Ecological behavior of velvet longhorned beetle *Trichoferus campestris*

(Coleoptera: Cerambycidae), including hosts and phenology

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Dedication

I dedicate this thesis to my nieces, Matilda and Astrid (and my budding nephew Lucián). You were an integral part of naming beetles, and I love you all very much.

Thank you for reminding me why environmental stewardship is so important.
Thesis Abstract

Velvet longhorned beetle *Trichoferus campestris* (Faldermann) (Coleoptera: Cerambycidae) is a longhorned beetle native to eastern Russia and Asia. It has established populations in countries far beyond its native range, including the United States, but its ecological impacts remain unclear. The biology and behavior of *T. campestris*, such as preferred hosts and seasonal phenology, have not been thoroughly studied. *Trichoferus campestris* is thought to be polyphagous and univoltine, with a peak flight period in mid-summer, but few if any studies exist on these topics. Knowledge of these characteristics can assist with effective strategies for monitoring and managing nonnative species. Here, we investigate hosts and phenology of *T. campestris*. We quantify ovipositional preferences and larval/developmental performance using choice and no-choice assays comparing the suitability of material harvested from different tree species of the upper midwestern United States: black walnut, *Juglans nigra*; the ‘Honeycrisp’ apple cultivar, *Malus domestica*; sugar maple, *Acer saccharum*; and eastern white pine, *Pinus strobus*. We study differences in oviposition between *Malus domestica* branches with vs. without cankers as well. We complement these laboratory studies with catch data from field trapping experiments, analyzing trends among traps hung in various genera of trees. Finally, we used trap catch numbers and local temperature data to elucidate the phenology of *T. campestris* in Minneapolis and St. Paul, Minnesota, USA in the summers of 2019 and 2020.
Thesis Introduction

As the impacts of human disturbance and other invasive species compound, ecosystems may become more vulnerable, leading to greater potential damage to landscapes (Mehta et al., 2007). Some species have devastating impacts to novel hosts in their introduced ranges, such as *Anoplophora glabripennis* (Motschulsky) (Coleoptera: Cerambycidae), known as the “starry sky beetle” in its native range, and *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), or emerald ash borer (Haack et al., 1997; Haack, 2006; Haack et al., 2015). Other species appear much more benign, or can exhibit a long delay between arrival and impact (Epanchin-Niell and Liebhold, 2015). For this reason, knowledge of an introduced species’ biological attributes is crucial to developing effective management strategies in the event that the species becomes an ecological or economic problem (Suckling et al., 2012; Crimmins et al., 2020).

Risk assessments and responses to introduced insects should be informed by the insect’s potential and realized hosts in the novel environment. In the case of mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Curculionidae), laboratory studies confirmed the potential susceptibility of novel pine hosts as climate change allowed the beetle’s geographic range to expand (Rosenberger et al., 2018). Likewise, field observations of larval *Agrilus planipennis* infestations on novel host white fringetree, *Chionanthus virginicus* L., complemented laboratory observations of adult feeding on the same species (Cipollini, 2015). Elucidation of an insect’s phenology can also improve pest detection, suppression, and management for insect species whose visibility, susceptibility to control measures, and ability to spread differ across life stages.
Wood borers complete their larval stage beneath the bark of their tree hosts, for example.

The velvet longhorned beetle, *Trichoferus campestris* (Faldermann) (Coleoptera: Cerambycidae), is a beetle introduced to North America whose potential impacts have not yet been determined with certainty. Native to northeast Asia, *T. campestris* was first intercepted in North America in the 1990s and has since been intercepted in 15 US states and 3 Canadian provinces. In the USA, it has known established populations in Utah and Illinois and a presumed established population in Minnesota (Iwata and Yamada, 1990; Cocquempot, 2006; Dascălu et al., 2013; Pennacchio et al., 2016; Keszthelyi et al., 2019). This Cerambycid beetle lays eggs on tree trunks and branches as well as stored products made of wood, including solid wood packing material and wooden furniture (Keszthelyi et al., 2019). Upon eclosion, the larvae tunnel underneath the bark to consume the phloem and xylem tissues, but sources contain conflicting information regarding the pest potential of *T. campestris*. Several studies refer to it as a drywood or lumber pest (Kostin, 1973; Iwata and Yamada, 1990; Ximing and Miao, 1998); other studies disagree about whether the beetle causes minor damage (Guo and Yin, 2005; Liu and Li, 2014; Gui et al., 2016) vs. weakness and death in its tree hosts (Makhnovskii, 1966; Dascălu et al., 2013). To date, the only study with an in-depth investigation on the impacts of *T. campestris* of which we are aware found it to be only a secondary factor in the death of a Norway maple (*Acer planatoides*) in Mississauga, Ontario, Canada, with *Armillaria* root rot as the primary cause of death (Bullas-Appleton et al., 2014).
Studies from the native range of *T. campestris* suggest that this beetle is highly polyphagous. It can infest trees in over 40 genera, including both softwoods and hardwoods (Makhnovskii, 1966; Kostin, 1973; Svacha and Danilevsky, 1987; Wang et al., 1988; Iwata and Yamada, 1990; Liu and Li, 2014; Bullas-Appleton et al., 2014). In some instances, infestations of *T. campestris* have even been found in dried plant materials such as milkvetch *Astragalus* spp., a forb of importance in traditional Chinese medicine (Jiang and Zhang, 1996). The beetle’s preferences between hosts is unclear. There is some evidence that *T. campestris* prefers apple when given a choice between apple and other fruit trees (Zhang et al., 2017), and *T. campestris* is frequently observed in cultivated or wild orchards (Kostin, 1973; Bai and Zhang, 1999; Bullas-Appleton et al., 2014; Zhang et al., 2017). Additionally, multiple sources report a preference for trees in the genera *Malus* and/or *Morus* (Kostin, 1973; EPPO, 2008; EPPO, 2009; Bullas-Appleton et al., 2014). However, these claims do not come from comparative studies that make use of host choice assays.

There are also discrepancies between sources as to whether *T. campestris* prefers stressed trees, healthy trees, or dead wood (Bai and Zhang, 1999; Li et al., 2009; Dascălu et al., 2013; Bullas-Appleton et al., 2014; Pennacchio et al., 2016). One study noted that *T. campestris* tends to lay eggs on rougher bark (Zhang et al., 1977), which may be associated with reduced tree vigor if caused by phytopathogens (Guyon et al., 1996; Desprez-Loustau et al., 2006; Nabi et al., 2018). Few of these studies on *T. campestris* originate from North America, however, where there is limited knowledge on its reproductive ecology and behavior.
Similarly, knowledge of the life cycle of *T. campestris* is sparse; sources report variable voltinism, ranging from one to three years (Makhnovskii, 1966; Li et al., 2009; Hegyessy and Kutasi, 2010; Pennacchio et al., 2016). Factors influencing this life cycle variability, such as climate or host characteristics, have yet to be investigated (Cherepanov, 1981; Svacha and Danilevsky, 1987). In its native range, adults of *T. campestris* emerge between May and September (Cherepanov, 1981; Xinming and Miao, 1998; Grebennikov and Gill, 2010). Previous work on theoretical worldwide species distribution of *T. campestris* has assumed 699 cumulative degree days above 10˚C are necessary for successful development from egg to adult (Keszthelyi et al., 2019), although we are not aware of any published studies that document the developmental temperature thresholds of *T. campestris* in a laboratory setting. Because behavior can differ between historical and novel ranges (Yan et al., 2005; Formby et al., 2018), it is important to complement such theoretical studies with applied local field studies using local climatic conditions (Keszthelyi et al., 2019).

The goal of my thesis is to study the behavior and reproductive ecology of *T. campestris*, particularly in its introduced range in Minnesota. In chapter 1, I assessed host suitability of cut branches of four North American tree species that I identified as potential hosts. I conducted host choice assays comparing oviposition rates between species and no choice assays assessing larval development on and adult emergence from cut branches of the same four tree species. I also tested whether or not *T. campestris* would preferentially oviposit and develop more rapidly on cut branches with cankered bark than on cut branches with no cankers, exploring potential associations between bark
roughness and oviposition. In chapter 2, I describe temporal patterns of T. campestris in Minnesota using adult abundance as monitored by Lindgren funnel traps baited with species-specific pheromones over the course of two summers (2019-2020). I describe variation in seasonal flight using seasonal accumulation of degree days over a calendar year as calculated using local temperature data. I also compare rates of capture of T. campestris in traps hung in trees of various genera. Both of these chapters aim to inform management goals should they become necessary.

The chapters of my thesis were prepared for publication in peer-reviewed journals. Although I am the lead author, this research reflects the combined efforts of multiple authors, so I use the plural voice throughout. Chapter 1 is being prepared for submission to Agricultural and Forest Entomology, and Chapter 2 is being prepared for submission to Environmental Entomology. Any redundancy between the frameworks for these chapters is included to preserve the integrity of each chapter as a stand-alone unit.
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Chapter 1

Ovipositional behavior and larval performance of *Trichoferus campestris* (Faldermann) (Coleoptera: Cerambycidae) on cut logs of four North American tree species
Synopsis

Velvet longhorned beetle *Trichoferus campestris* (Faldermann) (Coleoptera: Cerambycidae) is native to eastern Europe and Asia. It has established populations in several states in the United States. This beetle is capable of feeding on and reproducing within a wide variety of tree species in its native range. However, ovipositional preferences and developmental performance in potential hosts have not been studied in its introduced range.

