of wild chili populations, field experimentation, and the use molecular tools to broadly characterize the fungal community of wild chili seeds. We focus on *Capsicum chacoense,* a basal chili species native to the semi-arid Chaco region of Bolivia and Argentina that produces both pungent (spicy) and non-pungent fruits. Specifically we ask the following questions: How diverse are communities of fungi that infect the fruits and seeds of wild chilies? Are these fungi responsible for seed mortality? How does fruit pungency impact the diversity and community structure of fruit-inhabiting fungi? At what spatial scales are fungal communities structured? To address this last question, we examine fungal community structure

at multiple scales: across chili populations, across two field sites with local and non-local plants, and across fruit pungency. Finally, we focus on the presumptive insect vectors of seed fungi and ask: are specific fungal taxa associated with fruit damage caused by foraging insects?

Methods:

Study system

The wild chili pepper, *Capsicum chacoense,* Hunz., is a solanaceous perennial plant native to the Chaco region of southeast Bolivia, Argentina and Paraguay. It produces red fleshy fruit that is approximately 10 mm in length and 5.5 mm in width and contains an average of 17 seeds (Tewksbury et al., 2008). Birds are the primary seed dispersers and typically eat fruits whole. *C. chacoense* produces a family of chemicals called capsaicinoids in fruits, most of which (> 90%) are in the form of capsaicin and dihydrocapsaicin. Capsaicinoids are produced exclusively in fruit and are responsible for the fiery (pungent) taste of chili peppers. These compounds remain present in the fruit through development and maturity (Prasad et al., 2006), and have documented antimicrobial properties (Billing & Sherman, 1998; Molina-Torres et al., 1999; Kurita et al., 2002). While most *Capsicum* species do not show intraspecific variation in pungency (Eshbaugh, 1980), *C. chacoense* is polymorphic for capsaicinoid production (Tewksbury et al., 2008; Haak et al., 2011). Two distinct, and genetically determined (Blum et al., 2002) chemical phenotypes coexist: plants with pungent (capsaicin-producing) fruits occur alongside plants whose fruits completely lack pungency. Across our 300 km^2 study area in Bolivia, polymorphic populations of *C. chacoense* vary considerably in the proportion of plants that bear pungent fruit (34%-100%). This variation extends from the dry lowlands in the northeast, where most chilies lack

pungency, to moister, high elevation regions in the southwestern end of our transect where all chilies are completely pungent (Tewksbury et al., 2006; Tewksbury et al., 2008). The polymorphism in capsaicin production and the geographic gradient in "population-level" pungency in *C. chacoense* provides a unique opportunity to explicitly test the impact of capsaicin on the community structure and diversity of seed fungi.

Survey and fruit collection in natural C. chacoense populations

We surveyed wild chili populations distributed across a 300 km^2 area in southeastern Bolivia to determine how the polymorphism in capsaicin production varies across the landscape. To determine the proportion of pungent plants at each population, we classified each plant in a population as pungent or non-pungent by tasting at least one fruit per plant. Non-pungent plants completely lack any capsaicin and are easy to distinguish from pungent plants in the field. We determined plant population pungency in each location by recording the proportion of pungent plants observed over the total number of plants surveyed between 2003-2008. Between 2006-2008, we collected fruits from 6 populations that captured the natural variation present in plant population pungency. At each location, we randomly selected mature fruits from plants, collecting from up to 10 pungent and 10 non-pungent plants per population when possible. Fruits were placed in labeled paper envelopes and stored in a cool, dry and well-ventilated environment prior to transport to the lab. Fruits dried in their envelopes during field storage due to the naturally low ambient moisture content of *C. chacoense* fruits.

Fungal isolation from natural populations

In the lab, we stored dry fruits in paper envelopes at 6° C prior to isolation. We isolated fungi by surface sterilizing and placing fruits on agar plates containing half-strength potato dextrose agar (PDA) with a 0.2% solution of the antibiotics tetracycline, ampicillin, and streptomycin. Since fruits were dry at the time of isolation, they quickly absorbed any solutions we placed them in for purposes of surface sterilization. To prevent total saturation of the fruits, we used sterile forceps to dip whole fruits into a 70% ethanol solution for 2 seconds, followed by a 2 second dip into a 30% bleach solution. After wetting the fruits with the bleach solution, we allowed the bleach-covered fruits to sit for 2 minutes in an empty sterile Petri dish, followed by a triple rinse with sterile DI water. We used sterile scalpels to cut the fruits in half prior to transferring them to PDA plates. We incubated fruits in the dark at 25° C and checked them daily for fungal growth for up to two months. We subcultured isolates until axenic, transferred them onto agar slants for storage, and refrigerated them at 6° C.

Fruit infection field experiment

We conducted a field experiment to investigate the impact of environment, plant pungency, and insect damage on fungal community composition in more detail. We focused our experiment on two sites: a wet site and a dry site. At our wet site (21.520°S, 63.781°W), where all plants are pungent, insect pressure on chili plants is high, with an average of 11 punctures per fruit, and relatively high average rainfall (about 950 mm / year). At our dry site, (20.018°S. 63.100°W) insect pest pressure is much less extreme, with < 1.5 punctures per fruit, substantially lower annual rainfall (about 650 mm / year), and only 34% of the *C. chacoense* are pungent (J. Tewksbury et al., 2008). Study sites were located about 180 kilometers apart in the Gran Chaco

region, Santa Cruz, Bolivia. We used only non-pungent plants from the dry site and only pungent plants from the wet site. To investigate the effects of field site, fruit pungency, and fruit damage (and their interactions) on fungal community composition, we conducted fruit wounding experiments at both sites in common gardens that contained both plant types. The most common insect seed predator at both our sites was *Acroleucus coxalis* (Stäl) (Lygaeidae), a hemipteran seed bug. *A. coxalis* targets the endosperm of seeds by using its stylus to forage, repeatedly puncturing the skin of the fruit. In natural populations, puncture sites frequently develop signs of fungal infection, thus we focused on *Acroleucus* as a potential fungal vector in our experiment. Fruits on experimental plants were assigned to one of three infection treatments: "insect," "needle," or an un-manipulated control. For the insect treatment, we placed a locally caught adult instar of *A. coxalis* in a mesh bag with a fruit for 7 days, where they were allowed to feed naturally by puncturing fruits. For the needle treatment, we punctured fruits with a sterile needle. To control for the size of the holes we created, we measured the diameter of the stylus of *Acroleucus*, and used sterile acupuncture needles of equal width (Sierin, JT.12x15; 0.12mm) to wound fruit. We used these needles to introduce 5 holes into each fruit (average number of holes left by bagged insects in a pilot study, unpublished data) before covering the fruit with a mesh bag. Control fruits were not damaged, but they were handled and covered with a mesh bag to exclude insect damage. We applied one treatment per fruit and each plant had a balanced set of treatments. During setup, we wore latex gloves and surface sterilized them with 70% isopropanol to prevent cross contamination between fruits. After one week, we collected and individually placed each fruit in a sealed paper envelope. We stored fruits in a cool, dry, and well-ventilated environment prior to transport to the lab.

