Love at First Flight: Mating Errors, Population Genetics, and the Slow Spread of an Invader

by

Jennifer L. Anderson

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Supervisor: Stephen B. Heard, PhD, Biology

Examining Board: Mike Duffy, PhD, Biology

Joe Nocera, PhD, Forestry and Environmental Management

Deepa Pureswaran, PhD, Adjunct Professor, Biology

External Examiner: Brent Sinclair, PhD, Department of Biology, University of Western Ontario

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Abstract

This thesis investigates mating interactions and population genetics of the invasive *Tetropium fuscum* Fabricius (Coleoptera: Cerambycidae) and its native congener, *Tetropium cinnamopterum* Kirby (Coleoptera: Cerambycidae) in Nova Scotia, Canada, to help explain the slow spatial spread of *T. fuscum* in North America. *Tetropium fuscum* first appeared in North America in about 1990 and has since outcompeted the native *T. cinnamopterum* in the sympatric zone and spread approximately 150 km from its point of entry but has since halted its spread. *Tetropium fuscum* and *T. cinnamopterum* have common host plants, overlapping flight periods and share the same male-produced sex-aggregation pheromone, fuscumol, that attracts both sexes. These species would have ample opportunity to encounter each other in the field due to these similarities suggesting hybridization is a possibility. Our mating behaviour experiments showed males of both species make mating errors under no-choice mating conditions. Under choice mating conditions *T. cinnamopterum* males show a strong preference for same-species females but *T. fuscum* is less discriminating. We designed a species discriminating SNP assay with the goal of detecting hybridization to confirm that mating errors also take place in the field. We found low levels of introgression in the sympatric zone as well as the presence of cryptic individuals that morphologically present as *T. cinnamopterum* while genetically presenting as *T. fuscum*. We investigated the possibility that female body size rather than species influences mate choice in *Tetropium*. *Tetropium cinnamopterum* females are significantly larger than *T. fuscum* females although body size was not a determining factor in male mate choice for these species. Our findings suggest heterospecific matings between these species happen but may be rare and the resulting

offspring may not be as fit as their parents, which could exacerbate Allee effects at the edge range of *T. fuscum* and reinforce its apparently pinned range border.

Dedication

To my ever loving and forever patient son: this is not my accomplishment, but ours.

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Chapter 1 - General Introduction

Key Terms Defined

Hybrid: offspring resulting from the interbreeding of individuals from two distinct species, subspecies, or genetically divergent populations. Hybrids usually display a combination of traits from both parent lineages.

Introduced species: organisms intentionally or accidentally brought into an ecosystem outside their native range by human actions. These introductions can be deliberate, like species introduced for agricultural purposes, or accidental through global trade or transport.

Introgression: genetic material from one species or population enters and becomes part of the gene pool of another through repeated backcrossing. This process can lead to the transfer of advantageous or deleterious traits between species or populations.

Invasion biology: the study of non-native species that have been introduced to novel environments and how the species interacts within those environments. Invasion biology explores the impacts, dynamics, and consequences of these introductions on ecosystems, focusing on the spread, establishment, and influence of non-native organisms on established species.

Invasive species: introduced organisms that thrive and substantially alter their new environment. They typically outcompete native species, reproduce rapidly, and disrupt ecosystems, causing ecological, economic, or societal harm.

Mating error: a mating attempt between individuals of different species that is not typically beneficial or intended based on the species' ecological and evolutionary context. A heterospecific mating is considered a mating error if it results in no fertilization, poor fertilization, or if the offspring are not viable, fertile or display hybrid depression.

Early Biological Invasions

Invasion biology is a vital branch of ecology that was first conceptualized by Charles Elton in his 1958 book "The Ecology of Invasions by Animals and Plants." Since the writing of this book, invasion biology has grown into an indispensable science that helps predict and mitigate impacts of nonindigenous species in novel habitats. Invasion biology has far-reaching implications both ecologically and economically. We used an invasive forest pest in eastern North America to investigate a particularly important issue in invasion ecology: what determines whether an invasion is fast or slow?