We conducted choice and no-choice host experiments in 2019 and 2020. For ovipositional choice assays, mated female *T. campestris* were allowed to freely oviposit on cut branches of four different North American tree species: black walnut, *Juglans nigra*; the ‘Honeycrisp’ apple cultivar, *Malus domestica*; sugar maple, *Acer saccharum*; and eastern white pine, *Pinus strobus*. We separately investigated oviposition between apple branches with and without cankers from *Cytospora* spp. and *Phomopsis* spp. Half of the choice trials were exposed to winter chill temperatures. For no-choice assays, eggs were placed on cut branches of these same four species. In the no-choice assays, half of the branches were dissected at intermediate points to measure insect development. The other half of the branches from our host suitability assays were exposed to winter chill temperatures and *T. campestris* emergence was quantified.

*Trichoferus campestris* laid the most eggs on *J. nigra* and the least on *P. strobus*. It laid twice as many eggs on cankered apple wood as on uncankeraded apple wood. Across the four species, larvae were least likely to be recovered alive on *P. strobus* six weeks following oviposition. Larvae grew largest on *J. nigra* and *M. domestica* after 5 months.
of development. More adults emerged from *J. nigra* and *M. domestica* than from *A. saccharum* or *P. strobus* in both 2019 and 2020 trials. Choice assays demonstrated benefits of a chill treatment on larval development and emergence. Our results suggest that *T. campestris* is polyphagous, but that its oviposition and development occur more readily on *J. nigra* and *M. domestica* than on *A. saccharum* or *P. strobus*. 
**Introduction**

Every year, nonindigenous forest insects are introduced to the United States via solid wood packing material such as pallets or dunnage, often associated with international trade (Haack, 2006). Coleoptera are most often associated with wood packing material, as phloem and wood boring beetles are overrepresented among those introduced forest pests that cause notable impacts (Haack, 2006; J.E. Aukema et al., 2010). Though it is rare for these insects to establish, those that do can have devastating effects on ecosystems. Some well-publicized examples include *Anoplophora glabripennis* (Montschulsky) (Coleoptera: Cerambycidae) and *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), which have led to widespread death of their tree hosts in the United States (Haack et al., 1997; Haack, 2006; Haack et al., 2015). This damage to ecosystems is exacerbated by increasing ecosystem vulnerability over time, as the impacts of human disturbance and other invasive species compound (Mehta et al., 2007).

Despite high-profile examples of exotic wood borers with profound impacts in their new range, many more insects establish with no notable impacts to their novel ecosystems. For instance, the Palearctic beetle *Agrilus cyanescens* (Ratzeburg) (Coleoptera: Buprestidae) has yet to cause any noticeable impact in North America, where it is long established and feeds on plants in the genera *Lonicera* and *Rhamnus* (J.E. Aukema et al., 2010; Maier, 2012; Hallinen et al., 2021). It can be difficult to predict whether or not an introduced insect species will negatively impact its novel environment, particularly because there can be a delay between initial interception at a port of entry.
and evidence of establishment (Bullas-Appleton et al., 2014). In the event that an introduced species becomes problematic, knowledge of its biological attributes, such as host range, degree of damage, and mobility, is crucial to developing effective management techniques (Suckling et al., 2012).

*Trichoferus campestris* (Faldermann) (Coleoptera: Cerambycidae) is one such introduced beetle whose potential impacts have not yet been determined with certainty. Native to northeast Asia, *T. campestris* was first intercepted in North America in the 1990s and has since been intercepted in 15 US states and 3 Canadian provinces. In the USA, established populations occur in Utah, Wisconsin, and Illinois and presumably in Minnesota as well (Iwata and Yamada, 1990; Cocquempot, 2006; Dascălu et al., 2013; Pennacchio et al., 2016; Keszthelyi et al., 2019). This Cerambycid lays eggs on tree trunks and branches. Some authors report that it can only develop on wood when bark is present (Iwata and Yamada, 1990), likely because of reports of emergence of *T. campestris* from stored products made of wood with bark, including solid wood packing material and furniture (Keszthelyi et al., 2019). This also reflects the beetle’s ability to tolerate low moisture conditions. Like some other Cerambycinae, *T. campestris* lays its eggs on the outer surface of its hosts in clusters, in contrast to the Lamiinae, which typically chew an oviposition niche (Haack et al., 2017). Upon eclosion, the larvae tunnel underneath the bark to consume the phloem and xylem tissues. The life cycle of *T. campestris* varies between one and three years (Makhnovskii, 1966; Li et al., 2009; Hegyessy and Kutasi, 2010; Pennacchio et al., 2016).
Host range studies for *T. campestris* suggest that this beetle is highly polyphagous. It can allegedly infest trees in over 40 genera, including softwoods and hardwoods (Makhnovskii, 1966; Kostin, 1973; Svacha and Danilevsky, 1987; Wang et al., 1988; Iwata and Yamada, 1990; Liu and Li, 2014; Bullas-Appleton et al., 2014). In some instances, it has even been found infesting dried plant materials such as milkvetch *Astragalus* spp., a woody, perennial forb of importance in traditional Chinese medicine (Jiang and Zhang, 1996). The beetle’s preferences between hosts are unclear. *Trichoferus campestris* may prefer apple when given a choice between apple and other fruit trees (Zhang et al., 2017), and *T. campestris* is frequently observed in cultivated orchards or among uncultivated fruit trees (Kostin, 1973; Bai and Zhang, 1999; Bullas-Appleton et al., 2014; Zhang et al., 2017). Indeed, multiple sources report a preference for trees in the genera *Malus* and/or *Morus* (Kostin, 1973; EPPO, 2008; EPPO, 2009; Bullas-Appleton et al., 2014). However, these claims do not come from comparative studies that make use of host choice assays.

Likewise, there are discrepancies between sources as to whether *T. campestris* prefers stressed trees, healthy trees, or dead wood (Bai and Zhang, 1999; Li et al., 2009; Dascălu et al., 2013; Bullas-Appleton et al., 2014; Pennacchio et al., 2016). Several studies refer to *T. campestris* as a drywood or lumber pest (Kostin, 1973; Iwata and Yamada, 1990; Xinming and Miao, 1998), while others claim the beetle causes minor damage to live hosts (Guo and Yin, 2005; Liu and Li, 2014; Gui et al., 2016). Other works state the beetle can cause weakness and death in its hosts (Makhnovskii, 1966; Dascălu et al., 2013). To date, the only study with an in-depth investigation on the
impacts of *T. campestris* of which we are aware found it to be only a secondary factor in the death of a Norway maple (*Acer platanoides*) in Mississauga, Ontario, Canada, with *Armillaria* root rot as the primary cause of death (Bullas-Appleton et al., 2014). That study indicated that *T. campestris* can coexist alongside other biotic stress factors but did not show evidence of the beetle as a mortality agent on its own. Another study noted that *T. campestris* tends to lay eggs on rougher bark (Zhang et al., 1977), which may be associated with phytopathogens that reduce tree vigor. In apple trees, cankered bark can often present as rough, sunken areas that may progress into lesions and swollen tissue (Nabi et al., 2018). These symptoms can be caused by fungi such as *Cytospora* spp. and *Phomopsis* spp. that have been linked to underlying stresses such as drought and defoliation (Guyon et al., 1996; Desprez-Loustau et al., 2006; Nabi et al., 2018). Associations between oviposition and changes in bark texture that may explain putative preference of *Malus* spp. by *T. campestris* have not been explored, however.

The purpose of this study was to assess ovipositional preferences and larval performance of *T. campestris* in cut branches of four North American tree species that we identified as potential hosts. We also tested whether or not *T. campestris* would preferentially oviposit and develop more rapidly on cut branches with cankered bark than on cut branches with no cankers. We hoped to explore potential associations between canker presence and oviposition, including but not limited to bark roughness that can be associated with canker presence. Moreover, we assessed larval development after host material was artificially infested with eggs. Our goal was to collect data to confirm potential susceptibility of North American tree species to *T. campestris* infestation.
Materials and Methods

Experimental Insects

In 2019, insects were collected in public parks in areas of south Minneapolis, MN that had exhibited naturalized T. campestris within the previous year. To capture sufficient numbers of live adults for our laboratory assays, we also set traps in public parks and golf courses around Salt Lake City, UT; the city in the United States in which T. campestris numbers have been highest (Krishnankutty et al., 2020). In 2020, we expanded our collection sites to additional public parks and private businesses in Minneapolis, MN and wood waste locations in St. Paul, MN. We were unable to supplement our beetle captures with T. campestris from Salt Lake City, UT in 2020 due to COVID19 pandemic travel restrictions.

To collect insects, we deployed Lindgren funnel traps baited with lures containing Trichoferone 100 mg, a sex pheromone synthesized from male T. campestris (release rate: 1-3 mg per day, Alpha Scents, Canby, OR) (Ray et al., 2019). Trapping began between 4 June and 10 June in 2019 and between 12 June and 17 June in 2020. Traps were removed by the second week of September in both years. In both 2019 and 2020, traps were attached to tree branches and were checked weekly for live beetles until the first interception of T. campestris. After the initial catch, traps were checked at least every two days. Once collected, beetles were stored alone in plastic holding containers (ca. 12 cm in diameter and 8 cm tall) alongside a piece of approximately 2 cm long absorbent cotton soaked in a 10% by volume honey water solution. The cotton was checked at least once every 48 hours and the honey water was replenished when the
cotton was dry. When the cotton began to darken, it was replaced with a fresh piece. A twig of crabapple (*Malus*) under 10 cm long was also placed in the plastic holding containers to serve as a food source (H. Nadel, Otis Laboratory, USDA APHIS, pers. comm.). Beetles were stored in these containers in the laboratory until assay initiation.

