Temperature-Dependent Genotype-by-Genotype Interaction between a Pathogenic Fungus and Its Hyperparasitic Virus

Sarah Franziska Bryner* **and Daniel Rigling**

Swiss Federal Institute for Forest, Snow, and Landscape Research (WSL), CH-8903 Birmensdorf, Switzerland

Submitted May 3, 2010; Accepted September 30, 2010; Electronically published November 30, 2010

Online enhancements: appendixes.

ABSTRACT: The outcome of host-parasite interactions may depend not only on the genotypes of the species involved but also on environmental factors. We used the fungus *Cryphonectria parasitica*, the causal agent of chestnut blight, and its hyperparasitic virus, *Cryphonectria* hypovirus-1 (CHV1), to test for genotype-by-genotype-by-environment interactions in a host-parasite system. In *C. parasitica*, infection with CHV1 induces a hypovirulent phenotype with reduced virulence toward the chestnut tree (*Castanea* spp.) and thus controls chestnut blight in many European regions. In contrast, uninfected virulent *C. parasitica* have nearly eradicated the American chestnut in North America. We applied a full factorial design and assessed the fungal growth and sporulation of four *C. parasitica* strains, uninfected and infected with each of the four known CHV1 subtypes, at 12°, 18°, 24°, and 30°C. We found a significant ($P \leq .0001$) genotype-by-genotype-by-environment interaction, demonstrating the potential for a selection mosaic. As a consequence, different host and parasite genotypes would be selected under different climatic conditions, affecting the coevolutionary dynamics of the host-parasite interaction and the course of chestnut blight epidemics. Genotype-by-genotype-by-environment interactions are essential to take into account when designing biological control strategies.

Keywords: host-parasite interaction, coevolution, *Cryphonectria parasitica*, fungal virus, biological control, climate change.

Introduction

Parasites and hosts attempt to manipulate each other's physiology and fitness. Ultimately, the outcome of the interaction between a host and a parasite may depend on particular combinations of genotypes (genotype-by-genotype interaction; Browder 1985; Thompson and Burdon 1992; Peever et al. 2000; Carius et al. 2001). Coevolutionary theory states that the antagonistic relationship between hosts and parasites results in an evolutionary arms race (Dawkins and Krebs 1979). Furthermore, it has been hypothesized that this arms race is influenced by environmental factors (Little 2002; Thomas and Blanford 2003; Mitchell et al. 2005; Wolinska and King 2009). One important environmental variable is temperature, which has been shown to alter infection-related traits of hosts and parasites (Wolinska and King 2009). On the one hand, temperature alone can generally increase or decrease the effect of parasitism (Mitchell et al. 2005; Vale et al. 2008*b*). On the other hand, temperature may specifically interact with certain host genotypes, influencing their relative resistance (Browder 1985; Blanford et al. 2003), and with particular parasite genotypes, influencing their relative virulence (genotype-by-environment interaction; Laine 2008; Vale and Little 2009). This raises the question of whether temperature also alters the interaction between specific host and parasite genotypes (genotype-by-genotype-by-environment interaction). To date, the vast majority of studies have included only environmental effects on different genotypes in one of the two interacting species and have therefore not enabled statistical testing of genotype-by-genotype-by-environment interactions (Thomas and Blanford 2003; Lazzaro and Little 2009; Wolinska and King 2009). We are aware of no more than three studies in host-parasite systems that statistically analyzed this three-way interaction (Laine 2007; Tétard-Jones et al. 2007; Vale and Little 2009). A significant genotype-by-genotype-by-environment interaction was found in only one of these studies, which investigated the interaction with a biotic environmental factor (rhizobacteria; Tétard-Jones et al. 2007).

Demonstrating the existence of genotype-by-genotype-by-environment interactions may be of interest for two different reasons: first, to predict phenotypic expression and the outcome of host-parasite interactions in different environments, and second, to understand how natural selection acts on host-parasite interactions in different environments. The effect of the environment on the phenotype is described by the reaction norm, which displays the series of phenotypes expressed by a genotype across a range of environments (Gomulkiewicz and Kirkpatrick

* Corresponding author; e-mail: sarah.bryner@wsl.ch.

Am. Nat. 2011. Vol. 177, pp. 65–74.
DOI: 10.1086/657620

1992). The reaction norm of the interaction between two species is called an interaction norm (Thompson 1988). Genotype-by-genotype-by-environment interactions are indicated by nonparallel slopes of interaction norms. Crossing host-parasite interaction norms would further indicate that no combination of host and parasite genotypes exists that outperforms all the others across a range of environments and that different combinations of coevolving host and parasite genotypes are favored in different environments. Such variation in natural selection among ecosystems is hypothesized by the geographic-mosaic theory of coevolution (Thompson 1999; Gomulkiewicz et al. 2000). This theory assumes the presence of a selection mosaic in which genotype-by-genotype-by-environment interactions govern the extent and the direction of natural selection on interacting species. Despite growing evidence for the presence of geographic selection mosaics (Benkman 1999; Zangerl and Berenbaum 2003; Toju and Sota 2006; Laine 2009), testing for their existence is not trivial (Gomulkiewicz et al. 2007). Most empirical studies have assessed only whether selection on coevolving species differs in different environments and not whether differences are due to variation in how selection acts on the same genotype-by-genotype interactions in different environments (Hoeksema et al. 2009). A first step in this direction would be to demonstrate the potential for a selection mosaic by proving the existence of genotype-by-genotype-by-environment interactions (Gomulkiewicz et al. 2007; Hoeksema et al. 2009).

The pathogenic fungus *Cryphonectria parasitica*, the causal agent of chestnut blight, and its hyperparasitic virus, *Cryphonectria* hypovirus (CHV), constitute a model system for studying fungus-virus interactions. This system provides a textbook example of a viral disease that significantly reduces fungal virulence, a phenomenon called hypovirulence (Van Alfen et al. 1975; Taylor et al. 1998; Nuss 2005). Among the four CHV species described, the most attention has been given to CHV1 because of its role in the biological control of chestnut blight and its high prevalence in Europe (Hillman and Suzuki 2004). Within CHV1, four genetically distinguished subtypes have been identified: Italian subtype I, German/Spanish subtype D, and French subtypes F1 and F2 (Gobbin et al. 2003).