Although species introductions are not always the result of anthropogenic influence, this is the predominant mechanism. One of the earliest examples is the dumping of ballast from ships transporting goods around the globe. Early ships used soil, sand, and dirt as ballast rather than water, and when a ship arrived at its destination to get

its cargo, the ballast was dumped into the water or onto the land, releasing whatever organisms were living in the ballast into the environment (Mills et al. 1993). At present, ships have switched to using water as a ballast and although many species still get transported around the globe through this mechanism, there are now laws in place about proper treatment of ballast water to minimize this effect (Ivče et al. 2021). Before we came to understand the potential negative consequences of introducing non-native species into novel habitats, people frequently intentionally did so (Nairn et al. 1996, Whitney and Gabler 2008). An ongoing method is the introduction of various plant species that people want to keep as garden specimens and ornamentals. One such introduction in New Jersey (USA) in 1916 led to the accidental introduction of Japanese chafer beetles, *Popillia japonica* Newman (Elton 1958), which are now considered a major agricultural pest in the USA and Europe (Marianelli et al. 2018). The European starling, *Sturnus vulgaris* Linnaeus, was intentionally introduced multiple times in New York (USA) in order to get it to establish but has since become ubiquitous in North America (Elton 1958) where it is considered an agricultural pest of orchards and vineyards (Khatri-Chhetri et al. 2020). Intentional introduction of non-native species has decreased but still occurs in the release of aquarium fish (Padilla and Williams 2004, Della Venezia et al. 2017) and the stocking of game fish into waterways (Kerr et al. 2005).

Consequences of invasive species

Introduction and establishment of invasive species steadily increased for many years (Kolar and Lodge 2001, Seebens et al. 2017) and caused major global changes (Vitousek et al. 1996, Sakai et al. 2001). This was due at least in part to the rise in

international trade and transport of untreated wood products (Haack 2006) and was happening despite efforts at shipping ports and border entries to intercept non-native species (Suh et al. 2013, Turner et al. 2021). Of particular concern are wood-boring beetles, which are cryptic in nature. Despite international regulations (e.g., International Phytosanitary Measure 15) that now require heat-treatment or fumigation of wood packaging, subsequent audits found < 1% of wood packaging contained live insects. Consequently, the millions of shipping containers that arrive in international ports annually ensure the continued arrival of non-native woodboring beetles.

Invasive species have been a problem since long before Elton published his 1958 book, and we know well that they have serious impacts both economically (Evans 2003) and ecologically (Elton 1958, Lodge 1993, Simberloff 1996, Sakai et al. 2001), altering community structure (Fritts and Rodda 1998, O'Dowd et al. 2003, Whitney and Gabler 2008), and negatively impacting biodiversity (Williamson and Fitter 1996, Wilcove et al. 1998, Parker et al. 1999, Sala et al 2000, Whitney and Gabler 2008). Invasive species can negatively impact the community they invade through competition with native species (Brown et al. 2002, Tennessen et al. 2016), may spread disease (Linz et al. 2007, Beltrán-Beck et al. 2011), and can facilitate native species (Rodriguez 2006). Their most direct effect is the attack of crop plants which negatively impacts harvests and results in less product available for sale (Evans 2003).

While some invasives arrive in a new ecosystem and find no close relatives there, others invade alongside native congeners, which may lead to invasive/native hybridization. The implications of hybridization between an invasive species and its native congener are various and although invasives can have a significant impact on native species, native species can also affect the invasion success of the invader. Invasives may impact community structure through hybridization with native congeners (Abbott 1992, Ellstrand and Schierenbeck 2000, Bleeker et al. 2007, Whitney and Gabler 2008). This may cause population decline or extinction in either the invasive species, the native species or both due to introgression (Levin et al. 1996, Rhymer and Simberloff 1996). Asymmetric hybridization can cause genetic swamping in the population and possible extinction of whichever species is being diluted by the other (Rhymer and Simberloff 1996, Ellstrand and Schierenbeck 2000, Bleeker 2007, Whitney and Gabler 2008). Hybrids may experience increased fitness (hybrid vigour or heterosis) compared to their parental species (Lippman and Zamir 2007, Birchler 2015) and have been shown to outcompete them in plants (Campbell et al. 2006, Whitney and Gabler 2008) - a feature commonly exploited in agriculture (Ravi et al. 2007, Deshmukh et al. 2015). However, they may also exhibit decreased fitness compared to their parents (outbreeding depression) (Lynch 1991) which can result in increased disease susceptibility (Goldberg et al. 2005). Hybrids can be nonviable or infertile in the F1 or F2 generation (Barton and Hewitt 1981, Templeton 1981, Coyne and Orr 1989, Lynch 1991), which would result in gamete wastage and hinder the growth of the populations producing them.

