

THESIS

CHARACTERIZING THE PINE WILT DISEASE PATHOSYSTEM IN THE FRONT RANGE  
REGION OF COLORADO

Submitted by

David Atkins

Department of Forest & Rangeland Stewardship

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Advisor: Seth Davis

Co-advisor: Jane Stewart

Diana Wall

Wade Tinkham

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## ABSTRACT

### CHARACTERIZING THE PINE WILT DISEASE PATHOSYSTEM IN THE FRONT RANGE OF COLORADO

Pine wilt disease, caused by the pinewood nematode (*Bursaphelenchus xylophilus*; PWN), is one of the most damaging invasive species in Asia. Tree mortality associated with PWN has recently been reported in Colorado and surrounding states. There remains little documentation on PWN incidence in native pine species or the biology of potential vectors in the Rocky Mountain region.

Here we regionally surveyed for PWN in host trees and two putative insect vectors (*Monochamus clamator* & *M. scutellatus*) for two years to develop vector flight phenology models and test the hypothesis that disturbance factors predict vector abundance across the landscape.

Flight phenology was similar between vectors: flight initiated in mid-July and continued into October for both species. PWN was distributed throughout the Front Range at rates lower than those reported in the putative native range (Host: 3.6%; Vector 4.2%). Infection rate in hosts varied among sites (0-89%), and four ‘epicenters’ of vector infectivity were identified. We also report the first incidence of PWN-*M. clamator* association in the U.S.

The four identified epicenter sites varied in the timing of anomalous infection frequency, and flight phenology of infective vectors differed between epicenter and peripheral sites.

*Monochamus* populations were found primarily in natural forest areas and seasonally migrate in

small numbers to urban areas. Landscape factors such as proximity to burned area were positively correlated with *Monochamus* abundance.

Synthesis and applications: Our study describes PWN infection frequency to be greater than that expected of a newly introduced pathogen, but lesser than the eastern United States and Canada where PWN is known to be established. Our findings provide tools that can predict exposure windows of disease exposure, which were observed to be highest in the early season in Colorado. We also describe the threat that populations of PWN in wildland forests pose to urban landscapes, and how this risk varies seasonally. These findings collectively serve characterize PWN distribution in the native ecosystem and provide tools that can be used by decision-makers and managers to proactively manage the spread of pine wilt disease.

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## TABLE OF CONTENTS

ABSTRACT.....	ii
ACKNOWLEDGEMENTS.....	iv
Chapter 1 – Literature Review .....	
Introduction.....	4
Pine wilt disease in Asia and Europe.....	4
Pine wilt disease in North America .....	8
Pine wilt disease pathosystem.....	10
Vector biology and ecology .....	13
Problem statement and knowledge gaps .....	15
Chapter 2 – Description of pinewood nematode distribution and identification of disease epicenters .....	22
Introduction.....	23
Materials & methods.....	26
Study design & site description .....	26
Data analysis .....	29
Results.....	32
Discussion.....	34
TABLES & FIGURES.....	46
APPENDIX.....	55

# CHAPTER 1

## LITERATURE REVIEW

### 1.1 Pine wilt disease: an introduction

Pine wilt disease is a lethal wilting disease in susceptible conifer species caused by the pinewood nematode (*Bursaphelenchus xylophilus* Steiner and Nickle; hereafter, PWN). The nematode reproduces rapidly after introduction into a host tree causing disease symptoms that can appear in 2-3 weeks. Initial symptoms include needle chlorosis and wilting, decreased rate of transpiration, and reduced oleoresin flow which progress rapidly to hydraulic failure and mortality in as little as two months for extremely susceptible hosts. Pine wilt disease system consists of three key trophic levels; the PWN, insect vectors (primarily Cerambycid beetles), and the fungi upon which the nematodes feed. Blue- and black-stain Ophiostomatoid fungi are commonly associated with PWN-killed pines. These fungi are also one of the best naturally occurring substrates for PWN-growth compared to other co-occurring fungi (Kobayahsi, Sasaki, & Mamiya 1974, 1975; Sriwati et al. 2007). The fungi serve as a food source for the nematode after the resources of the host tree are exhausted post-mortality (Fukushige 1991). PWN has an obligate vector relationship with long-horned beetles (Coleoptera; Cerambycidae), specifically the pine sawyer beetles of the genus *Monochamus*. *Monochamus* spp. are cosmopolitan and sympatric with host pines throughout the northern hemisphere. These beetles are typically secondary pests in their native range, however their relationship with PWN has greatly increased interest into their biology. Here, I review the literature regarding the PWN pathosystem and disease ecology along with the resulting impacts of PWN in its introduced and native ranges and address knowledge gaps that have become apparent with emphasis on recent work that has been done here in the Front Range Region of Colorado.

## 1.2 Pine wilt in Asia and Europe

Beginning in the early 20<sup>th</sup> century, widespread mortality in pines without an attributed cause was documented in Japan. Scientists initially placed the blame for this mortality on the naturally occurring sawyer beetles, *Monochamus alternatus* Hope, that were commonly found within downed pines (Yano 1913). In 1970, PWN was first isolated from dead pines in Japan (*Pinus thundbergii* Parl. & *P. densiflora* Siebold & Zucc.) and subsequently identified as the causal agent of pine wilt disease (Kiyohara & Tokushige 1971). These interactions between the nematode and *Monochamus* launched an investigation into the disease ecology of plant-insect-nematode interactions.

Although the nematode was not discovered to be the causal agent of pine wilt disease until 1970, detailed records of tree mortality allowed researchers to construct a post hoc record of disease spread. Pine wilt disease was first reported on Kyushu Island in 1905 (Yano 1913). This island is ~500 m from mainland Japan and contains patches of dense pine forests. Although managers at the time were unaware of what was causing the disease, they were able to effectively suppress the disease through aggressive sanitation measures. The disease reappeared several years later, however, and reached mainland Japan in 1921. This outbreak remained in Hyogo Prefecture for almost a decade before spreading into several neighboring prefectures. In the late 1930's and 1940's, Japan became focused on World War II and forest management efforts were abandoned almost entirely. During this period pine wilt disease caused widespread forest mortality and spread into other regions of Japan, moving into the northern remote forested areas. The Japanese government instituted a program aimed to reduce the impact of pine wilt disease which eventually led to the identification of the pinewood nematode as the causal agent (Kiyohara & Tokushige 1971). The knowledge that PWN was vectored by *Monochamus* spp. (Morimoto & Iwasaki, 1972)



led to spraying of insecticides that were aimed to prevent transmission of PWN. This slowed the rate at which the disease was spreading, but by the mid 1980's PWN was found in all but the two most northern prefectures (Futai 2008). During this same time period the first cases of PWN were reported in the Jiangsu province of China (Cheng et al. 1986). The disease spread rapidly and now is found in almost 30 prefectures in Jiangsu province. During the late 1990's and 2000's PWN spread throughout 10 other provinces in China. The Chinese Ministry of Forestry has estimated the damaged area at almost 2 M ha, with over 100 M trees succumbing to the disease (Zhang, Jianghua, & Baoxu. 2006; Zhao et al. 2008; Yu, Xu, & Ding 2011). The rapid movement of PWN through China was accompanied by the first reports of PWN in South Korea, where it was detected in 1988 (Yi et al. 1989). The nematode spread rapidly through South Korea, and although likely the first introduction was via trade channels, the disease spread naturally into 6 provinces within less than a decade (Shin, 2008). PWN was first detected in Europe in 1999 after introduction from East Asia (Mota et al. 1999; Burgermeister et al. 1999; Viera et al. 2007).

Since that time, PWN has established itself as one of the major threats to European forestry (Vincente et al. 2012; Soliman et al. 2012). Many pine species in the Iberian Peninsula, particularly Scots pine (*Pinus Sylvestris* L.), are extremely susceptible to pine wilt disease (Bakke, Anderson, & Kvamme 1991). This fact, coupled with a widely distributed and abundant vector in *Monochamus galloprovincialis* Oliver caused EPPO to classify PWN as an A1 pest (non-native, high risk, full quarantine; EPPO 2013). This was recently changed to an A2 designation (post-introduction) after scientists acknowledged that it was unlikely the disease would ever be eradicated from Portugal (EPPO 2018; Økland 2010). Containment efforts in Portugal have been extreme, with the most aggressive measure involving clearcutting a 3 km wide strip of forest bisecting one of the largest tracts of public land (EPPO 2018). PWN has been subsequently

introduced into Spain from Portugal on four separate occasions, although eradication efforts have been successful each time thus far (Robertson et al. 2011). Recent studies have estimated that if left unchecked PWN could cause \$1.2 B in damage annually to the European forestry industry over the next 30 years (Soliman et al. 2012).

In Asia there are two species of *Monochamus* that are known to vector PWN; *M. alternatus* is the primary vector both in Japan and throughout eastern Asia and *Monochamus saltuarius* Gebler, which is restricted to areas of Japan (Maehara & Futai 1996). Up until the introduction of PWN into Japanese forests, *M. alternatus* was considered a rare species. However, the increase in available oviposition sites due to trees killed by PWN is considered the most likely reason for the rise in population. Fire management practices to remove standing dead wood had made oviposition space a limiting resource before the increased mortality due to pine wilt disease (Togashi & Shigesada 2006; Togashi & Jikumaru 2007). This was documented in the occurrence patterns observed in Japanese forests where PWN outbreaks were localized in areas that had corresponding increases of *Monchamus* populations in years following PWN outbreaks (Mamiya 1984; Togahsi 1990). In forests where there is an active outbreak, mortality often exceeds 30% of the total standing trees compared to below 10% in non-infested stands (Kishi 1995). *Monochamus alternatus* life history varies greatly throughout its range. Individuals in northern Japan are regularly semivoltine, while individuals in southern China can be multivoltine and may complete 2 to 3 generations per year (Song et al. 1991). The timing of flight and flight duration also varies between species from different climates. Flight initiation occurs in April for populations in temperate zones (Asia - including Korea, Japan, & China and Portugal) and in June and July in areas with longer winters (>37°N in North America). Earlier emergence and a prolonged flight season are generally associated with multivoltine populations (Togashi & Magira 1981).

The epidemic of PWN in Asia has severe ecological and economic impacts. Economically, the losses to the timber resources have been estimated at millions of cubic meters per year in Japan. Expenditures by the Chinese government to manage PWN have exceeded \$100 M in some outbreak years (Yu, Xu, & Ding 2011). This has been part of an integrated management plan that also includes the reduction of disease reservoirs through sanitation and increasing host resistance through selective breeding programs (Kamata 2008; Kwon et al. 2011). In natural forest stands, sanitation and planting of resistant trees are difficult to implement because of the often-remote locations of PWN outbreaks and the impracticality of replacing entire stands or forests with resistant or tolerant host species. Sanitation and replanting are extremely costly, and most countries with at-risk pine populations have implemented strict regulations to prevent the introduction of PWN. The most effective management option for PWN is to control the spread of beetle vectors primarily *M. alternatus* (Kamata 2008). Regulations vary based on country, but the practice of spraying insecticides can complicate and compound the effects of what is already a very difficult ecological problem. Studies have documented spraying insecticides and deterrents often has consequences for off-target species in the treatment zones, with aerial spraying leading to large decreases in non-target arthropod populations for 3 consecutive years in one Japanese forest (Watanabe 1983).

### **1.3 Pine wilt disease in North America**

The majority of research on PWN in North America was completed in the 1980's in response to EPPO's initial designation of PWN as an A1 pest. PWN is believed to be endemic to the southeastern U.S. because many of the native pine species are resistant to pine wilt disease (Wingfield et al. 1982). In Canada, surveys were completed on potential hosts and vectors found in the country (Bergdahl 1988; Bowers et al. 1992; Sutherland 2008; Blatt, Bishop & Sweeney

2017). These studies found PWN was present in every Canadian province infesting a wide array of hosts. Canadian researchers encountered both the mucronated 'm' and round-tailed 'r' forms of the nematode, although the more virulent 'r' type was only associated with pines. Trees that were found to contain PWN were thought to have precluded stress by some abiotic factor or secondary infection. The occurrences of PWN were in single or pairs of trees that appeared otherwise isolated from any disease centers. The same study also examined ~1,300 *Monochamus* and only encountered PWN in a single individual of *Monochamus clamator* Leconte (Bowers et al. 1992). This has been the only documented report of a PWN-*M. clamator* association. Researchers attempted to produce infective vectors by rearing immature insects in logs inoculated with PWN but were unsuccessful. These findings resulted in the conclusion that the likelihood of exporting PWN in Canadian packing material was low (Bowers et al. 1992). Since that time, PWN in *Monochamus scutellatus* Say in Canada has been reported (Akbulut & Stamps 2012) but little attention has been aimed at management. Low summer temperatures found in Canada compared to other outbreak areas likely influence the low severity of the disease (Rutherford, Mamiya & Webster 1990).

PWN emerged as a pest of urban forests in the U.S. shortly after it was identified in Missouri in 1979 (Dropkin & Foudin 1979). Mortality in several exotic urban-use pine species, primarily Scots (*P. sylvestris*) and Austrian pine (*P. nigra*), was attributed to pine wilt disease. Initial evaluations deemed it was unlikely that PWN posed a threat to natural forests in the U.S. (Dropkin 1981; Wingfield et al. 1982 & 1983). Since that report, PWN has appeared along an increasingly westward-moving front. PWN had been documented in Kansas (Robbins 1979), Indiana (Marshall & Favinger 1980), Illinois (Malek & Appleby, 1984), and Nebraska (Gleason et al. 2000) prior to a first report in Colorado in 2006 (Blunt et al. 2014). Similar to previous

reports, PWN in Colorado was only associated with mortality in exotic pines. A study by Pimentel et al. (2014) surveyed for PWN in its numerous vectors present along both the eastern and western U.S. In the eastern U.S. study sites were established in Vermont, New Jersey, and Louisiana. Western study sites were also established in Arizona and California. For eastern sites; in Vermont, *M. scutellatus* and *M. notatus* Drury were captured. *M. titillator* Fabricius and *M. carolinensis* Oliver were captured in New Jersey and Louisiana. In California, *M. obtusus* Casey and *M. clamator* were captured, while only *M. clamator* was captured in Arizona. In eastern forests, the researchers reported PWN phoresy as being common in all *Monochamus* vectors and across study sites, with average rates of phoresy between 20-30%. This is slightly higher, but still comparable to what was reported in the Canadian survey which studied the same vectors present in Vermont (Bowers et al. 1992). In western forests, captures were significantly lower than in the east (~100 vs. ~2,000), and no individuals of either western species were found to carry PWN. These findings further support the idea that while PWN may be common in eastern North America, there is little evidence that it is native or common in the areas west of the Great Plains states.

#### **1.4 Pine wilt disease pathosystem**

The pinewood nematode belongs to the genus *Bursaphelenchus*, whose members are known to persist primarily as plant parasites or as mycophages. Some nematodes are capable of switching between the two states depending on their developmental stage (Mamiya 1975; Kanzaki & Futai 2001; Wang et al. 2005), and *B. xylophilus* is capable of both strategies. PWN feeds on parenchyma cells of plant resin canals until resources are exhausted, then can begin feeding on fungi by employing the same families of hydrolytic enzymes used in the digestion of plant material (Baojun 1997). This strategy allows the nematode to persist in dead hosts until they are able to colonize a new vector. PWN is only capable of feeding on certain groups of fungi (Kobayahsi ,

Sasaki, & Mamiya 1974, 1975; Fukushige 1991), and their preference for feeding on fungal tissue or the parenchyma walls of the host tree change depending on time-since-mortality of the host tree (Maehara & Futai 2000). Blue- and black-stain fungi of the genus *Ophiostoma* are associated with almost all PWN-killed pines and are also one of the best naturally occurring substrates for PWN-growth compared to other co-occurring fungi (Kobayashi, Sasaki, & Mamiya 1974, 1975; Sriwati et al. 2007). These fungi play an important part in the disease cycle of PWN beyond simply serving as a food source. The intensity of fungal infection in the wood surrounding pupal chambers has been linked to both the number of nematodes in the wood and the nematode-load of the emerging vectors (Maehara et al. 2005, 2006). These vectors are also capable of carrying a fungal inoculum on their bodies which is transmitted concurrently with PWN (Maehara and Futai, 1996), and fungal growth may occlude xylem tracheids. The timing of this inoculation relative to other wood-decaying fungi is also important for PWN reproduction. *Trichoderma* spp. and *Verticillium* spp. that were present in samples were able to outcompete *Ophiostoma* and effectively reduce PWN populations in both artificial pupal chambers (Maehara et al. 2006) and in *P. densiflora* trees (Maehara 2008). Fungi have also been demonstrated to be influential to the within-host distribution and virulence of PWN (Suzuki et al. 2005; Wang et al. 2008), and mycophagous feeding is used as a behavioral symptom to delineate pine wilt disease into two distinct stages of disease progression (Mamiya 1983; Fukuda 1997; Zhao et al. 2007b & 2013).