Seed infection and germination study

To quantify infection in seeds from the field experiment and investigate the impact of infection on seed germination and seedling survival, we initiated seed germination in the lab, checked seeds for fungal infection, and followed their survival to seedling stage. We used sterile technique remove a subset of six seeds from each fruit. We assigned each seed an identification number and placed them in Petri dishes on sterile cellulose pads. We watered seeds with 1.5 ml sterile water every 3 days to keep them evenly moist. We incubated dishes in an environmental chamber that mimicked field temperature and light conditions: dishes were kept on a daily 11 hour light/13 hour dark cycle at 33 C°/22 C° (2 hour ramp) and were rotated daily. After 7 days, we used a dissecting microscope to inspect and score each seed for the presence or absence of filamentous fungal growth. All scoring was blind; observers had no knowledge of seed source, treatment, or fruit pungency. We replaced seeds in the chamber after scoring and allowed them to incubate until germination. Any seeds that germinated and had exposed cotyledons were transplanted into 72-well seed trays filled with Sunshine soil mix. We transferred germinated seeds with the seed coat attached and handled all seeds with sterile forceps. We kept seedlings in the environmental chamber for 20 additional days and checked tracked their survival at the seedling stage. We tracked germination for 6 weeks total, seeds that had still not germinated after this point were considered non-viable.

Fungal isolation from experimental seeds

To characterize the fungi responsible for killing seeds from the field experiment, we isolated fungi seeds that did not survive the germination. While infections among multiple seeds per fruit were common, fungal morphology suggested that within fruit diversity was very low. In almost

all cases, the seeds appeared to be infected by the same fungus. As a result, we isolated one seed per fruit. We used sterile forceps to transfer each seed to an agar plate with half strength PDA containing a 0.2% solution of the antibiotics tetracycline, ampicillin, and streptomycin. We incubated seeds in the dark at 25° C and checked them daily for fungal growth. We subcultured isolates until axenic, transferred them onto agar slants for storage, and refrigerated them at 6° C.

DNA extraction and sequencing

We grew isolates in liquid culture prior to DNA extraction. We transferred a small amount of mycelia to 15 mL falcon tubes filled with 10 mL of potato dextrose broth and incubated the sealed tubes in a dark shaker at 25 ° C for approximately 1 week. We added 0.5 mL of mycelia to 1.5 mL microcentrifuge tubes containing 0.5 mL of lysis buffer and briefly vortexed tubes before initiating a freeze/thaw protocol to break open fungal cells. We placed tubes in a dry ice/ethanol solution for 30 seconds, moved them to a 25 ° C water bath for 30 seconds, followed by a 65° C water bath for 3 minutes. We repeated this twice, but on the second round we held tubes in the 65° C bath for 20 minutes. We then centrifuged tubes at 14,000 rpm for 10 minutes before transferring 400 μL of the supernatant to new microcentrifuge tubes. We added 240 μL of polyethylene glycol (PEG) to each tube and gently inverted them to mix. We centrifuged tubes again for 5 minutes at 10,000 rpm to pellet the DNA. We discarded the supernatant and let the tubes drip dry before adding 450 μL of 10mMTris pH8 buffer to each. We resuspended the DNA by briefly vortexing and then added 50 μL of 0.5 M NaCl and 1 mL 95% EtOH to each tube. We allowed them to sit for 20 minutes at room temperature before centrifuging for 5 minutes at 10,000 rpm to pellet DNA. We again discarded the supernatant and let tubes completely air dry before resuspending DNA in μL of 10mM Tris buffer pH 8.

We amplified the ITS1–5.8s-ITS2 region of nuclear ribosomal RNA with 1µl each of 25µM ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) primers in a 50µl reaction mixture consisting of 1-3 µl DNA template, 5µl of 10x PCR reaction buffer, 1µl of 10mM dNTP, 2.5µl (2.5 units) ChoiceTaq Blue™ DNA Polymerase and water to volume. We set up all reactions on ice. We initiated PCRs with an initial 2 minutes denaturation at 94°C, followed by 30 cycles of 1 minute denaturation at 94°C, 1 minute annealing at 62°C, and 1.5 minute extension at 72°C. The final extension was 10 minutes at 72°C. To confirm a single product, amplified DNA fragments were visualized in a 1.0% agarose gel stained with ethidium bromide. We cleaned PCR products using an enzyme mixture consisting of 1 part (1-10 units/ µl) exonuclease, 2 parts shrimp alkaline phospitase (1 unit/ µl) and 3 parts buffer (0.5 M KCl, 0.1M Tris HCl pH 8.3 and 0.025 M $MgCl_2$), and added 2 µl to each 5 µl PCR aliquot. We incubated them at 37°C for 30 minutes followed by 80°C for 15 minutes. We submitted samples for automatic sequencing in both directions to the Penn State Genomic Core Facility, Univeristy Park, PA. An ABI 3739XL (Applied Biosystems, Foster City, CA) was used to produce sequences. We edited sequence data and assembled consensus sequences using Sequencher (Gene Codes Corporation, Ann Arbor, Michigan, USA).

We estimated taxonomic placement at the class and genus level for each isolate by BLAST searching the NCBI GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/BLAST/). To assign a genus to each isolate, we used the highest GenBank sequence similarity scores available, and assigned a genus name if the sequence similarity score was 90% or higher and the E-value was 0.0. The Expect-value (E-value) represents a threshold of statistical significance for matches

made by the BLAST algorithm. For the majority of isolates (80.0%), sequence similarity scores were above 97%.

Data analysis

To analyze patterns of fungal infection and seed survival, we used linear mixed-effects models (LMERs) to characterize the effects of field site, plant population origin, and treatment on infection and survival. Plant was included as a random effect in all analyses. For fungal infection, rather than analyzing individual seeds (which were not independent because we sampled 6 seeds per fruit), we scored fungal infection as present or absent in each fruit and used a binomial error distribution with a logit link function. For seed survival, we scored seeds as "alive" or "dead," created a proportional response variable (*n*/6 seeds to survive), and used a binomial error distribution in models. We analyzed both infected and uninfected seeds together in survival models but added seed infection as an additional fixed factor, which we characterized as a yes/no variable. We used Akaike's Information Criterion (AIC_c) to conduct stepwise model simplification to find the minimal best-fit model. We compared the models by using likelihood ratio tests (LRT) to determine which factors contributed significantly to the fit of the minimal model (Crawley, 2012).

We analyzed the overall diversity of all isolates from natural and experimental groups by calculating exact values of richness, abundance, Shannon (Shannon & Weaver, 1949) and inverse Simpson (Simpson, 1949) diversity indices. We also compared the diversity of subsets of isolates. For isolates from natural populations, we compared the diversity of pungent vs. non-

pungent fruits and looked diversity trends across populations. For pungent vs. non-pungent fruits, we reported mean diversity values for pooled samples of isolates in subsets grouped by pungency. For population comparisons, we report exact values of diversity statistics for each population except for richness, which we rarified using subsamples of 6 (the smallest number of isolates recovered in a population). For experimental isolates, we compared the diversity of pungent vs. non-pungent isolates, the diversity of insect, needle and undamaged treatments, and the diversity of isolates from the wet vs. dry field site. For these comparisons, we pooled isolates into bins based on the pungency, field site, and treatment they were from. These bins were treated as samples in our analyses. In addition, we used Renyi diversity profiles (Tóthmérész, 1995) to rank the diversity of all compared groups.

