Probability of occurrence and phenology of pine wilt disease transmission by insect vectors in the Rocky Mountains

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Abstract
1. Pine wilt disease, caused by pinewood nematode (Bursaphelenchus xylophilus; abbreviated ‘PWN’), is a damaging and globally distributed insect-vectored forest pathogen. Native forest tree mortality associated with PWN is newly reported from the Front Range of Colorado, but there is no regional information on PWN frequency or biology of local insect vectors, limiting management options.
2. A sampling array was established to survey PWN in native pines (Pinus ponderosa) and longhorn beetles (Monochamus clamator and M. scutellatus) over 2 years and across natural and urban forest landscapes. We developed flight phenology models and evaluated effects of landscape factors on vector abundance and probability of infection.
3. Flight phenology was similar for vectors; Monochamus flight initiated in mid-July and continued into October for both species. We report the first M. clamator–PWN association in the United States. PWN was distributed in the region at rates lower than reported from its putative native range: 3.6 and 4.2% of sampled pines and beetles, respectively, tested positive for PWN. Many host trees were outwardly asymptomatic; infection frequency in tree populations varied considerably and four epicentres of vector infectivity were identified.
4. Epicentres varied in timing of anomalous infective vector frequency – some epicentres had high abundances of infected beetles early in the growing season whereas others had high abundances of infected beetles late in the growing season, though PWN-positive beetles were captured at all sites. Monochamus populations were found primarily in natural forest stands but migrated to urban areas late in the growing season. The only landscape factor positively correlated with abundances of both Monochamus species was distance to previous wildfire.
5. PWN epicentres in the southern Rocky Mountains exhibit specific temporal windows of vector activity that differ from proximal sites. Urban forests, where the disease was initially observed in the region, do not support vector populations. Our results suggest that natural forest landscapes in the region are important reservoirs of PWN, and vector populations are especially abundant near burned stands. Collectively, our findings...
INTRODUCTION

Pine wilt disease is a lethal vascular wilt of conifers caused by an infection of the pinewood nematode (*Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle; hereafter PWN) (Kiyohara & Tokushige, 1971). This nematode is among the greatest biotic threats to pine forests globally (Vicente, Espada, Vieira, & Mota, 2012; Webster & Mota, 2008). The pathogenicity of PWN varies greatly depending on differences in host susceptibility, abiotic conditions, and secondary infections (Rutherford, Mamiya, & Webster, 1990). The PWN is hypothesized to be native to America, ranging from the south-eastern United States northward into the Great Lakes area (Dwinell, 1989) and throughout Canada (Bowers et al., 1992). In these areas, PWN is found in the natural landscapes where the native conifers are highly resistant to pine wilt disease (Dropkin, 1981; Wingfield, Blanchette, Nichols, & Robbins, 1982). PWN has been introduced to Asia, where it has spread through Japan, China, and Korea (Zhao et al., 2008), before it was first detected in 1982. PWN has been introduced to Asia, where it has spread through Japan, China, and Korea (Zhao et al., 2008), before it was first detected in Europe in both Portugal and Spain (Robertson et al., 2011). Pine species found in these introduced ranges are highly susceptible to pine wilt disease and projections of uncontrolled spread of PWN exceed $1B (USD) per year in losses to timber resources (Soliman et al., 2012).

In North America, concern for PWN first arose after pine wilt disease was reported in 1979 in Missouri on Austrian pine (*Pinus nigra* Arnold) (Dropkin & Foudin, 1979). Pine species that are highly susceptible to pine wilt disease, including Scot’s pine (*P. sylvestris* L.), Mugo pine (*P. mugo* Turra), and Austrian pine (*P. nigra*), are commonly planted as ornamentals in urban forests. The disease has moved westward with cases reported in Kansas (Robbins, 1979), Indiana (Marshall & Favinger, 1980), Illinois (Malek & Appleby, 1984), and Nebraska (Gleason et al., 2000). The first report of pine wilt disease in the Rocky Mountain region was made in the Front Range region of Colorado in 2006 on Scot’s pine (*P. sylvestris* L.) (Blunt, Jacobi, Appel, Tisserat, & Todd, 2014), with subsequent detections in symptomatic native ponderosa pine (*P. ponderosa* Douglas ex. Lawson) in 2016 (Atkins et al., 2020). These observations of pine wilt disease and associated mortality in ponderosa pine in the Rocky Mountain region suggests that this pathosystem is rapidly spreading (Ostfeld, Glass, & Keesing, 2005). Consequently, there is a need to describe the factors that contribute to probability of pine wilt disease transmission in the Front Range region, as many species that are considered susceptible to pine wilt are present in both wildland and urban settings throughout the western United States (Mamiya, 1983).