In the early stage of pine wilt disease, the initial defense response from the host tree is sufficient to suppress extreme growth of the PWN population (Pimentel et al. 2017). The formation of tyloses occurs as attempts to block movement of PWN through the main stem (Fukuda, Hogetsu & Suzuki 1992; Fukuda 1997). A chemical defense of oleoresin production occurs and there is an increase of secondary defense metabolites, namely terpenoids and phenolics (Takeuchi 2008).

Nematodes continue to feed on epithelial and ray parenchyma cells which are responsible for producing these defense compounds, compromising the ability of the host to produce these defenses and preceding eventual cessation of their production (Myers 1988; Yamada & Ito 1993; Yamada 2008). The point at which oleoresin production at wound sites ceases has proven to be a precursor to the development of external symptoms in host trees (Odani et al. 1985). The first symptoms that become apparent are the wilting of needles, which change from green to reddish then a light straw color. These symptoms typically coincide with the arrival of drought conditions and rising summer temperatures (Kuroda et al. 1988). The life cycle of PWN is sensitive to changes in temperature, and it has been demonstrated that PWN typically only persists in areas where mean summer temperatures are greater than 20°C (Rutherford & Webster 1987; Zhao et al. 2007b).

The life cycle for PWN is typically completed in 4-5 days at 25°C but can occur in as little as 3 days if mean temperatures rise to above 30°C (Tomminem, Halik, & Bergdahl 1991). This increase in reproduction, coupled with the weakening of defense response commonly associated with drought stress in conifers, is likely one of the ways in which PWN is able to rapidly overcome tree defenses during summer months. The onset of summer temperatures is when progression into the second stage of the disease occurs. After the defense response from the host tree is halted, PWN populations begin to increase exponentially (Kuroda 2008). PWN individuals are capable of moving up to 150 cm per day in infected trees (Kuroda & Ito 1992) and they quickly migrate throughout the main stem of the host. PWN continue to feed on tissue in the xylem resulting in tracheid cavitation leading to dysfunction (Myers 1986 & 1988). This leads to blockage of water transport within the xylem and rapid mortality of the host tree. This entire process can occur in as little as 2 months in many susceptible host tree species (Fukuda 1997; Kuroda 2008; Wang et al. 2010).

## 1.5 Vector biology & ecology

All members of the PWN genus *Bursaphelenchus* have a phoretic relationship with insects, although the insect vector varies between species and location (Evans et al. 1996). The most competent vectors of PWN are sawyer beetles (*Monochamus*), which introduce nematodes into the tips of branches during spring maturation feeding (Akbulut & Stamps 2012) or during oviposition (Wingfield et al. 1983). These insects colonize dead and dying wood of coniferous tree species that have ceased oleoresin production for their oviposition sites and are only considered to be secondary pests in their native ranges (Walsh & Linit 1985). Sawyer beetles have a synergistic relationship with the phoresy of PWN. By carrying PWN to new, healthy hosts that are then killed, beetles benefit from an increase in oviposition sites (Togashi 1990a; Aikawa 2008). Nematodes first enter the beetles through the spiracles during the eclosion of pupating individuals. The nematodes reside in the trachea of emerging beetles as dauerlarvae until exiting through the mouthparts and spiracles of adult beetles during maturation feeding or oviposition (Mamiya & Enda 1972; Naves et al. 2007). Transmission that occurs during feeding is termed ‘primary’ transmission and that which occurs during oviposition as ‘secondary’ (Wingfield et al. 1982). The mechanism for causing the transition from the dauerlarvae to adult stage and subsequent egress from the beetles remains unclear, although both host volatiles (Ishawaka, Shuto, & Watanabe 1986) and CO<sub>2</sub> concentrations (Wu et al. 2019) have been implicated in PWN egress from vector beetles.

*Monochamus* spp. benefit from trees that are killed by pine wilt disease as they can then serve as oviposition sites during the following year, leading to increased *Monochamus* reproduction following PWN introduction. In systems where PWN is not present, the limiting resource for *Monochamus* spp. is considered to be viable oviposition space (Togashi 1990a).



Larvae are unable to survive in trees with active oleoresin flow, making their search for dead and dying trees a critical aspect of their biology. Studies have documented landscape disturbances, particularly fire, as highly predictive of *Monochamus* abundance (Saint-Germain et al. 2004; Costello, Negrón, & Jacobi 2011). Upon emergence, adult *Monochamus* are reproductively immature, and beetles must undergo a period of maturation feeding before they begin to find a mate. The duration of this pre-reproduction feeding period has been linked to ambient temperature conditions, with warmer air temperatures correlated with a shorter time before individuals begin to mate (Akbulut & Stamps 2012). Maturation feeding takes place on new and 1-year old growth of healthy adult trees of available conifers with *Pinus* species typically preferred (Allison & Borden 2001). *Monochamus* individuals will chew on the phloem and green growth tissue of small twigs, leaving feeding wound scars 2-6 cm in length. Dispersal occurs randomly via flight in immature adults or over short distances by crawling. While flight distance is typically short (<40 m; Togashi 1990b; Akbulut & Linit 1999), adults have been recorded to travel up to several kilometers in mark-recapture studies (Ido 1975). Mature adults are chemotactic and attracted to kairomones emitted by stressed or dying trees and aggregation pheromones emitted by reproductive adult males (Parajes et al. 2004; Macias-Samano et al. 2012). After copulation, females chew oviposition niches into the bark of trees to lay eggs. These niches are frequently contested by other females, and the original insect often leaves behind a pheromone to discourage others from attempting to utilize the egg niche (Anbutsu & Togashi 2000 & 2001). Fecundity in females varies greatly (range: 30-500 eggs), and egg abundance is correlated with emergence timing and female body size (Togashi & Magira 1981; Koutroumpa et al. 2008). Following oviposition, larvae emerge after five days and begin to feed on the phloem and inner bark of host trees. The larvae go through four developmental instars, and tunnel into the xylem of their host

trees after transition to the third instar. The fourth instar larvae excavate a pupal chamber curving back towards the outer area of the tree where they overwinter before beginning pupation. The pupation period usually lasts for around 2 weeks (Akbulut & Stamps 2012; Naves et al. 2008; Jikumaru et al. 1994). During this time, PWN is attracted to the pupal chamber via emissions and volatiles excreted by the eclosing adult and host tree. PWN enters *Monochamus* individuals through their spiracles and begin to form dauerlarvae in the tracheae of the beetle's ventilation system (Zhao et al. 2007a). These infective vectors emerge in spring and begin maturation feeding, completing the disease cycle.

### **1.6 Problem statement & knowledge gaps**

In 2016, the first reports of PWN associated with dead ponderosa pine in Colorado (*Pinus ponderosa* Douglas ex L.) were made (Tisserat and Stewart, personal communication). This launched an effort to screen for PWN in symptomatic ponderosa pine in forested and urban sites and to increase outreach efforts to stakeholders to raise awareness of PWN. Several ponderosa pines that had no other obvious causes of mortality were found to contain large populations of PWN similar to those found in exotic pines that had succumbed to pine wilt disease. No survey had been conducted for PWN in the natural forests or potential vectors found in Colorado prior to these reports.

PWN is a clear threat to many pines planted in urban settings across the U.S. and to species common in arboriculture. Temperatures have been rising across the Front Range of Colorado with many counties now reporting average summer temperatures above 20°C - a critical threshold for PWN development (Rutherford & Webster 1987; Zhao et al. 2007a&b). The incidence of mortality of native pines associated with PWN has increased markedly in the Colorado Front Range since 2016 with efforts to increase public awareness, leading to new cases documented each summer.

However, these reports are only made after a tree has died and occurrences are not representative of the *in situ* occurrence rate of PWN. Colorado also has two species of *Monochamus* that are putatively capable of vectoring PWN. Little work has been done to understand the biology of these species or the factors driving their distributions across the landscape. Knowledge of the frequency of PWN in hosts and vectors across landscape types will be critical for managers to determine if PWN is an issue that needs to be addressed and make an informed decision before a persistent disease cycle becomes established.

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## CHAPTER 2

### DESCRIPTION OF PINWOOD NEMATODE DISTRIBUTION AND IDENTIFICATION OF DISEASE RESERVOIRS IN THE FRONT RANGE REGION OF COLORADO

#### 2.1 Introduction

Pathogens play a fundamental role in many terrestrial ecosystems as contributors to changes in species composition, competitive interactions, and overall productivity (Gilbert & Parker 2006; Garbelotto et al. 2010; Metz et al. 2012; Flower & Gonzalez-Meler 2015). Novel diseases often arise from the introduction of an organism or from an emergent pathogen. The introduction of new diseases is becoming more frequent with ever-increasing globalization (Hulme 2009). Emergent diseases result from changes in environmental conditions that modify contextually dependent interactions between host, vector(s), and pathogen(s) (Woolhouse et al. 2002; Davis et al. 2015; Cobb & Metz 2017). The impact of these introduced and emergent pathogens is often extreme because ecosystems are not evolutionarily equipped to confront unprecedented disease pressure (Woolhouse et al. 2002; Parker & Gilbert 2004; Boyd et al. 2013; Young et al. 2017). Climate models predict an upcoming period of rapid ecological change and accompanying increase in cases of pathogen outbreaks (Harvell et al. 2002; Anderson et al. 2004; Stenlid & Oliva 2016). While the distinction between an introduced or emergent pathogen is difficult to make, patterns in disease distribution and occurrence timing can be used to discern between novel pathogens and those that are ecologically integrated into ecosystem processes. Spatiotemporal clustering, particularly in the instance of vector-borne pathogens, is consistent with the high frequency of infections observed near a disease reservoir. (Woolhouse & Gowtage-Sequeria 2005; Meentemeyer et al. 2008; Fisher et al. 2012). This distinction is important to make to develop appropriate integrated pest management strategies (Ghelardini et al. 2016; Grant et al. 2017).

Pine wilt disease is a lethal wilting disease of conifer species in the Pinaceae family caused by the pinewood nematode (*Bursaphelenchus xylophilus* Steiner and Nickle; hereafter, PWN) (Kiyohara & Tokushige 1971). *Bursaphelenchus* spp. are known to persist primarily as plant parasites or as mycophages; PWN is capable of both strategies (Mamiya 1975; Kanzaki & Futai 2001; Wang et al. 2005; Zhao et al. 2013). The PWN has an obligate vector relationship with long-horned beetles (Coleoptera; Cerambycidae), specifically, PWN relies on pine sawyer beetles of the genus *Monochamus* for transmission between hosts (Akbulut & Stamps 2012). *Monochamus* spp. are cosmopolitan and multiple sympatric species that colonize pines throughout the northern hemisphere (Bergdahl 1992). Beetles are attracted to weakened or damaged trees, or forest stands which have recently experienced disturbance including fire (Saint-Germain et al. 2004; Costello et al. 2011). Nematodes orient to and colonize beetles during pupation and are subsequently introduced to susceptible hosts during early-season maturation feeding (Linit 1990) and later-season oviposition (Wingfield et al. 1983).

In the early stages of infection, the initial defense response from the host tree is sufficient to suppress exponential growth of PWN populations (Pimentel et al. 2017). Defenses include formation of tyloses (Fukuda, Hogetsu & Suzuki 1992; Fukuda 1997) and upregulation of oleoresin production and secondary defense metabolites such as terpenoids and phenolics (Takeuchi 2008). Nematodes feed on epithelial and ray parenchyma cells which are responsible for producing these defense compounds, compromising the ability of the host to produce defenses, resulting in death (Myers 1988; Yamada & Ito 1993; Yamada 2008). The first visible symptoms may become apparent 15-20 d following infection and include wilting of needles and changes in color from green to reddish to a light brown. These symptoms typically coincide with rising summer temperatures and high evapotranspirative demand (Kuroda et al 1988).

Accordingly, the life cycle of PWN is sensitive to changes in temperature and it has been demonstrated that PWN infections are most severe in areas where mean summer temperatures regularly exceed 20°C (Rutherford & Webster 1987; Zhao et al. 2007a&b).

The pinewood nematode is one of the most damaging forest pests in Asia and may be among the greatest biotic threats to pine forests globally (Webster & Mota 2008; Mota et al. 2009). Pine wilt disease has caused billions (\$USD) of economic damages since it was introduced into Japan in the early 20<sup>th</sup> century, with projections of uncontrolled spread costing \$1B per year in losses to timber (Soliman et al. 2012; Vicente et al. 2012). Since then, PWN has spread into China (Cheng et al. 1986), Korea (Yi et al. 1989) and Portugal (Mota et al. 1999); PWN is well established in the southeastern United States where it is not considered a pest (Dropkin 1981; Wingfield et al. 1982; Wingfield et al. 1983). In North America, the first cases of mortality in Austrian pine (*Pinus nigra* Arnold) resulting from PWN-infection were reported in Missouri in 1979 (Dropkin & Foudin 1979). Since that report, PWN has moved westward, with documented tree mortality in Kansas (Robbins 1979), Indiana (Marshall & Favinger 1980), Illinois (Malek & Appleby, 1984), Nebraska (Gleason et al. 2000), and throughout Canada (Bowers et al. 1992). The first report of PWN in Colorado was made in 2006 (Blunt et al. 2014), and in 2016 the first reports of PWN associated with ponderosa pine (*Pinus ponderosa* Douglas ex. Lawson) mortality were made in Colorado (Tisserat and Stewart, personal communication).

Many pine species commonly planted in urban settings across Colorado face pressure from PWN introductions, particularly Scot's pine (*Pinus sylvestris* L.), Austrian pine (*P. nigra*), and Mugo pine (*Pinus mugo* Turra). However, it remains unclear how widespread the nematode is in native forest ecosystems or vector populations. Temperatures have been rising across the Front Range region of Colorado with many municipalities reporting average summer

temperatures above 20°C (PRISM Climate Data Group: Oregon State University) - which is expected to correspond with increased PWN reproductive rates (Rutherford & Webster 1987; Rutherford et al. 1992; Zhao et al. 2007a&b). Colorado has two species of *Monochamus* that are putatively capable of vectoring PWN; *Monochamus clamator* Leconte and *Monochamus scutellatus* Say. It will be critical to understand the degree of PWN establishment and the relationships between PWN, regional *Monochamus* vectors, and native ponderosa pines to make appropriate management decisions. Here we address three objectives to describe the pine wilt disease pathosystem in the Front Range of Colorado, including (1) describing vector abundances and flight phenology across the region; (2) characterizing PWN infection rates of insect vectors and host tree populations, and (3) modelling disease epicenters and drivers of infection probability. Our results provide new fundamental information on temporal windows of exposure to PWN in both wildland and urban locations and demonstrate that disease epicenters are distributed widely, and probability of infection is related to several landscape factors. Collectively, these findings can be used to develop new control applications for PWN in Colorado and elsewhere.

## **2.2 Materials & Methods**

### *2.2.1 Study design & site description*

Sites were selected from both wildland-urban interface (WUI; N=32; Stewart et al. 2007) and urban (N=12) landscapes (Table S1). Sites were located between 50-500 m from a road with ponderosa pine (*P. ponderosa*) as the dominant canopy species. Other species included Douglas-fir (*Pseudotsuga menziesii* var. *glauca* Mayr), lodgepole pine (*Pinus contorta* var. *latifolia* Douglas ex. Loudon), limber pine (*Pinus flexilis* E. James), and quaking aspen (*Populus tremuloides* Michx.) Plots were used to characterize forest structural characteristics and select

sample trees to test for PWN presence on a per unit area basis. Urban sites were located within an area of publicly owned land in the cities of Fort Collins, Loveland, Boulder, and Golden, CO where mature Austrian pine (*P. nigra*) or Scots pine (*P. sylvestris*) were planted and dominant in the canopy (3 locations in each municipality).

