Temperature mediates vector transmission efficiency: inoculum supply and plant infection dynamics

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Abstract
Climate, particularly environmental temperature, frequently plays an important role in disease epidemiology. This study investigated the role of environmental temperature on transmission of the generalist plant pathogen Xylella fastidiosa by its leafhopper vectors. In this system temperature is known to influence both vector performance and feeding rate, yet the implications for pathogen transmission have not been documented. Experiments were conducted over a range of temperatures to document effects on transmission efficiency of the California native Graphocephala atropunctata Signoret (blue–green sharpshooter) and the invasive Homalodisca vitripennis Germar (glassy-winged sharpshooter). Inoculation efficiency of H. vitripennis was positively related to temperature. G. atropunctata mortality and transmission responded non-linearly to temperature, with the highest rates of both at the highest temperature. The experiment also evaluated whether differences in inoculum supply contributed to plant infection level using quantitative PCR. Although total X. fastidiosa population within G. atropunctata was not related to plant infection, the number of infectious vectors was a strong predictor of plant infection level—suggesting that the number of inoculation events is important in the development of systemic infection of X. fastidiosa in grapevines. These results, along with existing evidence from the literature, point to wide-ranging impacts of climate on the epidemiology of X. fastidiosa diseases.

Introduction
In disease ecology, an understanding of the transmission process is fundamental to describing the spatial epidemiology and dynamics of host–pathogen interactions (Jeger et al., 1998; Ng & Perry, 2004). This view is fuelled by the recognition that the effective implementation of disease management practices requires knowledge of the numerous biological features that mediate pathogen transmission (Almeida et al., 2005). In the case of vector-borne pathogens, sources of ecological variability in transmission rate include: vector abundance (Ng & Perry, 2004; Daugherty & Almeida, 2009), vector species (Palermo et al., 2001; Daugherty & Almeida, 2009), host species (Wistrom & Purcell, 2005), pathogen strains (Lucio-Zavaleta et al., 2001) and local climate (Shih et al., 1995; Dohm et al., 2002). One way in which the last of these, climate, is frequently important is in the effect of temperature, which can influence all aspects of the vector–pathogen–host interaction.

Temperature-dependent transmission efficiency is a common feature of many vector-borne disease systems. Local environmental temperature is known to affect the competence of mosquito (Dohm et al., 2002) and tick (Shih et al., 1995) vectors of human and wildlife pathogens. Moreover, such effects are well documented on the transmission efficiency of numerous insect-borne plant pathogens (Sylvester, 1964; Murral et al., 1996; Lucio-Zavaleta et al., 2001; Anhalt & Almeida, 2008). The temperature at which transmission occurs can
mediate the efficiency of this process in at least four ways, by affecting: (a) pathogen multiplication in source hosts (Feil & Purcell, 2001), (b) pathogen multiplication within vectors (i.e. vector latent period—Dohm et al., 2002), (c) the establishment of infection in the host (i.e. minimum infective dose—Chu & Volety, 1997) or (d) vector behaviour [e.g. feeding rate (Su & Mulla, 2001), probing behaviour (Sylvester, 1964), or movement (Vail & Smith, 2002)]. One system for which these effects of temperature may be prevalent is in the transmission of the generalist plant pathogen *Xylella fastidiosa* by sharpshooter leafhoppers.

*Xylella fastidiosa* is a xylem-limited bacterium that is pathogenic to several economically important agricultural crops in both North and South America, including grape, alfalfa, almond, citrus, coffee, peach and plum (Hopkins & Purcell, 2002). In grapevines, *X. fastidiosa* causes Pierce’s disease, which is characterised by progressive leaf scorching, defoliation and cane or whole plant death of susceptible vines within a few years. Pierce’s disease has been present in vineyards in the western USA since at least the 1880s (Purcell, 1989), and studies of pathogen transmission and vector efficiency have been conducted in this system since at least the 1940s. Consequently, much is known about the transmission biology of *X. fastidiosa* and its vectors.