The PWN has an obligate vector relationship with long-horned beetles (Coleoptera; Cerambycidae). Specifically, PWN relies on beetles in the genus *Monochamus* (sawyer beetles) for transmission between hosts (Akbolut & Stamps, 2012). *Monochamus* spp. are cosmopolitan and multiple sympatric species colonize pines throughout the northern hemisphere (Bergdahl, 1988). Colorado has two species of *Monochamus* that are putatively capable of vectoring PWN: *M. clamosus* Leconte and *M. scutellatus* Say. Beetles are attracted to weakened or damaged trees and may be abundant in recently disturbed stands (Costello, Negrón, & Jacobi, 2011; Saint-Germain, Drapeau, & Hébert, 2004). Nematodes colonize beetles during pupation and are subsequently transmitted to susceptible hosts during early-season adult maturation feeding (Linit, 1990) or following dispersal and oviposition (Wingfield & Blanchette, 1983). Visible symptoms are apparent 15–20 d following infection and include wilting of needles and changes in colour from green to reddish to light brown.

Symptom expression coincides with rising summer temperatures and high evapotranspirative demand (Kuroda, Yamada, Mineo, & Tamura, 1988). Accordingly, the life cycle of PWN is sensitive to shifts in ambient temperature and PWN infections are most severe in areas where mean summer temperatures regularly exceed 20°C (Rutherford & Webster, 1987). Temperatures are rising across Colorado with many municipalities exhibiting average summer temperatures above 20°C (Figure S1), which could correspond with increased PWN detection and tree mortality (Rutherford & Webster, 1987). In addition, many of the pines planted in urban areas are highly susceptible (e.g. Scot’s pine and Austrian pine). Due to the proximity of Colorado to areas where PWN is established and the concurrent westward-advancing disease front, it is unclear whether PWN is newly introduced to the system or if rising temperatures and drought are inciting pathogenesis. This distinction between an introduced or emergent pathogen will inform best management procedures as an established population of a latent pathogen may be nearly impossible to eradicate. Native pines in the Rocky Mountain region are far too numerous to survey with any effect; however, previous work in this pathosystem has yielded a highly attractive lure for capturing *Monochamus* (Miller et al., 2013). Comparing the frequency of PWN-phoresy in *Monochamus* vectors to that of areas where PWN is known to be established and describing landscape-level distribution of the vectors will yield insight into the status of PWN in native systems (Kitron, 1998). In the case of a newly introduced pathogen, a patchy distribution with foci of high infection frequencies, or ‘epicentres’ of disease is more likely to be observed (Anderson et al., 2004).

Presently, regional prevalence of PWN in native forest trees (ponderosa pine) and vector populations is poorly understood, and there
is little information on the dispersal behaviours of putative vectors or the landscape factors associated with their densities. However, an understanding of PWN infection rates and the factors that may predict disease transmission are crucial for developing integrated pest management strategies to curtail establishment of a persistent disease cycle and limit potential westward expansion of PWN within native forests in the United States. Our objectives were to (1) describe vector abundances and flight phenology across the region; (2) characterize PWN infection rates in insect vectors and host tree populations (ponderosa pine) in natural landscapes; and (3) identify disease epicentres and model drivers of infection probability. This knowledge will provide new information on temporal windows of exposure to PWN in both wildland and urban locations, as well as landscape and spatial factors associated with vector abundances and disease prevalence, with implications for management of an economically and ecologically injurious agent of forest disease.

2 | MATERIALS AND METHODS

2.1 | Site measurements and sampling of PWN in trees and beetles

A sampling array was established to regionally survey for PWN in host trees and insect vectors. Sites were established in wildland-urban interface (WUI; N = 32; Stewart, Radeloff, Hammer, & Hawbaker, 2007) and urban (N = 12) greenspaces (Table S1). Sites were located 50–500 m from roads where ponderosa pine (P. ponderosa) was the dominant canopy species. Urban greenspaces were located in municipalities of Fort Collins, Loveland, Boulder, and Golden (Colorado, USA) where Austrian pine (P. nigra) or Scots pine (P. sylvestris) were dominant canopy species (three locations in each municipality). Urban greenspaces were selected to be as close to WUI forests (west) as possible while still having >5 trees within the urban greenspace. Sites elevation ranged from 1725 to 2567 m (GIS, ARCMAP 10.4, ESRI, Inc.).