### Site and landscape surveys

The WUI sites were 0.04 hectare fixed-area plots, ranging from 1725-2567 m in elevation. Basic forestry measurements were taken in the summer of 2018 including a census of tree species and their diameters at breast height (DBH at 1.3 m), a census of crown-class (suppressed, intermediate, co-dominant, or dominant) for all recorded trees, and the presence/absence of visible fungal infection, mechanical damage, or fire damage was recorded from all trees with a DBH > 2 cm. In addition, site aspect, hillslope, and other landscape variables including distance to recent (post 2000) fire (km), distance to the closest eastern boundary (forest margin) of ponderosa pine canopy (km), % canopy cover in a 250 m radius, and distance to nearest city (population >2,000; km) were derived for each site to develop predictive models of beetle abundance. Landscape variables were collected 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 ( $\text{MJ}\cdot\text{cm}^{-1}\cdot\text{year}^{-1}$ ) incorporating slope, aspect, and latitude, was also calculated for each site. In addition, maximum daily temperature was recorded (HOBO Pendant Model: UA-002-08 Onset Computer Corporation, Bourne, MA) for a random subset (N=20) of WUI sites and for all urban sites during 2019.

To estimate PWN infection frequency in host trees at sample sites, branch and sawdust samples were taken from a subset of 7-10 randomly selected trees per site with a DBH > 10 cm. Samples were collected as follows: a 20 cm section proximal to the bole from each of 2 branches

was taken from each randomly selected tree using a pole pruner. Sawdust was collected from 2 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 grouped into a composite sample for testing PWN presence using molecular assays.

### Insect trapping

Each site contained a single, centrally located black crossvane trap as described in Morewood et al. (2002) utilizing a diffuse pesticide (No-Pest 2 Strips; Dichlorvos; 18.6% 2,2-dichlorovinyl dimethyl phosphate; Hot Shot Corp., St. Louis MO) to kill captured insects. Traps were baited with lures containing host-tree volatiles, ethanol, and *Monochamus* and *Ips* pheromones including monochamol and ipsdienol (Monochamus lite combo lure - lot #546371; Synergy Semiochemicals, Victoria BC). 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 only visited during 2019. All captured members of the family Cerambycidae were identified to the species level and the sex was recorded. All captured *Monochamus* spp. were subsequently analyzed for the presence of PWN using a molecular assay, described below.

### Molecular assays to test for PWN presence in beetles and tree tissues

Both tree tissues and trapped beetles were tested for the presence of PWN using Loop-mediated isothermal **AM**plification (LAMP) assay (Bx Detection Kit, Lot #'s 29000H-L, Nippon Gene Co., Tokyo Japan), a highly sensitive molecular method. Tissues collected from tree samples (N=289) were tested according to the protocol outlined in Kikuchi et al. 2009. For insect samples, the same assay was used but the following modifications were made to the extraction process to increase efficiency: a batch screening approach was developed by bisecting a subset of 100 beetles

and testing them individually according to regular protocol. For method development, the remaining half of a single positive beetle was selected and grouped with known negatives into batches of 5, 10, 25, and 50 during the extraction process and ground using a mortar and pestle to determine the sensitivity of the method. These batches were then tested, all of which resulted in a positive result, confirming test sensitivity up to 50 samples per batch. Following this confirmation, insects were bisected longitudinally and grouped into batches of ~10 (6-16) beetles based on sample location and date of capture before an initial screening. Remaining portions of beetles were stored at -20°C. Any batches that tested negative were recorded and those that tested positive were revisited and resolved to the individual level. Using this approach, infection frequency for each *Monochamus* species was determined and compared between species.

### 2.2.2 Data analysis

#### Vector distribution

To evaluate the effects of landscape factors on vector abundances, beetle trap capture abundance for each species was modeled using a multiple-regression model selection (function ‘dredge’) to test the hypothesis that *Monochamus* will be more abundant near areas that have a higher quantity of available oviposition sites (i.e. disturbed areas) and that temperature may be a limiting factor for beetle activity. Distance to fire, site elevation, heat-load index, distance to eastern ponderosa pine cover boundary, distance to city edge, and average canopy cover within 250 m of sample location were included as predictors. Variables were root-transformed where necessary in order to meet model assumptions of normality and heteroscedasticity. Sampling year was included as a random effect even though it was not significant in either model (package ‘lme4’ & ‘MuMIn’, R ver. 3.6). Models were selected via minimization of AIC (Akaike 1974).

#### Flight phenology



Flight phenology for *M. clamator* and *M. scutellatus* was modeled using a 2-parameter logistic regression (function ‘nplr’) with ordinal day as the predictor and cumulative proportion of site captures as the response (Dell & Davis 2019) (Figures S1 & S2). Initiation, peak, and termination of flight were estimated using 10%, 50%, and 90% cumulative capture for each species and year to evaluate differences in flight patterns between vectors. Flight synchrony was estimated using the growth rate of the logistic curve at 50% capture—highly synchronous flight periods have rapid curve growth rates, whereas asynchronous flight periods are associated with a low curve growth rate. Estimates of flight initiation, peak, and termination were made for each species at each site and for each species in aggregate. Only sites with 10 or more captures recorded for each species were considered for species-level flight phenology models (*M. clamator* N=30, *M. scutellatus* N=18). These estimations were made by solving the logistic curve (function ‘getEstimates’) generated for each site to estimate the date on which each flight period (10%, 50%, and 90% of captures) occurred (package ‘nplr’ R ver. 3.6). Comparisons of flight timing between beetle species were made using a 2-sample Student’s *t*-test to test the hypothesis that the timing of flight and flight synchrony differs between species (R ver. 3.6).

#### PWN frequency in vectors and hosts

Infection frequency was compared between vector species using a Chi-squared test. Infection frequency was compared between vectors captured at WUI and urban centers using a Fisher’s Exact test (R ver. 3.6).

#### Disease epicenter identification

The recency of first reports of pine wilt disease in Colorado indicate that the disease may not be established uniformly in the study system, with infections radiating from central locations or ‘epicenters’. Identification of these disease reservoirs will be critical to preventing the

establishment of a persistent disease cycle. Here, disease epicenters were identified as any site with an occurrence of spatiotemporal outlier for the frequency of infective vector captures during either year. If PWN is not uniformly established in the forest, this can be observed through differences in patterns of disease incidence throughout the growing season. Initial identification of potential epicenters was made using a scanning statistic to identify sites or aggregate zones where the rate of infection is dissimilar to others. Any sites identified in this manner were further included as a factor in a generalized linear mixed-effects model to test the hypothesis that the likelihood of infection is greater during the beginning of vector flight as observed in studies conducted in the southeastern United States (Pimentel et al. 2014) where PWN is known to be established.

Epicenters were identified using a Type I error rate of  $\alpha=0.05$  for assigning statistical significance using the function ‘scan\_eb\_poisson’ with 999 Monte-Carlo iterations (package ‘scanstatistics’, R ver. 3.6). This function computes an expectation-based Poisson scan statistic useful for identifying anomalous spatiotemporal clusters of disease incidence (Kulldorff et al. 2005). The method compares all possible temporal windows for each group in a given zone list to test a null hypothesis of spatiotemporal randomness using a likelihood ratio statistic. A custom zone list was used that included all possible levels of nearest-neighbor combinations for sites grouped within an area while excluding combinations that would include a nearest-neighbor from a geographically discrete (>5 km distance) area based on vector flight capacity as reported in other studies (Akbulut & Linit 1999; Togashi & Shigesada, 2006; Van Nguyen et al. 2017).

### Infection probability modeling

The likelihood of vector association with PWN was calculated using a log-likelihood model selection approach. Factors considered included a full factorial of first order interactions between vector species (n=2 factor levels), sex (n=2 factor levels), day-of-year of capture

(continuous effect), and whether or not the capture occurred at a previously identified epicenter (n=2 factor levels, 'yes' or 'no'). The final model was selected via the minimization of AICc (Akaike 1974). To evaluate differences in disease exposure risk between areas surrounding epicenter and peripheral sites, flight phenology was modeled as described above using only cumulative proportion of captured infective vectors as the response. Peak relative exposure-risk was calculated for each year (2018 & 2019) and site type (epicenter or peripheral) by solving the logistic curves for 50% of cumulative infective vector capture.

### 2.3 Results

#### *Vector abundances and flight periodicity across the region*

Across two years of collection, a total of 5,146 beetles were captured at WUI sites: 4,068 *M. clamator* (2018, N=1,822; 2019, N= 2,246) and 1,078 *M. scutellatus* (2018, N=364; 2019, N=714). In 2019, 41 *M. clamator* and 12 *M. scutellatus* were captured at urban sites. The frequency of each species was similar between WUI and urban sites for 2019 ( $\chi^2=0.01$ ,  $P=0.92$ ). Mean trap captures at study sites were more than an order of magnitude greater at WUI sites ( $92.5 \pm 10.3$ ) than urban sites ( $4.5 \pm 1.3$ ) for 2019 ( $t = 9.1$ ,  $P<0.001$ ). M:F sex ratio was different between *M. clamator* (0.6) and *M. scutellatus* (0.51) in WUI sites ( $\chi^2=5.5$ ,  $P=0.019$ ) but similar between species (0.64) at urban sites ( $\chi^2=0.2$ ,  $P=0.65$ ).

Peak flight occurred earlier at WUI (day  $254 \pm 2d$ ) sites than urban ( $266 \pm 3d$ ) sites. The timing of flight periods was similar between species in WUI sites but varied between years (Figure 1). Flight phenology logistic curve models showed a goodness of fit between 0.79-0.9 for both vector species over both years (Figure 2). Flight models for both vector species over both years at all sites shown in Figures S1 & S2. Flight synchrony was similar between species ( $t = 1.38$ ,  $P=0.18$ ).

Abundance of *M. clamator* varied with distance to fire and elevation. Abundance had a negative correlation with both distance to fire ( $\beta = -0.028$ ,  $P = 0.002$ ) and elevation ( $\beta = -0.386$ ,  $P = 0.006$ ). Abundance of *M. scutellatus* was negatively correlated with distance to fire ( $\beta = -0.018$ ,  $P = 0.005$ ; Figure 3). The effects of year, average canopy cover (250m radius), heat-load index (HLI) and distance to forest edge or nearest city did not significantly predict the abundances of either species (Table 2). Non-significant predictors collectively explained less than 2% of the variance in the data.

#### *Characterize of infection frequency in vector insect and host tree populations*

PWN was detected in 3.1% of *M. clamator* individuals and 7.8% of *M. scutellatus* individuals captured throughout the study. Frequency of infection was lower in *M. clamator* than *M. scutellatus* ( $\chi^2=26.8$ ,  $P<0.001$ ) PWN infection rate was higher in WUI (4.2%) than urban (1.9%) beetle captures (Fisher's Exact Test  $P=0.73$ ) PWN was detected in 3.6% of *P. ponderosa* trees sampled (3 out of 32 WUI sites). PWN-infection frequency ranged from 10-89% for the three sites where PWN-positive hosts were identified. PWN was not encountered in any trees tested from urban sites (Figure 4).

#### *Modeling of disease epicenters and evaluation of drivers of infection probability*

Spatiotemporal outliers in infection rates were identified at a total of 4 sites ('epicenters') across both years (Sites 1-4, Figure 5). Anomalies were identified at sites 1, 2, and 3 in the early flight season (site 1: week 3,  $P=0.019$ ; site 2: weeks 1-3,  $P<0.001$ ; site 3: weeks 1-3,  $P<0.032$ ), as opposed to much later in the season for site 4 (week 11,  $P=0.032$ ) A generalized linear model (GLM) to estimate the likelihood of infection included significant associations between beetle species and if the capture site was an identified epicenter. Probability of infection was ~2.5X

higher for *M. scutellatus* than *M. clamator* individuals on average. The probability of infection in vector insects was related to whether a given location was identified as an epicenter, and this interacted with seasonality: beetles from locations identified as epicenters were ~20-fold more likely to be infected early in the vector flight period, but through the flight period (August-October) this difference continuously decreased. The lower order day of year term was not significantly associated with likelihood of infection, indicating that this early season increase in infection probability is exclusive to identified epicenters (Table 4). Infection rates and mean captures per site varied seasonally between epicenter and peripheral sites (Figure 6). Peak relative risk-exposure occurred earlier for epicenter sites (2018: Day 196 [Jul 15]  $\pm$  5d; 2019: 235 [Aug 23]  $\pm$  11d) than at peripheral sites (2018: 247 [Sep 4]  $\pm$  1d; 2019 255 [Sep 12]  $\pm$  2d; Figure 7).

## 2.4 Discussion

A multi-year trapping study showed that vectors of PWN are abundant throughout the Colorado Front Range region of Colorado. While infection frequency of vectors was lower than reported in other studies, the frequency of association with PWN was not insignificant, ranging widely from 0-38% (mean: 4.2%) depending on time of year and vector species. Infective vectors were found across all study sites, although only 50% of the sites recorded positive captures in both years. In addition, we report for the first time that *M. clamator* is a common vector of PWN in the Rocky Mountain region. Our analysis indicates *M. scutellatus* individuals were 2.5 times more likely to be vectors of PWN than *M. clamator*. *Monochamus scutellatus* is known to be associated with PWN, while only a single *M. clamator* individual has been previously associated with PWN in Canada (Bowers et al. 1992). Our study represents the first report of *M. clamator*-PWN association in the United States, and our research shows that not only is *M. clamator* commonly

associated with PWN, it is by far the more abundant of the two vectors in Colorado. In addition, our analysis identifies four epicenter areas that may serve as regional reservoirs of PWN disease and confirms that vectors captured from epicenters are more likely to be infected early-on in the growing season during flight initiation. Peak relative risk-exposure periods for peripheral sites lagged significantly behind that of epicenter sites. Accordingly, near disease epicenters the risk of PWN transmission is higher in the early season but at sites distal to epicenters transmission is likely to be later in the growing season following flight and dispersal of infective vector insects (Kitron 1998). The increased likelihood of infection of *Monochamus* spp. at epicenter sites early in the season matches trends observed in the eastern U.S. from areas where PWN is long-established (Pimentel et al. 2014). This finding has important implications for PWN management, as measures to limit PWN transmission must match with early-season windows of exposure.

Beetle vectors positive for PWN were collected from all sampled locations in the present study and suggest cause for concern as *Monochamus* spp. are capable of travelling relatively large distances (compared to other insects) during a growing season. These findings also reveal that PWN disease in urban areas along the Front Range region of Colorado is likely due to dispersal of infected vectors from nearby forested sites. Collections from replicated trapping arrays indicate that vector populations do not apparently persist in urban areas, but instead migrate to urban sites after peak flight is observed in wildland areas. The capture of PWN-positive vectors at urban sites demonstrates that while the disease may not yet persist in urban areas, any established populations in WUI areas pose a threat to trees planted within city limits, which are more commonly exotic pines that are more susceptible to the disease (Mamiya 1983; Nunes da Silva et al. 2015). This underscores the importance of management for pine wilt disease in wildland-urban interfaces where high-value trees may be at risk of exposure. Disease pressure radiating from forests may be

effectively managed by preventing dispersal into urban areas through trapping lines or pesticide applications in areas closer to forests (Coyle et al. 2005; Brockerhoff et al. 2006). Another implication for this finding is that any introductions of PWN into the urban landscape may prove unsuccessful due to the minimum vector population requirements to prevent an Allee effect as observed in other systems. Yoshimura et al. (1999) demonstrated that the successful establishment of PWN is dependent on the density of both susceptible host trees and *Monochamus* vectors.

Vector flight phenology in the Front Range region of Colorado is much later than flight phenology described from warmer regions. *Monochamus galloprivincialis* found in Portugal and *M. alternatus* in Japan often begin their flight period by the first week of May, while both *M. clamator* and *M. scutellatus* were not observed consistently until mid-July of both years. Climatic and elevational differences between seasonal temperatures are likely the cause for this observation. These findings will help outline a useful framework for disease management. Descriptions of flight timing and peak flight periods will help managers deploy pesticides and/or trapping arrays during periods of peak vector activity. In contrast to other areas where PWN is established, no vectors were observed before July 1<sup>st</sup>. Approximately 50% of total vector flight occurred during the three-week period from August 15<sup>th</sup> through the first week of September. Identified epicenters can serve as foci for integrated pest management efforts, preventing dispersal of vectors with a high frequency of PWN-infection early in the season. These identified epicenters all occur near areas frequented by out of state visitors where recreational activity is high. Because of the distance between the nearest reported cases in Kansas and the first reports of Colorado, it is probable that human movement of firewood is responsible for PWN introduction. Carefully monitoring firewood transportation and employing ‘burn where you buy’ practices may be effective at curbing further satellite introductions of PWN. Similarities observed in flight timing here indicate that

management practices will be equally effective among both vector species. However, continuously rising temperatures may impact the voltinism of vectors or increase the duration of the flight period by reducing flight synchrony (Kishi 1995; Azrag et al. 2020). This will further increase exposure windows and overall disease pressure. Lengthening exposure windows may cause trees that are in a defense-compromised to become more frequently exposed to PWN likely resulting in increased tree mortality (Pershing & Linit 1986; Kwon et al. 2019).