The goals of this study were to elucidate the influence of temperature on the host-parasite interaction between *C. parasitica* and CHV1 and to test for a genotype-by-genotype-by-environment interaction, thereby exploring the potential for a temperature-dependent selection mosaic. In a full factorial design, we used four *C. parasitica* strains as virus-free controls and in combination with each of the four CHV1 subtypes, and we assessed fungal growth and sporulation at four temperatures.

Material and Methods

The Study System

Cryphonectria parasitica is a tree pathogen originating in East Asia that causes lethal bark cankers on susceptible *Castanea* spp. It was introduced to both North America and Europe during the past century. In a devastating epidemic, it has nearly eradicated American chestnut (*Castanea dentata*) in North America (Anagnostakis 1982). In contrast, chestnut blight incidence is high on European chestnut (*Castanea sativa*) in Europe but is maintained at low severity in most regions (Heiniger and Rigling 1994) due to the infection of *C. parasitica* with CHV1, an unencapsidated double-stranded RNA virus of the genus *Hypovirus* (Choi and Nuss 1992). CHV1 significantly decreases canker growth, strongly attenuates asexual sporulation, and almost completely inhibits sexual reproduction of its fungal host (Elliston 1985; Zhang et al. 1998; Peever et al. 2000). It is dispersed by asexual fungal spores (Prospero et al. 2006), and its transmission from one fungal individual to another by hyphal fusion (anastomosis) theoretically allows for the spread of hypovirulence within the fungal population (Milgroom and Cortesi 2004). Natural dissemination and biological control efforts have led to a high prevalence of hypovirulence in many areas in Europe (Heiniger and Rigling 1994). The failure of hypovirulence in North America, however, may have resulted from environmental differences, higher blight susceptibility of the American chestnut, differences in the fungal population structure, and/or differences in the virulence of the hypoviruses (MacDonald and Fulbright 1991; Dawe and Nuss 2001; Milgroom and Cortesi 2004).

The lack of an extracellular phase and the resulting complete dependence of CHV1 on *C. parasitica* suggest a significant genotype-by-genotype interaction (Peever et al. 2000) and indicate reciprocal selection on fitness traits. Genetic variation in natural populations of *C. parasitica* (Liu et al. 1996; Breuillin et al. 2006) and CHV1 (Gobbin et al. 2003) further provides the basis for natural selection. Additionally, both chestnuts (Anagnostakis 1987; Conedera et al. 2004) and *C. parasitica* (Roane et al. 1986) grow under a wide range of temperatures, and an effect of environmental factors such as temperature (Anagnostakis and Aylor 1984) or light intensity (Hillman et al. 1990) on the expression of hypovirulence has been suggested.

Host and Parasite Isolates

We used four fungal strains in combination with each of the four CHV1 subtypes and with no virus (in total, 20 combinations). To create these fungus-virus combinations, virus-infected *C. parasitica* isolates obtained from four different geographic regions in Europe and stored in glycerol

at −80°C were used. They harbored four different subtypes of CHV1 (Gobbin et al. 2003). Isolate M1372, obtained from Oberkirch (Germany) in 1992, harbored CHV1 subtype D (Gobbin et al. 2003) and was given the name "baw" to identify the fungal strain. Isolate M4357, obtained from Dordogne (France) in 2003, harbored CHV1 subtype F1 and was given the name "dor." Isolate M2021, obtained from Var (France) in 1999, harbored CHV1 subtype F2 (Robin et al. 2010) and was given the name "var." Finally, isolate M4042, obtained from Ticino (Switzerland) in 2004, harbored CHV1 subtype I and was named "tic." From each virus-infected isolate, we obtained a virus-free culture through single conidial isolation (Prospero et al. 2006). Each hypoviral subtype was then transmitted from the infected fungal donor isolate to each of the virus-free recipient strains by hyphal anastomosis (Rigling et al. 1989; Peever et al. 2000; Robin et al. 2010). A small piece of mycelium was taken from the edge of the infected recipient culture to initiate the stock cultures for the experiment. The history of subcultivation was identical for all stock cultures. Each culture was checked for presence or absence of CHV1 by extraction of double-stranded RNA, and the identity of the CHV1 subtype was verified by sequence analysis as described in Gobbin et al. (2003).

Experimental Setup

We took three phenotypic measures in two experiments with identical design. Fungal growth and sporulation were assessed on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) in the same colonies, and fungal growth was also measured on dormant chestnut stems. In each experiment, we exposed six replicates of each of the 20 fungus-virus combinations to four different temperatures (12°, 18°, 24°, and 30°C) that fall within the thermal range of chestnut growing regions. Replicates were assigned to one of six blocks and randomized within blocks. The four climate chambers used in the experiment were of identical type and were ventilated with outside air through a common air duct. The temperatures were kept constant at 12°, 18°, 24°, or 30°C, and relative humidity was set to 70% in all chambers (DICON SM Universeller Kompaktregler, JUMO, Fulda, Germany). Actual temperature and relative humidity (rH) values were recorded with data loggers (DL-120TH Humidity/Temperature, Voltcraft, Hirschau, Germany) at 5-min intervals during the course of the experiment; mean values (±SD) were as follows: 12.1° ± 0.2°C at 72.2% ± 1.5% rH, 18.0° ± 0.4°C at 72.7% ± 3.1% rH, 24.0° ± 0.2°C at 71.4% ± 2.5% rH, and 30.0° ± 0.3°C at 70.3% ± 3.3% rH.

Growth and Sporulation on PDA

For each fungal colony in the experiment, we used an individual sterile 9-cm petri dish (84-mm inner diameter) containing 25 mL of PDA. These PDA plates had been prepared from the same lot 10 days before inoculation and were kept at 4°C. On the starting day of the experiment, we inoculated the PDA plates by placing into the center of the plate a mycelial plug (6-mm diameter) taken from the growing edge of 5-day-old precultures. The plates were wrapped with Parafilm and arranged in adjacent blocks on a shelf in each chamber. Each shelf was illuminated by 12 new fluorescent tubes (Philips Master PL-L-36W/840/4P-ICT, Koninklijke Philips Electronics, Amsterdam) set for a 14L : 10D photoperiod. Light intensity (mean ± SD) received by the fungal colonies was 3,330 ± 160 lx (illuminance meter, Minolta, Osaka, Japan). At 30°C, all cultures belonging to block 1 displayed severe growth deficiencies and were therefore excluded from analysis, resulting in the use of five rather than six replicates of each isolate.