At WUI sites several forest measurements were conducted on 0.04 hectare fixed-area plots including tree species and diameters-at-breast-height (DBH; 1.3 m), crown-class (suppressed, intermediate, co-dominant, or dominant), presence/absence of visible fungal infection, and fire damage for all trees with DBH > 2 cm (Figures S2–S5). Site aspect and hillslope were recorded. Landscape variables including distance to recently burned stands (km), distance to edge of ponderosa pine canopy (km), per cent canopy cover (250 m radius), and distance to nearest urban area (population > 2000; km) were derived for each study site using a geographic information system (GIS, ARCMAP 10.4; ESRI, Inc.) (USDA Forest Service). Heat-load index (McCune & Keon, 2002), a metric of radiative forcing (MJ·cm⁻¹·year⁻¹) incorporating slope, aspect, and latitude, was also calculated for each site. These variables were used to develop predictive models of beetle abundance.

To estimate PWN infection frequency in host trees at sample sites, branch and sawdust samples were taken from a subset of 6–10 randomly selected ponderosa pine trees per site (DBH > 10 cm). A 20-cm section proximal to the bole from each of two branches was taken from each selected tree using a pole pruner. Sawdust was collected from two holes drilled on opposing N and S aspects at 1.3 m height on the bole with an auger-style drill bit (15 mm) to a depth of 6 cm. Tissues samples from each tree were homogenized into a composite sample and nematodes were extracted using the Baermann funnel method (Viglierchio and Schmitt, 1983); extracted nematodes were stored at ~20°C until molecular testing. To sample insect vectors, black crossvane traps were centrally placed in each plot and (described in Morewood, Hein, Katinic, & Borden, 2002) supplied a diffuse pesticide (No. Pest 2 Strips; Dichlorvos; 18.6% 2,2-dichlorovinyl dimethyl phosphate; Hot Shot Corp., St. Louis, MO, USA) to kill captured insects. Traps were baited with lures containing host tree volatiles, ethanol, monochamol and ipsdienol (Monochamus lite combo lure - lot #546371; Synergy Semichemicals, Victoria, BC, Canada). In 2018 and 2019, traps were visited weekly after commencement of the beetle flight season (July) until flight termination (October) (2018: N = 13 weeks; 2019: N = 15 weeks). Urban sites were sampled only during 2019. Collected specimens were stored at ~20°C until molecular testing to preserve nematode DNA.

All captured Monochamus beetles and Baermann extracts of wood tissues (WUI N = 289; urban N = 42 samples) were subsequently analysed for the presence of PWN using a molecular assay. Beetles were bisected longitudinally and homogenized using a sterile micropesctle prior to analysis. Baermann extracts of tree tissues and homogenized beetle tissues were tested for PWN using a loop-mediated isothermal amplification (LAMP) assay (Bx Detection Kit, Lot #’s 29000HL, Nippon Gene Co., Tokyo, Japan) according to methods of Kikuchi, Aikawa, Oeda, Karim, and Kanzaki (2009). Samples were resolved to the individual level (i.e. all sampled trees and captured beetles were tested). This molecular assay is commonly employed in the invasive range of PWN and is 1000 times more sensitive than traditional PCR approaches (Kikuchi et al., 2009). Presence/absence data were recorded via this assay as opposed to attempting to quantify nematode load/beetle for two reasons: (1) the captured vectors were far too numerous, and (2) the aim of the study was to identify areas where PWN was present rather than evaluate vector competency.

2.2 | Data analysis

All analyses were performed in the R statistical programming environment and unless otherwise stated use a Type I error rate of α = 0.05 for assigning statistical significance (R Core Team, 2019).

Flight phenology for M. clator and M. scutellatus was modelled using a two-parameter logistic regression (function ‘nplr’, Commo & Bot, 2016) with ordinal day as the independent variable and cumulative proportion of captures as the response variable. Initiation, peak and termination of flight were approximated using 10%, 50% and 90% cumulative capture for each species and site × year combination to evaluate differences in phenology between vectors and years (Tables S2 and S3). Flight synchrony was estimated by solving growth rate of logistic curves at 50% capture – greater flight synchrony is consistent with more rapid logistic growth (Dell & Davis, 2019). Only sites with 10 or more captures recorded for each species were considered reliable
for informing species-level flight phenology models (M. clamator N = 30 sites, M. scutellatus N = 18 sites). Phenology thresholds (mean dates of flight initiation, peak and termination) and flight synchrony were compared between beetle species using a two-sample Student’s t-test.