Habitat distribution modelling reinforced previous findings that fire plays an important role in *Monochamus* life history while also supporting the hypothesis that temperature is an important predictor of beetle activity (Saint-Germain et al. 2004; Costello et al. 2011). The abundances of both vector species were positively correlated with increasing proximity to burned areas. In areas where PWN causes widespread tree mortality, outbreaks of PWN are often strong predictors for *Monochamus* abundance due to the increase in defensively compromised trees that serve as oviposition sites and larval food resources (Togashi 2002, 2006). PWN presence has previously been associated with other disturbances such as the advancing edge of southern pine beetle (*Dendroctonus frontalis* Zimmermann) infestation in the southeastern U.S. (Kinn & Linit 1992). In another instance, a *Bursaphelenchus* sp. was found to be associated with spruce beetle (*Dendroctonus rufipennis* Kirby), affecting their feeding capacity (Cardoza et al. 2008). Collectively, these associations with disturbance from different systems indicates any cause of tree mortality or stress should be considered as potential contributing factor for PWN outbreaks. Increasing site elevation was associated with decreasing *M. clamator* abundance, but this was not the case with *M. scutellatus*. Study sites spanned an elevational range of 850 m and are likely associated with a substantial thermal gradient which may become further exacerbated by changing climate (Pepin & Losleben 2002). *Monochamus clamator* is more likely than *M. scutellatus* to be



found in areas with higher average temperatures, although both species are abundant at  $>45^{\circ}$  N latitude (Bowers et al. 1992, Saint-Germain et al. 2004). These findings are applicable to management of PWN in other areas as they serve to help define areas of high vector abundance that may serve as likely points of disease establishment.

Observations of PWN incidence across the landscape suggest a patchy distribution of PWN in both *P. ponderosa* and beetle vectors. No obvious regional pattern was observed in the distribution of PWN-positive hosts, however small study site area and limited sampling within sites relative to the region may have obscured any such signal. PWN-positive hosts were encountered at three out of 32 WUI study sites, with 80% of the infected host individuals contained within a single study site that was also the only identified epicenter of infective vector activity during both years of observation. Infective vectors were documented at every WUI site over the two years of the study, although only 50% sites had positive vectors in both years and many instances were of a single infected individual. This suggests some degree of transiency for PWN exposure at sites that may be located further from identified epicenters and links asymptomatic hosts to high vector infection frequencies. The identification of spatiotemporal outliers of PWN-infection frequency in vectors and the range of infected hosts at each site further support the hypothesis that PWN is not uniformly distributed in the native landscape.

The timing of anomalous infection frequencies varied among epicenter sites and could be explained by re-emergence of infective vectors following initial dispersal and oviposition. Similarly, an emergence of a second generation would also corroborate this pattern. Multiple emergences or generations per year may cause additional pulses of infective vectors throughout the season, complicating management efforts targeting specific temporal windows. Additionally, emergences later in the season expose trees to PWN that may already be in a defense-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 (Wang 2012; Roques et al. 2015). All documented cases of asymptomatic PWN-infection during this study were in mature (>14in DBH) otherwise vigorous *P. ponderosa* trees that had no evidence of other infections or damage. While PWN is a known threat to susceptible species used in urban forests, the threat to native pines remains undescribed. Here, we show that exposure windows to PWN vary based on vector flight activity and proximity to disease reservoirs. High infection frequencies early in the season near epicenters indicate elevated risk to trees located nearby. Additional emergences of infective vectors complicate the calculation of true ‘risk’ of PWN infection as this will be a result of both the exposure experienced and conditional susceptibility, both of which fluctuate seasonally. Accordingly, it will be important to determine whether vector beetles re-emerge after dispersal or are capable of achieving multiple generations per year. Experimental inoculations with PWN should also be conducted under controlled abiotic conditions to more accurately assess the threat that PWN poses to native pines after exposure. These efforts will help elucidate what conditions are requisite for symptom expression in *P. ponderosa* and further the understanding of the mechanisms involved in disease development.

New diseases resulting from the introduction of an exotic pathogenic organism (i.e. ‘introduced pests’) or a change in environmental conditions or novel species interaction that causes disease (i.e. ‘emergent pests’) pose unique challenges to forest managers. (Dobson & Foufopoulos 2001; Daszak, Cunningham & Hyatt 2000). In the case of pine wilt disease, there are several biotic factors (fungi, bacteria, and host susceptibility) and environmental variables (increased temperatures and drought) that collectively contribute to mortality rates in pines (Ouyang & Zhang

2003; Lee et al. 2017). Within 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; Rutherford et al. 1992; Zhao et al. 2007a&b); if these thermal trends continue, it could indicate heightened pressure from this disease complex is likely for *P. ponderosa* forests in the Rocky Mountain region. It remains unknown whether PWN is native to the region the emergence of PWN is driven by environmental changes and host stress or is a new introduction; additional studies to analyze PWN genetic structure across the region could address this question and may have key implications for integrated pest management strategies. Our findings collectively serve to define the distribution of the pinewood nematode in the Front Range of Colorado and provide tools that can be used by decision-makers and managers to proactively manage the spread of pine wilt disease.

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**Table 1.** Day of year (mean  $\pm$  SE) on which each flight period occurred. Values generated from solving the logistic curve function for values of 10% (Start), 50% (Peak), and 90% (End) of cumulative vector capture.

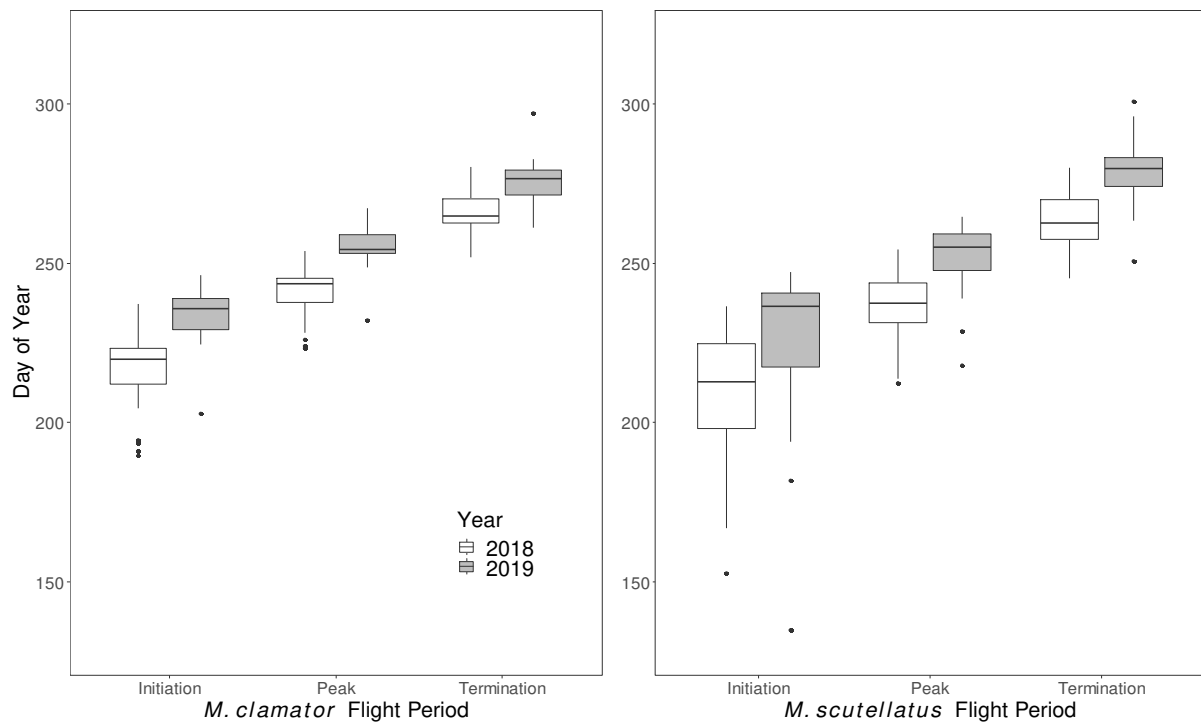
<b>Flight</b>	<i>M. clamator</i>		<i>M. scutellatus</i>	
	2018	2019	2018	2019
Initiation (10%)	215[Aug 03] $\pm$ 2d	230 [Aug 18] $\pm$ 2d	206 [Jul 25] $\pm$ 4d	230 [Aug 18] $\pm$ 8d
Peak (50%)	242[Aug 30] $\pm$ 1d	254 [Sep 11] $\pm$ 1d	237 [Aug 25] $\pm$ 2d	255 [Sep 12] $\pm$ 3d
Term. (90%)	269[Sep 26] $\pm$ 1d	278 [Oct 05] $\pm$ 1d	269 [Sep 26] $\pm$ 2d	280 [Oct 07] $\pm$ 3d

**Table 2.** Generalized linear mixed-effects model results for predicting abundances of two *Monochamus* species from the Front Range region of Colorado. Significant results are indicated in bold. Distance to fire was a significant predictor of abundance for both species, along with elevation for *M. clamator*. Canopy cover, heat-load index (HLI), and distance to forest edge and city were not significant for either species.

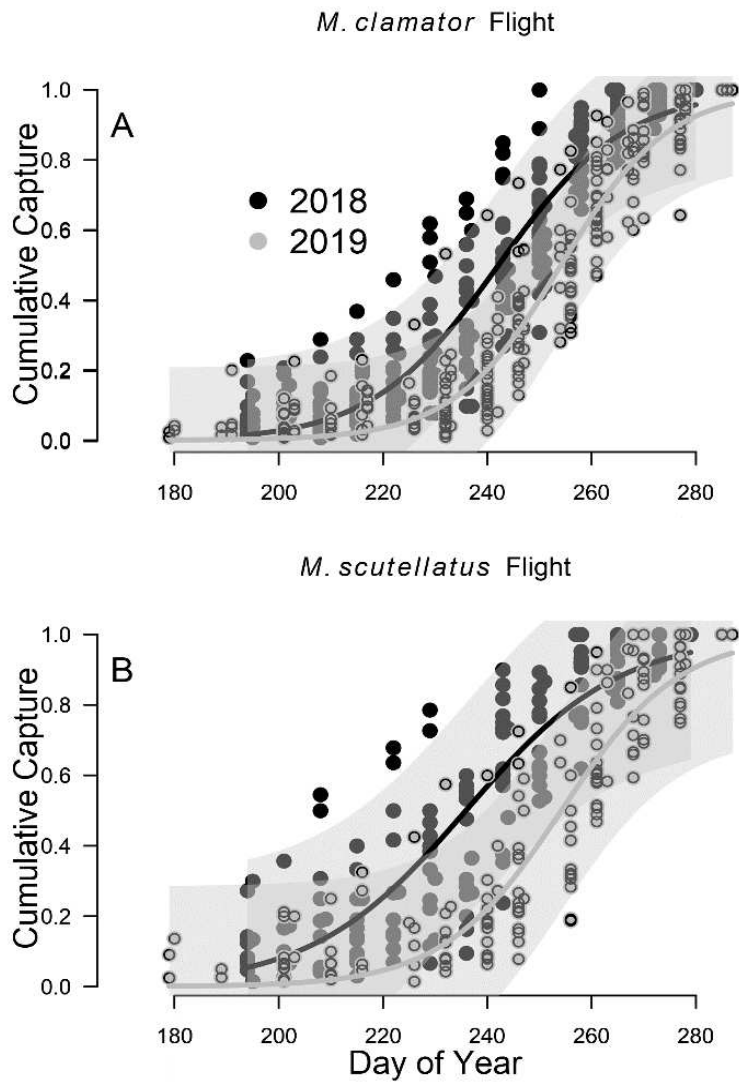
Parameter	<i>Monochamus clamator</i>				<i>Monochamus scutellatus</i>			
	Est.	SE	<i>t</i> -score	<i>P</i>	Est.	SE	<i>t</i> -score	<i>P</i>
Random Effects								
Year (Intercept)	0.003	0.003	--	--	0.001	0.002	--	--
Residual variance	0.002	0.001	--	--	0.003	0.002	--	--
Fixed Effects								
(Intercept)	25.96	7.77	3.342	<0.001	4.819	0.389	12.365	<0.001
<b>Distance to fire (km)</b>	<b>-28.0</b>	<b>9.0</b>	<b>-3.145</b>	<b>0.002</b>	<b>-0.018</b>	<b>0.006</b>	<b>-2.94</b>	<b>0.005</b>
<b>Elevation (m)</b>	<b>-0.386</b>	<b>0.141</b>	<b>-2.737</b>	<b>0.006</b>	0.196	0.122	1.612	0.11
Canopy cover (%)	0.009	0.019	0.516	0.61	0.001	0.016	0.028	0.978
HLI (MJ/cm <sup>2</sup> )	-0.161	3.764	-0.043	0.97	2.319	3.246	0.715	0.478
Dist. forest edge (km)	-12.0	12.0	1.071	0.28	3.0	10.0	0.325	0.746
Dist. nearest city (km)	-8.0	10.0	-0.819	0.41	-18.0	18.0	1.09	0.283

**Table 3.** Mixed-model results for predicting probability of infection amongst all vectors captured in the Front Range region of Colorado. Significant results are indicated in bold.