During the first 10 days of the experiment, we assessed the radial growth on PDA every 24 h. Two cardinal diameters of each colony through two orthogonal axes previously drawn on the bottom of each plate were measured with a ruler, using a millimeter scale. As the shapes of the colonies were not perfect circles, we calculated the geometric mean diameter of an ellipse. Radial growth of *C. parasitica* cultures on PDA has been described as undergoing a phase of linear growth (Anagnostakis and Aylor 1984), and we determined this phase of linear growth for each temperature separately. Linear regression implemented in Microsoft Excel 2007 was performed on the geometric mean diameter calculations from the first 10 days of the experiment. The criterion $R^2 > 0.980$ of the linear regression fit was applied to define the time period during which all cultures at a given temperature were growing linearly. The increment of the fitted regression line was taken as the growth rate during the linear growth phase and was used for all further analyses of growth on PDA. We chose to assess growth rate because it includes data from an extended period of time, which is biologically more informative and meaningful than fungal colony size at a single time point.

After 31 days of incubation, we assessed sporulation by liberating conidia (asexual spores) with a glass rod in 10 mL of 0.15% Tween 80 (Sigma-Aldrich) according to Hillman et al. (1990). Serial 10-fold dilutions were made from each spore suspension, and the number of conidia was quantified by direct counting under a light microscope (DMIL, Leica, Solms, Germany) with 400 × magnification, using a hemocytometer (Thoma, 0.1 mm × 0.0025 mm^2). The measured concentration in conidia per milliliter was

multiplied by the volume of the suspension recovered from the fungal colony.

Growth on Chestnut Stems

Six plastic containers (57 cm × 37 cm × 13 cm), each holding five dormant chestnut stems (50 cm in length, 5–10 cm in diameter), were used as blocks in each of the above-mentioned chambers. Twenty healthy stems were cut from each of six individual *C. sativa* sprout clusters in the Ticino (Switzerland) at the beginning of November 2009, a few days before the start of the experiment. Sprout clusters growing up from a single stump are clones of the same tree. Each sprout cluster was assigned to a block, and five stems of the same sprout cluster were put together in one container. Both ends of the stems were sealed with paraffin. Along the axis of the stem, four circular wounds (6-mm diameter) were made with a cork borer to the depth of the cambium. The wounds were arranged 12 cm apart from each other and 7 cm from the two ends of the stems, thereby assuring that the fungal cultures would not influence one another. Within each block at each temperature, the 20 fungus-virus combinations were assigned to wounds at random, and we filled each wound with two mycelial mats (6-mm diameter) obtained from the growing edge of the 5-day-old precultures. The holes were then covered with transparent adhesive tape to prevent desiccation. Within the containers, the stems were placed horizontally on plastic grids located 5 cm above the bottom of the container. The containers were filled with water to a depth of 2 cm and covered with a nontransparent lid. At 12°C, one stem had to be removed from the experiment due to contamination with another fungus, and at 24°C, one inoculation was missing, resulting in a total of five missing values in this experiment. After 18 days of incubation, we determined the lesion diameters on the chestnut stems with a millimeter scale. Two diameters of each lesion were measured, one along the longitudinal axis and a second along the lateral axis of the stem. As the shapes of the lesions resembled ellipses, we calculated the geometric mean diameter and used it as a phenotypic value for growth on chestnut stems in all further analyses.

Data Analysis

We analyzed separately the three variables growth on PDA, sporulation on PDA, and growth on chestnut stems, using a general linear model (GLM) in SPSS 17.0 (SPSS, Chicago) with the fixed factors temperature, fungus, and virus and their interactions. Block was included as a random factor. In the GLM for the experiment on PDA, block was nested within temperature. In the experiment on dormant chestnut stems, stems originating from the same sprout cluster were assigned to a block. As all replicate measurements at a particular temperature were obtained within the same chamber, we cannot exclude the possibility that uncontrolled chamber effects confounded the effects we attributed to temperature. However, any potential effect of the chamber was minimized by ensuring identical growth conditions (apart from temperature). Residuals of the GLM were normally distributed and displayed constant error variances for growth on PDA and growth on chestnut stems, but not for sporulation. Therefore, we log transformed the sporulation data, which stabilized error variances.

We also applied GLMs on the data within each temperature. The fixed factors of this reduced linear model were fungus and virus, and block was a random factor. Tukey's test was then implemented to detect significant (at $\alpha < 0.01$) differences among fungus-virus combinations at the same temperature.

We calculated Pearson's correlation coefficients to test for a linear relationship between growth and sporulation on PDA and between growth on PDA and growth on chestnut stems. In the latter case, the mean values for each isolate were used.

To study the effect of virus infection on the fungal host, we calculated the difference between the phenotypic mean values of the virus-infected strain and those of the corresponding uninfected strain for each fungus-virus combination at all temperatures. The resulting value was given as a proportion of the phenotype value of the uninfected strain and termed the virus effect. The output of Tukey's test implemented in the within-temperature GLMs was used to determine whether the virus effect was significant (at $\alpha < 0.01$). Tukey's tests and GLMs were also performed on virus effects to test for significant (at $\alpha < 0.01$) differences in the effectiveness of virus infection among temperatures and among viral subtypes as well as between home and away host-parasite combinations. Residual analysis revealed no violation of GLM assumptions.