To evaluate effects of landscape factors on vector abundances, beetle capture abundances were modelled for each species using a multiple-regression model selection using distance to burned stands (burned since year 2000), elevation, heat–load index, distance to ponderosa pine cover boundary (USDA Forest Service, GTAC, 2008), distance to city edge, and canopy cover as predictors. Trap-capture data were root-transformed where necessary in order to meet assumptions of normality and heteroscedasticity. Sample year was included as a random effect, and models were selected via minimization of Akaike’s information criterion, AIC (Akaike, 1974) with a ΔAIC threshold of 2 (function ‘drdreg’, Barton, 2019).

Vector beetle species and sex ratios were compared using Chi-squared tests. The probability of vector association with PWN was evaluated using a log-likelihood mixed-effects modelling approach (Bates et al., 2015). Factors considered included both vector species (N = 2 factor levels) and sex (N = 2 factor levels), as well as day-of-year of capture (continuous effect) and capture year (N = 2 factor levels). Site (N = 44) was included as a random effect and evaluated using a likelihood ratio test (P = 1).

The recency of first reports of PWN in Colorado indicate that the disease may not be established uniformly in the region, with infections radiating from central locations or (i.e. epicentres). This may be observable via differences in patterns of disease incidence throughout the growing season. Here, disease epicentres were defined as sites with an occurrence of spatiotemporal outliers in the frequency of infection in vector captures during either year (Kitron, 1998). Identification of epicentres was made using a scanning statistic to identify sites or aggregate zones where the rate of infection is dissimilar to proximal areas. Epicentres were identified using the function ‘scan_{eb:poisson}’ with 999 Monte Carlo iterations (package ‘scanstatistics’, Allévius, 2018). This function computes an expectation-based Poisson scan statistic useful for identifying anomalous spatiotemporal clusters of disease incidence and is commonly employed in human epidemiological studies for a similar purpose (Kulldorff, Heffernan, Hartman, Assunção, & Mostashari, 2005). The method compares all possible temporal windows for each group in each zone list to test a null hypothesis of spatiotemporal randomness using a likelihood ratio statistic. This analysis allows for the possibility of sampling from within a population of vectors twice by considering similarities in infection frequencies between nearest neighbour groups and across the flight season. The site list used all possible levels of nearest neighbour combinations for sites grouped within an area while excluding combinations that would include a nearest neighbour from a geographically discrete (> 5 km distance) area based on reported vector flight capacity (Akbulut & Linit, 1999; Togashi & Shigesada, 2006). To validate findings, a second model using sites with an identified spatiotemporal anomaly with an interactive site-by-date term was used to test the hypothesis that the likelihood of capturing infected vectors is higher early in the vector flight season. This pattern is consistent with results reported from the south-eastern United States where PWN is long-established (Pimentel, Ayres, Vallery, Young, & Streett, 2014). Observing significant interactive effects with date would serve as further evidence that previously identified epicentres reflect patterns observed in established systems.

### RESULTS

#### 3.1 Objective 1: Describe vector abundances and flight phenology across the region

In total 5146 beetles were captured at WUI sites: 4068 M. clamator (2018, N = 1822; 2019, N = 2246) and 1078 M. scutellatus (2018, N = 364; 2019, N = 714). In 2019, 42 M. clamator and 12 M. scutellatus were captured at urban sites. The species ratio was similar between WUI and urban sites for 2019 (N = 3014; \( \chi^2 = 0.01; p = 0.92 \)). Mean trap captures at study sites were ~95% greater at WUI sites (92.5 beetles ± 10.3) than urban sites (4.5 beetles ± 1.3) (\( t_{42} = 9.13; p < 0.001 \)). Sex ratios differed between M. clamator (60% M:40% F) and M. scutellatus (51% M:49% F) in WUI sites (N = 5199; \( \chi^2 = 5.54; p = 0.02 \)) but was similar between species (64% M:36% F) at urban sites (N = 53; \( \chi^2 = 0.23; p = 0.65 \)).