When a species is introduced to a novel habitat, there are three possible outcomes in terms of establishment and spread. The first outcome is that the individuals in the founder population die off due to predation, inability to find a suitable mate or food source, or simply not being well-suited to the environment. Many die before arriving at the new habitat or shortly thereafter (Kolar and Lodge 2001). There is limited information on invasions that failed to take hold (Zenni and Nuñez 2013) since our interests are usually in studying invasive species that directly impact humans and unsuccessful invasions rarely do so. The second possibility is that the species establishes by founding a self-sufficient population (Lockwood et al. 2013) and successfully spreads into other areas, often having a significant economic and ecological impact. This is the most well-studied case because of the direct impact these species have on humans. Many introduced insects in North America have gone on to be major pests, such as the emerald ash borer *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) (Bauer et al. 2008), the Asian longhorned beetle *Anoplophora glabripennis* Motschulsky (Coleoptera: Cerambycidae) (Sjöman et al. 2014), the European spongy moth *Lymantria dispar* Linnaeus (Lepidoptera: Erebidae) (Paini et al. 2018), the Argentine ant *Linepithema humile* Mayr (Hymenoptera: Formicidae) (Wetterer et al. 2009), and the spotted lanternfly *Lycorma delicatula* White (Hemiptera: Fulgoridae) (Dara et al. 2015). The third and likely least well understood scenario is that an introduced species successfully establishes but does not spread far or become a major pest. This can be observed in the case of the Brown Spruce Longhorn Beetle, *Tetropium fuscum* Fabricius (Coleoptera: Cerambycidae) (Rhainds et al. 2011).

Characteristics of successful invaders

It is important to examine which traits are common among successfully invasive species to make better predictions about future invasions and prevent or mitigate their negative impacts. A lot of research has been done to investigate which life history traits allow a species to be a successful invader, although most of these studies focus on plants and birds (Hayes and Barry 2008) and research focus on insect invasiveness traits is scarce. Many traits have been tied to invasiveness in various taxa including large body size (Ehrlich 1989, Hayes and Barry 2008, Catford et al. 2019), tolerance to a wide array of environmental conditions (Moyle 1986), phenotypic or physiological plasticity (Ehrlich 1989, Rejmánek et al 2005, Kelley 2014, Boltovskoy et al. 2021), high dispersal rate (Moyle 1986, O'Connor 1986, Fournier et al. 2019), generalist diet (Ehrlich 1989, Shik and Dussutour 2020), and competitiveness (Moyle 1986, O'Connor 1986, Cassini 2020, Boltovskoy et al. 2021). Another factor that has been tied to invasion potential is high reproduction rate - which could mean large broods or many broods per year (O'Connor 1986, Veltman et al. 1996, Daehler 1998, Sakai et al. 2001, Boltovskoy et al. 2021), but may also include a short juvenile stage (Ehrlich 1989, Richardson et al. 1990, Rejmanek and Richardson 1996, Reichard and Hamilton 1997). The trait most tied to invasiveness in the literature is association with humans (Moyle 1986, Newsome and Noble 1986, Ehrlich 1989, Sakai et al. 2001, Gippet et al. 2019). Multiple studies have also found that species are more likely to become invasive if the novel habitat is more like their native habitat (Newsome and Noble 1986, Hayes and Barry 2008). Of all the traits suggested to be associated with establishment and invasiveness, propagule pressure is the one that comes up most often and across various taxa. Propagule pressure can be in

the form of repeated introductions into the novel habitat or in large population sizes being released and is strongly associated with invasiveness, establishment, and range expansion (Newsome and Noble 1986, O'Connor 1986, Williamson 1989, Mulvaney 2001, Lockwood et al. 2005, Hayes and Barry 2008, Blackburn et al. 2015, Levin 2020, Thomaz 2022).