Results

Identification of Significant Effects and Factor Interactions

Growth and sporulation on PDA were significantly affected by all main factors (fungus, virus, and temperature) and all interactions (table 1). Growth on chestnut stems was also significantly affected by all main factors and by the virus-by-temperature interaction. When the data were restricted to the virus-infected cultures, the respective GLM terms remained significant for the same factors (results not shown). Figure 1 shows not only that the fungus-by-virus-by-temperature interaction was significant, as indicated by nonparallel interaction norms, but also that the

Table 1: General linear models on growth and sporulation of *Cryphonectria parasitica* strains infected with *Cryphonectria* hypovirus-1 subtypes or uninfected, at different temperatures

		Growth rate on agar medium			Sporulation on agar medium				Growth on chestnut stems		
Source	df	MS	*F*	*P*	MS	*F*	*P*	df	MS	*F*	*P*
T	3	2,336.088	487.090	≤.0001***	111.903	517.308	≤.0001***	3	6,747.715	237.913	≤.0001***
F	3	22.703	76.814	≤.0001***	.489	5.764	.002**	3	195.869	6.409	.005**
V	4	360.291	1,912.597	≤.0001***	10.756	107.746	≤.0001***	4	6,427.216	344.347	≤.0001***
T × F	9	9.214	31.175	≤.0001***	1.508	17.767	≤.0001***	9	80.334	3.249	.004**
T × V	12	48.244	256.102	≤.0001***	3.266	32.714	≤.0001***	12	404.638	16.689	≤.0001***
F × V	12	6.169	30.347	≤.0001***	.861	11.723	≤.0001***	12	29.905	1.271	.259
T × F × V	36	1.868	9.188	≤.0001***	.884	12.037	≤.0001***	36	20.233	.923	.599
Error	228	.203			.073			175	21.928		

Note: The effects of the fixed factors temperature (T), fungal strain (F), virus subtype or no virus (V), and their interactions were tested on growth on agar medium (growth rate in colony diameter in mm day^{-1} during the phase of linear growth), sporulation on agar medium (log number of conidia produced after 31 days of incubation) and growth on chestnut stems (lesion diameter after 18 days of incubation). The blocking factor was included in the model as a random term, and the associated mean square values are reported in table A1 in the online edition of the *American Naturalist*. df = degrees of freedom.

** $P \leq .01$.

*** $P \leq .001$.

fungus-virus interaction norms even crossed. Crossing interaction norms included different fungal strains infected with the same virus subtype. The majority of crossings occurred between 24° and 30°C and were most reliably observed for growth rate on PDA (fig. 1*A*), the phenotypic variable that could be most accurately measured (mean coefficient of variation for growth rate on PDA, 0.06; for sporulation on PDA, 0.51; for lesion diameter on chestnut stems, 0.21). Ranking of the fungus-virus combinations according to their phenotypic values revealed that some fungus-virus combinations switched their ranks substantially among temperatures, reaching high ranks at some temperatures and low ranks at others.

In the separate GLMs for each temperature, the fungus-by-virus interaction exhibited a significant effect on growth and sporulation on PDA at all temperatures. Growth on chestnut stems was significantly affected by the fungus-by-virus interaction at 12°C, but not at the other temperatures, while the main effect of the virus was significant at all temperatures. Growth and sporulation tended to increase with increasing temperature, but not all fungus-virus combinations reached their highest growth or sporulation at the same temperature. Differences were related to virus treatment (fig. 1). On PDA, the cultures reached their highest growth rate at 24°C if they were uninfected or were infected with the viral subtypes D or I. If they were infected with subtypes F1 or F2, they grew, on average, most rapidly at 30°C (fig. 1*A*; table B1 in the online edition of the *American Naturalist*). No distinctive pattern was observed for sporulation, but the majority of cultures produced the largest number of spores at 24°C (fig. 1*B*; table B2 in the online edition of the *American Naturalist*). Growth on chestnut stems of most cultures was highest at 30°C. The increase in lesion diameter from 24° to 30°C was generally greater for strains infected with subtypes D, F1, and F2 than it was for strains infected with subtype I or for uninfected strains (fig. 1*C*; table B3 in the online edition of the *American Naturalist*).

Correlation of Phenotypes

Growth on PDA and growth on chestnut stems displayed the same trend across temperatures and were correlated at $r = 0.673$ ($P \leq .01$). The major inconsistency between the growth measurements on the two types of media was found in the performance of the uninfected cultures in relation to that of the virus-infected cultures. Exclusion of the virus-free cultures from the data set increased the correlation ($r = 0.751$, $P \leq .01$). Growth of the uninfected strains on PDA was intermediate when compared with that of the virus-infected strains, whereas the uninfected strains generally outperformed the virus-infected strains when grown on chestnut stems (fig. 1*A*, 1*C*). The ranking of each viral subtype with respect to the growth of its host averaged across all temperatures and fungal strains was I > D > F2 > F1 on both PDA and chestnut stems, with subtype I allowing the highest growth and subtype F1 the lowest. Growth and sporulation (log-transformed data) on PDA were highly correlated ($r = 0.738$, $P \leq .01$). Both variables displayed a trend of higher values with increasing temperatures up to 24°C (fig. 1*A*, 1*B*).

Effect of Virus Infection

The effect of each virus on each strain was expressed by the relative difference in performance between the virus-infected strain and the corresponding uninfected strain at each temperature. In figure 2, the effects of the four virus