**Host Plants**

Tree species were selected for host screening based on their cultural, ecological, or economic importance to the midwestern United States. *Malus domestica* was included in the experiment due to reports that it is a preferred host of *T. campestris*. We opted to represent *M. domestica* with the ‘Honeycrisp’ cultivar, given its economic value (USDA, 2021). *Juglans nigra* was included because antique furniture made of walnut wood was found to be infested with *T. campestris* after being imported to a cabin in Wisconsin in 2016, which necessitated seizure and destruction of the shipment from Asia by state and federal regulators. *Acer saccharum* and *Pinus strobus* were included because both species hold significant cultural and ecological value in eastern North America (Yamasaki, 2003; Whitney and Upmeyer, 2004; Lovett et al., 2004; Uprety et al., 2013; Bishop et al., 2015; Costanza et al., 2018). *Pinus strobus* has the added benefit of representing softwoods among a group of hardwood trees. All four genera are reportedly possible hosts for *T. campestris* (Iwata and Yamada, 1990; Bai and Zhang, 1999; Hegyessy and Kutasi, 2010; Bullas-Appleton et al., 2014; Zhang et al., 2017).

Branches of *Malus domestica* ‘Honeycrisp’, *Juglans nigra*, and *Acer saccharum* were collected from the Minnesota Landscape Arboretum Horticultural Research Center in Chaska, MN on 13 June 2019 and 12 June 2020. Branches of *Pinus strobus* were
acquired from the University of Minnesota Cloquet Forestry Center in Cloquet, MN on 9 July 2019 and 15 June 2020. We selected only branches that were between 2.5 cm - 8 cm in diameter and had current growth of green needles or leaves. With the exception of *M. domestica* ‘Honeycrisp’, whose trees are simply identical clones within a cultivar, branches were selected from ten different trees to ensure independence among genotypes. After being harvested, we dipped the ends of all branches in paraffin wax to reduce desiccation before placement in trials. In the second year of our trials, we expanded the study to compare oviposition on cankered and uncankered *M. domestica* ‘Honeycrisp’ wood after noticing a tendency of *T. campestris* to lay eggs deep in bark crevices. Both types of wood were selected from a commercial orchard in Woodbury, Minnesota, to ensure consistency in site conditions across treatments. The presence of *Cyotospora* and *Phomopsis* cankers were visually assessed, then confirmed by the University of Minnesota Plant Disease Clinic. The ends of these branches were also dipped in paraffin wax to reduce desiccation prior to placement in trials.

Species Choice Assays

In each trial, a ten-centimeter section of branch from each of the four tree species was placed into the corners of a rectangular plastic container measuring approximately 33.5 cm long x 20 cm wide x 11.5 cm tall. Branch sections rested against container sides to maximize distance between them and to prevent movement as much as possible. The paraffin wax was removed from the ends of each branch before placement. One gravid female *T. campestris* was placed in the center of each container and allowed to oviposit freely on the branch sections. Once beetles were placed in trials, they were no longer
provisioned with food or soaked cotton. Plastic containers were kept at a constant indoor temperature both years (approximately 22°C). In trials set in 2019 (henceforth referred to as “2019 choice trials” for the year when eggs were laid), plastic containers were set next to a window to allow natural light:dark cycles until all adult beetles died. In trials set in 2020 (henceforth referred to as “2020 choice trials” for the year when eggs were laid), due to restricted university access caused by the COVID19 pandemic, procedures were altered such that some trials were placed in rooms without natural light:dark cycles prior to the completion of oviposition. After each female died, eggs were counted on each branch section. The choice study was replicated 36 times in 2019 and 16 times in 2020.

Following egg censuses, each branch section was placed into an individual triangular cardboard rearing tube. The sides of the triangular tubes measured approximately 7.5 cm in height and length was adjusted to each branch. A mesh screen of approximately 7.1-by-6.3 apertures per square centimeter (18-by-16 apertures per square inch) covered the ends to allow for ventilation.

To test the combined impacts of a chilled period and tree species on T. campestris emergence, we exposed half of our species choice tests to an unheated room during the winters during which our study took place (2020 and 2021). One half of the 2019 choice trials were moved to an unheated room on 29 January 2019. During the winter (from placement on 29 January lasting through 21 March), the temperature fluctuated between -1°C and 10°C. Temperatures in the spring of 2020 (from 22 March through 26 May) fluctuated between 6°C and 30°C. Our 2019 choice trials exposed to cold were moved back into the laboratory on 26 May in 2020. The other half of the 2019 choice trials were
kept in the laboratory (averaging 22°C). Both sets of branches were monitored for emergence of adults the following summer/fall, once each week, from August of 2020 to 19 December 2020. On 19 December 2020, the half of our 2019 choice trials that had been exposed to an unheated period were moved back to the unheated room to simulate their second winter.

Similarly, one half of the 2020 choice trials were moved to an unheated room on 19 December 2020. In 2020, winter temperatures (from placement on 19 December lasting through 21 March) ranged from -2°C to 14°C and spring temperatures (from 22 March through 16 April) from 7°C to 21°C. On 16 April 2021, all 2019 and 2020 choice trials were moved once again to our laboratory, where they were checked for emergence weekly. All branches—2019 choice trials and 2020 choice trials that were exposed to winter temperatures or kept at consistent temperatures—were peeled and dissected between 28 July 2021 and 18 August 2021 to assess the number and lengths of surviving larvae that had not yet developed to adult life stages and emerged.

Choice Assays with Branches with Cankers

After noticing that *T. campestris* laid eggs deep in bark crevices when present, we investigated this tendency further in a host choice assay examining oviposition among branch sections with and without cankers. Sections of 10.16 cm from each of two types of branch (cankered vs. uncankered) were cut from *M. domestica* ‘Honeycrisp.’ To characterize bark roughness, tracing paper was wrapped around each section and colored over with a crayon to create a two-dimensional image of the branch’s bark relief prior to placement in the canker choice assay (Fig. 1.1).
In each trial, a ten-centimeter section of uncankered *M. domestica* branch and a ten-centimeter section of cankered *M. domestica* branch were placed on either end of a rectangular plastic container measuring approximately 33.5 cm long x 20 cm wide x 11.5 cm tall. Branch sections rested against container sides to maximize distance between them and to prevent movement as much as possible. The paraffin wax was removed from the ends of each branch before placement. One gravid female *T. campestris* was placed in the center of each container and allowed to oviposit freely on the branch sections. Once beetles were placed in trials, they were no longer provisioned with food or soaked cotton. Eggs were counted on each branch section following the adult’s death. The canker choice study was replicated 20 times and conducted only in 2020. Similar to the species choice assay in 2020, some trials were placed in rooms without natural light:dark cycles prior to the completion of oviposition. Branch sections were again placed in triangular cardboard rearing tubes following egg censuses. Half of the containers were kept in the laboratory at a constant indoor temperature, while the other half were stored with a simulated winter period similar to the species choice assay. Emergence was checked, branches were peeled, and larval abundance and lengths were documented as in the species choice assay.

**No Choice Assays**

For the no choice assays to assess host suitability, branch sections between 45-60 cm and 25-38 cm in length were selected in 2019 and 2020, respectively. Branches varied in length to ensure reasonably consistent diameters of branch sections across tree species and year, as branches of *M. domestica* ‘Honeycrisp’ tended to be much narrower than
branches of *Juglans nigra*. By host suitability, we mean ability to produce offspring that complete development to adulthood in a given species of tree. In no-choice trials set in 2019 (henceforth referred to as “2019 no-choice trials” for the year when eggs were laid), branches were left waxed prior to placement, while wax was removed in no-choice trials set in 2020 (henceforth referred to as “2020 no-choice trials” for the year when eggs were laid) prior to placement. On each section of branch, eggs were adhered using adhesive bandages or gauze and medical tape to ensure that they remained on the bark until hatch. Eggs were taken from plastic holding containers where *T. campestris* mating groups had laid them prior to placement in the host choice assays each year. The *T. campestris* in our trials oviposited rapidly following mating, even in the absence of potential host material; this meant that they laid eggs in their holding containers, often on the plastic sides or on cotton, if we were unable to place them in trials immediately following mating. Three eggs were placed such that approximately 7-13 cm space was left between the eggs and the ends of the branches and eggs were laid approximately 15 cm and 7 cm apart in assays from 2019 and 2020, respectively. Branch sections of each species were isolated in triangular cardboard rearing tubes as previous.

In both 2019 and 2020, half of the branch sections were peeled partway through the insects’ development (see below), while the other half of branch sections were left to allow insects to complete development and assess emergence. Eggs were placed on 2 August 2019; between 3 and 10 September 2019 (between 4.5 and 5.5 weeks after infestation), we peeled the bark from one half of the logs and recorded whether a larva successfully eclosed from each egg, whether any larvae were live or dead, and their
lengths. There was very little variation in these metrics among the different branches used in 2019 (see Results). Therefore, in 2020, the period of time larvae were left to develop prior to peeling the logs was extended to five months. Eggs were placed between 7 and 20 July 2020, and branches were dissected between 24 November and 17 December 2020. Unfortunately, in both years some larvae were found hatched but unable to tunnel because they had crawled to sticky portions of the medical tape or band-aid adhesive strips. These cases were excluded from analysis (in 2019, \( n = 5 \), or 8.47% of the larvae; in 2020, \( n = 10 \), or 12.82% of the larvae).