On average peak flight occurred approximately 2 weeks earlier at WUI sites (day 254 ± 2 d) than at urban sites (day 266 ± 3 d) sites. Flight phenology thresholds (initiation, peak and termination) were similar between beetle species but varied between years (\( F_{2,60} = 17.13; p < 0.001 \)). Flight typically initiated in late July or early August, peaked in late August or early September, and ended by October (Table 1). Flight phenology models showed a goodness-of-fit between 0.79 and 0.90 for both vector species over both years (Figure 1), and there was no difference in flight synchrony between species (\( t_{46} = 1.38, p = 0.18 \)).

| Flight threshold | Monochamus clamator | Monochamus scutellatus |
|------------------|---------------------|-----------------------|
|                  | 2018 | 2019 | 2018 | 2019 |
| Initiation (10% capture) | 215[Aug 03] ± 2 d | 230[Aug 18] ± 2 d | 206[Jul 25] ± 4 d | 230[Aug 18] ± 8 d |
| Peak (50% capture) | 242[Aug 30] ± 1 d | 254[Sep 11] ± 1 d | 237[Aug 25] ± 2 d | 255[Sep 12] ± 3 d |
| Termination (90% capture) | 269[Sep 26] ± 1 d | 278[Oct 05] ± 1 d | 269[Sep 26] ± 2 d | 280[Oct 07] ± 3 d |

### TABLE 1 Estimated day of year (mean ± SE) of flight phenology thresholds for each vector species and sample year, derived from solutions of logistic models of beetle accumulations at trapping sites
FIGURE 1  Aggregate flight phenology models derived from all site-by-week captures for (a) *M. clamator* (*N* = 30 sites; 4068 beetles) and (b) *M. scutellatus* (*N* = 18 sites; 1078 beetles) for 2018 (*N* = 13 weeks) and 2019 (*N* = 15 weeks) in the Front Range region of Colorado

Trap captures of both beetle species were predicted by landscape factors, particularly distance to recent fires. Abundances of *M. clamator* varied with distance to fire and elevation. Abundances were negatively correlated with distance to fire (β = −0.028, *p* = 0.002) and elevation (β = −0.386, *p* = 0.006), and abundances of *M. scutellatus* were similarly negatively correlated with distance to fire (β = −0.018, *p* = 0.005; Figure 2). There was no effect of collection year, canopy cover (250 m radius), heat–load index (HLI) or distance to forest edge or nearest city on abundances of either species (Table 2). Non-significant predictors collectively explained less than 2% of the variance in the data.

3.2  Objective 2: Characterize PWN infection rates in host tree and vector populations

PWN was detected in 3.6% of ponderosa pine trees sampled and at 9% of study sites (3 out of 32 WUI sites). The PWN-infection frequency ranged from 10 to 89% for the three sites where PWN-positive hosts were identified. PWN was not encountered in any trees tested from urban sites (*P. nigra*, *N* = 28; *P. sylvestris*, *N* = 14; Figure 3).

PWN was detected in 3.1% of sampled *M. clamator* and 7.8% of sampled *M. scutellatus*. Infection frequency in captured vectors ranged from 0 to 38% (mean 4.2%). Across both years, at least one positive vector was captured at all WUI sites. For both 2018 and 2019, 16/32 (50%) of WUI sites had at least one positive vector.

3.3  Objective 3: Identify disease epicentres and model drivers of infection probability

Analysis with a generalized linear model revealed that infection probability of insect vectors captured during flight was significantly related to beetle species, study year and day of year. The infection rate was similar between males and females (β = 0.002, *p* = 0.99) among both species. *M. scutellatus* was significantly more likely to vector PWN than *M. clamator* (β = 1.03, *p* = < 0.001), but there was no interaction between vector sex and species (β = −0.42, *p* = 0.18; Table 3). Mean probability of infection was ∼2x higher for *M. scutellatus* than *M. clamator*. Beetles captured during 2019 were roughly twice as likely to be infective as those captured in 2018. Overall, beetles captured earlier in the season were more likely to be infective than those captured near flight termination (*p* < 0.001). Beetle sex and site landscape type did not affect the probability of infection (Table 3).

Four instances of spatiotemporal outliers in infection rates of captured vectors were identified from four unique sites in 2018 and 2019. In 2018, a single site (14) was identified as having a higher frequency of infection in captured vectors during the first 3 weeks of vector flight. In 2019, three areas (sites 4, 14 and 15, and 23) were identified as having a higher frequency of infection in captured vectors (Figure 4). These areas varied in the timing of anomalous infection frequency and in one instance two neighbouring sites (14 and 15) were reported to have similar elevated infection rates during the same temporal window, indicating that these sites represent a single population. The site identified in 2018 was part of this pair identified again in 2019 (Table 4). Two of the four sites identified in this manner showed significant seasonal patterns in vector infectivity consistent with the native range of PWN (Table 5).