To test if the factors of treatment, plant pungency, field site or population pungency had an impact on the composition of the fungal community, we narrowed our focus to the most highly represented genera in both sets of isolates: *Phomopsis* and *Fusarium*. In the natural population isolates, we counted the number of times each genus was represented in samples of pungent and non-pungent plants for every population. In the experimental isolates, we counted the number of times these genera were represented in samples from each treatment, pungency and field site. For both groups, we converted these count data into proportions by treating the presence of the genus as "successes" and all other genera as "failures." We used general linear models (glms) with a binomial error distribution to examine the impact of fruit pungency and population pungency on the occurrence of *Fusarium* and *Phomopsis* in natural population isolates, and the impact of treatment, fruit pungency, and field site on their occurrence in experimental isolates (Crawley, 2012). We began each analysis with fully saturated models and included all interactions, and

used AIC_c values to conduct stepwise model simplification to find the minimal best-fit model. We compared the models by using likelihood ratio tests (LRT) to determine which factors contributed significantly to the fit of the minimal model (Crawley, 2012). We conducted all analyses in R (R Development Core Team, 2011). We built mixed models using the lme4 package (Bates & Maechler, 2013) and conducted diversity analyses using the packages vegan (Oksanen et al., 2011) and BiodiversityR (Kindt & Coe, 2005).

Results:

Diversity and community structure from natural C. chacoense populations

We recovered a total of 54 isolates from whole fruits across 6 natural *C. chacoense* populations. These isolates represented 5 genera (plus 2 unknown isolates that were identified only as Sordariomycetes, Table S1). All isolates belonged to the class Sordariomycetes, except for two *Phoma* isolates, which are Dothideomycetes. All genera (with the exception of *Daldinia*) were isolated from multiple fruit. *Phomopsis* was the most common genus represented, making up a total of 52% of isolates and *Fusarium* was the second most common, comprising 33% of the isolates (Fig. 1a). Both of these genera were isolated from every population we sampled. Richness of genera appeared to be somewhat asymptotic (Fig. 2a), but the large confidence intervals caused by the relatively small number of isolates make estimation of the curve shape difficult. Diversity indices suggest a community relatively low in diversity at the genus level: Shannon = 1.19 and inverse Simpson= 2.59.

Overall, isolates recovered from pungent fruit were more diverse than those recovered from non-pungent fruit (Fig. 3a). While the number of isolates was nearly the same for both groups (28 isolates from pungent fruits and 26 from non-pungent fruits), pooled samples from pungent fruits had higher mean richness, Shannon and inverse Simpson scores than those from non-pungent fruits (n = 6 for both groups; Table 2). We also found differences in diversity when we compared isolates between populations. With the exception of one population (46% pungent), we found that the diversity of isolates was generally higher in populations that had more pungent plants (Fig. 3b; Table 2).

We examined the impact of fruit and population pungency on the occurrence of the two most highly represented genera: *Fusarium* and *Phomopsis.* We found that *Phomopsis* was significantly more common in populations that had fewer pungent plants (Fig. 4a; GLM population main effect; LRT, p= 0.039) and more common in non-pungent plants (Figure 4b; GLM fruit pungency main effect, p=0.09). In contrast, proportions of *Fusarium* did not differ across populations. While *Fusarium* tended to be more common in pungent fruits (data not shown), the difference between fruit types was not significant, likely due to the relatively small number of *Fusarium* isolates in total.

Fungal infection and seed survival in experimental plants

We followed germination and survival for a total of 2794 seeds: 1041 from non-pungent fruits and 1753 from pungent fruits. Overall, 28.8% of seeds were infected with filamentous fungal growth, and fungal infection significantly lowered seed survival (lmer infection main effect; LRT, $p < 0.0001$ compared to null). While 48.3% of uninfected seeds survived, survival for

infected seeds was only 13.6%. When we looked at the background infection rates at our sites (using fruits in needle and undamaged treatments), we found that infection rates were nearly twice as high at our wet field site (lmer site main effect; LRT p= 0.003). Overall, fruit pungency did not significantly lower seed infection in any treatment; infection rates between pungent and non-pungent fruits were similar. However in undamaged fruits, once seeds were infected, they were more likely to survive if they came from pungent fruits (lmer fruit pungency main effect; LRT, p = 0.009 compared to null). Seeds from pungent fruits were not more likely to survive if they came from the insect or needle treatment. When we compared insect and needle treatments, the presence of insect vectors significantly increased the occurrence of infections only at the dry site; infections for both treatments were equally high at the wet site (lmer site × treatment interaction; LRT p= 0.04). Once infected, seeds in the insect treatment were the least likely to survive; only 9.0% of infected seeds from the insect treatment survived compared to 16.0% in the needle treatment (lmer treatment main effect; LRT p< 0.0001). In addition to significant survival differences caused by fungal infection, a significant decrease was seen in the survival of uninfected seeds from the insect treatment. While 57% of uninfected seeds from the needle treatment survived (n= 779), only 26% of seeds from the insect treatment survived (lmer treatment main effect; p< 0.001), suggesting that damage caused directly by insects was responsible for mortality.

Diversity of seed fungi from experimental plants

We obtained ITS sequences for a representative total of 98 isolates from infected, non-germinating seeds, which represented 17 genera from 3 classes (Table S2). The majority of seed-associated fungi were identified as Sordariomycetes (84 isolates representing 10 genera),

followed by Dothiedeomycetes (13 isolates representing 6 genera). We isolated 6 genera from more than one seed; they comprised 88.8% of all isolates. The rest of the genera (11.2%) were isolated only once. *Phomopsis* was the most commonly isolated genus (41.8%), followed by *Fusarium* (21.4%) and *Colletotrichum* (13.2%)(Fig.1b). Richness of genera is non-asymptotic (Fig. 2b) and diversity indices suggest a relatively diverse community at the genus level: Shannon = 1.86 and inverse Simpson=4.08.

Overall, isolates recovered from pungent fruit were more diverse than those recovered from non-pungent fruit (Fig. 5a). Pooled samples of isolates from pungent fruits (n = 6 for both groups) had higher mean richness, mean abundance, mean Shannon and inverse Simpson scores than those from non-pungent fruits (Table 2). In addition, the consistently higher, non-intersecting Renyi diversity profiles of isolates from pungent fruit indicated higher diversity than those from non-pungent fruits (Fig. S3). High profile values of alpha = 0 also suggested that richness was greater than evenness for both sets of isolates.

Diversity was also impacted by the presence of vectors (Fig 5b). Isolates recovered from the insect treatment (in which insects were allowed to wound fruits with their stylus) were lower in overall diversity than those recovered from the needle treatment (in which fruits were experimentally wounded with sterile needles). Pooled samples of isolates from the insect treatment (n = 4 for both groups) had lower mean richness, mean abundance, mean Shannon and inverse Simpson scores than those from the needle treatment (Table 1). Renyi diversity profiles were also consistent with lower diversity in insect fruits: the profile of isolates recovered from the insect treatment was always lower than that of isolates from the needle treatment. For both

groups of isolates, higher profile values of alpha = 0 indicated that overall richness was greater than evenness (Fig. S4).

Comparisons between pooled isolates from the wet and dry field sites suggested that overall diversity did not differ. While isolates from the wet site had higher mean richness and abundance, mean Shannon and inverse Simpson indices indicated no difference in diversity. This was likely driven by the large difference in the number of isolates recovered at each site; we recovered nearly twice the number of isolates at the wet field site. Renyi profiles of isolates from each site intersected near alpha = 0 and nearly overlapped, (data not shown) indicating that diversity between sites was not significantly different.

The impact of vectors, fruit pungency and field on fungal community composition

We examined the impact of vectors, fruit pungency and field site on the presence of the three most abundant genera in our study: *Phomopsis, Fusarium, and Colletotrichum. Phomopsis* was the only genus that was impacted by treatment; there were significantly more *Phomopsis* isolates in the insect treatment than the needle treatment (Fig. 6a; treatment main effect, LRT, $p = 0.016$ when compared to null). *Fusarium* was more common in fruits that came from non-local plants (Fig. 6b). At the wet site, where all native plants are pungent, *Fusarium* was 3 times more common in non-pungent plants. Conversely, at the dry site where non-pungent plants dominate, *Fusarium* was twice as likely to be found in pungent plants. GLM models supported these results; field site, plant pungency, and their interaction were all significant predictors of *Fusarium* presence (LRT; $p = 0.030$). *Colletotrichum* appeared to be randomly distributed; the null model was most highly supported for this genus.