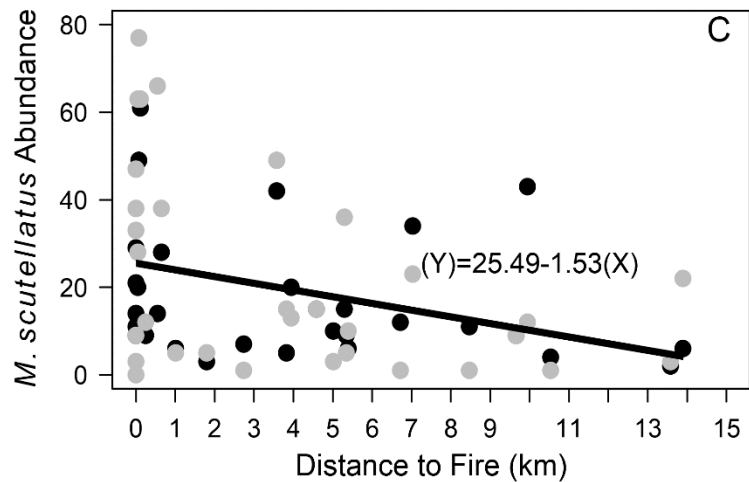
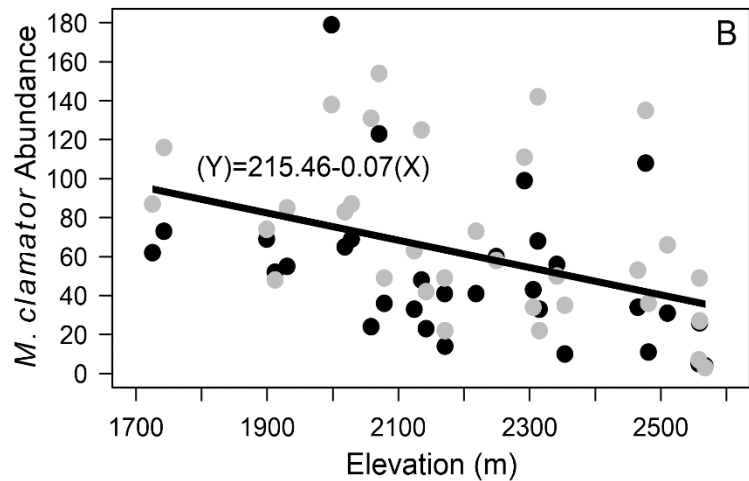
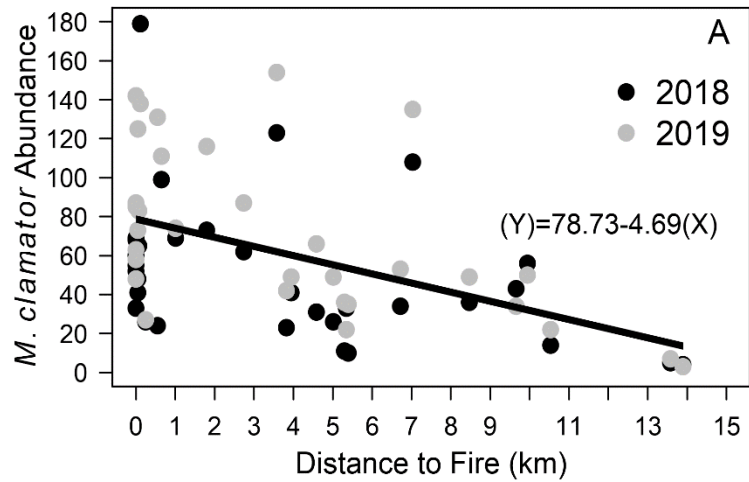
<b>Parameter</b>	<b>Estimate</b>	<b>SE</b>	<b>z-score</b>	<b>P</b>
(Intercept)	-4.368	1.058	-4.130	<0.001
<b>Epicenter</b>	<b>7.369</b>	<b>1.603</b>	<b>4.597</b>	<b>&lt;0.001</b>
Day of Year (DOY)	0.003	0.004	0.788	0.431
<i>M. scutellatus</i>	<b>0.91</b>	<b>0.146</b>	<b>6.237</b>	<b>&lt;0.001</b>
<b>Epicenter:DOY</b>	<b>-0.028</b>	<b>0.007</b>	<b>-4.251</b>	<b>&lt;0.001</b>



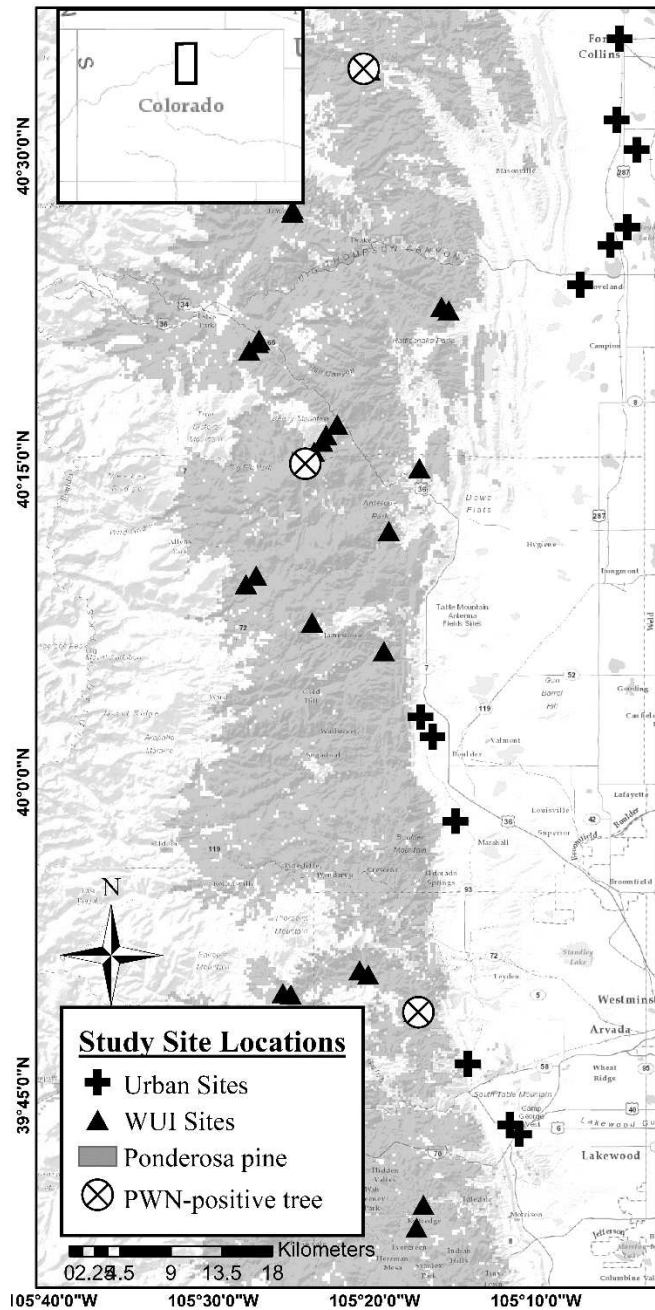
**Figure 1.** Boxplots indicating the distribution of ordinal date for 10% (initiation), 50% (peak), and 90% (termination) of flight of each *Monchamus* species in each study year from 32 sites.



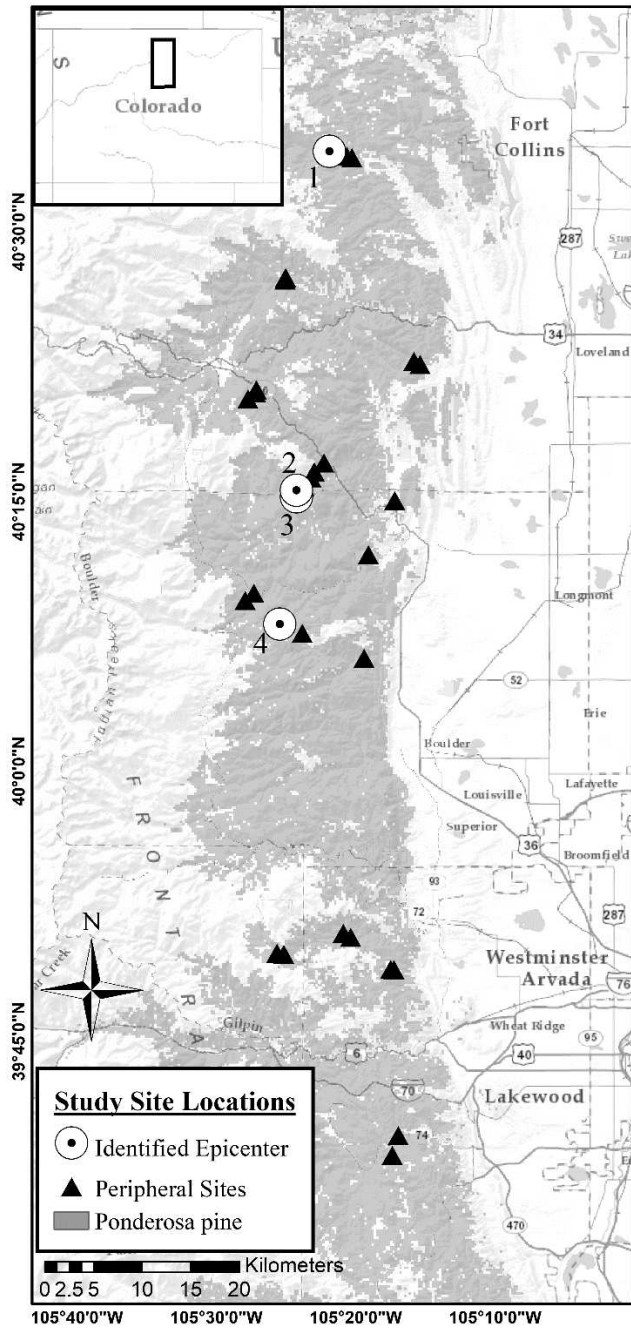
**Figure 2.** Flight phenology models for both species of vector. (A) *Monochamus clamator* and (B) *M. scutellatus* are both found in the Front Range region of CO. Flight phenology represented as 2-parameter logistic regression with cumulative proportion of captures as the response and day of year as the predictor. Data collected over 2 years in the Front Range region of Colorado.



**Figure 3.** Least-squares regression shown for the response in *Monochamus clamator* abundance to distance to fire (A), elevation (B) and *M. scutellatus* response to distance to fire (C) from 32 sites over 2 years in the Front Range region of Colorado.

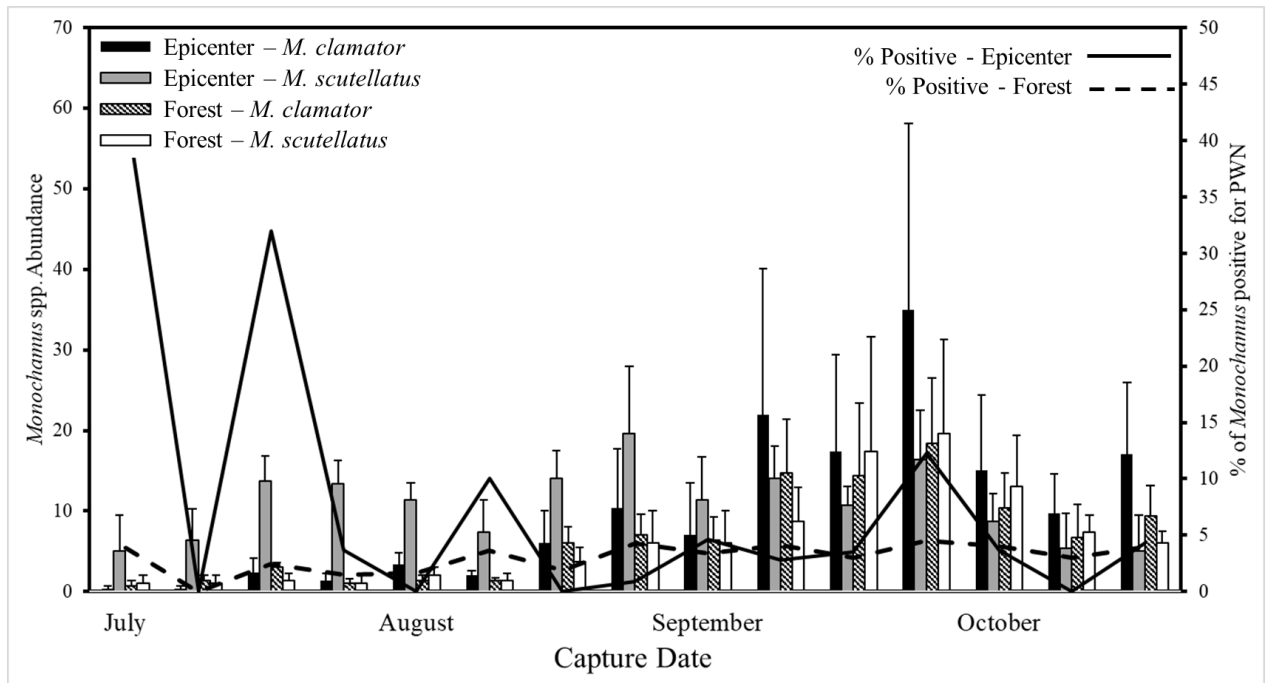


**Figure 4.** Survey for PWN in host pines. This map shows all urban and WUI sites from which tree samples were taken during 2018 & 2019. Areas where a host pine tested positive for PWN are indicated (N=3).

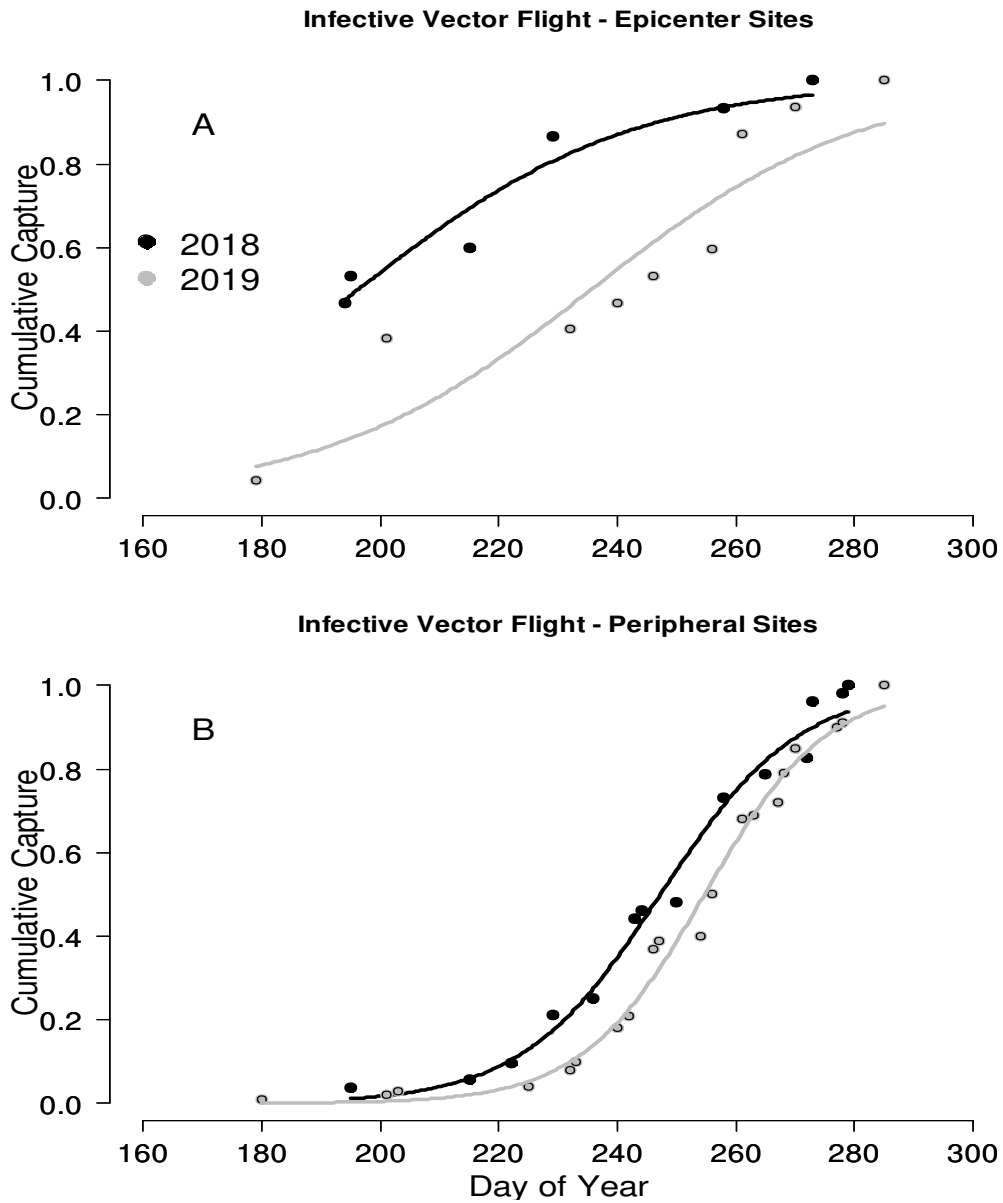


**Figure 5.** Epicenters identified at WUI Study Sites in the Front Range of Colorado. This map shows the location of the four epicenters of infective vector activity identified from the 32 WUI study sites.