4  DISCUSSION

Our study shows that both *Monochamus* spp. found abundantly along the Front Range of Colorado are vectors of PWN, but the frequency of association with PWN ranged widely across sites (0–38%; mean 4.2%) depending on time of year and beetle species. Infective vectors were captured at all locations, but only 50% of sites recorded positive captures in both years. We report for the first time that *M. clamator* is a common vector of PWN in the southern Rocky Mountain region, but *M. scutellatus* were ∼2 times more likely to be associated with PWN. However, *M. clamator* was 73% more abundant over the course of the
FIGURE 2  Regression models showing relationship between (a) *M. clamator* abundance per site as a function of distance to burned stands and (b) elevation and (c) *M. scutellatus* abundance per site as a function of distance to burned stands. Outer lines show the 95% confidence interval of each fit.

TABLE 2  Summary of a generalized linear mixed-effects model for predicting abundances of *Monochamus* species in the Front Range region of Colorado. Significant effects are indicated in bold.

| Parameter                        | *Monochamus clamator* | *Monochamus scutellatus* |
|----------------------------------|-----------------------|--------------------------|
|                                  | Estimate  | SE    | t-score | p   | Estimate  | SE    | t-score | p   |
| Random Effects                   |           |      |         |     |           |      |         |     |
| Year (Intercept)                 | 0.003     | 0.003 | –       | –   | 0.001     | 0.002 | –       | –   |
| Year residual variance           | 0.002     | 0.001 | –       | –   | 0.003     | 0.002 | –       | –   |
| Fixed Effects                    |           |      |         |     |           |      |         |     |
| (Intercept)                      | 25.96     | 7.77  | 3.342   | <0.001 | 4.819     | 0.389 | 12.365  | <0.001 |
| Distance to fire (km)            | –28.028   | 9.047 | –3.145  | 0.002 | –0.018    | 0.006 | –2.943  | 0.005 |
| Elevation (m)                    | –0.386    | 0.141 | –2.737  | 0.006 | 0.196     | 0.122 | 1.612   | 0.112 |
| Canopy cover (%)                 | 0.009     | 0.019 | 0.516   | 0.613 | 0.001     | 0.016 | 0.028   | 0.978 |
| HLI (MJ/cm²)                     | –0.161    | 3.764 | –0.043  | 0.971 | 2.319     | 3.246 | 0.715   | 0.478 |
| Distance to forest edge (km)     | –12.033   | 10.022 | 1.071   | 0.285 | 3.012     | 10.021 | 0.325   | 0.746 |
| Distance to nearest city (km)    | –8.023    | 10.014 | –0.813  | 0.414 | –18.034   | 18.019 | 1.094   | 0.283 |

study and overall comprised 60% of PWN-positive vectors, indicating *M. clamator* as the primary vector of PWN in the region. Our analysis identified epicentre areas that may serve as regional reservoirs of PWN disease and confirms that vectors captured from epicentres are more likely to be infected during flight initiation with delayed risk of exposure at proximal sites. Accordingly, probability of disease transmission is higher at epicentres early in the flight period while proximal sites are exposed following beetle dispersal (Kitron, 1998). This pattern matches trends observed in areas with well-established PWN populations (Pimentel et al., 2014). Epicentres occurred near areas frequented by tourists and recreationists and should serve as foci for integrated pest management efforts.

Monochamus flight phenology in the southern Rocky Mountains occurs later than has been described from warmer regions. For instance, *M. galloprovincialis* in Portugal and *M. alternatus* in Japan often fly in early May, while we did not observe flight of either focal species until mid-July in both years of study. The same flight period is observed in two sympatric vector species (*M. carolinensis* and *M. titillator*) located
FIGURE 3  Map of the locations of urban and WUI sites where tree tissues and beetles were collected in the Front Range region. This figure shows the abundance and relative proportion of captured vector species (i), percentage of vectors that tested positive for PWN (ii) and the percent of sampled trees that tested positive for PWN along with the four identified anomalous infection frequency sites (iii). Distribution of *P. ponderosa* is also shown in each panel.

TABLE 3  Summary of a generalized linear mixed-effects model for predicting probability of infection among all *Monochamus* spp. captured in the Front Range region of Colorado. Significant effects are indicated in bold.

| Parameter       | Estimate | SE    | z-score | P    |
|-----------------|----------|-------|---------|------|
| (Intercept)     | -1.903   | 1.217 | -1.563  | 0.118|
| Sex: Male       | 0.002    | 0.184 | 0.010   | 0.992|
| Sp. *M. scutellatus* | 1.03  | 0.176 | 5.865   | <0.001|
| Year 2019       | 0.605    | 0.158 | 3.826   | <0.001|
| Site type WUI   | 1.379    | 1.022 | 1.336   | 0.182|
| Day of year (DoY) | -0.013 | 0.003 | -4.165  | <0.001|
| Sex: Species    | -0.418   | 0.315 | -1.326  | 0.185|