In California, *X. fastidiosa* is transmitted to grapevines by several species of xylem sap-feeding insects, the most significant being sharpshooter leafhoppers (Hemiptera: Cicadellidae; Severin, 1949). Within this group of leafhoppers, two of the most important vector species are the native *Graphocephala atropunctata* Signoret (blue–green sharpshooter; Purcell, 1975; Purcell, 1989) and, since the 1990s, the invasive *Homalodisca vitripennis* Germar (glassy-winged sharpshooter; Almeida et al., 2005). There is no vertical transmission of *X. fastidiosa* (Freitag, 1951). Sharpshooters must acquire the pathogen by feeding within-vessel elements colonised by *X. fastidiosa*. Successful transmission requires the bacterium to attach to the foregut of vectors (Almeida & Purcell, 2006). Following acquisition, sharpshooters are able to inoculate *X. fastidiosa* successfully with no apparent latent period (Purcell & Finlay, 1979). Finally, although there is no evidence for specificity in interactions between *X. fastidiosa* and xylem sap-feeding insects (Purcell, 1989), transmission rates of this pathogen vary greatly among vector species and as a function of plant access period (Daugherty & Almeida, 2009).

Evidence suggests that temperature may contribute to variation in sharpshooter transmission efficiency. *Xylella fastidiosa* growth rate, for example, is positively associated with temperature up to 31°C (Feil & Purcell, 2001). This is noteworthy because *X. fastidiosa* acquisition by sharpshooters is related to plant infection level (Hill & Purcell, 1997), suggesting that acquisition efficiency should be temperature-dependent. With respect to pathogen inoculation, both sharpshooter survival and feeding rate are known to depend on temperature (Johnson et al., 2006). Although vector abundance is clearly related to inoculation rate (Daugherty & Almeida, 2009), the effect of behavioural shifts such as changing feeding rates are not well understood. It is plausible that, as is the case for other vector-borne disease systems (McElroy et al., 1997; Su & Mulla, 2001), higher sharpshooter feeding rates or number of probing events increase pathogen inoculum supply, thereby increasing transmission success or plant infection level. This hypothesis, however, has not been tested explicitly in the *X. fastidiosa*–sharpshooter system.

Two experiments were conducted to test the hypothesis that environmental temperature mediates sharpshooter transmission efficiency of *X. fastidiosa* to grapevines. These experiments were conducted at multiple temperatures with both *H. vitripennis* and *G. atropunctata*. The second experiment also evaluated whether differences in inoculum supply affect plant infection dynamics.

### Materials and methods

#### Homalodisca vitripennis transmission

A first experiment tested for the effects of temperature on the *X. fastidiosa* transmission efficiency of *H. vitripennis*. The individuals used in this experiment were the F1 progeny of insects collected earlier in the summer from a citrus orchard in Riverside, California, USA, and raised on mixed plantings of healthy sweet basil (*Ocimum basilicum* L.), grape (*Vitis vinifera* L. var. Cabernet Sauvignon) and mugwort (*Artemisia douglasiana* Bess.) that were replaced weekly. Sharpshooter nymphs shed their foregut lining between molts and hence lose any attached *X. fastidiosa*; thus, regular replacement of host plants ensures that developing adults are clear of infection.

*H. vitripennis* adults were caged in groups of approximately 40 individuals on *X. fastidiosa*-infected (Stag’s Leap strain—STL: Napa Valley, CA, USA) rooted grapevine cuttings for a 4-day acquisition access period (AAP). During the first 3 days of this AAP, source plants were all housed in a single shared insectary greenhouse at the Oxford Tract facilities at UC Berkeley (Berkeley, CA, USA). For the fourth day, the plants and insects were moved to an environmental chamber set to a constant temperature of 25°C to facilitate their acclimation. Afterwards, *H. vitripennis* were confined in pairs on healthy grape seedlings (var. Cabernet Sauvignon) grown in 10-cm pots covered with fine mesh sleeve cages (10 cm × 20 cm)
and were placed in environmental chambers set to one of three temperature regimes: 20°C, 25°C or 30°C day; 20°C night; 14:10 (L:D) photoperiod. This experiment was conducted in environmental chambers because of severe restrictions on working with *H. vitripennis* in field or non-insectary greenhouse settings. After a 4-day inoculation access period (IAP), all insects were removed and plant infection status was determined 12–15 weeks later via plate culturing (Hill & Purcell, 1995). Temperatures were recorded hourly over this period. There were 30–32 individual plants with insects at each of the three temperatures, which were assumed to be independent replicates for the purpose of statistical analysis. In addition, there were five plants caged without insects at each temperature to serve as non-inoculated controls.