The remainder of no-choice trials were stored with a cold period similar to the species choice assay. Emergence was checked and branches were peeled as in the species choice assay. Larval length was documented for analysis. The no choice study was replicated 20-22 times per species in 2019 (depending on availability of host material) and 19 times per species in 2020.

**Statistical Analysis**

For the species choice assays, a mixed-effects ANOVA was used to analyze the effect of the species of tree (i.e., a fixed effect) on the number of eggs laid, incorporating a term for the experimental container as a random effect. A two-way ANOVA was used to analyze the effects of chilled vs. unchilled (i.e., a fixed effect) and tree species (i.e., a fixed effect) on the number of adult *T. campestris* emerged, incorporating a term for the interaction between chill and tree species. After the conclusion of the experiment in August 2021, larval survival and larval lengths were recorded for insight into the development of insects that had not reached adulthood. We used a two-way ANOVA to
analyze the effects of both chilled vs. unchilled (i.e., a fixed effect) and tree species (i.e.,
a fixed effect) on the number of larvae that survived or larval lengths as separate response
variables, incorporating in both analyses a term for the experimental container as a
random effect.

For the choice assays using cankered vs. uncankered wood, we also used a mixed-
effects ANOVA to analyze the effect of presence or absence of canker (i.e., a fixed
effect) on the number of eggs laid, again incorporating a term for the experimental
container as a random effect. We also explored whether bark roughness in the cankered
vs. uncankered choice assay might explain variation in oviposition. To measure bark
roughness, we used the program GIMP to pixelate each bark relief scan (Fig. 1.1), then
calculated prevalence of dark pixels (i.e., pixels with a value of 100 or below in
greyscale) as a proportion of the whole, with more dark pixels corresponding to rougher
bark. We used ANCOVA to assess the effect of bark roughness (i.e., a covariate;
proportion of dark pixels) and the presence or absence of canker (i.e., a factor) on the
number of eggs laid. We also used ANOVA to assess the effect of chilled vs. unchilled
treatments on lengths of larvae found when we peeled remaining branches at the study’s
conclusion.

For the no-choice assays, the effects of species on the probability of egg eclosion
or live recovery of larvae (i.e., yes/no, a binary variable) partway through development
were analyzed using logistic regression. We used ANOVA to determine the effects of
species on the number of emerged adult *T. campestris*. As in the choice assay, larval
lengths were recorded to assess development of insects that did not reach adulthood. We used ANOVA to determine the effects of species on larval lengths.

Response variables were square root transformed as necessary to reduce heteroscedasticity in the residuals, except for binary response variables. Where significant treatment effects occurred ($\alpha \leq 0.05$), pairwise comparisons were performed with a Tukey adjustment to account for multiple comparisons. We used the emmeans package (Lenth, 2020) to perform these post-hoc comparisons. All data analyses were performed in R v4.0.3, and used ggplot2 for graphical work (Wickham, 2016; R Core Team, 2020).

**Results**

**Choice Assays**

Female *T. campestris* demonstrated different rates of oviposition by tree species that were relatively consistent in both years of the study. Individual female beetles showed wide variation in the numbers of eggs laid overall. Beetles laid between 1 total and 98 total eggs in various containers in 2019, with a median of 31 eggs, and between 4 total eggs and 262 total eggs in various containers in 2020, with a median of 25 eggs. In 2019, gravid females oviposited most on cut branches of *J. nigra*, followed by *M. domestica* and *A. saccharum* (Fig. 1.2A; $F_{3,99} = 32.84$, $P < 0.0001$). Some insects in each of the walnut, apple, and maple treatments in 2019 laid more than 40 eggs on a branch section in less than two weeks, while other insects laid fewer than five. The least preferred host was white pine. In 2019, only two *T. campestris* females laid any eggs on *P. strobus*, with five or fewer eggs laid each time. In 2020, differences in oviposition
followed a similar pattern (Fig. 1.2B; $F_{3, 44} = 9.79, P < 0.0001$). The most preferred tree species still included $J. \text{nigra}$, and $P. \text{strobus}$ remained least preferred. The number of eggs laid per branch section remained highly variable, with over 200 eggs laid on one $J. \text{nigra}$ branch section and under five eggs laid on others. Female $T. \text{campestris}$ showed an ovipositional preference for $J. \text{nigra}$ over $M. \text{domestica}$ and $P. \text{strobus}$, with no other apparent differences between tree species. Eggs in both years were consistently laid on branches, rather than loose in our containers. Nearly all of the infested branches began to show signs of egg eclosion and larval development, in the form of frass and loosening bark, within the first month.

Through both years of choice assays, only 20 adult $T. \text{campestris}$ total emerged from the 208 branch sections we monitored for emergence. No emergence in our host choice assays was noted in the year 2020, when 2019 trials had undergone approximately a single year of development. However, in the year 2021, when 2019 trials had undergone two years of development and 2020 trials had undergone only one year of development, we observed 15 adult $T. \text{campestris}$ emerge from 2019 trials and 5 from 2020 trials. Of the 20 adult $T. \text{campestris}$ that emerged, 18 came from chilled trials and only 2 from unchilled trials. Thirteen emerged from $M. \text{domestica}$ (all from chilled branches), six from $J. \text{nigra}$ (4 chilled branches, 2 unchilled branches), and one from $P. \text{strobus}$ (chilled). We observed no emergence in 2019 trials or 2020 trials from branches of $A. \text{saccharum}$. See appendices for detailed information on number of larvae found on various tree species (Appendix A) and different larval lengths between species treatments (Appendix B) and chill treatments (Appendix C).
When we analyzed these data, we noted significant differences among different tree species in 2019 choice trials (Fig. 1.3A; $F_{3,136} = 5.99, P < 0.001$). More insects also emerged from chilled branches compared to those left at room temperatures (Fig. 1.3A; $F_{1,136} = 13.52, P < 0.001$). However, emergence within species varied when these species were subjected to a chill treatment (Fig. 1.3A; interaction term: $F_{3,136} = 5.99, P < 0.001$). We observed higher emergence numbers from chilled *M. domestica* branches than any other treatment combination. In 2020 choice trials, we observed no statistically significant differences (Fig. 1.3B; $F_{3,56} = 0.94, P = 0.43$).

When presented with an option between cankered and uncankered *M. domestica* ‘Honeycrisp’, *T. campestris* primarily oviposited on branch segments with cankers (Fig. 1.4; $F_{1,19} = 8.39, P = 0.0093$). On average, we found more than twice as many eggs on cankered wood (approximately 44 eggs laid per branch section) versus uncankered wood (approximately 20 eggs laid per branch section). Canker branches had rougher texture than uncanker branches according to our image pixelation analysis (Fig. 1.1, $Z = 2.04, P = 0.041$). Roughness was associated with the number of eggs laid ($F_{1,38} = 4.55, P = 0.039$) when the term was evaluated as the sole explanatory variable, but when bark roughness was fit after a term for the presence/absence of cankers in an ANCOVA, bark roughness lost its significance ($F_{1,37} = 0.0044, P = 0.95$), while canker presence/absence remained highly significant ($F_{1,37} = 8.86, P = 0.0051$). Thus, canker presence or absence was a stronger predictor of the number of eggs laid than bark roughness using our image metric. Only two adult *T. campestris* emerged from our canker choice study by early August, when all branches were peeled (see Methods). One emerged from a cankered
branch and the other from an uncankered branch. Both branches had been stored with a cold period. For detailed information on the effects of chill treatment on lengths of larvae at the study’s conclusion, see appendices (Appendix D).

**No Choice Assays**

Eggs of *T. campestris* were equally likely to eclose on all represented tree species used in no choice assays in both 2019 ($\chi^2 = 5.41, \text{df} = 3, P = 0.14$) and 2020 ($\chi^2 = 1.21, \text{df} = 3, P = 0.75$). Overall, the mean eclosion rate was 61% ± 0.49 in 2019 and 93% ± 0.26 in 2020. When given no choice between different tree species, larvae were least likely to survive on *P. strobus* and most likely to survive on *J. nigra*, in both 2019 (Fig 1.5A; $\chi^2 = 18.87, \text{df} = 3, P < 0.001$) and 2020 (Fig. 1.5B; $\chi^2 = 25.44, \text{df} = 3, P < 0.0001$). In 2019 no-choice trials, we recovered far fewer larvae on *P. strobus* than any other species tested. We recovered five larvae on *P. strobus*, but only two of these were alive. Several of the others were observed dead in their entry holes on branch sections of *P. strobus*. The probability of recovering live larvae was significantly less on *P. strobus* than on *M. domestica*, *A. saccharum*, or *J. nigra* (Fig. 1.5).

In 2020 no-choice trials, larvae were more likely to survive on *J. nigra* versus *M. domestica*, *A. saccharum*, and *P. strobus*. Though larval survival was lowest on *P. strobus* compared to the other three species numerically, a statistically significant difference was not observed between *P. strobus* and *A. saccharum* or *M. domestica*.

When we peeled the bark to examine larval size after six weeks in 2019, we did not find any significant differences in larval lengths (Fig. 1.6A; $F_{3,46}=2.43, P = 0.077$). The larvae recovered in 2019 were all under five millimeters in length at this early stage.
of development. In 2020, when we delayed debarking until five months post-infestation, the larval size ranged from 4 – 24 mm, demonstrating developmental differences between larvae on different hosts (Fig. 1.6B; $F_{3, 49} = 11.55$, $P < 0.0001$). Larvae that developed on *A. saccharum* were the shortest, contrary to what was observed in the choice assay (Fig. 1.2) and probability of larval survival (Fig. 1.5). Larvae on *M. domestica* and *J. nigra* were both significantly larger than larvae recovered from *A. saccharum*.