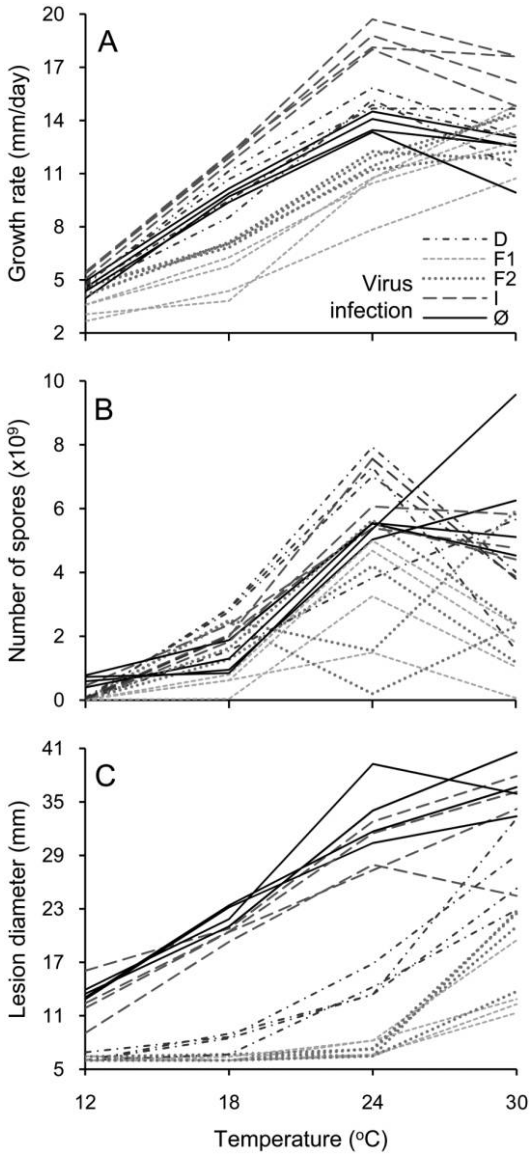

Figure 1: Interaction norms for the growth rate (in colony diameter) on agar medium during the phase of linear growth (*A*), the number of spores produced on agar medium after 31 days of incubation (*B*), and growth (lesion diameter) on dormant chestnut stems after 18 days of incubation (*C*). Lines represent the mean performance ($n = 6$) of each combination between four *Cryphonectria parasitica* strains and the four *Cryphonectria* hypovirus-1 subtypes. Different line styles refer to different subtypes (D, F1, F2, and I) and uninfected control strains (Ø). A color version of this figure is available in the online edition of the *American Naturalist*.

subtypes on the host phenotype averaged across all fungal strains are displayed for each temperature. The effect of virus infection on all three measured traits differed significantly (at $\alpha < 0.01$) among temperatures and viral subtypes. When focusing on each fungus-virus combination at each temperature separately (table B1), both qualitative and quantitative differences were evident (tables B1–B3). The growth rate on PDA of nearly all strains at all temperatures was significantly (at $\alpha < 0.01$) increased after infection with subtype I. Subtype D did not significantly (at $\alpha < 0.01$) alter the growth rate of most strains at all temperatures. The subtypes F1 and F2 significantly (at $\alpha < 0.01$) reduced growth of most strains at 12°, 18°, and 24°C, but at 30°C they significantly increased growth in four cases, significantly reduced growth in one case, and exhibited no significant effect in three cases. The sporulation of most fungal strains on PDA was significantly (at $\alpha < 0.01$) reduced by all viral subtypes at 12°C when compared with the uninfected strains. At 18°C, sporulation was generally increased after virus infection, but this effect was not significant (at $\alpha < 0.01$). At 24° and 30°C, only a few significant (at $\alpha < 0.01$) effects were observed, and most of these resulted from an infection with the subtypes F1 or F2, reducing sporulation of the host. Growth on chestnut stems of all fungal strains was not significantly (at $\alpha < 0.01$) affected by infection with subtype I at any temperature. Subtypes D, F1, and F2 always significantly (at $\alpha < 0.01$) reduced lesion diameter in combination with all fungal strains at 12°, 18°, and 24°C. In contrast, at 30°C, only subtype F1 reduced the growth of all strains significantly (at $\alpha < 0.01$), while subtype F2 significantly (at $\alpha < 0.01$) reduced growth of only two strains and subtype D did not exhibit any significant (at $\alpha < 0.01$) effect on any of the strains.

With our fully reciprocal set of fungus-virus combinations, each virus was infecting its home and three away hosts, allowing us to test for local adaptation. The effect of virus infection did not differ significantly (at $\alpha < 0.01$) between home and away hosts for all three measured traits, with a single exception of subtype F1, which reduced the growth rate on PDA of its home host more than of the away hosts (results not shown).

Discussion

Significance of the Genotype-by-Genotype-by-Environment Interaction

In this study, we found a highly significant genotype-by-genotype-by-environment interaction between fungal strains, hypovirus subtypes, and temperature in the chestnut blight pathosystem. Furthermore, we observed crossing interaction norms (i.e., rank switches of genotype-by-genotype combinations across temperatures), suggesting the potential for a selection mosaic in the studied host-parasite system. To our knowledge, this is the first report of a genotype-by-genotype interaction in a host-parasite system

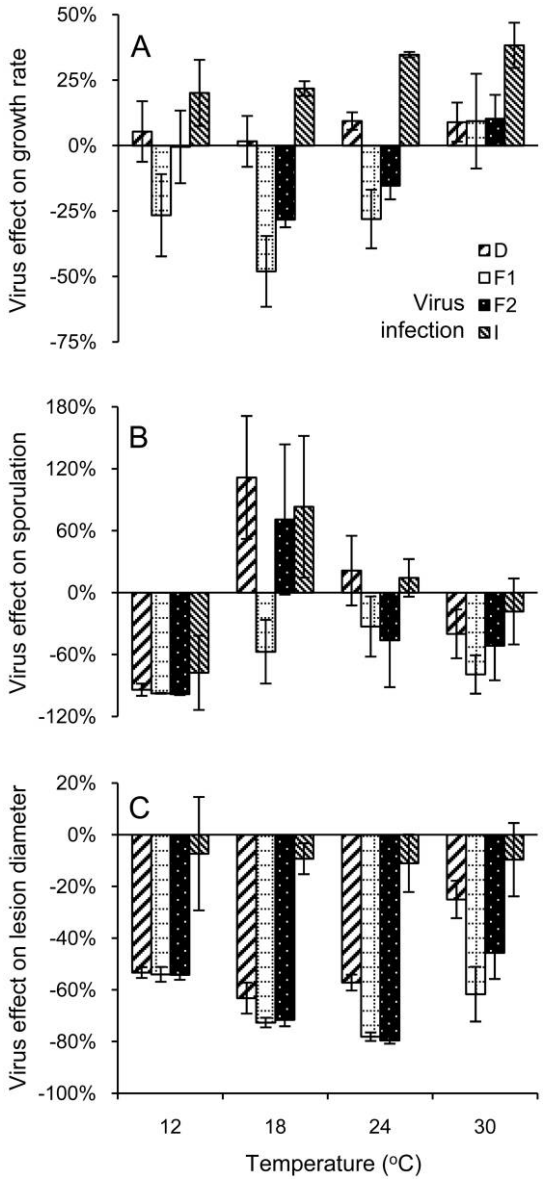

Figure 2: Effect of virus infection on growth rate (in colony diameter) on agar medium during the phase of linear growth (*A*), sporulation on agar medium after 31 days of incubation (*B*), and growth (lesion diameter) on chestnut stems after 18 days of incubation (*C*). The virus effect is the difference in performance between the virus-infected colony and the corresponding uninfected control as a proportion (%) of the performance of the uninfected control. For each *Cryphonectria* hypovirus-1 subtype (D, F1, F2, and I), the average effect across four strains of *Cryphonectria parasitica* at each temperature is displayed. Error bars represent standard deviations.

that is modified by the abiotic environment. These results provide evidence that temperature potentially impacts the coevolutionary trajectory of host-parasite interactions. At each of the four experimental temperatures, a significant genotype-by-genotype interaction between the host and the parasite was found on agar medium, affecting the phenotypic expression of the infection. Therefore, the ability of a certain hypovirus subtype to manipulate the physiology of a particular fungal strain seems to be temperature dependent. Interestingly, most of the crossing interaction norms were observed between 24° and 30°C, while interaction norms at 12°–24°C were nonparallel but mostly noncrossing. This indicated that most qualitative differences in the outcome of the host-parasite interaction in this pathosystem can be expected at increased temperatures.