**Graphocephala atropunctata transmission**

A second experiment tested effects of both temperature and inoculum supply on transmission by *G. atropunctata* and on *X. fastidiosa* infection dynamics. *X. fastidiosa*-free *G. atropunctata* adults were caged on known *X. fastidiosa*-infected grape cuttings (STL strain) in groups of approximately 40 (approximately 200 total insects split among five source plants). These sharpshooters were from a greenhouse colony established from insects collected earlier that summer along the Russian River near Forestville, California, USA, and then raised on healthy sweet basil plants. After a 4-day AAP, *G. atropunctata* were removed from source plants, then either one or four insects were confined on healthy grape cuttings (var. Cabernet Sauvignon) inside a 10 cm × 20 cm mesh sleeve cage. These different vector numbers were used as a natural manipulation of potential inoculum supply to plants. Grape cuttings were in 3.8-L pots with Supersoil potting soil (Rod Mclellan Company, San Mateo, CA, USA), and plants were pruned 1 week before similar sizes. These caged plants were then placed in one of the three rooms at the Jane Gray Research Greenhouse (Berkeley, USA), and plants were pruned 1 week before similar sizes. These suspensions were used for plate serial dilutions to quantitate *X. fastidiosa* population contained within insects—as a proxy for the potential inoculum supplied into each replicate grapevine cutting. After removing the insects, plants were returned to the temperature-controlled rooms for 10 weeks post-insect introduction. At 3, 6 and 9 weeks' post-insect introduction, petioles were sampled from each plant to estimate *X. fastidiosa* populations in the plant. At each sampling, two petioles were randomly selected from the region of the vine covered originally by the sleeve cage. After 10 weeks, the vine stem was sampled at the point where the top of the mesh cage originally was placed. qPCR was used to estimate *X. fastidiosa* populations in both petiole and stem samples for each vine.

**Quantitative PCR**

For the *G. atropunctata* experiment, qPCR was used to quantify *X. fastidiosa* populations in insects and grapevines. For plant samples, 150 mg of stem or petiole tissues were processed. The two petioles sampled per vine were pooled, then these samples were processed separately for each replicate vine for each sampling period—and separately from stem samples. For insects, all *G. atropunctata* were processed individually, with the whole insect body used in the extraction process. DNA from plant and insect samples was extracted according to Francis et al. (2006). For the standard curve, *X. fastidiosa* DNA was extracted from suspensions of cultured cells using the Qiagen DNeasy blood and tissue kit (Hilden, Germany) with the pretreatment for gram-negative bacteria, according to the manufacturer’s instructions. These suspensions were used for plate serial dilutions to correlate the starting number of cells with DNA content. *Xylella fastidiosa* was quantified in samples extracted from plants and insects by absolute quantification on a 7500 realtime thermocycler (Applied Biosystems, Foster City, CA) using *Xylella*-specific primers GL5 and GL6 designed by Francis et al. (2006) and SYBR Green. Individual samples were run in triplicate 20 μL reactions. For the standard curve, DNA from five dilutions of plate-cultured *X. fastidiosa* (3, 30, 300, 3000, 30 000 colony forming units) was diluted in DNA extracted from healthy grape (for the quantification of *X. fastidiosa* in grape samples) or uninfected sharpshooter DNA (for the quantification of *X. fastidiosa* in *G. atropunctata* samples). Each of these five standards was included in triplicate in each plate. PCR mixes with DNA from healthy plants and healthy insects were included as negative controls. Water controls, devoid of template, were also included. At the end of the PCR run, the dissociation curves were inspected to ensure that quantitation was not affected by non-specific amplification products. *Xylella fastidiosa*
populations in plant and insect samples were then expressed as concentrations of *X. fastidiosa* cells relative to total extracted DNA (# *X. fastidiosa* cell equivalents μg⁻¹ total DNA).

**Statistical analyses**

The R statistical software v. 2.8.1 was used for all statistical analyses. For the *H. vitripennis* experiment, the proportion of plants that became infected at different temperatures were compared using a generalised linear model with binomial errors (Crawley, 2007), with mean temperature as a continuous variable. This approach is equivalent to logistic regression analysis, which is necessary to describe adequately responses that are categorical in nature (e.g. infected/not infected). Source plant was included initially as a blocking factor, but was found to be non-significant (P > 0.05) and was therefore dropped from the final analyses.