Discussion:

Fungal transmission and impact on seed survival

We found that fungal infection of *C. chacoense* seeds was relatively common, and that infection had a significant negative effect on survival. Overall, only 13.6% of infected seeds survived, providing further evidence that fungi are important seed predators in wild chilies. We also found that there was significantly more infection in seeds at our wet field site. In previous work, Tewksbury et al. (2008) showed that capsaicin production in polymorphic *C. chacoense* populations increased with increased risk of microbial attack. In that study, however, microbial pressure was not measured directly and the positive relationship between fungal seed infection and insect scars was used to infer increased microbial attack. Here, we confirmed that microbial pressure is indeed higher in populations at the wetter end of the biogeographic gradient in pungency, where insect pressure is high and most fruits are pungent.

Acroleucus, the dominant insect found on wild *Capsicums* in Bolivia, feeds by using its stylus to inject digestive enzymes into the seed endosperm and suck up the partially-digested result (Beck et al., 1958). We provide direct evidence that insect damage leads to increased fungal infection, although in our study this was only true at the dry site where background fungal infection was lower. This suggests that insects are likely fungal vectors, and that their impact becomes more pronounced in populations with lower microbial pressure and fruit pungency. We also showed that insect foraging was associated with overall lower fungal diversity and a higher proportion of *Phomopsis* isolates than expected by chance. Combined, these data suggest that insects may transmit a specific subset of the larger fungal community. Thus, insects impact both the infection

and composition of fungal communities in the fruits they visit. In addition, we provided evidence that insects are important seed predators themselves; uninfected seeds were twice as likely to survive if they were not from fruits damaged by insects. Estimates of the direct impact of Lygeid seed bugs on seed survival in non-agricultural settings are few and vary dramatically (Crawley, 1989), but seed losses greater than 50% have been documented in several plant species (Flemion, 1958). In our study, the combined impact of insects and fungal seed infection reduced seed survival to below 10%.

Antifungal effects of capsaicin

The antimicrobial and antifungal properties of capsaicin are well documented (Cordell & Araujo, 1993; Molina-Torres et al., 1999; Kurita et al., 2002), and decreased fungal infection of pungent *C. chacoense* seeds has been demonstrated in the field. Tewksbury et al. showed that non-pungent fruits were infected at twice the rate of pungent fruits and that capsaicin added to media slowed the growth of *Fusarium* by about 30% (J. Tewksbury et al., 2008). However, our results provided mixed evidence for the antifungal role of capsaicin on seeds. Although overall infection rates were slightly lower in seeds from pungent fruits, the difference was not significant. Pungency did significantly increase the survival of seeds of undamaged fruits once they were infected, but not in fruits from the insect or needle treatment. A possible explanation for these discrepancies may lie in the duration of *in situ* incubation time for the fungus. One of the main differences in methodology between our study and the Tewksbury et al. study was the time fruits (and the fungi inside) were left in the field. In the previous study, seeds remained in the fruit and incubated *in situ* for an entire dry season, while we collected fruits from plants one week after wounding treatments were applied. The effects of capsaicin may be most apparent over longer

time periods. While the source of fungal infection in our undamaged experimental fruits is not well understood, it is possible that these fungi were present in the fruit prior to the initiation of our experiment. If undamaged fruits contained endogenous fungi, they were exposed to capsaicin in pungent fruits for a longer time than fruits that were experimentally wounded, resulting in a detectable antifungal effect.

Additionally, we induced germination in our study by removing seeds from fruit and incubating them in Petri dishes. By removing seeds from the fruit, we reduced seed contact with the capsaicin-producing tissues that may contribute to decreasing fungal growth rates *in situ.* Fungal exposure to capsaicin in our experiment was limited to residues that remained on seeds after fruit removal. In addition, we placed seeds on moist filter paper to induce germination, creating a humid chamber with a rich capsaicin-free carbon source for the fungi. It is possible that under conditions that are very favorable for fungal growth, the antifungal effects of capsaicin become difficult to detect. Finally, many phenolic compounds are liable to oxidation after prolonged exposure to light and oxygen, which can induce changes in their structure and properties. The rapid turnover and degradation of capsaicin has been documented during fruit development (Bernal et al., 1993), but the antimicrobial effects of the oxidative products have not been studied. Our experimental setup minimized seed contact with placental tissues, provided an alternate energy source for the fungus, and exposed capsaicin remaining on seeds to possible oxidation. Combined, these factors may have contributed to the limited antifungal effects we observed in pungent seeds.

Diversity of fungi associated with C. chacoense

Overall, the diversity fungi recovered from experimental seeds was higher than that of fungi recovered from naturally occurring whole fruits. Out of the 53 isolates recovered from whole fruits, we found 5 genera plus two unknown Sordariomycetes. *Phomopsis* and *Fusarium* were found in every *C. chacoense* population we sampled, and accounted for 85% of fruit isolates. Of the 98 isolates we recovered from non-viable seeds, *Phomopsis* and *Fusarium* were also dominant, but the number of singletons we recovered was higher. Out of 17 genera, we recovered 11 singletons, which contributed to their higher overall richness. The genus accumulation curve for seed isolates (Fig. 2b) did not reach an asymptote, and suggests that increased sampling would have revealed additional diversity at the genus level. Greater sampling of fruits from natural populations is needed determine if richness of those isolates reached an asymptote (Fig. 2a), although the higher diversity we observed in seed fungi suggests that this is unlikely. By limiting our study to the genus level, our estimates of diversity are conservative and almost certainly underestimate the presence of rare taxa occurring within a single genus. The pattern of a few dominant and many rare taxa that we observed is common in fungi and has been reported in endophytic (Arnold, 2007), mycorrhizal (Gehring et al., 1998) fungal communities. Studies of the diversity and community structure of fungi that are presumably plant pathogens are comparatively rare (but see Gallery et al., 2007; Hersh et al., 2011).

The vast majority of the isolates we recovered belong to the Sordariomycetes, one of the largest monophyletic clades in the Ascomycota, containing over 600 genera and 3000 described species (Kirk et al., 2001). *Phomopsis* and *Fusarium* are widely distributed genera and include known endophytes, saprotrophs, and plant pathogens. While many of the fungi we recovered appeared

to be pathogenic based on reduced seed survival rates, inoculation trials are the only way to confirm their pathogenicity. In general, caution should be used when inferring the ecological roles of fungi associated with plants. Many genera contain both pathogens and endophytes and studies have shown that the outcomes of plant-fungal symbioses are context- dependent and can shift according to host identity, physiology, or environment (Redman et al., 2001; Kogel et al., 2006). We recovered many isolates from presumably undamaged fruits, and it is possible that fruit colonization occurred from fungi present asymptomatically in host tissues. It is also possible that some of these fungi are primarily saprotrophic and target seeds made non-viable by insect damage, but can act as pathogens when they consume viable seeds in the same fruit.