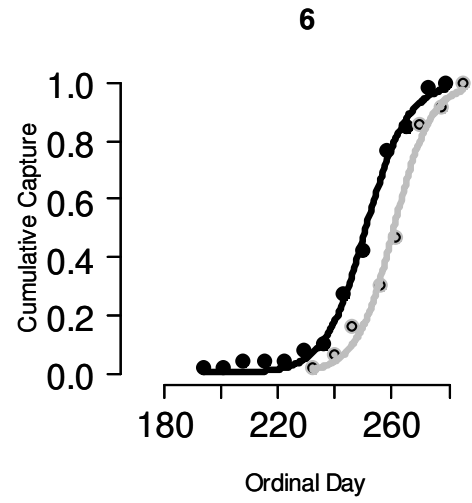
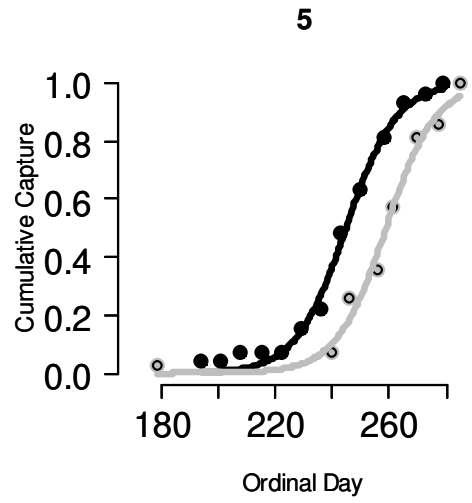
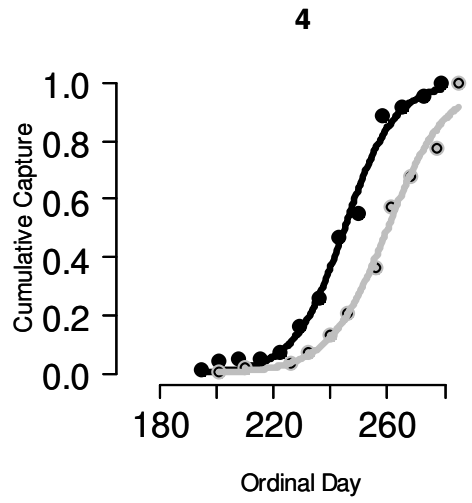
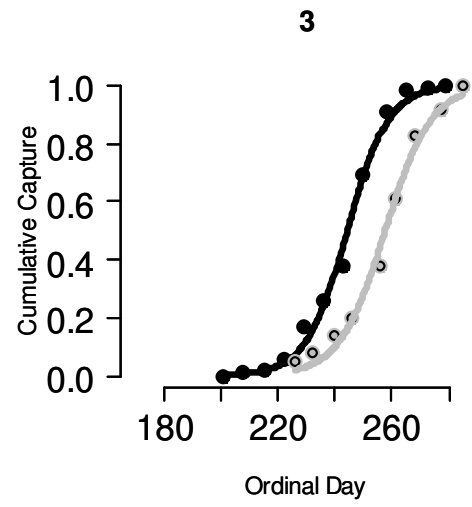
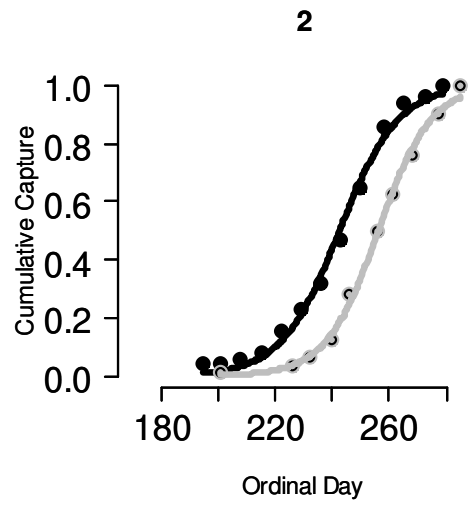
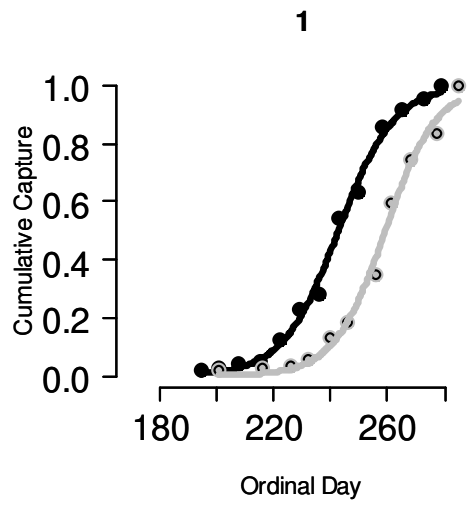
**Figure 6.** Seasonal variation in average *Monochamus clamator* and *M. scutellatus* captures per site (left axis) and overall infection rate of vectors (right axis) between identified epicenter and forest sites.

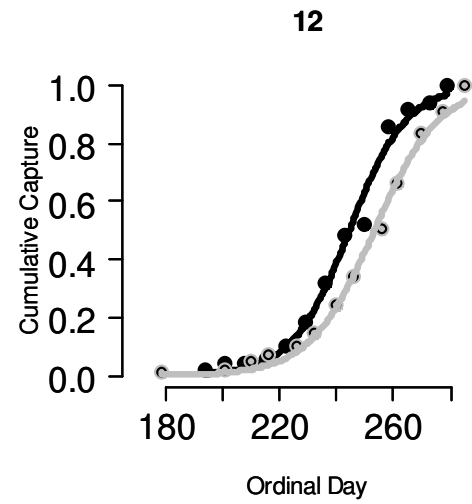
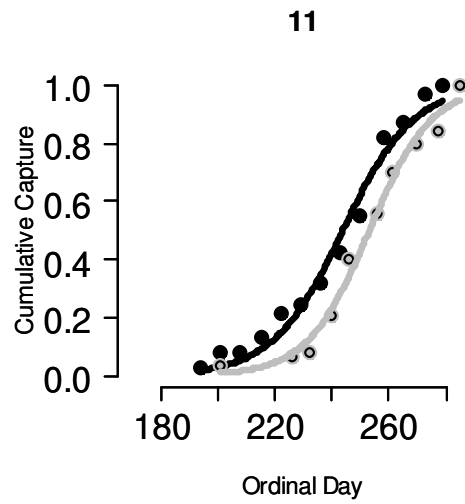
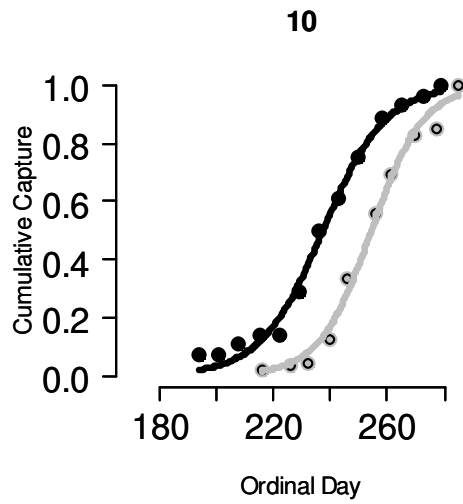
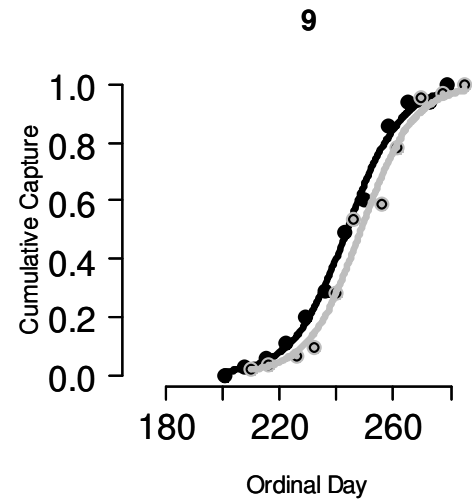
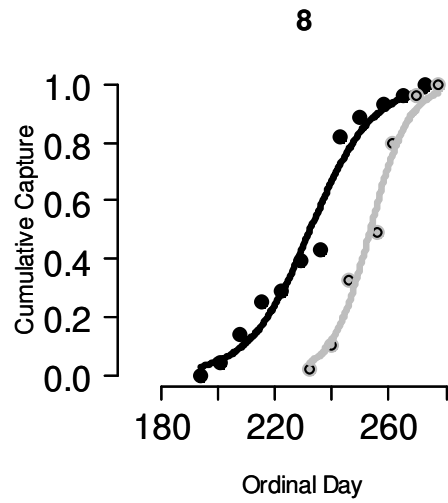
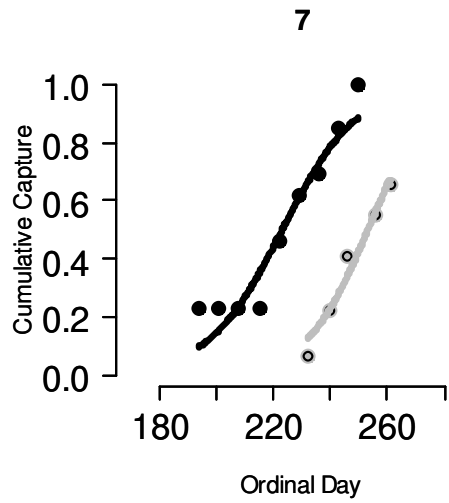


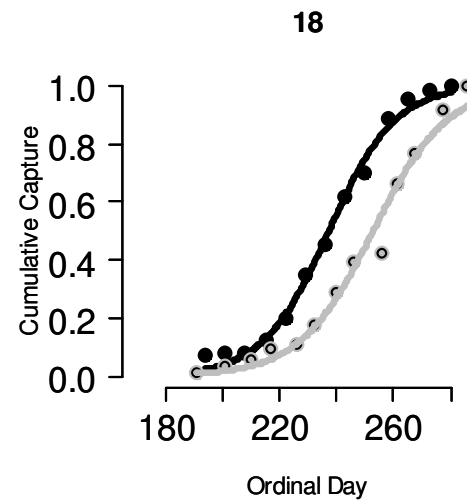
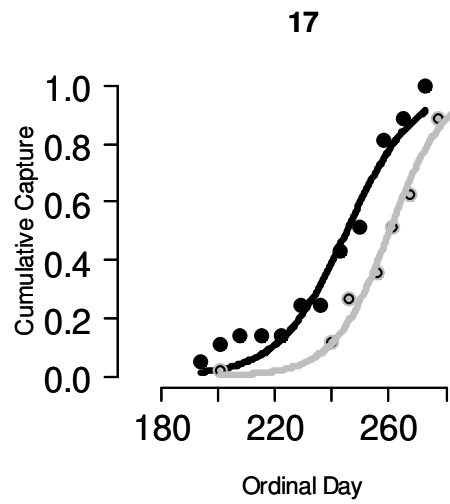
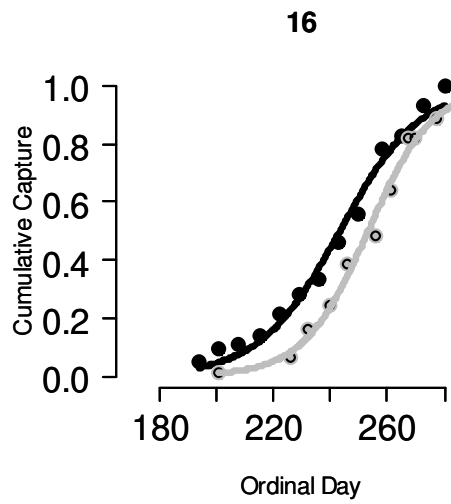
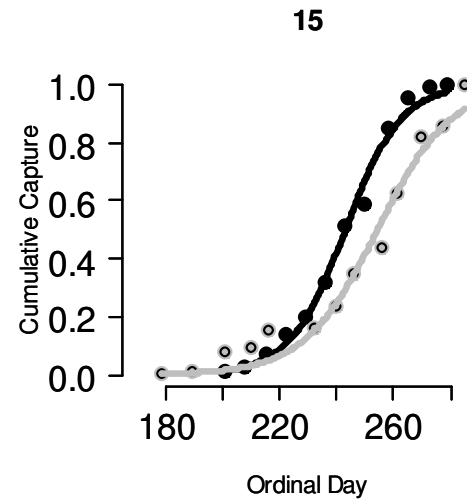
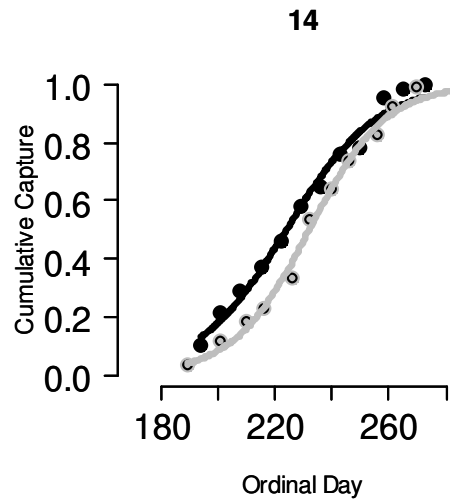
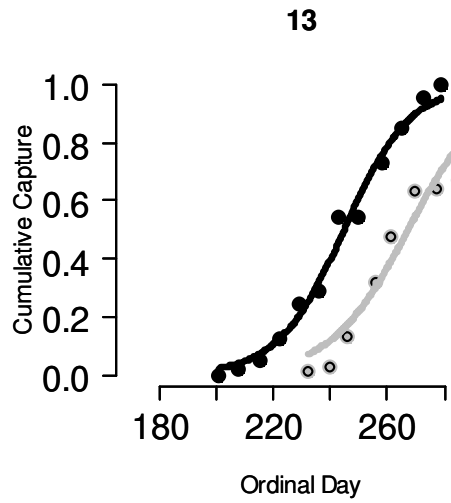
**Figure 7.** Flight phenology models for PWN-positive *Monochamus* spp. found in the Front Range region of CO. Flight phenology represented as 2-parameter logistic regression with cumulative proportion of infected captures as the response and day of year as the predictor for both epicenter (N=4) and peripheral (N=28) WUI study sites.

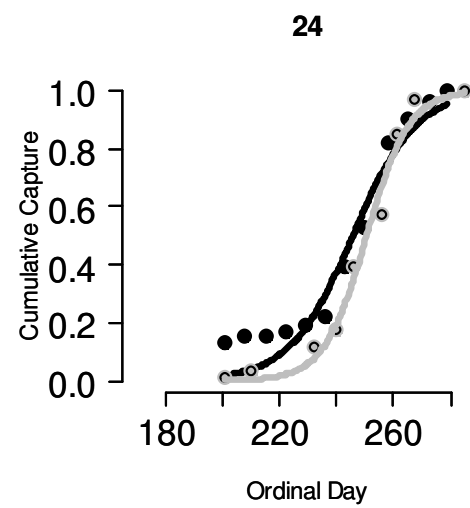
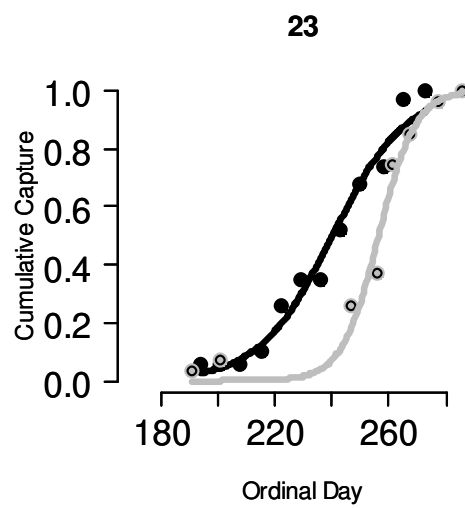
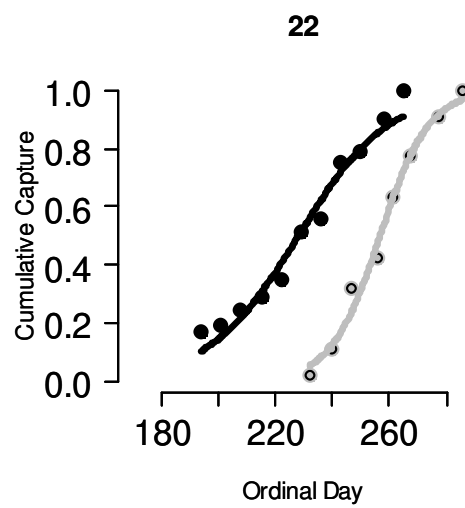
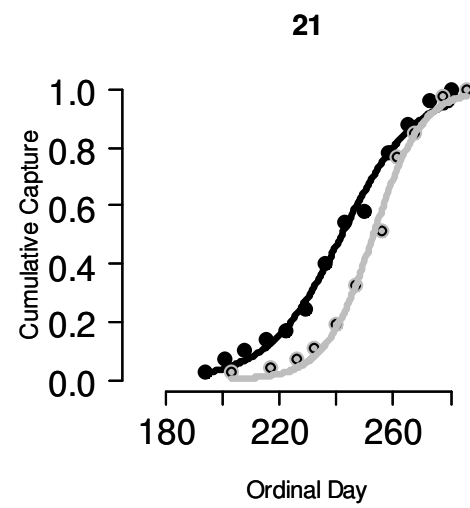
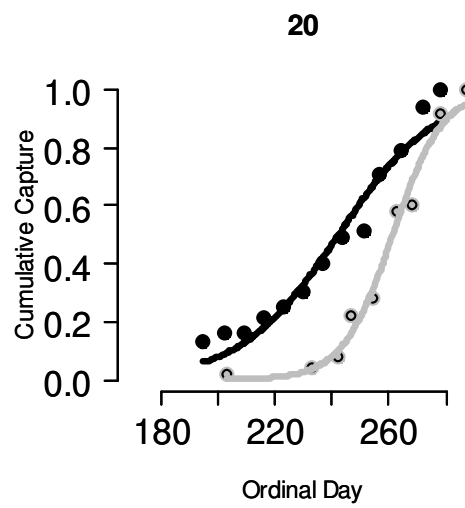
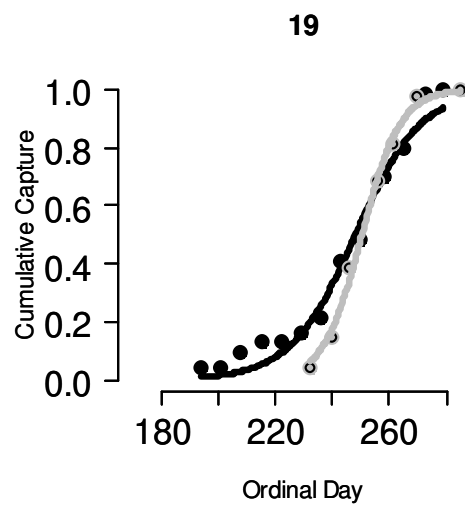
**Table S1.** Study site descriptions. This table shows the coordinates, landscape type, total vector captures, and % of captures positive for PWN across both years of the study. Numbers in parentheses indicate a site was an identified epicenter (Figure 4).