For the *G. atropunctata* experiment, because of non-linear effects of temperature, mean temperature was treated as a categorical variable for all analyses. In addition, model simplification techniques (Crawley, 2007) were used when appropriate to determine the most parsimonious adequate description of response variables. For this experiment, first the proportion of *G. atropunctata* that died during the IAP as a function of temperature and vector number (one or four insects) were first compared using a generalised linear model with binomial errors. Next, vector infection levels were compared in two separate ways. A two-way generalised linear model with binomial errors was used to compare the proportion of vectors in each replicate plant that tested positive for *X. fastidiosa* according to qPCR among temperatures and vector numbers. As a second test of vector infection level among treatments, the total *X. fastidiosa* population detected by qPCR were compared among temperatures and vector group sizes using two-way analysis of variance (ANOVA), with the total population in insect groups (one or four insects) log transformed to meet test assumptions. The ANOVA tested the overall main effects of and the interaction between temperature and vector number. Differences between means for significant (P < 0.05) effects were investigated using the least significant difference (LSD) at P = 0.05.

The second set of analyses for the *G. atropunctata* experiment relates to plant infection levels. Effects of vector number and temperature on the likelihood of plant infection were tested using a generalised linear model with binomial errors. For this test, a plant was considered positive if any of its three petiole or one stem samples tested positive according to qPCR. Significant (P < 0.05) effects were followed up with comparisons between means using the LSD at the P = 0.05 level. To determine whether vector survival affected transmission, mean *G. atropunctata* mortality was correlated with the proportion of *X. fastidiosa*-infected plants among the three temperatures. Finally, analysis of covariance (ANCOVA) was used to compare plant infection levels among the three temperatures, as determined by qPCR analyses, using two separate metrics of inoculum supply as covariates: the number of infectious *G. atropunctata* in each replicate and the total *X. fastidiosa* population in vectors in each replicate (populations pooled in the four insect replicates). This last test considered only infected plants, and plant infection level was defined by *X. fastidiosa* population in the stem at week 10.

**Results**

*Homalodisca vitripennis* transmission

For the *H. vitripennis* experiment, temperatures in the three chambers averaged 20.5 °C, 24.0 °C and 28.5 °C over the 4-day IAP (range: 18.3–22.1 °C, 17.6–26.3 °C and 19.2–33.0 °C, respectively). Vector mortality was low, with less than 4% (7 of 190) of the *H. vitripennis* dying during the experiment. None of the negative control plants became infected. There was a significant effect of temperature on infection rate (χ² = 15.427, d.f. = 1, P < 0.001). Plants at the highest temperature were 13 times more likely to become infected than at the lowest temperature, and infection rates of plants in the middle temperature were intermediate between the other two (Fig. 1).

*Graphocephala atropunctata* transmission

For the *G. atropunctata* experiment, observed temperatures in the greenhouse rooms were different than targeted, averaging 17.4 °C, 21.4 °C and 24.3 °C over the course of the 2-day IAP (range: 14.4–21.9 °C, 16.6–28.1 °C and 17.3–31.1 °C, respectively). Vector mortality was higher than in the previous experiment. The best-fit model included effects of temperature but no effect of vector number. The effect of temperature on mortality was significant (χ² = 15.326, d.f. = 2, P < 0.001). Mortality at 21.4 °C did not differ significantly (P > 0.05) from 17.4 °C (Diff. = −1.027, LSD = 1.043, d.f. = 101), but it was lower and significantly different (P < 0.05) than at 24.3 °C (Diff. = 2.027, LSD = 0.989, d.f. = 100; Fig. 2). With respect to vector infectivity, the best-fit model included only a constant, with no effects of temperature or vector number. The value of this constant (±SE) is −0.355 (±0.167), meaning that the back-transformed proportion of infectious vectors was,
on average, 0.412. However, there were significant effects of temperature ($F_{2,55} = 5.471$, $P = 0.007$), vector number ($F_{1,55} = 33.343$, $P < 0.001$), and a temperature by vector number interaction ($F_{2,55} = 5.736$, $P = 0.006$) on the total X. fastidiosa population. Xylella fastidiosa populations in individual G. atropunctata ranged from approximately 3 to 800 X. fastidiosa cells μg$^{-1}$ total DNA. Bacterial populations were consistently low across temperatures in the single vector replicates but were higher and significantly different ($P < 0.05$) in the four vector replicates, especially at the highest temperature (Fig. 3). An increase from one to four vectors increased total pathogen populations in vectors, on average, by 2.865 log (# X.f. cells μg$^{-1}$ total DNA) at 17.4$^\circ$C, 0.808 at 21.4$^\circ$C and 4.793 at 24.3$^\circ$C.