More adult *T. campestris* emerged from *M. domestica* and *J. nigra* than *A. saccharum* or *P. strobus*. By 27 July 2021, 19 adult *T. campestris* had emerged from our 2019 no-choice trials. Significantly more adult *T. campestris* from 2019 no-choice trials emerged from branches of *M. domestica* than branches of *A. saccharum* or *P. strobus* (Fig. 1.7A; $F_{3, 41} = 5.024$, $P = 0.0047$). Our 2020 no-choice trials showed a different pattern, with significantly more adult *T. campestris* emerging from branches of *J. nigra* than branches of *P. strobus* (Fig. 1.7B; $F_{3, 31} = 3.36$, $P = 0.031$). See appendices for detailed information on the effects of species on lengths of larvae at the study’s conclusion (Appendix E).

**Discussion**

Our results that *T. campestris* is able to oviposit, begin development, and emerge from the four tree species used in these experiments are consistent with previous reports of a high degree of polyphagy exhibited by this beetle (Makhnovskii, 1966; Kostin, 1973; Svacha and Danilevsky, 1987; Wang et al., 1988; Iwata and Yamada, 1990; Liu and Li, 2014; Bullas-Appleton et al., 2014). This characteristic has likely facilitated the ability of *T. campestris* to successfully invade and establish in many places around the world, as
polyphagy is associated with likelihood of insect establishment after an introduction to a novel environment (Cocquempot and Lindelöw, 2010). Given that frequent introductions of *T. campestris* to the US have already been documented (Keszthelyi et al., 2019; Wu et al., 2020), there is a distinct possibility that this beetle will establish over a wide geographic area in the U.S., if it has not done so already. The wide spread of *T. campestris* in Europe (Hegyessy and Kutasi, 2010; Dascălu et al., 2013; Pennacchio et al., 2016) demonstrates a capacity to inhabit diverse ecosystems, such as those in the United States. This inference is consistent with recent research investigating likely future spread of *T. campestris* (Keszthelyi et al., 2019; Krishnankutty et al., 2020). However, establishment may vary between different landscapes. Our finding that *T. campestris* can, but usually does not, oviposit on and emerge from *P. strobus* suggests that landscapes in which *Pinus* spp. is more widespread may face lower susceptibility to invasion by *T. campestris* than landscapes with more desirable host species, such as *J. nigra*, *M. domestica*, and *A. saccharum*.

Other beetles in the family Cerambycidae are known to display disparate preferences between coniferous and deciduous trees as well. For example, *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) primarily infests coniferous trees but has been known to infest *Malus* spp. as well (Fan and Sun, 2006). In contrast, *Anoplophora chinensis* (Forster) (Coleoptera: Cerambycidae) mainly infests deciduous hosts but has occasionally been seen on *Pinus* spp. (Sjöman et al., 2014). Both stimulation and repulsion by volatile terpenes from coniferous trees have been shown in longhorned borers such as *M. alternatus* (Fan and Sun, 2006), and similar mechanisms may be
facilitating ovipositional choices made by *T. campestris*. *Pinus* spp. have strong constitutive defenses, with well-developed networks of resin ducts containing oleoresin (Lewinsohn et al., 1991). Volatiles may be emitted for weeks after white pine has been cut (Dyer and Seabrook, 1978). Wide variation has been found in terpene concentration among *Pinus* spp. individuals even within a single species (Dyer and Seabrook, 1978; B.H. Aukema et al., 2010); this intra-species variation may have contributed to the oviposition on *P. strobus* by some *T. campestris* females but not others.

Our data suggest that the optimal conditions for *T. campestris* emergence are development on *M. domestica*, consistent with existing reports (Kostin, 1973; EPPO, 2008; EPPO, 2009; Bullas-Appleton et al., 2014; Zhang et al., 2017), along with exposure to a seasonal cold treatment (Fig. 1.3A). Cold periods have been demonstrated to break diapause in multiple species of Cerambycids that undergo true diapause, often by accelerating their development and ultimately leading to greater incidence of adult emergence (Gardiner, 1970; Rogers and Serda, 1979; Yoon et al., 2000; Koutroumpa et al., 2008; Haack et al., 2017). Indeed, in branches that were peeled in the choice assays, larvae that had not yet emerged were significantly larger in chilled vs. unchilled branches (Appendices C and D). Further studies are needed to draw conclusions regarding incidence of diapause and define parameters of an optimal cold period to break a potential diapause in *T. campestris*. Not only is this information valuable for establishing laboratory rearing protocols for *T. campestris*, but it would inform management in locations where *T. campestris* is currently regulated. For instance, the state of Hawaii is one of few locations within the United States where *T. campestris* remains
actionable/reportable, as *T. campestris* has yet to be intercepted there as of the time of this publication (USDA, 2019). Our findings may be useful in determining the threat level of *T. campestris* to Hawaiian landscapes, as much of the state never experiences temperatures lower than 15°C (according to NOAA weather stations in Hawaii with IDs 51-5575, 51-1919, 51-6534, 51-2572, and 51-1492). These temperatures did facilitate complete development of *T. campestris* in our trials in Minnesota.

A number of factors likely influence the patterns we observed in ovipositional choice and larval performance. A lack of coevolutionary history between hosts and an insect, for example, can decrease associations between oviposition and larval performance (Thompson, 1988). With the exception of *M. domestica*, which stems from multiple *Malus* spp. largely originating in northeastern Asia (Robinson et al., 2001), the historical ranges of the tree species represented in our studies do not overlap with the native range of *T. campestris* (USDA Forest Service, 1990; Burns and Honkala, 1990). Polyphagy in phytophagous insects tends to obscure host fidelity anyway (Gripenberg et al., 2010). Females may also choose to oviposit on hosts that maximize the fitness of their offspring, a concept known as the preference-performance hypothesis (Mayhew, 1997). Our results indicate some support for the preference-performance hypothesis, as not only were *T. campestris* less likely to oviposit on *P. strobus* (Fig. 1.2), but larvae that developed on *P. strobus* also had the highest rates of mortality (Fig. 1.4) and lowest rates of emergence (Fig. 1.7). Likewise, *A. saccharum* was not the most preferred species for oviposition (Fig. 1.2A), larvae on *A. saccharum* were significantly smaller than those on branch sections of other species (Fig. 1.5), and larval emergence was among the lowest
on branch sections of *A. saccharum* (Fig. 1.7). Our observation that larvae can still develop to adulthood in hosts in which they experience slow development is supported by previous findings (Flaherty et al., 2011), such as the ability of *T. campestris* to complete development in other *Acer* spp. under field conditions (Bullas-Appleton et al., 2014). The slow larval development we observed on *A. saccharum* may relate to nutritional conditions of the wood, as has been suggested to explain slow development of *T. campestris* on *Fagus* spp. (Iwata and Yamada, 1990).

Our findings that canker presence or absence is a stronger predictor of oviposition than bark texture suggest that apple cankers have an influence that extends beyond bark texture. Trees experiencing stress are associated with higher incidence of *Cytospora* spp. canker (Adams and Jacobi, 2016), lowered plant defenses, and higher nutrient contents more favorable to phytophagous insects (Mattson and Haack, 1987; Flaherty et al., 2011). As such, *Cytospora* spp. and *Phomopsis* spp. fungi may have denoted trees with lowered host defenses and/or higher nutritional value in addition to altering the texture of *M. domestica* bark. Several studies (Bullas-Appleton et al., 2014; Pennacchio et al., 2016; Krishnankutty et al., 2020) indicate that *T. campestris* is able to infest trees with varying degrees of vigor (i.e. stressed, weakened, or not stressed), but there are few if any comparative studies documenting a preference between them or elucidating mechanisms.

We note several potential limitations in this study. First, while branches were not placed in our study until 3.5 weeks after harvest, the reduced oviposition and larval performance on *M. domestica* branches in 2020 species assays as compared to those from 2019 (Figs. 1.2 & 1.5) could have been due to residual chemical treatment with
Delegate® WG insecticide (a.i. 25% spinetoram, used for control of lepidopterous larvae, dipterous leafminers, thrips, and some psyllids). This pesticide was applied to the orchard block within 24 hours of our harvest of branch sections. While we would have preferred untreated wood, the COVID-19 pandemic impacted our species choice assays by limiting field access. Fortunately, the branches in our assays investigating host vigor through an ovipositional choice between cankered and uncankeried *M. domestica* wood (Fig. 1.4) had not undergone chemical treatment within the growing season. Second, we are unsure of the lower limit of branch diameter necessary for successful *T. campestris* reproduction. Infestation and development of *T. campestris* larvae has been recorded in branches as small as 2.5 – 3 cm in diameter (EPPO, 2009), which was smaller than any of our branches; however, no mention of emergence appeared in these reports. Third, it is possible that we did not expose the insects to appropriate thermal regimes to meet overwintering requirements of all individuals. Although many individuals emerged over the two years, development of *T. campestris* individuals can exceed two years (Appendices A, B, C, and E). Finally, we allow that the use of the term “no-choice” entails development of the immature and not adult ovipositional behavior. Further studies examining ovipositional choice in a no-choice context would be valuable.