Fruit chemistry and fungal diversity

For both sets of isolates, we found that diversity was higher overall in pungent seeds and fruits. This finding was consistent across natural populations as well; populations that were dominated by pungent plants also had significantly higher diversity than populations dominated by non-pungent plants. While on its face, higher isolate diversity in pungent fruits appears counterintuitive, increased diversity is likely driven by increased heterogeneity of pungent fruit relative to non-pungent fruits. The hypothesis that habitat heterogeneity is positively related to diversity is foundational in ecology (Simpson, 1949; MacArthur & Wilson, 1967; Ricklefs, 1977) and is well-supported in animal systems (Tews et al., 2004). It posits that complex habitats provide more niches and ways of exploiting resources, thus increase opportunities for greater species diversity (Bazzaz, 1975). For fruit-inhabiting fungi, fruits provide both habitat and resources. While non-pungent fruit uniformly lack capsaicin; the capsaicin present in pungent fruit is variable on multiple scales: pungency varies across the landscape (Tewksbury et al.,

2006), plants vary in the amount of capsaicin they produce (J. Tewksbury et al., 2008), and within a single pungent fruit the spatial distribution of capsaicin is heterogeneous. For example, seeds near the top of the fruit are closer to the source of capsaicin synthesis and likely have higher amounts of capsaicin on their surface, creating the potential for a capsaicin gradient within each fruit. Variation in capsaicin can provide opportunities for fungal colonization by taxa that vary in their tolerance of capsaicin. In addition, the stress placed on fungi by capsaicin may provide competitive release from the most competitive fungi present in non-pungent fruits. Previous work in this system (see Chapter 2) demonstrated that a fungal tolerance of capsaicin comes with tradeoffs; isolates that are affected least by capsaicin grow slowest in the absence of capsaicin. In fungi, rapid growth rates are often associated with greater competitive ability, thus capsaicin-tolerant isolates may be poor competitors in less pungent environments. The positive role of disturbance or stress on diversity is a central idea in ecology, and many studies support the role of stress in reducing the dominance of strong competitors and in shaping the diversity and structure of communities (Janzen, 1970; Grime, 1973; Connell, 1978; Menge & Sutherland, 1987).

Host plant defensive chemistry is a stressor frequently encountered by fungal plant inhabitants. Despite the ubiquity of intimate plant-fungal relationships, the impact of host plant chemistry on the diversity of fungal inhabitants has only been addressed by a handful of studies on endophytes. In an analysis of the diversity of endophytes recovered from medicinal plants rich in phenolic compounds, diversity was lowest in plants that had the lowest flavonoid content and highest in plants with flavonoid content in mid-ranges (Huang et al., 2008). Other studies of endophytic fungi have suggested that host specificity and diversity of many endophytes is driven

in part by their sensitivity to plant chemistry (Arnold et al., 2000; Saunders & Kohn, 2009). In a study of tropical endophytes, fungi were insensitive to host leaf extracts but were inhibited by leaf extracts of local non-host species (Arnold & Herre, 2003). In our study, we found that *Phomopsis* was more likely to be recovered from non-pungent fruits and more common in populations where non-pungent plants dominated. While we were unable to demonstrate that *Fusarium* was significantly more likely to be recovered in pungent fruits, trends in our data suggest that *Fusarium* species may likely candidates for particularly capsaicin-tolerant isolates.

Conclusions

Selection by microbial seed predators has been frequently invoked as an explanation for the presence of defensive chemistry in fruit ((Janzen, 1977; Cipolini, Martin L. & Stiles, Edmund W., 1993; Cipollini & Levey, 1997; Cazetta et al., 2008), yet the effect of defensive chemistry on the occurrence, diversity and community structure of microbes is largely unknown. We report that a diverse community of fungi infects the fruits and seeds of *C. chacoense*, significantly reducing seed survival and supporting the hypothesis that fungal seed consumers place selection pressure on plants. We also show that multiple factors- site, the activity of insect vectors, and the production of capsaicin- all contribute significantly to the diversity and community structure of fungal fruit and seed inhabitants. The increase in fungal diversity associated with pungent fruits suggests that heterogeneity and stress, factors long associated with increasing diversity in animal systems, may also apply on much smaller scales: to plants and their fungal symbionts. Additional experiments of other host plants are needed to test the possibility that plant defensive compounds are a primary mechanism determining the diversity and community structure of their fungal inhabitants.

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Figures:

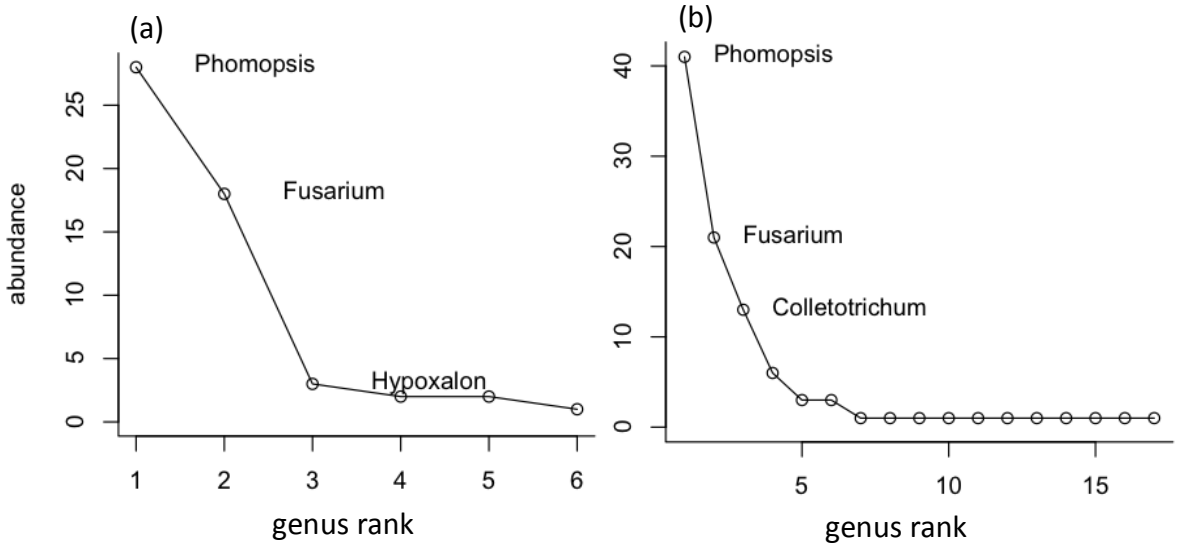

Figure 1. The rank abundance of genera associated with all fungi isolated from (a) fruits from natural *C. chacoense* populations and (b), non-viable experimental seeds of *C. chacoense.*