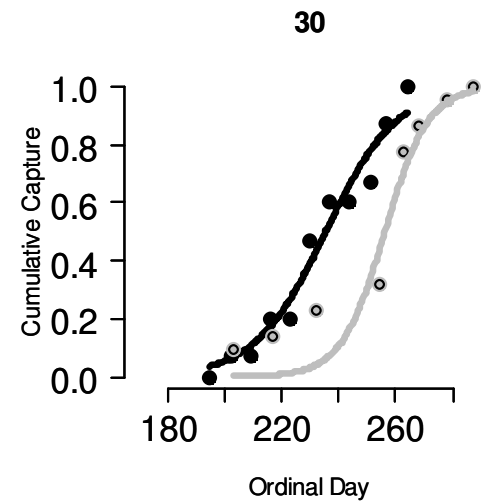
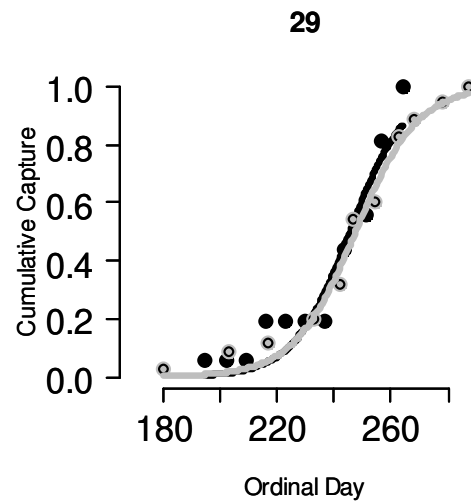
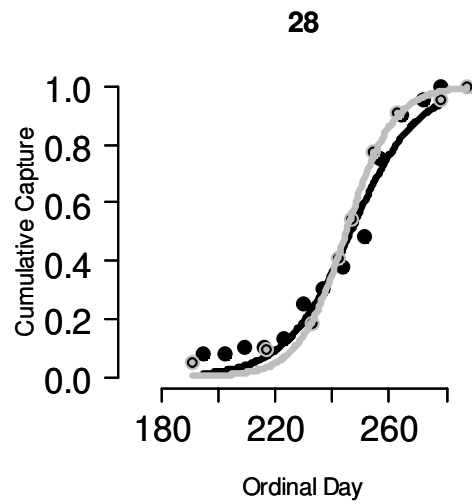
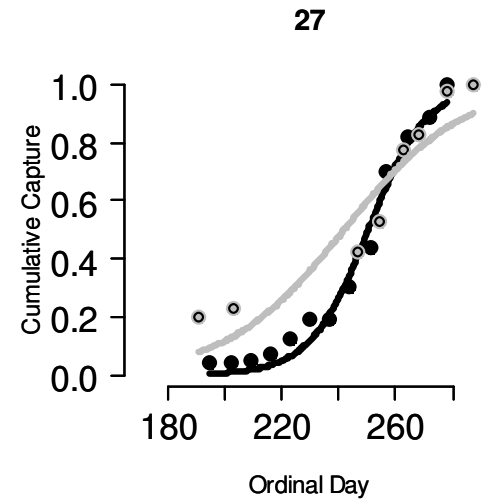
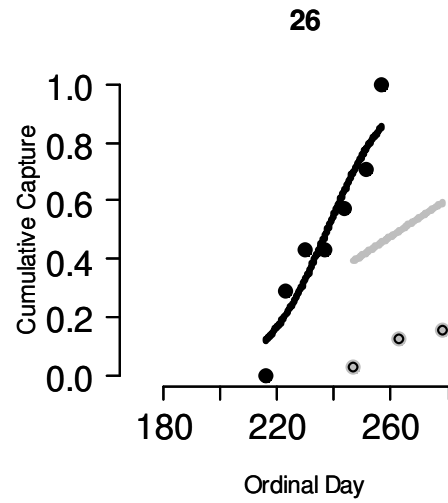
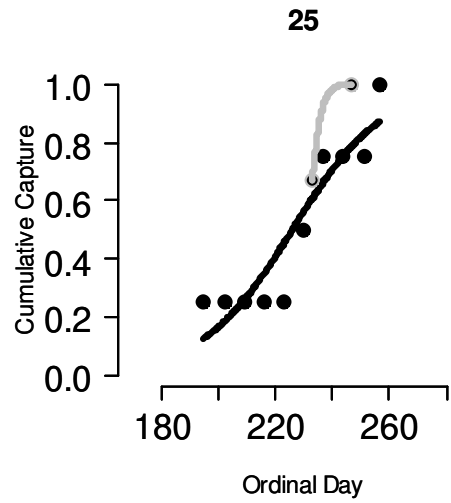
Site	Latitude	Longitude	Landscape	<i>M. clamator</i>	<i>M. scutellatus</i>	% vector with PWN	% host with PWN
1	40°34'11.52"	-105°20'36.06"	WUI	334	98	6.5	11
2	40°34'16.37"	-105°20'58.21"	WUI	162	113	5.1	0
3	40°34'20.04"	-105°21'9.36"	WUI	186	55	2.9	0
4 (1)	40°34'33.12"	-105°22'14.34"	WUI	286	79	3.6	0
5	40°27'30.84"	-105°25'23.58"	WUI	68	20	3.4	0
6	40°27'22.62"	-105°25'25.02"	WUI	96	28	6.5	0
7	40°21'16.38"	-105°27'28.38"	WUI	49	48	5.2	0
8	40°21'6.60"	-105°27'32.04"	WUI	76	8	2.4	0
9	40°20'44.94"	-105°28'4.74"	WUI	99	29	3.9	0
10	40°17'13.86"	-105°22'32.70"	WUI	158	82	4.2	0
11	40°16'43.98"	-105°23'14.46"	WUI	101	58	3.1	0
12	40°16'24.12"	-105°23'29.16"	WUI	177	72	3.2	0
13	40°15'54.66"	-105°23'59.22"	WUI	113	49	2.5	0
14 (2)	40°15'20.40"	-105°24'31.38"	WUI	211	65	9.8	89
15 (3)	40°15'42.72"	-105°24'31.74"	WUI	217	39	5.1	0
16	40°12'8.16"	-105°19'17.76"	WUI	197	7	2.5	0
17	40°9'58.26"	-105°27'34.26"	WUI	90	10	1	0
18	40°9'33.66"	-105°28'12.42"	WUI	253	47	4.7	0
19	40°22'43.02"	-105°15'34.98"	WUI	104	0	1.9	0
20	39°50'52.38"	-105°20'28.68"	WUI	112	32	2.8	0
21	40°6'22.92"	-105°19'34.44"	WUI	146	9	1.3	0
22	40°7'44.64"	-105°24'3.30"	WUI	120	20	2.1	0
23 (4)	40°8'15.78"	-105°25'40.80"	WUI	58	19	11.7	0
24	40°15'9.00"	-105°17'23.82"	WUI	158	2	1.3	0
25	39°49'58.62"	-105°25'47.88"	WUI	5	32	13.5	0
26	39°49'54.84"	-105°25'17.10"	WUI	13	4	5.9	0
27	39°51'4.86"	-105°21'0.72"	WUI	91	13	1.9	0
28	39°49'5.16"	-105°17'20.82"	WUI	62	8	1.4	1
29	39°49'5.40"	-105°17'35.34"	WUI	51	16	3	0
30	39°38'45.78"	-105°17'25.20"	WUI	37	3	2.5	0
31	39°39'51.66"	-105°16'59.6"	WUI	88	5	1.1	0
32	40°22.887'	-105°16'2.7"	WUI	150	8	4.4	0
33	40°35'44.53"	-105°4' 53.0"	Urban	1	0	0	0
34	40°31'51.67"	-105° 5' 3.91"	Urban	1	0	0	0
35	40°30'25.83"	-105°3' 48.2"	Urban	0	0	0	0
36	40°26'43.47"	-105°4' 23.01"	Urban	0	0	0	0
37	40°25'51.72"	-105°5' 27.38"	Urban	2	0	0	0
38	40°23'57.68"	-105°7' 20.81"	Urban	8	6	7.1	0
39	40°3'14.29"	-105°17' 15.84"	Urban	0	1	0	0
40	40°2'17.97"	-105°16' 30.68"	Urban	2	2	0	0
41	39°58'15.12"	-105°15' 4.06"	Urban	8	0	0	0
42	39°46'37.78"	-105°14' 15.48"	Urban	2	0	0	0
43	39°43'42.5"	-105°11' 37.91"	Urban	6	0	0	0
44	39°43'15.85"	-105°11' 4.218"	Urban	7	0	0	0



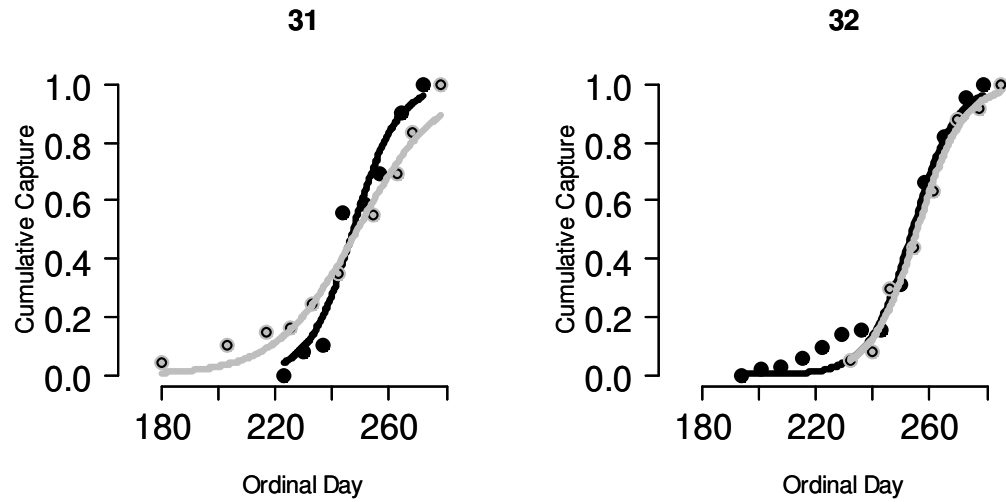




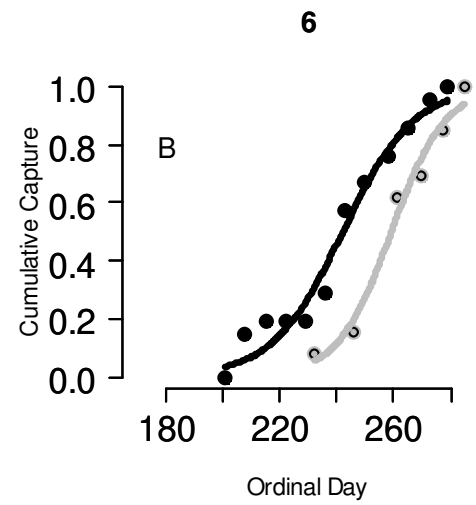
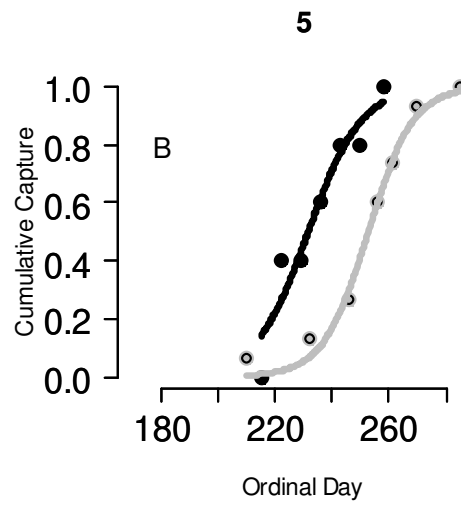
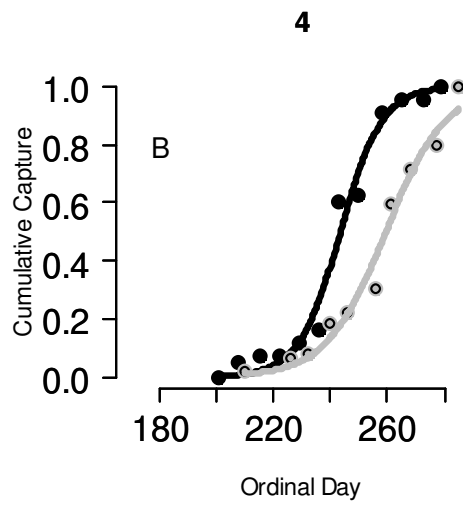
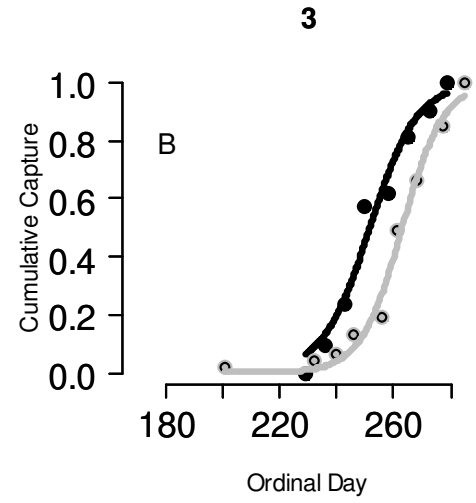
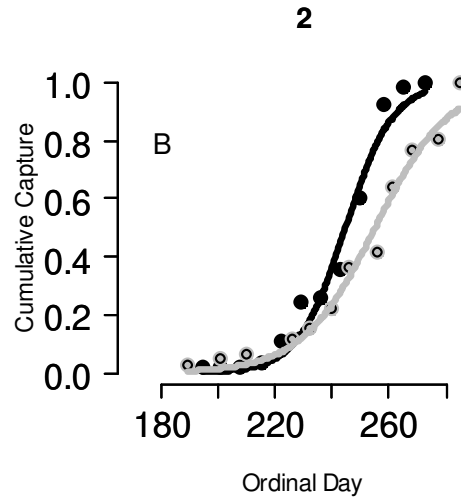
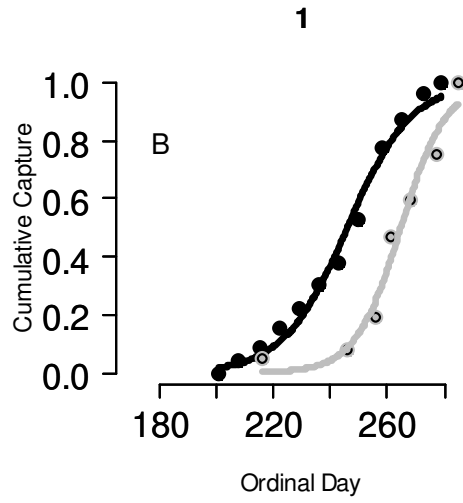