While our results provide useful information to make inferences about the host preferences and suitability of *T. campestris*, further study on live hosts is necessary for a broader picture of this beetle’s host preferences in its novel range. Live host assays are needed to investigate host trees’ induced defenses, and further host preferences can be elucidated with additional preference-performance assays on additional tree species.
Likewise, factors that may predispose trees to colonization, such as canker pathogens (Fig. 1.4), are not fully investigated. Finally, host assays on additional agricultural commodities are necessary to assess potential consequences of *T. campestris* introduction in locations such as Hawaii, where the beetle is not yet established and is therefore still regarded as reportable/actionable.
Figure 1.1. Examples of bark roughness relief images of *M. domestica* prior to the pixelation of photos using the program GIMP. (A) Uncankered branch. (B) Cankered branch.
Figure 1.2. Box plot of number of eggs laid on branches of the four tree species by adult *T. campestris* in host choice assays, 2019-2020. Each datapoint is an individual branch section. The boxes represent 50% of observations, ranging from the 1st quartile to the 3rd quartile, with the intermediate representing the median. The vertical lines show the spread of the data from minimum to maximum, excluding outlying values (defined as values outside the box by at least 1.5 times the interquartile range), which are represented by individual points. Different letters indicate significant treatment differences (Tukey procedure). (A) 2019 assay (*N* = 136 total; *F*$_3$,99 = 32.84, *P* < 0.0001). (B) 2020 assay (*N* = 63 total; *F*$_3$,44 = 9.79, *P* < 0.0001).
Figure 1.3. Box plot of number of adult *T. campestris* emerged from our host choice study in 2019-2020. Each datapoint represents the emergence from a single branch. The boxes represent 50% of observations, ranging from the 1st quartile to the 3rd quartile, with the intermediate representing the median. The vertical lines show the spread of the data from minimum to maximum, excluding outlying values (defined as values outside the box by at least 1.5 times the interquartile range), which are represented by individual points. Within each species, the left side represents those branches not exposed to colder temperatures through the winter. These averages are denoted by a grey box where the average was greater than zero. The right side represents those branches that were exposed to colder temperatures through the winter. These averages are denoted by a white box where the average was greater than zero. The asterisk marks the only significant treatment difference, which was significantly greater than all other treatments (Tukey procedure). (A) 2019 assays (*N* = 144, *F*<sub>3,136</sub> = 5.99, *P* = 0.00073). (B) 2020 assays (*N* = 64, *F*<sub>3,56</sub> = 0.94, *P* = 0.43).
Figure 1.4. Box plot of number of eggs laid on cankered and uncANKERed branches of *M. domestica* by adult *T. campestris* in host choice assays 2020. Each datapoint is an individual branch. The boxes represent 50% of observations, ranging from the 1st quartile to the 3rd quartile, with the intermediate representing the median. The vertical lines show the spread of the data from minimum to maximum, excluding outlying values (defined as values outside the box by at least 1.5 times the interquartile range), which are represented by individual points. Different letters indicate significant treatment differences (Tukey procedure) (*N* = 40 total, *F*1, 19 = 8.39, *P* = 0.0093).
Figure 1.5. Probability of recovering live *T. campestris* larvae on branches of the four tree species in no choice assays 2019-2020. The center points represent the estimated probability, with the lines marking the upper and lower 95% confidence intervals around each estimate. (A) 2019 assay, evaluated after 6 weeks of development (*N* = 112 total, $\chi^2 = 18.87$, df = 3, $P < 0.001$). (B) 2020 assay, evaluated after 5 months of development (*N* = 110 total, $\chi^2 = 25.44$, df = 3, $P < 0.0001$).
Figure 1.6. Box plot of larval *T. campestris* length in millimeters on branches of the four tree species in no-choice assays 2019-2020. Each datapoint is an individual larva. The boxes represent 50% of observations, ranging from the 1st quartile to the 3rd quartile, with the intermediate representing the median. The vertical lines show the spread of the data from minimum to maximum, excluding outlying values (defined as values outside the box by at least 1.5 times the interquartile range), which are represented by individual points. Different letters indicate significant treatment differences (Tukey procedure). (A) 2019 assay, evaluated after 6 weeks of development. No statistically significant differences were found between treatments (*N* = 50, *F*$_{3, 46}$ = 2.43, *P* = 0.077). (B) 2020 assay, evaluated after 5 months of development (*N* = 53, *F*$_{3, 49}$=11.55, *P* < 0.0001).
Figure 1.7. Box plot of number of adult *T. campestris* emerged from our host no-choice study in 2019-2020. Each datapoint represents the emergence from a single branch. The boxes represent 50% of observations, ranging from the 1st quartile to the 3rd quartile, with the intermediate representing the median. The vertical lines show the spread of the data from minimum to maximum, excluding outlying values (defined as values outside the box by at least 1.5 times the interquartile range), which are represented by individual points. Different letters indicate significant treatment differences (Tukey procedure). (A) 2019 assay (*N* = 45, *F*$_3$,41 = 5.024, *P* = 0.0047). (B) 2020 assay (*N* = 35, *F*$_3$,31 = 3.36, *P* = 0.031).
Chapter 2

Seasonal phenology of *Trichoferus campestris* (Coleoptera: Cerambycidae) in park trees in Minneapolis and St. Paul, Minnesota
Synopsis

Improved detection methods for nonindigenous forest insects can improve the timeliness and effectiveness of other response strategies that aim to limit damages from these nonnative insects. In particular, knowledge of a species’ phenology can assist with timing accurate surveys to detect presence and density in a novel environment.

*Trichoferus campestris* (Faldermann) (Coleoptera: Cerambycidae) has been intercepted in the United States since the 1990s, but its biology and behavior remain poorly understood. This study investigated the phenology of *T. campestris* in Minneapolis and St. Paul, Minnesota, USA, based on local temperature data. In the summers of 2019 and 2020, 30 and 40 pheromone-baited traps, respectively, were deployed in trees in suburban parks to monitor capture of local individuals. Traps were suspended from branches of mature trees selected at random, with nine genera of trees selected over both years. Early, peak, and late adult abundance were characterized, and the impact of tree genus in which each trap was hung was evaluated. Abundance was found to be unimodal both summers with a peak around 650 degree days (base 10°C ) in early July. Significantly more adult *T. campestris* were caught in traps hung in trees of the genus *Tilia* than in trees of the genus *Quercus*. These findings are important first steps to improving monitoring of *T. campestris* presence and conducting risk assessments.
Introduction

*Trichoferus campestris* (Faldermann) (Coleoptera: Cerambycidae), or the velvet longhorned beetle, is an insect that is frequently intercepted in novel environments around the world. This beetle is native to Asia and eastern Europe but has spread through much of Europe and North America. In North America, it was first intercepted in the 1990s and has since been intercepted in 22 states and 3 Canadian provinces. In the USA, it has known established populations in Utah, Wisconsin, and Illinois and a presumed established population in Minnesota (Iwata and Yamada, 1990; Cocquempot, 2006; Dascălu et al., 2013; Pennacchio et al., 2016; Keszthelyi et al., 2019).

Many species introduced to novel environments never establish, and many that do cause negligible harm to novel ecosystems. However, those that do establish and cause harm can be devastating. Problematic introduced forest species are frequently insects in the order Coleoptera (J.E. Aukema et al., 2010), as demonstrated by the widespread tree death caused by *Anoplophora glabripennis* (Motschulsky) (Coleoptera: Cerambycidae) and *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) and efforts associated with monitoring and controlling these two beetle species. When a novel species is introduced, monitoring and management can be improved through knowledge of their biology; the phenology of wood boring beetles in particular can inform pest detection (Suckling et al., 2012; Crimmins et al., 2020) as these insects complete their larval stage beneath the bark of their tree hosts and thus can only be trapped as adults. Information about the phenology and host species of *T. campestris* are not definitive, however.
Indeed, knowledge of the life cycle of *T. campestris* is sparse. Sources report variable voltinism, ranging from one to three years (Makhnovskii, 1966; Li et al., 2009; Hegyessy and Kutasi, 2010; Pennacchio et al., 2016). Factors influencing this life cycle variability, such as climate or host characteristics, have yet to be investigated (Cherepanov, 1981; Svacha and Danilevsky, 1987). In its native range, *T. campestris* adults emerge between May and September (Cherepanov, 1981; Xinning and Miao, 1998; Grebennikov and Gill, 2010). Previous work on theoretical worldwide species distribution of *T. campestris* has assumed a threshold temperature of 10˚C for development and necessary cumulative degree days of 669 for development from egg to adult (Keszthelyi et al., 2019), although we are not aware of any studies that have confirmed the developmental thresholds of *T. campestris* in a laboratory setting. Because behavior can differ between historical and novel ranges (Yan et al., 2005; Formby et al., 2018), it is important to complement such theoretical studies with applied local field studies using local climatic conditions (Keszthelyi et al., 2019). In Minnesota, *T. campestris* was first trapped in 2010. Numbers seem to be increasing, particularly around Minneapolis and St. Paul, Minnesota, but there is still considerable uncertainty around when and where the beetle is found in the state (MDA, 2020).

Few studies on the hosts of *T. campestris* originate in North America, and few studies are comparative in nature. Studies from the beetle’s native range report tree hosts to the beetle in over 40 genera, including softwood and hardwood trees (Makhnovskii, 1966; Kostin, 1973; Svacha and Danilevsky, 1987; Wang et al., 1988; Iwata and Yamada, 1990; Liu and Li, 2014; Bullas-Appleton et al., 2014), suggesting this beetle is
polyphagous. Many sources report that *T. campestris* prefers hosts in the genera *Malus* or *Morus* (Kostin, 1973; EPPO, 2008; EPPO, 2009; Bullas-Appleton et al., 2014), and in its native range, the beetle is said to occur frequently in cultivated orchards or among wild fruit trees (Kostin, 1973; Bai and Zhang, 1999; Zhang et al., 2017).