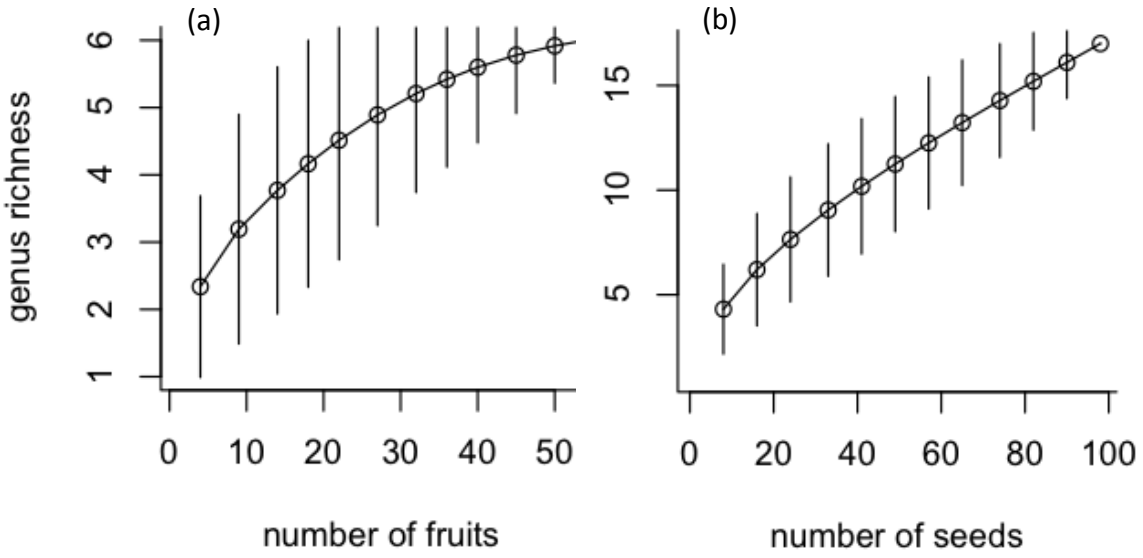

Figure 2. Mean accumulation of genera per fruit (a) and seed (a) isolates as determined by >90% ITS sequence similarity with sequences in the NCBI GenBank nucleotide database. The curve represents the mean accumulation of genera as randomized isolates were added; the bars represent 95% confidence intervals based on 100 randomizations.

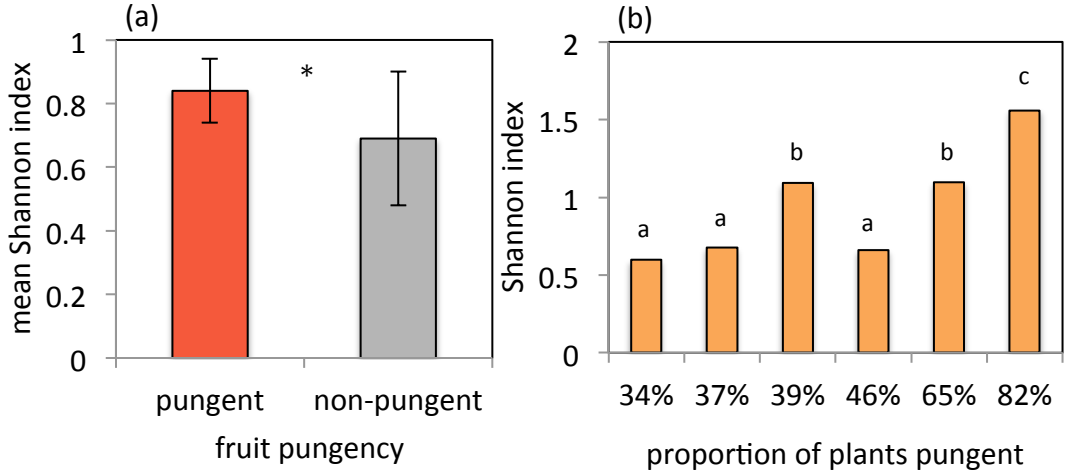

Figure 3. Shannon diversity index values of isolates recovered from natural *C. chacoense* fruits. Pooled sample means are shown for isolates from pungent vs. non-pungent fruits (a) and exact Shannon values are shown population comparisons (b). Each bar represents a population. We determined if communities were significantly different by comparing their Renyi diversity profiles. (See figures S1 and S2.) Error bars are ±1 SE.

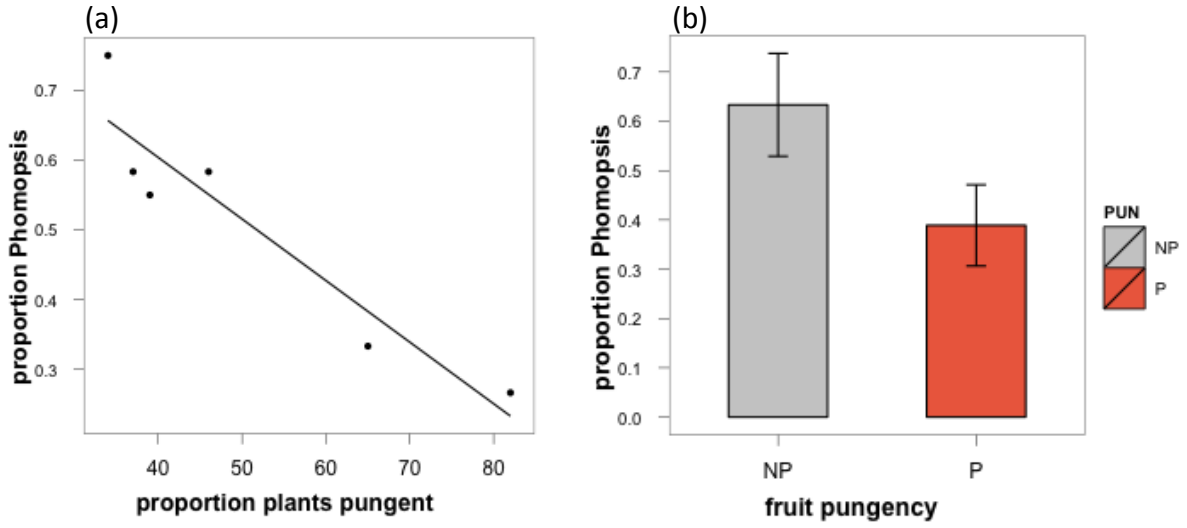

Figure 4. The proportion of isolates identified as *Phomopsis* from natural fruits across (a) populations of *C. chacoense* that vary in plant population pungency (GLM population main effect; LRT, p= 0.039) and (b) non-pungent and pungent fruits (GLM fruit pungency main effect; LRT, p=0.09*)*. Error bars are ±1 SE.

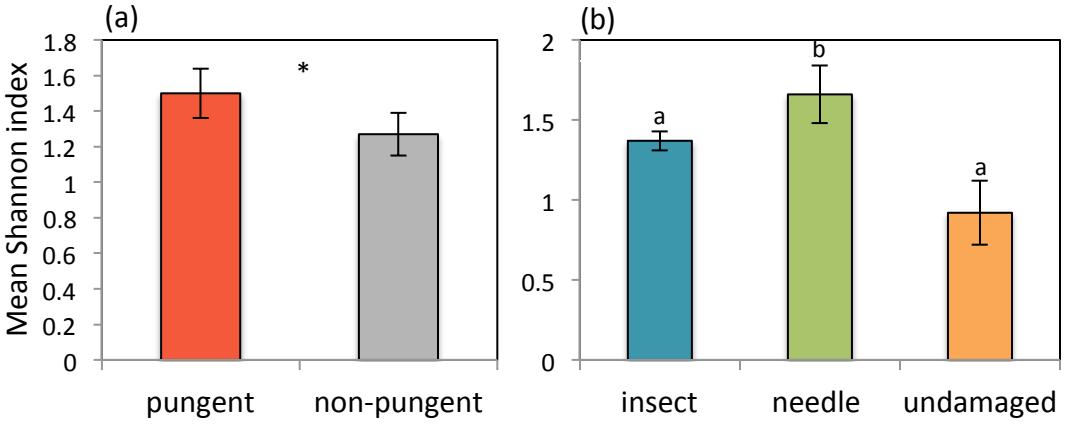

Figure 5. Mean Shannon index for comparisons of isolate diversity recovered from experimental fruits: (a) all pungent and non-pungent fruits and (b) experimental fruit wounding treatments. Fruits were subjected to insect foraging, simulated insect damage with sterile needle punctures or left undamaged. We determined if communities were significantly different by comparing their Renyi diversity profiles. (See figures S3 and S4.) Error bars are ±1 SE.