In the south-eastern United States the PWN’s putative native range (Alya & Hain, 1985). No flying beetles were captured before July 1. and approximately 50% of all vector flight activity occurred during the 3-week period from mid-August to September. We report similar flight phenology between *M. clamator* and *M. scutellatus*, suggesting that monitoring and management efforts (e.g. trapping or spraying) are likely to target both species simultaneously. However, continued warming may impact voltinism or increase duration of flight periods by reducing flight synchrony (Azrag et al., 2020; Dell & Davis, 2019), which could have negative consequences for host trees with high late season evaporative demands (Kwon et al., 2019). Differences between timing of flight periods observed in Colorado and those in areas where mortality due to pine wilt disease is more frequent warrant further investigation.

Habitat distribution modelling was consistent with previous findings that fire disturbances impact regional *Monochamus* densities (Costello et al., 2011; Saint-Germain et al., 2004) and the abundances of both beetle species increased with proximity to burned areas. Consequently, locations near recently burned stands may be at higher risk of PWN exposure. However, *Monochamus*-driven PWN transmission can also be affected by other forest disturbances.
TABLE 4 Spatiotemporal epicentres with identified anomalous frequency of vector infection

| Spatiotemporal epicentre | Sites         | Year (Weeks) | p-value |
|-------------------------|---------------|--------------|---------|
| A                       | Site 14       | 2018 (1–3)   | 0.02    |
| B                       | Site 4        | 2019 (3)     | <0.001  |
| C                       | Site 14 and 15| 2019 (1–3)   | <0.001  |
| D                       | Site 23       | 2019 (11)    | 0.03    |

This table shows the results from the function `scan_eb_poisson`, which is used to identify spatiotemporal windows where populations exhibit significantly higher rates of infection. For each identified spatiotemporal window, we report the associated study site(s) where the observation occurred and the temporal window – study year and seasonal week(s) of vector flight – during which the infection frequency was greater than other sites. When more than one site is listed, these sites were considered to be part of a single population. Pp-values are from an expectation-based test of observed values compared to the null Poisson distribution.

For example, PWN infections in *P. taeda* and *P. echinata* are associated with southern pine beetle (*Dendroctonus frontalis* Zimmermann, Coleoptera: Scolytinae) outbreaks in the south-eastern United States (Kinn & Linit, 1992). It is unknown whether similar patterns occur in the southern Rocky Mountains, but bark beetle outbreaks are regionally prevalent (Veblen, 2000) and associations with multiple disturbance types could further promote PWN transmission. Also, high elevations were associated with reduced *M. clamator* abundances, but this was not the case with *M. scutellatus*. Study sites spanned an elevational range of 850 m, which likely reflects a thermal gradient; *M. clamator* was more prevalent than *M. scutellatus* in warmer sites, although both species are abundant at > 45° N latitude (Bowers et al., 1992). These findings are applicable to management of PWN regionally and elsewhere as they define landscape factors that can be mapped to vector abundance.

PWN incidence across the landscape suggests a patchy distribution of PWN in both ponderosa pine stands and in *Monochamus* beetles (Figure 3). No obvious regional pattern was observed in the distribution of PWN-positive hosts, though relatively small fixed-area plots and uneven within-host distribution of PWN may have limited our ability to detect such a signal. *Monochamus* spp. can disperse 3–7 km per year (Akbulut & Linit, 1999), and the delayed and relatively low captures in...
urban areas indicate that in this instance urban disease pressure probably originates from proximal natural forest landscapes. In the case of PWN and ponderosa pine, the reason for rising tree mortality reported throughout the region is yet undescribed. Detection of asymptomatic host pines suggests that mortality in natural stands may either be dependent on abiotic stress or coupled with some contributing biotic factor. Consequently, PWN populations in natural forests may pose a threat to municipal trees, which are often susceptible exotic pines or horticultural species (Mamiya, 1983; Nunes da Silva, Solla, Sampedro, Zas, & Vasconcelos, 2015). However, it is unlikely that infected urban pines are able to serve as infective hosts as sanitation measures (host tree removal) are usually enacted before the vectors’ life cycle can be completed. This underscores the importance of regional management for pine wilt disease in natural forest stands to reduce potential for loss of high-value urban trees. Disease pressure radiating from forests is manageable by preventing dispersal into urban areas through trapping programs or targeted pesticide applications in areas near forests (Brockerhoff, Liebhold, & Jactel, 2006; Coyle, Nebeker, Hart, & Mattson, 2005). However, our results indicate that introductions of PWN into the urban landscape are unlikely to spread rapidly from tree to tree due to extremely low vector population densities (Yoshimura et al., 1999).