Overall 35% of plants (22 of 63) in the G. atropunctata experiment tested positive in at least one petiole or stem sample, whereas none of the control plants became infected. For four of these cases (three with a single vector, one with four vectors), the vectors did not test positive, suggesting false negatives occurred using qPCR as a detection method. Nine of the 22 infected plants had negative petiole samples but positive stem samples, suggesting petiole tissue samples are a less reliable indicator of plant infection level. For the proportion of plants infected, there were significant effects of temperature ($\chi^2 = 6.823$, d.f. = 2, $P = 0.033$), vector number ($\chi^2 = 10.910$, d.f. = 1, $P = 0.001$) and a temperature by vector number interaction ($\chi^2 = 9.290$, d.f. = 2, $P = 0.010$). The proportion of plants
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Figure 4 Proportion of plants to which G. atropunctata transmitted X. fastidiosa (±SE) versus number of vectors and temperature. The generalised linear model fit at different temperatures is denoted by the lines. Proportions are offset slightly for clarity. Standard errors of proportions were calculated as: \[ \sqrt{\frac{p(1-p)}{n}} \], where \( p \) is the overall proportion of plants infected, and \( n \) is the number of replicates [n(1 vs 4 vectors) = 12 vs 9, 10 vs 10 and 11 vs 10 for 17.4°C, 21.4°C and 24.3°C, respectively].

Infected was approximately 45% higher in four vector groups compared with single vectors (0.183 vs 0.548, respectively) and was highest at the highest temperature (0.306, 0.200 and 0.591 at 17.4°C, 21.4°C and 24.3°C, respectively). Yet the proportion of plants infected by 4 vs 1 G. atropunctata was 27.7% higher at 17.4°C, it was equivalent at 21.4°C, and it was 81.8% higher at 24.3°C (Fig. 4). The observed G. atropunctata mortality and plant infection rates were correlated strongly among temperatures, although not significantly so (r = 0.990, n = 3, F1,1 = 47.231, P = 0.092). Finally, for X. fastidiosa populations in stem samples of infected plants, the best-fit model included only an effect of the number of infectious vectors, with no effects of temperature or total pathogen supply in driving plant infection dynamics. The results suggest an important role of environmental temperature in mediating transmission in this system.

For both G. atropunctata and H. vitripennis, the proportion of plants that became infected with X. fastidiosa differed among temperatures. This effect was clearest in the consistent increase in the proportion of infected plants as a function of temperature in the H. vitripennis experiment. For G. atropunctata, transmission rates were non-linearly related to temperature, with the highest infection rates occurring at the highest temperature. These results support studies of other insect-borne plant pathogens, including both aphid-transmitted viruses (Sylvester, 1964; Lucio-Zavaleta et al., 2001; Anhalt & Almeida, 2008) and leafhopper-transmitted bacteria (Mural et al., 1996). It is important to note that the current two experiments, like most of these vector studies, were conducted in the laboratory or greenhouse over a limited range of temperatures. Such experiments can document whether climate-dependent transmission occurs and provide some insights into why it occurs. However, a broad understanding of the biological importance of this phenomenon requires more extensive field experiments over a wide range of conditions.

As noted earlier, effects of temperature on transmission efficiency can manifest through effects on vector latent period, source host infection level, minimum infective dose or vector behaviour. Given that X. fastidiosa transmission does not appear to have a latent period (Purcell & Finlay, 1979), this mechanism is not likely to

Discussion

The goals of this project were to quantify how temperature affects the transmission efficiency of two important X. fastidiosa vectors in California vineyards and to investigate the relative importance of inoculum