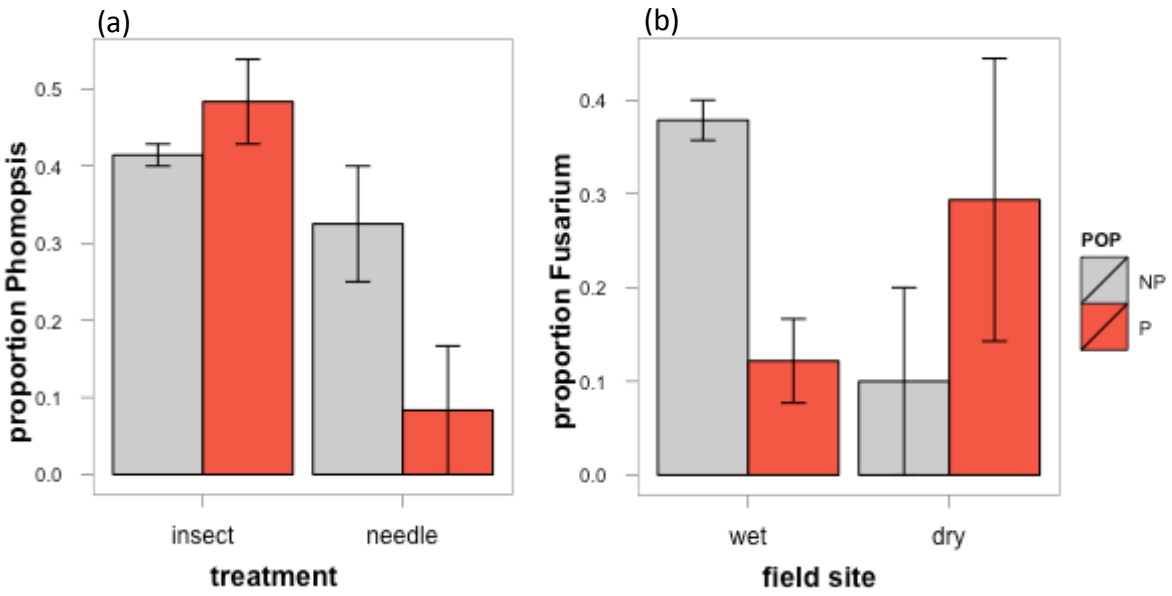

Figure 6. The proportion of isolates recovered from experimental seeds that were identified as (a) *Phomopsis* and (b) *Fusarium*. Gray bars represent non-pungent plants originating from the dry field site; red bars represent pungent plants originating from the wet field site. Isolates were subjected to insect foraging or simulated insect damage with sterile needles; *Phomopsis* was more likely to be associated with insect foraging. (GLM treatment main effect, LRT, $p = 0.016$). In comparisons between field sites, *Fusarium* was more common in non-local plants, regardless of pungency. (GLM field site × plant pungency interaction, LRT, $p = 0.030$). Error bars are ±1 SE.

Tables:

Table 1. Diversity statistics of fungi isolated from whole fruits that were collected from 6 natural populations of *C. chacoense,* including incidence, richness, and diversity indices. Exact values are reported for all isolates (top row) and for each population with the exception of richness. For population richness, samples were rarefied using a subsample of 6, the smallest number of isolates recovered in a population. For pungent vs. non pungent fruits, means ± 1 standard error are reported for pooled samples (6 samples) of isolates in subsets grouped by pungency.

		n	No. of isolates	Rarefied richness	Shannon index	Inverse Simpson
All isolates		na	54	6	1.19	2.59
Pungency	pungent	6	28	2.50 ± 0.34	0.84 ± 0.10	2.29 ± 0.21
	non-pungent	6	26	2.33 ± 0.56	0.69 ± 0.21	2.22 ± 0.57
Population pungency	34%	2	7	2.00 ± 0.00	0.598	1.69
	37%	2	12	1.99 ± 0.08	0.679	1.95
	39%	2	13	2.87 ± 0.67	1.091	2.52
	46%	2	8	2.00 ± 0.00	0.662	1.88
	65%	2	6	3.00 ± 0.00	1.099	3.00
	82%	2	8	4.39 ± 0.55	1.560	4.57

Table 2. Diversity statistics of fungi isolated from the non-viable seeds of *C. chacoense,* including incidence, richness, and diversity indices. Exact values are reported for all isolates (top row) and rarified means and ± 1 SE are reported for pooled samples of isolates in subsets grouped by pungency, fruit wounding treatment, and field site.

	pooled	*n*	No. of isolates	Richness	Shannon index	Inverse Simpson
All isolates		na	98	17	1.86	4.08
Pungency	pungent	6	61	5.83 ± 0.74	1.50 ± 0.14	3.60 ± 0.73
	non-pungent	6	39	4.33 ± 0.33	1.27 ± 0.12	2.80 ± 0.29
Treatment	insect	4	38	5.00 ± 0.00	1.37 ± 0.06	2.95 ± 0.26
	needle	4	36	6.25 ± 1.11	1.66 ± 0.18	4.29 ± 0.44
	undamaged	4	24	3.00 ± 0.41	0.92 ± 0.20	2.35 ± 0.29
Field site	wet	6	64	5.33 ± 0.80	1.36 ± 0.18	3.33 ± 0.77
	dry	6	35	4.83 ± 0.48	1.42 ± 0.01	3.06 ± 0.28

Supplemental information:

Table S1. Genus, class and frequency of isolates obtained from whole fruits obtained from 6 natural populations of *C. chacoense.* A total of 54 isolates were identified by sequencing the ITS1–5.8s-ITS2 region of nuclear ribosomal RNA.

Genus	Class	Number of isolates
Phomopsis	Sordariomycetes	29
Fusarium	Sordariomycetes	18
Hypoxylon	Sordariomycetes	2
Phoma	Dothideomycetes	2
Daldinia	Sordariomycetes	1
Unknown	Sordariomycetes	1
Unknown	Sordariomycetes	1

Table S2. Genus, class, and frequency of isolates obtained from non-viable seeds of *C. chacoense.* A total of 98 isolates were identified by sequencing the ITS1–5.8s-ITS2 region of nuclear ribosomal RNA.

Genus	Class	Number of isolates
Phomopsis	Sordariomycetes	41
Fusarium	Sordariomycetes	31
Colletotrichum	Sordariomycetes	14
Alternaria	Dothideomycetes	6
Nigrospora	Sordariomycetes	3
Phoma	Dothideomycetes	3
Botyrospheria	Dothideomycetes	1
Cephalotheca	Sordariomycetes	1
Chaetomium	Sordariomycetes	1
Epicoccum	Dothideomycetes	1
Guignardia	Dothideomycetes	1
Leptosphaerulina	Dothideomycetes	1
Neosartorya	Eurotiomycetes	1
Ophiostoma	Sordariomycetes	1
Pestalotiopsis	Sordariomycetes	1
Phialemonium	Sordariomycetes	1
Unknown	Sordariomycetes	1