To evaluate in a more natural system the relevance of the results obtained on agar medium, we applied the same full factorial design in an experiment on dormant chestnut stems. As observed in previous experiments (Chen et al. 2000; Robin et al. 2010), variation among replicates was substantially higher on dormant chestnut stems than on agar medium, where growth conditions can be better standardized. A high level of standardization is required for the resolution of factorial interactions. Variation in conditions can obscure existing effects, and this most likely explains why the genotype-by-genotype-by-environment interaction was not significant with the given sample size in our experiment on chestnut stems. On agar medium, the virus-free cultures performed worse than several virus-infected cultures, whereas they outperformed (almost all) virus-infected cultures when grown on chestnut stems, in accordance with other studies (Chen and Nuss 1999; Chen et al. 2000; Robin et al. 2010). However, as our main focus was the host-parasite interaction, we were mostly interested in the virus-infected cultures. In our experiment, the performance of the virus-infected cultures on the two types of media was correlated; in particular, the ranking among the CHV1 subtypes remained the same. We conclude that the pattern observed on dormant chestnut stems supports the biological relevance of the genotype-by-genotype-by-environment interaction obtained on agar medium. Results of previous research further showed that growth measures on agar medium explain the virulence of *Cryphonectria parasitica* in living chestnut sprouts (Dunn and Boland 1993) and the fitness and long-term persistence of CHV1 in the field (Robin et al. 2010). The fitness measures we assessed are key components of chestnut blight epidemics. The damage that *C. parasitica* causes to the tree is strongly related to the growth characteristics of the fungus (Bazzigher 1981; MacDonald and Fulbright 1991), while the degree of sporulation defines the spread of the disease as well as the potential for hypovirus dissemination.

In natural populations, an additional level of complexity is introduced by the third species in the tritrophic interaction of the chestnut blight pathosystem, the chestnut tree. Several surveys in natural populations of European and American chestnut have revealed a general suscepti-

bility to the disease, with little variation (Bazzigher 1981; Roane et al. 1986). The absence of significant resistance within populations can be explained by the lack of coevolution with the pathogen (Anagnostakis 1987), as *C. parasitica* is an introduced pathogen to both Europe and North America. The impact of hypovirulence on disease severity generally outweighs the impact of partial tree host resistance (Van Alfen et al. 1975; Fulbright 1984; Roane et al. 1986), and the tree genotype might therefore have little impact on the fungus-virus interaction. This could be different in resistant Asian chestnut populations, where the native chestnut species (*Castanea crenata* and *Castanea mollissima*) have coevolved with *C. parasitica* (Anagnostakis 1987). In resistant trees, growth and establishment of *C. parasitica* are highly constricted (Graves 1950), and a stronger selection could be expected against phenotypes of fungus-virus interactions with little growth and sporulation. Furthermore, the degree of expressed resistance might depend on the interaction between environmental factors and tree genotype (Browder 1985; Carson and Carson 1989). In resistant chestnut populations, the influence of genetic variation in resistance might therefore be complex and could strengthen or weaken the effect of the fungus-by-virus-by-temperature interaction.

With this study, we met the need to provide statistical confirmation of genotype-by-genotype-by-environment interactions and explore the potential for selection mosaics (Thomas and Blanford 2003; Gomulkiewicz et al. 2007; Piculell et al. 2008; Vale et al. 2008*a*; Lazzaro and Little 2009; Wolinska and King 2009). In line with the proposed stepwise approach for the dissection of the coevolutionary dynamics of host-parasite interactions (Gomulkiewicz et al. 2007; Piculell et al. 2008), our goal was not to demonstrate an actual selection mosaic in natural populations but to ask whether a selection mosaic could serve as a starting point for divergent selection in the particular host-parasite interaction under study. Our results suggest that different host and parasite genotypes could indeed be favored under different temperatures. The climatic conditions encountered in the chestnut growing regions are very diverse and include Mediterranean, oceanic, and continental climates. If the same *C. parasitica* and CHV1 genotypes are introduced to two regions that differ in temperature, the thermal specificity of the fungus-virus interaction would lead to different coevolutionary trajectories. Ultimately, genetically different local populations of *C. parasitica* and CHV1 would evolve, thus shaping the course and expression of local chestnut blight epidemics.

Potential Effects of Climatic Variation on the Chestnut Blight Pathosystem

Predicting the effect of environmental factors on phenotype expression is also important for biological control. In regions where chestnut blight is still active, biological control treatments with CHV1 could provide a means for containing the epidemic. However, the results of this study imply that the outcome of hypovirus applications on different fungal populations and under a different climate will be unpredictable, given the fungus-by-virus-by-temperature interaction. The suitability of a certain hypovirus subtype for biological control depends on both the temperature and the fungal host genotype. Therefore, different management strategies must be designed for different regions, and the most suitable biological control agent must be evaluated for each case individually.

In most European regions, chestnut blight does not currently pose a mortal threat to chestnut forests, because of the high prevalence of hypovirulence. However, the disease has not been eradicated and is still widespread, making it possible for a temperature increase to disturb the subtle host-parasite interaction between *C. parasitica* and CHV1. Climate change is expected to lead to more frequent temperature extremes during summer (Easterling et al. 2000). The significant fungus-by-virus-by-temperature interaction indicates that the outcome of any given fungus-virus interaction could change when temperatures increase and that an extreme summer heat event could turn a formerly hypovirulent fungus more virulent.