Figure 5 Xylella fastidiosa population in grapevines stems at 10 weeks [log (μg X.f. cells μg−1 total DNA)] versus the number of infectious G. atropunctata, according to qPCR. Regression line: \( y = 1.89x + 3.770 \), d.f. = 1, 20, \( s^2 = 2.733 \), \( R^2 = 0.292 \).
be important. Likewise, although transmission efficiency depends on pathogen population within plants (Hill & Purcell, 1997), which is in turn dependent on temperature (Feil & Purcell, 2001), source plants were held under the same conditions—thus this mechanism is not likely to explain the observed results. Instead, the observed effects of temperature occur because of the last two mechanisms, infective dose and vector behaviour. Results from a study that varied X. fastidiosa inoculum supply mechanically inoculated to coffee and citrus (Prado et al., 2008) are consistent with there being a minimum infective dose for infection, although how it is affected by temperature and what this dose is for Pierce’s disease in grapes have not been described. Observations of sharpshooter feeding show that temperature affects xylem sap intake rates (Johnson et al., 2006), which may affect pathogen transmission. The H. vitripennis experiment described effects during only the inoculation phase, for which effects of vector behaviour and perhaps infective dose may manifest. However, the G. atropunctata experiment included by design effects of temperature beyond transmission, potentially accentuating any effects of temperature on the minimum dose required for infection establishment. Although these studies did not quantify directly vector behaviour or manipulate infective dose, results from the G. atropunctata experiment may provide insights into their role in sharpshooter transmission and plant infection establishment.

Graphocephala atropunctata transmission rate was strongly correlated with mortality rates. This positive correlation was not significant, however, because of low statistical power associated with only three levels being correlated. Yet if this correlation is biologically meaningful, it suggests that stressed and dying insects were also more likely to transmit. Sharpshooters and other insect vectors alter their feeding behaviour (i.e. feeding rate, probing site) and movement under different environmental conditions (Sylvester, 1964; Su & Mulla, 2001; Vail & Smith, 2002; Johnson et al., 2006). Such differences in behaviour can have implications for pathogen transmission. More explicit measures of sharpshooter behaviour are needed to evaluate the precise mechanism by which temperature mediates inoculation efficiency.

The other notable outcome of the G. atropunctata transmission experiment relates to the role of inoculum supply in this system. For some plant pathogens, higher inoculum supply increases transmission success (Bressan et al., 2006). To date, the only identified predictor of sharpshooter transmission success is whether or not certain regions of a sharpshooter’s mouthparts are colonised by X. fastidiosa (Almeida & Purcell, 2006). The amount of X. fastidiosa within sharpshooters does not equate to higher transmission rate (Hill & Purcell, 1995; Jackson et al., 2008). This suggests that greater absolute pathogen supply is unlikely to explain why temperature affected sharpshooter transmission efficiency. The results of the current experiments compliment these studies in that higher potential inoculum supply by G. atropunctata did not affect plant infection level. The number of inoculation events appears to be a more relevant metric of inoculum supply in this system than is the absolute concentration of pathogen being introduced into plants. It is important to recognise the difference between potential inoculum supply, the total pathogen population within vectors, and actual inoculum supply, the number of pathogen cells inoculated into a host. This study measured potential inoculum supply in insect groups, which varied as a function of temperature and, especially, the number of G. atropunctata (Fig. 3). This distinction is important because potential supply can be a poor indicator of actual supply in both this system (Jackson et al., 2008) and for other vector-borne pathogens (Medica & Sinnis, 2005). Currently, an understanding is lacking of the factors governing release of bacteria from sharpshooter mouthparts (Jackson et al., 2008).

Thus, X. fastidiosa supply and infection establishment events appear stochastic in nature. If so, more infectious vectors should introduce redundancy into the inoculation process, promoting systemic infection.

Climate can affect the epidemiology of vector-borne diseases by affecting vector abundance or performance (Su & Mulla, 2001; Johnson et al., 2006), hosts recovery from infection (e.g. Palermo et al., 2001; Feil et al., 2003) or transmission efficiency (Sylvester, 1964; Murral et al., 1996; Lucio-Zavaleta et al., 2001). The current study concentrated on the importance of this last mechanism. Yet, all three of these mechanisms may play a role in this system, especially for H. vitripennis-mediated disease spread. Results in existing literature (Feil & Purcell, 2001; Al-Wahaibi & Morse, 2003; Hoddle 2004), in addition to the effects noted here, suggest the potential for wide-ranging and important effects of temperature on the epidemiology of Pierce’s disease in California.

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