Across both years, occurrences of anomalous infection frequencies were observed in vectors captured at four of the study sites; one was from a single site in 2018 (Site 14), while the other three occurred during 2019 (Sites 4, 15, 23). Site 14 was also identified in 2019; however, in 2019 the identified areas also include Site 15 positioned ~1.3 km away from Site 14. Site 14 also exhibited the highest percentage of trees that were positive for PWN. At Site 14, the seasonal pattern of vector infectivity was similar to those observed in areas where PWN is known to be established like in the south-east United States. Previous work has shown that there is an increase of infected vectors captured early in the flight season as compared to later in the season (Pimentel et al., 2014). Interestingly, however, collections at Sites 4 and 23—two other sites with anomalous infection occurrences—did not match this pattern. Several points of evidence highlight that Site 14 is likely a disease epicentre. First, Site 14 was identified in both years of the survey. It is unlikely this would occur by chance without a persistent population of PWN nearby. Second, Site 14 contained a large proportion of hosts that tested positive for PWN (89%). While no attempt was made to quantify the number of Monochamus that had infested these specific trees, it is more likely that infective vectors would be encountered in an area where there are more PWN-positive hosts. Finally, there was strong evidence for seasonal patterns that match those observed in the native range of the nematode. The vectors captured at this location also exhibited higher infection rates early in the flight season (weeks 1–3). The timing of higher infection frequencies varied compared to other identified areas. At epicentre Site 23, higher late-season abundance of infective vectors was observed. This could be explained by several potential patterns of emergence, including re-emergence of infective vectors following initial dispersal and oviposition or completion of a second beetle generation. Both patterns would complicate management efforts targeting specific temporal windows. In either case, late-season emergences or second flights may expose trees to PWN when they are in a defence-compromised state due to drought conditions and prolonged warmer temperatures (Kolb et al., 2016). The combined effects of a changing climate on the pathosystem may compound the severity of pine wilt disease via host-mediated effects (Roques, Zhao, Sun, & Robinet, 2015). Ultimately, the risk of pine wilt disease is a factor of the rate of exposure to PWN, the natural susceptibility of the tree species to the disease and what condition that tree is in when the exposure occurs. The environmental conditions (higher temperatures) that follow this exposure are also likely to influence whether nematode populations are able to establish and persist within a host. All documented cases of asymptomatic PWN infection during this study were in mature (>30 cm DBH), otherwise vigorous ponderosa pine trees that had no evidence of other infections or damage. It is likely that these trees would be capable of maintaining a successful defence against a pathogen in the absence of other stresses.

In summary, we conclude that PWN is present throughout the Front Range and may now pose a threat in both the WUI and urban settings. The presence of PWN in the native forests threatens both landscape types through wide dispersal of two capable native vectors. New diseases resulting from the introduction of an exotic pathogen or a change in environmental conditions or novel species interaction that causes disease pose unique challenges to forest managers (Daszak, Cunningham, & Hyatt, 2000; Dobson & Foufopoulos, 2001). In the case of pine wilt disease, there are several biotic (fungi, bacteria and host susceptibility, Zhao et al., 2013) and environmental factors (increased temperatures and drought) that collectively contribute to mortality rates in pines (Lee, Nam, Choi, & Park, 2017; Ouyang & Zhang, 2003).

During the past decade, mean summer temperatures in the study region have regularly exceeded 20°C, a critical threshold for PWN reproduction and vector flight activity (Rutherford & Webster, 1987; Zhao et al., 2007a, 2007b). If these thermal trends continue, it likely indicates heightened PWN pressure for ponderosa pine forests in the southern Rocky Mountains. It remains unknown whether PWN is native to the region or if recent observations of PWN-associated tree mortality are driven by some interaction between climate conditions and host tree physiology; accordingly, landscape genetic studies of PWN in the southern Rocky Mountains and elsewhere are merited. Understanding complex interactions between pathogen genetics, climate, host tree physiology and vector dispersal behaviours are critical for managing emergent diseases in forest ecosystems and can serve to elucidate our understanding of the complex ecology underlying the spread of infectious diseases in native tree populations.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.
AUTHORS’ CONTRIBUTIONS

TSD and JES conceived the study design and acquired funds for the project; DHA performed field and lab work and collected the data; DHA analysed the data and wrote the first draft of the manuscript; all authors contributed to editing the manuscript.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.sn02v6x32 (Atkins et al., 2021).

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