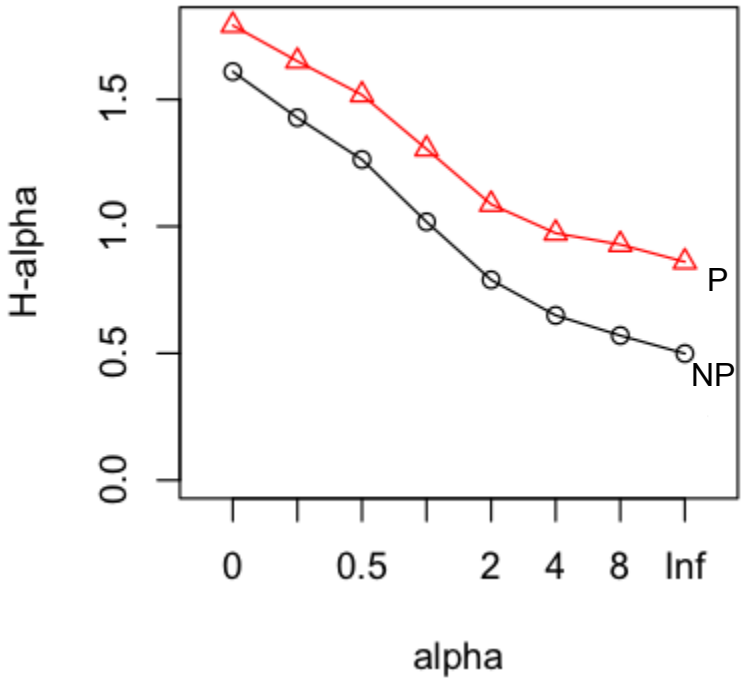

Figure S1. Renyi diversity profile of isolates from pungent (red line and symbols) and non-pungent fruits (red line and symbols) collected from natural chili populations. The consistently higher position of the pungent over the non-pungent indicates higher diversity in isolates recovered from pungent fruit. Curves were calculated by randomizing pooled samples and calculating diversity profiles for 100 permutations.

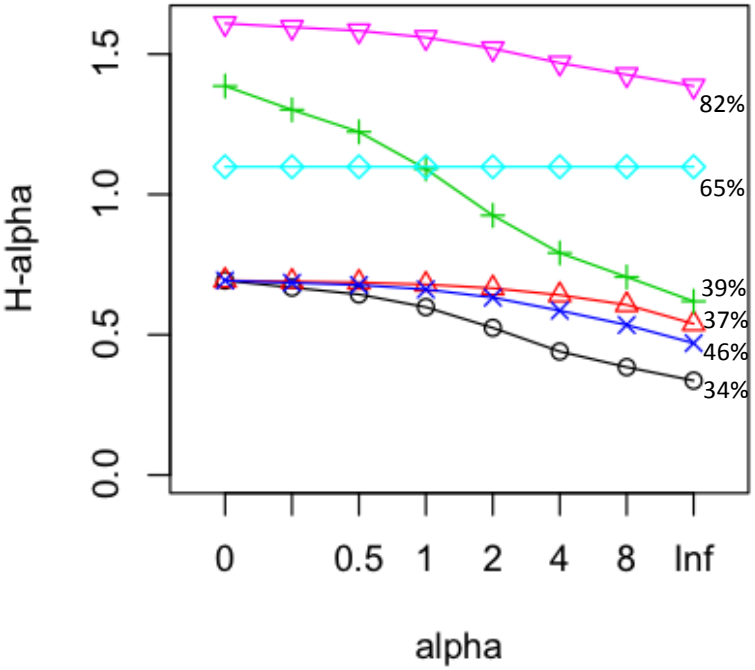

Figure S2. Renyi diversity profile of isolates recovered from natural chili populations that varied in the proportion of plants that were pungent. Curves that do not cross or overlap indicate

communities with significantly different diversities. Higher curve positions indicate higher diversity. We created profiles by randomizing pooled samples and calculating diversity profiles for 100 permutations.

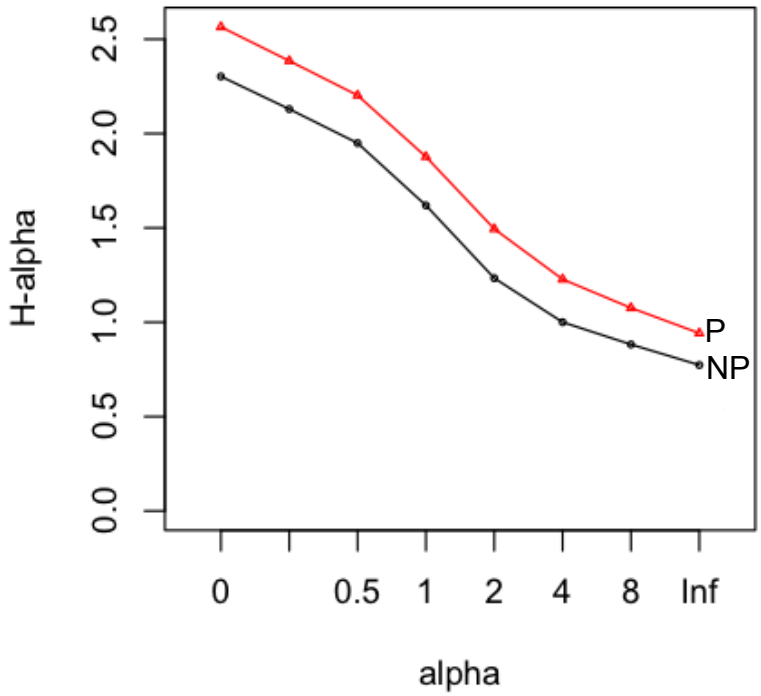

Figure S3. Renyi diversity profiles of isolates recovered from pungent (red line and symbols) and non-pungent seeds (black line and symbols) taken from experimental fruits. The consistently higher position of the pungent over the non-pungent curve indicates significantly higher diversity in isolates recovered from pungent fruit. We created profiles by randomizing pooled samples and calculating diversity profiles for 100 permutations.

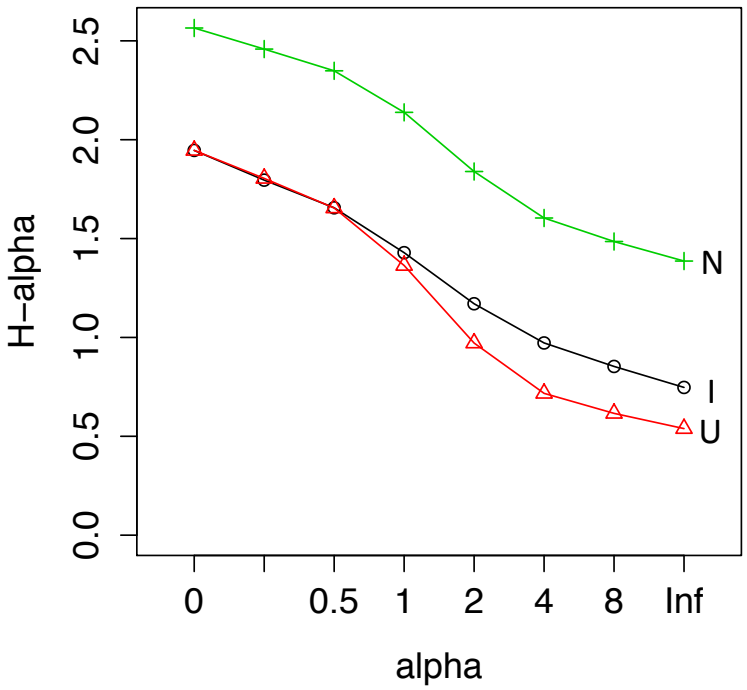

Figure S4. Renyi diversity profiles of isolates recovered from the needle (crosses, green line), insect (circles, black line), and undamaged experimental fruit treatments (triangles, red line). The consistently higher position of needle curve over the insect curve indicates significantly higher diversity in isolates recovered from the needle treatment. Curves that overlap or cross indicate non-significant differences in diversity. We created profiles by randomizing pooled samples and calculating diversity profiles for 100 permutations.