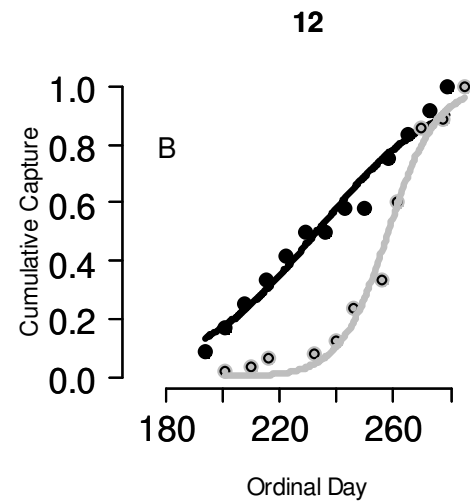
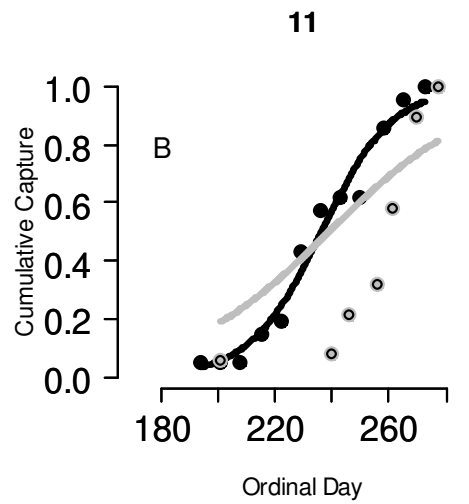
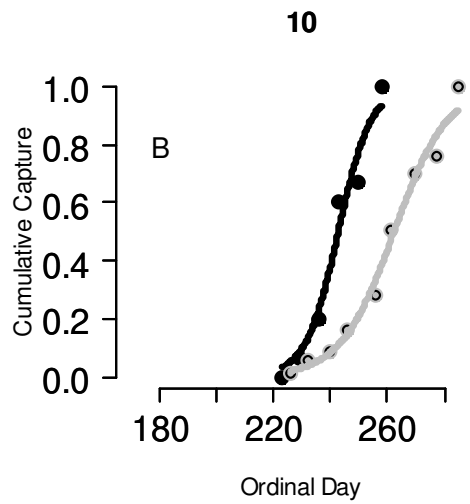
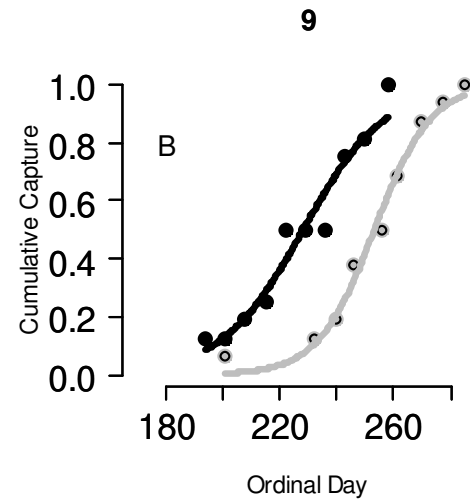
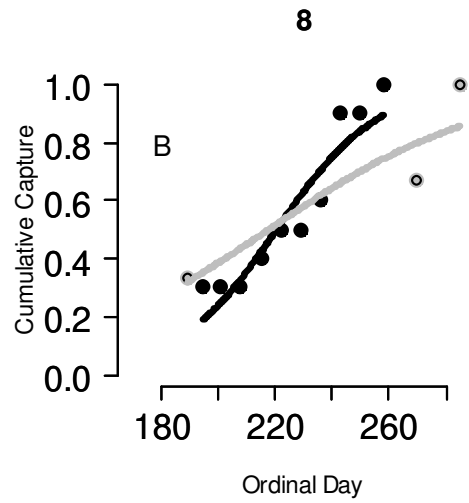
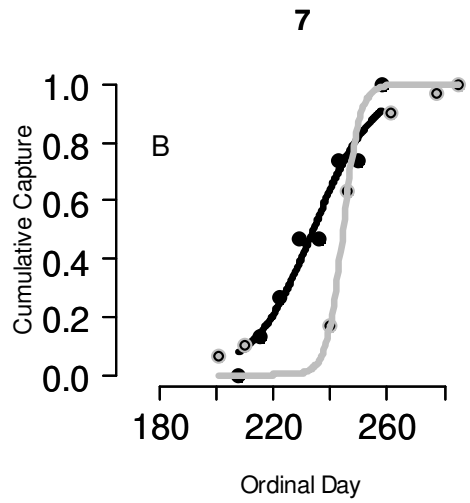


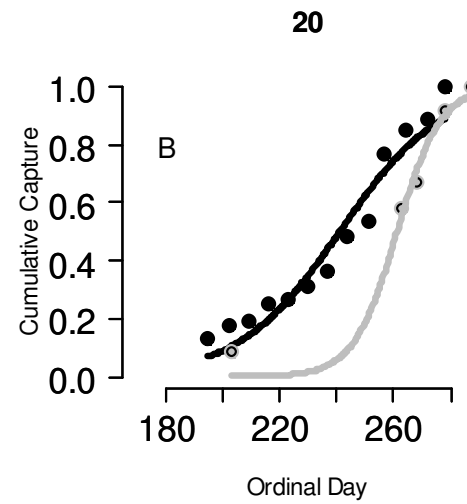
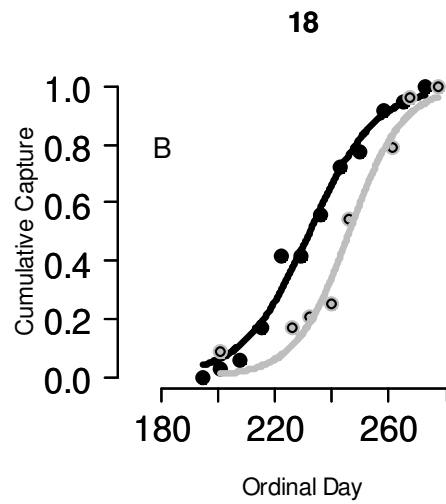
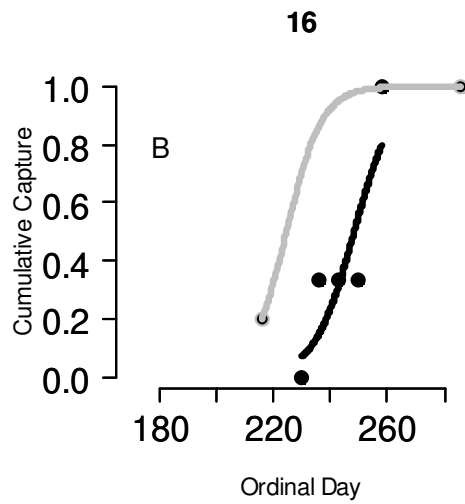
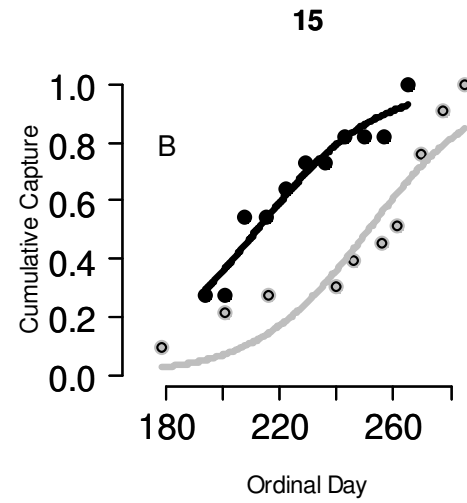
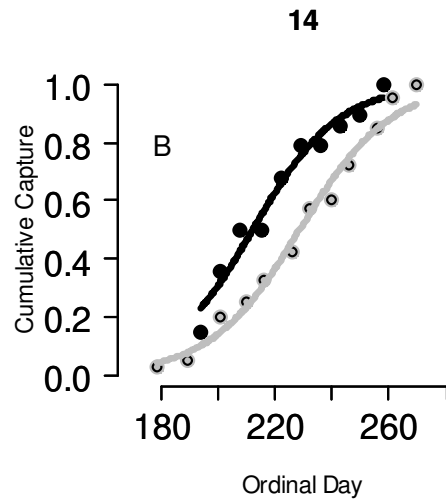
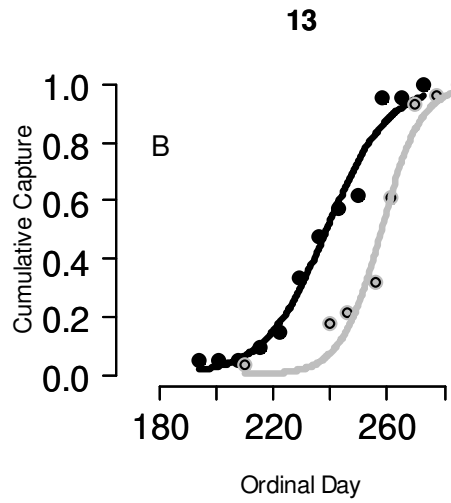


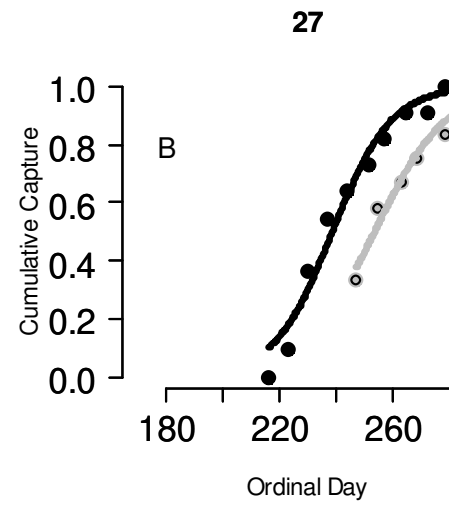
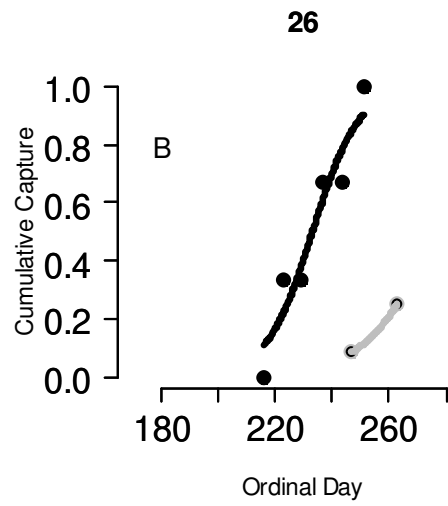
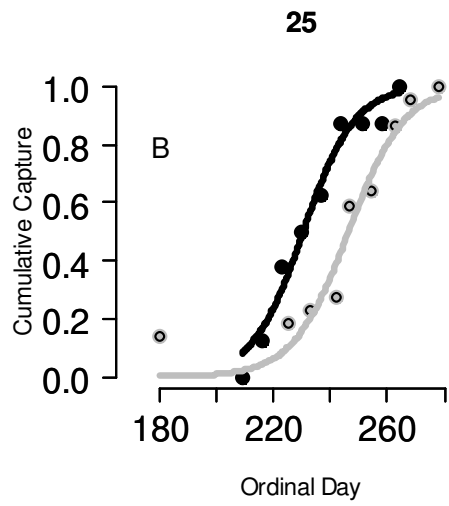
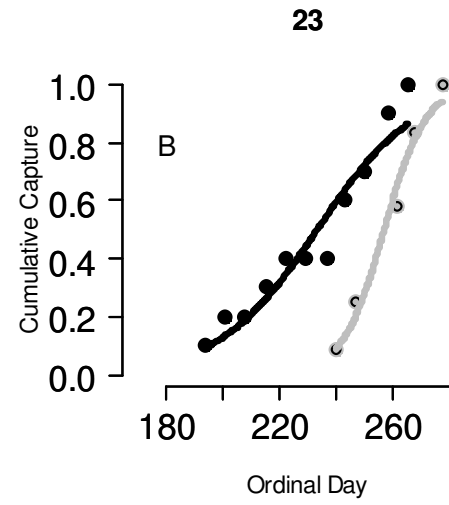
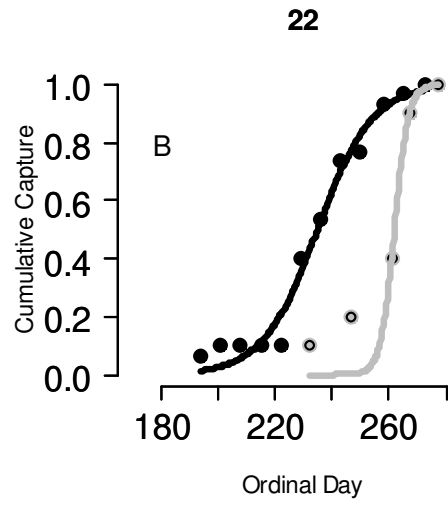
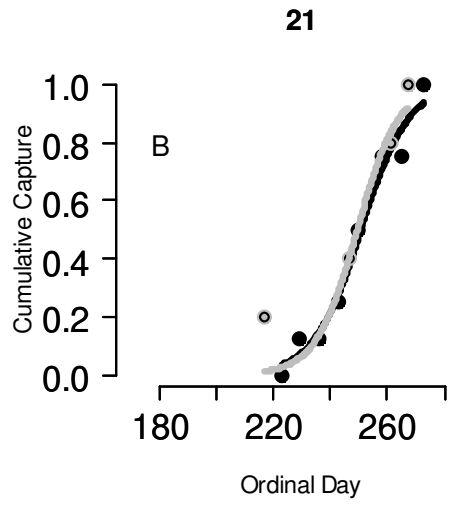


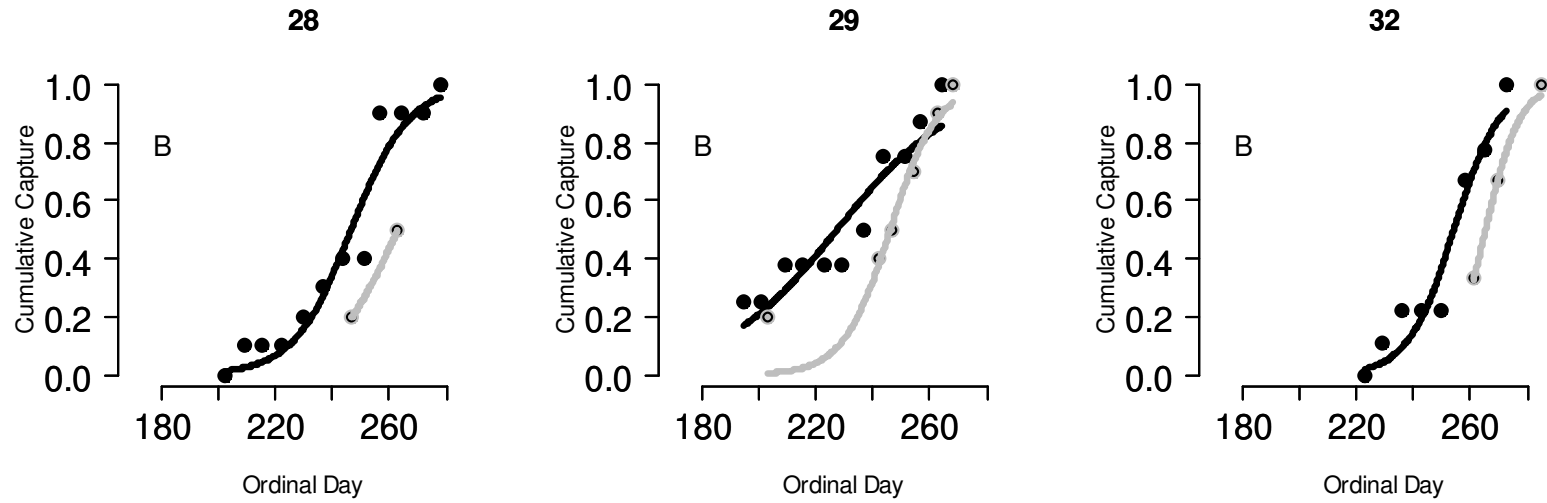
**Figure S1.** Flight phenology models for *Monochams clamator* at all sites. Flight phenology represented as 2-parameter logistic regression with cumulative proportion of captures as the response and day of year as the predictor. Data collected over 2 years in the Front Range region of Colorado.











**Figure S2.** Flight phenology models for *Monochams scutellatus* at all sites. Flight phenology represented as 2-parameter logistic regression with cumulative proportion of captures as the response and day of year as the predictor. Data collected over 2 years in the Front Range region of Colorado.