In evolutionary terms, chestnut blight is still a young disease in Europe, presumably explaining why we did not find evidence for local adaptation in our study. Little is known about the evolutionary trajectory of the fungus-virus interaction, and this makes predictions for the future difficult. Such knowledge, however, would be essential to design sustainable management strategies. Therefore, further research should be directed at investigating the fungus-virus coevolution in various environments and over an extended period of time. Our study is a first step in understanding how reciprocal selection acts in this pathosystem, and it highlights the importance of genotype-by-genotype-by-environment interactions for coevolution.

Acknowledgments

We thank D. Bühler, R. Holderegger, P. Spaak, and two anonymous reviewers for helpful comments on the manuscript. We acknowledge S. Ghosh, L. Rosinus, and H.-R. Roth for statistical advice and P. Barnett for the English revision of the manuscript. We are grateful to H. Blauenstein, S. Durot, and E. Jung for their help in the laboratory and to M. Conedera for providing the chestnut stems. This study represents part of the research project "The Role of Genetic Diversity in Host-Pathogen Interactions in Dynamic Environments" (GEDIHAP), funded by the Competence Center Environment and Sustainability (CCES) of the Eidgenössische Technische Hochschule Domain.

Literature Cited

Anagnostakis, S. L. 1982. Biological control of chestnut blight. Science 215:466–471.

———. 1987. Chestnut blight: the classical problem of an introduced pathogen. Mycologia 79:23–37.

Anagnostakis, S. L., and D. E. Aylor. 1984. The effect of temperature on growth of *Endothia* (*Cryphonectria*) *parasitica* in vitro and in vivo. Mycologia 76:387–397.

Bazzigher, G. 1981. Selection of blight-resistant chestnut trees in Switzerland. European Journal of Forest Pathology 11:199–207.

Benkman, C. W. 1999. The selection mosaic and diversifying coevolution between crossbills and lodgepole pine. American Naturalist 153:75–91.

Blanford, S., M. B. Thomas, C. Pugh, and J. K. Pell. 2003. Temperature checks the Red Queen? resistance and virulence in a fluctuating environment. Ecology Letters 6:2–5.

Breuillin, F., C. Dutech, and C. Robin. 2006. Genetic diversity of the chestnut blight fungus *Cryphonectria parasitica* in four French populations assessed by microsatellite markers. Mycological Research 110:288–296.

Browder, L. E. 1985. Parasite: host: environment specificity in the cereal rusts. Annual Review of Phytopathology 23:201–222.

Carius, H. J., T. J. Little, and D. Ebert. 2001. Genetic variation in a host-parasite association: potential for coevolution and frequency-dependent selection. Evolution 55:1136–1145.

Carson, S. D., and M. J. Carson. 1989. Breeding for resistance in forest trees: a quantitative genetic approach. Annual Review of Phytopathology 27:373–395.

Chen, B. S., and D. L. Nuss. 1999. Infectious cDNA clone of hypovirus CHV1-Euro7: a comparative virology approach to investigate virus-mediated hypovirulence of the chestnut blight fungus *Cryphonectria parasitica*. Journal of Virology 73:985–992.

Chen, B. S., L. M. Geletka, and D. L. Nuss. 2000. Using chimeric hypoviruses to fine-tune the interaction between a pathogenic fungus and its plant host. Journal of Virology 74:7562–7567.

Choi, G. H., and D. L. Nuss. 1992. Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. Science 257:800–803.

Conedera, M., P. Krebs, W. Tinner, M. Pradella, and D. Torriani. 2004. The cultivation of *Castanea sativa* (Mill.) in Europe, from its origin to its diffusion on a continental scale. Vegetation History and Archaeobotany 13:161–179.

Dawe, A. L., and D. L. Nuss. 2001. Hypoviruses and chestnut blight: exploiting viruses to understand and modulate fungal pathogenesis. Annual Review of Genetics 35:1–29.

Dawkins, R., and J. Krebs. 1979. Arms races between and within species. Proceedings of the Royal Society B: Biological Sciences 205:489–511.

Dunn, M., and G. Boland. 1993. Hypovirulent isolates of *Cryphonectria parasitica* in southern Ontario. Canadian Journal of Plant Pathology 15:245–252.

Easterling, D. R., G. A. Meehl, C. Parmesan, S. A. Changnon, T. R. Karl, and L. O. Mearns. 2000. Climate extremes: observations, modeling, and impacts. Science 289:2068–2074.

Elliston, J. E. 1985. Characteristics of dsRNA-free and dsRNA-containing strains of *Endothia parasitica* in relation to hypovirulence. Phytopathology 75:151–158.

Fulbright, D. 1984. Effect of eliminating dsRNA in hypovirulent *Endothia parasitica*. Phytopathology 74:722–724.

Gobbin, D., P. J. Hoegger, U. Heiniger, and D. Rigling. 2003. Sequence variation and evolution of *Cryphonectria* hypovirus 1 (CHV-1) in Europe. Virus Research 97:39–46.

Gomulkiewicz, R., and M. Kirkpatrick. 1992. Quantitative genetics and the evolution of reaction norms. Evolution 46:390–411.

Gomulkiewicz, R., J. N. Thompson, R. D. Holt, S. L. Nuismer, and M. E. Hochberg. 2000. Hot spots, cold spots, and the geographic mosaic theory of coevolution. American Naturalist 156:156–174.

Gomulkiewicz, R., D. M. Drown, M. F. Dybdahl, W. Godsoe, S. L. Nuismer, K. M. Pepin, B. J. Ridenhour, C. I. Smith, and J. B. Yoder. 2007. Dos and don'ts of testing the geographic mosaic theory of coevolution. Heredity 98:249–258.

Graves, A. H. 1950. Relative blight resistance in species and hybrids of *Castanea*. Phytopathology 40:1125–1131.

Heiniger, U., and D. Rigling. 1994. Biological control of chestnut blight in Europe. Annual Review of Phytopathology 32:581–599.

Hillman, B. I., and N. Suzuki. 2004. Viruses of the chestnut blight fungus, *Cryphonectria parasitica*. Advances in Virus Research 63: 423–472.