The purpose of this study was to describe the phenology of *T. campestris* in Minnesota, USA, a state within the introduced range of North America. Because hosts in natural conditions are as yet unknown, we focus on adult abundance as monitored by Lindgren funnel traps baited with species-specific pheromones over two summers of trapping, 2019-2020. We describe how seasonal flight varies with seasonal accumulation of degree days using local temperature data to calculate the number of degree days suitable for development of *T. campestris* in a calendar year. We also analyzed if *T. campestris* were more frequently captured in traps hung in different genera of trees. A deeper understanding of behavior will assist in monitoring and surveying locally and provide context to inform future work in other locations investigating the spread of *T. campestris*.

**Materials and Methods**

To assess seasonal abundance of *T. campestris* in Minneapolis and St. Paul, Minnesota, we deployed 12-funnel Lindgren funnel traps baited with lures containing Trichoferone 100 mg, a sex pheromone synthesized from male *T. campestris* (release rate: 1-3 mg per day, Alpha Scents, Canby, OR) (Ray et al., 2019). We used dry cups, collecting live *T. campestris* adults and bringing them back to our laboratory in Tupperware containers. In 2019, 30 traps were deployed to collect insects in public parks
in areas of south Minneapolis, MN. In 2020, we deployed 40 traps, expanding our survey sites to include adjacent public parks and private businesses in Minneapolis, MN and additional sites near wood waste locations in St. Paul, MN. One trap was hung per selected tree from basal branches such that the collection cup was suspended off the ground (height above the ground varied with tree species and age of tree). Traps were deployed between 4 June and 10 June to begin the experiment in 2019, between 185 and 259 degree days (see below for calculations), and established between 12 June and 17 June to begin the experiment in 2020, between 352 and 413 degree days. In 2019 and 2020, traps were checked weekly for live beetles until the first interception of *T. campestris*. After each season’s initial catch, traps were checked at least every two days. After two weeks with zero adult *T. campestris* captures in the late summer/early fall, traps were removed.

We collected data on daily maximum and minimum temperatures through the National Oceanic and Atmospheric Administration automated weather stations at the Minneapolis St. Paul International Airport (ID: GHCND:USW00014922). Because we were unable to find specific data on the development threshold of *T. campestris*, a lower developmental threshold of 10°C was used for degree day calculations. This threshold aligns with the lower end of the span of temperatures (10-12 °C) considered to be the average lower development threshold for Cerambycidae (Haack et al., 2017). The 10°C threshold has also been used for previous work on phenology of *T. campestris* (Keszthelyi et al., 2019). Degree days were calculated by averaging daily temperatures,
then subtracting the lower developmental threshold from each daily average, and then summing all positive values from each year beginning on 1 January 2019 and 2020.

Traps were hung in trees of 9 different genera: *Acer, Celtis, Gymnocladus, Malus, Morus, Pinus, Quercus, Tilia, and Ulmus*. These genera were present in the parks in the areas in which we knew *T. campestris* to be present, although some were represented in our study more frequently than others. Ultimately, 70 traps were deployed total (both years combined), with 13 traps hung in trees of the genus *Acer, 7 in Celtis, 1 in Gymnocladus, 12 in Malus, 16 in Morus, 1 in Pinus, 8 in Quercus, 5 in Tilia, and 7 in Ulmus*.

To compare catch rates among different tree genera, a mixed-effects ANOVA was used to analyze the effect of the genus of tree in which each trap was hung (i.e., a fixed effect) on total adult *T. campestris* capture, incorporating a term for year as a random effect. For purposes of analysis, the genera *Gymnocladus* and *Pinus* were excluded because they were unreplicated. Model assumptions were examined by graphical inspection of residual plots. Pairwise comparisons between genera were performed with a Tukey adjustment where significant treatment effects occurred. All data analyses were performed in R v4.0.3, with graphics generated using the package ggplot2 (Wickham, 2016; Team, 2020).

**Results**

**Phenology**

We observed consistent patterns in capture of *T. campestris* adults in 2019 and 2020 (Fig. 2.1). In total, we caught 84 adult *T. campestris*: 31 in 2019, or a mean 1 ± 0.3
SE beetles per trap, and 53 in 2020, or a mean of $1.3 \pm 0.3$ beetles per trap. In 2019, we captured the first *T. campestris* on 28 June, when approximately 448 degree days had accumulated. In 2020, the first *T. campestris* was captured on 16 June, when approximately 398 degree days had accumulated. Captures of *T. campestris* peaked at approximately 649 degree days on 12 July 2019 and approximately 666 degree days on 5 July 2020. Our final capture of *T. campestris* in 2019 occurred on 22 August, when approximately 1,193 degree days had accumulated. In 2020, the final adult was captured on 3 September, when approximately 1,453 degree days had accumulated. The cumulative collections of *T. campestris* were also consistent between 2019 and 2020 (Fig. 2.2), with 50% of *T. campestris* capture occurring between 680 and 700 degree days both years.

In 2019, degree day accumulation began on 27 March, which was the first day the daily average temperature reached 10°C (Fig. 2.3). Beginning on 21 May 2019, the daily average temperature did not drop below 10°C until 2 October, totaling 134 consecutive days during which *T. campestris* was likely able to develop. In 2020, the daily average temperature first reached 10°C on 8 March. The daily average temperature remained at least 10°C beginning on 13 May 2020 and lasted through September 8, totaling only 117 consecutive days of development, less than the 134 consecutive days we observed in 2019.

*Patterns in capture*

The average number of *T. campestris* captured per trap was $1.17 \pm 0.21$, with 84 adult *T. campestris* caught overall (both years combined). We observed high variation in
T. campestris captures between individual traps; nearly half of the traps (47%) did not catch any specimens. Three traps captured approximately 25% of the total number obtained, with 22 T. campestris caught between them. These traps were hung in trees of three different genera: Tilia, Ulmus, and Celtis. Average catch rates were highest among traps hung in trees of these same three genera, while lowest average catch rates occurred in traps hung in trees in the genera Acer and Quercus (Fig. 2.4; $F_{6, 61} = 2.44, P = 0.035$). We caught significantly more T. campestris in traps hung in trees of the genus Tilia than in traps hung in trees of the genus Quercus. No other statistically significant differences between tree species were observed.

Discussion

Our results that adult T. campestris abundance displayed a single peak between 649 and 700 degree days in both years of our study suggest that air temperature is a consistent predictor of T. campestris abundance. Knowledge of the emergence timing of T. campestris is crucial to the implementation of an effective pest management strategy, should the need arise. Though there is as yet no evidence of T. campestris causing significant damage in the United States, it has been noted as a lumber and orchard pest in its native range (Kostin, 1973; Iwata and Yamada, 1990; Bai and Zhang, 1999; EPPO, 2009; Zhang et al., 2017). Its potential impacts in the United States are difficult to predict; the Plant Pest Quarantine (PPQ) port policy regarding T. campestris is non-reportable/non-actionable in the continental United States due to the prohibitive costs of an eradication program, but it remains reportable/actionable for Hawaii and United States territories because it does not yet have established populations in those locations (USDA,
If the impacts of *T. campestris* in its introduced range are intensified, either by growing *T. campestris* populations in the United States or expansion into locations that are more vulnerable, such as Hawaii or United States territories, management strategies may become necessary. Multiple forms of pest management are dependent on the phenology of the pests which they aim to control (Wang, 2017). Certain insect pest infestations in lumber yards can be reduced or prevented by debarking bolts, for example. This strategy is most effective when timing of oviposition, eclosion, and other developmental parameters are taken into account (Meshkova et al., 2017).

The consistency of peak emergence at approximately 650 degree days each year suggests that if cold exposure requirements are met, climate change may introduce earlier *T. campestris* emergence each season (Chapter 1). Mean global temperatures are rising, particularly in North America, suggesting that insect developmental thresholds may be met increasingly early in the year (Bentz et al., 2010). While specific consequences of these climatic changes on any individual species are difficult to predict, developmental changes have been observed in several insect species in response to altered climate patterns (Dale et al., 2001; Bentz et al., 2010). For instance, the voltinism of *Ips typographus* (L.) (Coleoptera: Curculionidae) can vary between one and three generations of insects per year, depending on temperature (Lange et al., 2006). Similar responses to climate warming have been documented in the development of *Dendroctonus simplex* LeConte (Coleoptera: Curculionidae), which may also be capable of changing voltinism due to an increasingly early and warm spring season (Mckee and
Aukema, 2015). Whether warming temperatures will impact the voltinism and/or flight periods of *T. campestris*, and its potential impacts on host trees, remains to be seen.

Given the high degree of polyphagy of *T. campestris* and the fact that all traps were baited with the beetle’s pheromone, it is unsurprising that we did not observe differences in insect capture rates between most of the tree genera examined. Variation in host selection, such as a slight preference for traps deployed in *Tilia* spp. (Fig. 2.4), may be tied to various host plant characteristics. Cerambycid host preference can be inversely associated with lower wood density, and the lower average wood density of *Tilia* spp. than *Quercus* spp. is consistent with our results (Fig. 2.4); (Miles and Smith, 2009; Lanuza-Garay and Barrios, 2018). A more widely documented phenomenon is the ability of plant volatiles to synergize or antagonize the attraction of insects to their pheromones (Allison et al., 2004; Silk et al., 2007; Collignon et al., 2016). Compared to *Quercus* spp., *Tilia* spp. have higher emissions rates of *trans*-2-hexen-1-ol and *cis*-3-hexen-1-ol (Helmig et al., 1999), a green leaf volatile that has been shown to increase trap catches of the boll weevil *Anthonomus grandis* Boheman (Coleoptera: Curculionidae) in pheromone traps (Dickens, 1989). Examples of synergy occurring between plant volatiles and deciduophagous insects are not limited to the family Curculionidae; attraction of female *Hedypathes betulinus* (Klug 1825) (Coleoptera: Cerambycidae) to male *H. betulinus* pheromones is also enhanced by addition of volatiles from their host, green mate (*Ilex paracguariensis*) (Fonseca et al., 2010). Higher capture rates of *Anoplophora glabripennis* occur in traps baited with both pheromone and (deciduous) plant volatiles than in traps baited with only one or the other (Nehme et al., 2014). Though these
examples do not prove that the volatile profile of *Tilia* spp. has any impact on the attractiveness of the *T. campestris* pheromone (Trichoferone), they do demonstrate the potential for synergism between green leaf volatiles and Trichoferone that could be explored in future studies.

While our study provides vital information about *T. campestris* phenology and trends in trapping, further research into these topics can provide more definitive conclusions regarding *T. campestris* development and the role of host plant volatiles in this beetle’s host selection. The typical lower developmental threshold for cerambycids is 10-12°C, but dedicated laboratory trials are necessary to determine exact thresholds for a given species (Pershing and Linit, 1986; Adachi, 1994). Knowledge of exact developmental thresholds, along with additional years of catch data in multiple locations in the United States, might yield a more precise and thorough degree day model for *T. campestris* development. The role of host stress on attractiveness to *T. campestris* could also be investigated. Damaged vegetation, for example, can emit ethanol, a known generic attractant to Cerambycid beetles (Kimmerer and Kozlowski, 1982; Montgomery and Wargo, 1983; Geron et al., 2000). Future studies incorporating these factors of cerambycid host selection and development may improve our understanding of *T. campestris* considerably.
Figures 2.1. Average total number of adult *T. campestris* caught throughout the Twin Cities, Minnesota, USA, with standard errors, by the number of accumulated degree days. Degree days used a 10°C base. (A) 2019 (*n* = 29 traps). (B) 2020 (*n* = 40 traps).
Figure 2.2. Percentage of total annual capture of adult *T. campestris* reached at each accumulated degree day. Degree days are calculated as in Figure 2.1. (A) 2019 (*n* = 31 beetles total). (B) 2020 (*n* = 53 beetles total).
Figure 2.3. Maximum and minimum daily temperatures in the Twin Cities, Minnesota, USA, in degrees Centigrade. Data from NOAA Minneapolis-St. Paul International Airport weather station (ID: GHCND:USW00014922). Maximum daily temperatures are denoted by a darker grey color, while lighter grey corresponds to minimum daily temperatures. Julian day is used to denote date, with 1 corresponding to 1 January. Dashed lines depict the span of time during which the average daily temperature never dropped below 10°C. Arrows outside dashed lines depict the first and last dates annually on which the average daily temperature reached 10°C. a. 2019. b. 2020.
Figure 2.4. Box plot of number of *T. campestris* found in traps hung in trees of the seven genera in trapping experiments 2019-2020 (pooled data). Each datapoint is an individual trap. The boxes represent 50% of observations, ranging from the 1st quartile to the 3rd quartile, with the intermediate representing the median. The horizontal lines emerging from each box show the spread of the data from minimum to maximum, excluding outlying values (defined as values outside the box by at least 1.5 times the interquartile range), which are represented by individual points. Different letters indicate significant treatment differences (Tukey procedure). (*N* = 70 trees total; *F*_{6, 61} = 2.44, *P* = 0.035).
Thesis Conclusions

Chapter 1

1) *Trichoferus campestris* can oviposit on, develop in, and emerge from cut branches of at least four North American tree species. Polyphagy is associated with likelihood of insect establishments after introduction to a novel environment. Landscapes dominated by apple or walnut trees, such as orchards, may face higher susceptibility to invasion by *T. campestris* than landscapes dominated by pine trees, particularly in the face of a changing climate that could stress potential hosts.

2) More *T. campestris* emerged from tree branches that had undergone a late winter chilling treatment, particularly those of *M. domestica*. Northern temperate ecosystems that experience adequate cold temperatures seasonally may face higher risk of *T. campestris* establishment than ecosystems that experience consistently warm temperatures, such as Hawaii, U.S.

3) *Trichoferus campestris* was most likely to oviposit and emerge from branches of *J. nigra* and *M. domestica*. Branches on which lower oviposition was observed, such as *P. strobus* and *A. saccharum*, yielded lower emergence and smaller larvae. This evidence on the ovipositional behavior and development of *T. campestris* supports the preference/performance hypothesis.

Chapter 2

1) In both years of our study, patterns of *T. campestris* abundance displayed a single peak around 650-700 degree days. This result suggests that air
temperature is an effective tool for designing management and sampling strategies. With rising global mean temperatures and evidence of climate change impacting the phenology of other species of Coleoptera, it would not be surprising if increasing air temperatures led to an earlier peak flight period in *T. campestris*.

2) **We found significantly more *T. campestris* in traps hung in trees of the genus *Tilia* than the genus *Quercus*.** As synergy between host plant volatiles and other phytophagous beetles has been well-documented, synergy may be occurring between green leaf volatiles of *Tilia* spp. and *T. campestris* pheromones.


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Appendices

Appendix A. Box plot of number of *T. campestris* larvae found on branches of the four tree species in host choice assays, 2019-2020. Each datapoint is an individual larva found during branch peeling in August 2021 when the study reached its conclusion. The boxes represent 50% of observations, ranging from the 1st quartile to the 3rd quartile, with the intermediate representing the median. The vertical lines show the spread of the data from minimum to maximum, excluding outlying values (defined as values outside the box by at least 1.5 times the interquartile range), which are represented by individual points. Different letters indicate significant treatment differences (Tukey procedure). (A) 2019 assay (*N* = 140 total, *F*₃,₁₃₆ = 4.68, *P* = 0.0038). (B) 2020 assay (*N* = 64 total, *F*₃,₆₀ = 0.39, *P* = 0.76).
Appendix B. Box plot of larval *T. campestris* length in millimeters on branches of the four tree species in choice assays 2019-2020. Each datapoint is an individual larva found during branch peeling in August 2021 when the study reached its conclusion. The boxes represent 50% of observations, ranging from the 1st quartile to the 3rd quartile, with the intermediate representing the median. The vertical lines show the spread of the data from minimum to maximum, excluding outlying values (defined as values outside the box by at least 1.5 times the interquartile range), which are represented by individual points. Different letters indicate significant treatment differences (Tukey procedure). (A) 2019 assay (*N* = 51 total, *F*<sub>3,47</sub> = 5.652, *P* = 0.0022). (B) 2020 assay (*N* = 42 total, *F*<sub>3,38</sub> = 5.589, *P* = 0.0028).
Appendix C. Box plot of larval *T. campestris* length in millimeters on branches exposed or not exposed to chill in host choice assays 2019-2020. Data from larvae reared on *M. domestica, J. nigra, A. saccharum,* and *P. strobus* are pooled in chilled and not chilled categories. Each datapoint is an individual larva found during branch peeling in August 2021 when the study reached its conclusion. The boxes represent 50% of observations, ranging from the 1st quartile to the 3rd quartile, with the intermediate representing the median. The vertical lines show the spread of the data from minimum to maximum, excluding outlying values (defined as values outside the box by at least 1.5 times the interquartile range), which are represented by individual points. Different letters indicate significant treatment differences. (A) Length of larvae from 2019 trials (*N* = 51, *F*<sub>1,49</sub> = 0.25, *P* = 0.62). (B) Length of larvae from 2020 trials (*N* = 42, *F*<sub>1,40</sub> = 8.371, *P* = 0.0061).
Appendix D. Box plot of larval *T. campestris* length in millimeters on branches of *M. domestica* exposed or not exposed to chill in canker host choice assays 2020. Each datapoint is an individual larva found during branch peeling in August 2021 when the study reached its conclusion. The boxes represent 50% of observations, ranging from the 1st quartile to the 3rd quartile, with the intermediate representing the median. The vertical lines show the spread of the data from minimum to maximum, excluding outlying values (defined as values outside the box by at least 1.5 times the interquartile range), which are represented by individual points. Different letters indicate significant treatment differences. (*N* = 53, *F*$_{1,51} = 10.69$, *P* = 0.0019).
Appendix E. Box plot of larval *T. campestris* length in millimeters on branches of the four tree species in no choice assays 2019-2020. Each datapoint is an individual larva found during branch peeling in August 2021 when the study reached its conclusion. The boxes represent 50% of observations, ranging from the 1st quartile to the 3rd quartile, with the intermediate representing the median. The vertical lines show the spread of the data from minimum to maximum, excluding outlying values (defined as values outside the box by at least 1.5 times the interquartile range), which are represented by individual points. Different letters indicate significant treatment differences (Tukey procedure). (A) 2019 assay (*N* = 20, *F*₃,₁₆ = 0.91, *P* = 0.46). (B) 2020 assay (*N* = 25, *F*₃,₂₁ = 8.34, *P* = 0.00077).