Hillman, B. I., R. Shapira, and D. L. Nuss. 1990. Hypovirulence-associated suppression of host functions in *Cryphonectria parasitica* can be partially relieved by high light intensity. Phytopathology 80:950–956.

Hoeksema, J. D., B. J. Piculell, and J. N. Thompson. 2009. Within-population genetic variability in mycorrhizal interactions. Communicative and Integrative Biology 2:110–112.

Laine, A. L. 2007. Pathogen fitness components and genotypes differ in their sensitivity to nutrient and temperature variation in a wild plant-pathogen association. Journal of Evolutionary Biology 20: 2371–2378.

———. 2008. Temperature-mediated patterns of local adaptation in a natural plant-pathogen metapopulation. Ecology Letters 11:327–337.

———. 2009. Role of coevolution in generating biological diversity: spatially divergent selection trajectories. Journal of Experimental Botany 60:2957–2970.

Lazzaro, B. P., and T. J. Little. 2009. Immunity in a variable world. Philosophical Transactions of the Royal Society B: Biological Sciences 364:15–26.

Little, T. J. 2002. The evolutionary significance of parasitism: do parasite-driven genetic dynamics occur *ex silico*? Journal of Evolutionary Biology 15:1–9.

Liu, Y. C., P. Cortesi, M. L. Double, W. L. MacDonald, and M. G. Milgroom. 1996. Diversity and multilocus genetic structure in populations of *Cryphonectria parasitica*. Phytopathology 86:1344–1351.

MacDonald, W. L., and D. W. Fulbright. 1991. Biological control of chestnut blight: use and limitations of transmissible hypovirulence. Plant Disease 75:656–661.

Milgroom, M. G., and P. Cortesi. 2004. Biological control of chestnut blight with hypovirulence: a critical analysis. Annual Review of Phytopathology 42:311–338.

Mitchell, S. E., E. S. Rogers, T. J. Little, and A. F. Read. 2005. Host-parasite and genotype-by-environment interactions: temperature modifies potential for selection by a sterilizing pathogen. Evolution 59:70–80.

Nuss, D. L. 2005. Hypovirulence: mycoviruses at the fungal-plant interface. Nature Reviews Microbiology 3:632–642.

Peever, T. L., Y. C. Liu, P. Cortesi, and M. G. Milgroom. 2000. Variation in tolerance and virulence in the chestnut blight fungus-

hypovirus interaction. Applied and Environmental Microbiology 66:4863–4869.

Piculell, B. J., J. D. Hoeksema, and J. N. Thompson. 2008. Interactions of biotic and abiotic environmental factors in an ectomycorrhizal symbiosis, and the potential for selection mosaics. BMC Biology 6:23.

Prospero, S., M. Conedera, U. Heiniger, and D. Rigling. 2006. Saprophytic activity and sporulation of *Cryphonectria parasitica* on dead chestnut wood in forests with naturally established hypovirulence. Phytopathology 96:1337–1344.

Rigling, D., U. Heiniger, and H. Hohl. 1989. Reduction of laccase activity in dsRNA-containing hypovirulent strains of *Cryphonectria* (*Endothia*) *parasitica*. Phytopathology 79:219–223.

Roane, M., G. Griffin, and J. Elkins. 1986. Chestnut blight, other *Endothia* diseases, and the genus *Endothia*. APS Monograph Series. American Phytopathological Society, St. Paul, MN.

Robin, C., S. Lanz, A. Soutrenon, and D. Rigling. 2010. Dominance of natural over released biological control agents of the chestnut blight fungus *Cryphonectria parasitica* in south-eastern France is associated with fitness-related traits. Biological Control 53:55–61.

Taylor, D., A. Jarosz, D. Fulbright, and R. Lenski. 1998. The acquisition of hypovirulence in host-pathogen systems with three trophic levels. American Naturalist 151:343–355.

Tétard-Jones, C., M. A. Kertesz, P. Gallois, and R. F. Preziosi. 2007. Genotype-by-genotype interactions modified by a third species in a plant-insect system. American Naturalist 170:492–499.

Thomas, M. B., and S. Blanford. 2003. Thermal biology in insect-parasite interactions. Trends in Ecology & Evolution 18:344–350.

Thompson, J. N. 1988. Variation in interspecific interactions. Annual Review of Ecology and Systematics 19:65–87.

———. 1999. Specific hypotheses on the geographic mosaic of coevolution. American Naturalist 153:1–14.

Thompson, J. N., and J. J. Burdon. 1992. Gene-for-gene coevolution between plants and parasites. Nature 360:121–125.

Toju, H., and T. Sota. 2006. Imbalance of predator and prey armament: geographic clines in phenotypic interface and natural selection. American Naturalist 167:105–117.

Vale, P. F., and T. J. Little. 2009. Measuring parasite fitness under genetic and thermal variation. Heredity 103:102–109.

Vale, P. F., L. Salvaudon, O. Kaltz, and S. Fellous. 2008*a*. The role of the environment in the evolutionary ecology of host parasite interactions: meeting report, Paris, 5th December 2007. Infection Genetics and Evolution 8:302–305.

Vale, P. F., M. Stjernman, and T. J. Little. 2008*b*. Temperature-dependent costs of parasitism and maintenance of polymorphism under genotype-by-environment interactions. Journal of Evolutionary Biology 21:1418–1427.

Van Alfen, N. K., R. A. Jaynes, S. L. Anagnostakis, and P. R. Day. 1975. Chestnut blight: biological control by transmissible hypovirulence in *Endothia parasitica*. Science 189:890–891.

Wolinska, J., and K. C. King. 2009. Environment can alter selection in host-parasite interactions. Trends in Parasitology 25:236–244.

Zangerl, A. R., and M. R. Berenbaum. 2003. Phenotype matching in wild parsnip and parsnip webworms: causes and consequences. Evolution 57:806–815.

Zhang, L., R. A. Baasiri, and N. K. Van Alfen. 1998. Viral repression of fungal pheromone precursor gene expression. Molecular and Cellular Biology 18:953–959.

Associate Editor: Jukka Jokela
Editor: Judith L. Bronstein

European chestnut forest. Some trees were killed by virulent *Cryphonectria parasitica* strains, but most trees were healthy. They were infected with virus-infected *C. parasitica* strains. Photograph by Sarah F. Bryner.