Many highly successful invasive species display a subset of traits associated with invasion success; however, it is not necessary that they possess all of them to be invasive. The zebra mussel (*Dreissena polymorpha* Pallas (Myida: Dreissenidae)) has association with humans as it was likely first introduced from ballast water released by shipping vessels (Hebert et al. 1989), is rapidly reproducing (Ram et al. 1993), and has a high degree of phenotypic plasticity (Pathy and Mackie 1993, Rosenberg and Ludyanskiy 1994, Lajtner et al. 2004). The cane toad (*Rhinella marina* Linnaeus (Anura: Bufonidae)) also has association with humans and was intentionally introduced to eradicate agricultural pests in the Philippines (Sy and Heredero 2019), Australia, and several other Caribbean and tropical Pacific islands (Trumbo et al. 2016). It displays a high degree of plasticity in its body size (Cabrera-Guzmán et al. 2013), has a generalist diet (Brandt and Mazzotti 1999, Johnnides et al. 2016), a high rate of reproduction (Tingley et al. 2017), and tolerates a broad pH range (Wijethunga et al. 2006). The Asian longhorned beetle is associated with humans as it was probably first introduced into North America from solid wood packaging materials used in shipping (Haack et al. 1997). They have large bodies compared to other wood boring beetles (Hajek 2007) and can use a wide array of trees as host plants and food sources for their larvae (Haack et al. 1997). The European starling

has association with humans in the form of intentional introduction into North America (Cabe 1998). It had a high degree of propagule pressure as many attempts were made to introduce it before it finally took hold and became established (Elton 1958). It also competes strongly for nesting sites with other bird species and may consequently decrease the breeding success of those species (Nilsson 1984). There is a great deal of research dedicated to the study of successful invaders, presumably because our society puts more effort into researching species that have direct and serious impacts on our lives. There is, however, very little research published on introduced species that are poor or mediocre invaders. Our study examines the dynamics of mating errors and hybridization between an introduced species and a native congener, and the possible impacts on the ability of the introduced species to establish, adapt, evolve, and spread.

Study Species

Tetropium fuscum is a longhorn beetle native to Western Europe and Northern Eurasia (Juutinen 1955) that was first discovered in Point Pleasant Park in Halifax, Nova Scotia (Canada) in 1999. It had been collected and misidentified as a native *Tetropium* species in the collection at the Nova Scotia Museum of Natural History, indicating it had been present as far back as 1990 (Sweeney et al. 2004). In its native range, *T. fuscum* predominately attacks stressed or moribund Norway spruce (*Picea abies* (Linnaeus) Karsten) (Juutinen 1955), but in its invasive range, has been observed attacking apparently healthy red spruce (*Picea rubens* Sargent), white spruce (*Picea glauca* Moench (Voss)), black spruce (*Picea mariana* (Miller) Britton, Sterns and Poggenburg) and Norway spruce (Smith and Humble 2000). *Tetropium fuscum* emerges in the spring

starting in May and has a flight period lasting until August (Juutinen 1955). The adults are not known to feed and thus their life spans are limited to a couple of weeks. Males produce a mating and aggregation pheromone component, S-fuscumol, which combines synergistically with host volatiles to attract both males and females to the tree on which they are emitting (Silk et al. 2007, Rhainds et al. 2010, Sweeney et al. 2010). Once a female has mated with a male, she lays her eggs under the crevices of the bark of the tree. The eggs, which are approximately 1.0 – 1.2 mm in length, hatch about 2 weeks after being laid and the larvae burrow into the tree to feed on the phloem and will overwinter under the bark until spring, thus completing the life cycle (Juutinen 1955). *Tetropium fuscum* can be gathered for rearing by using a synergistic combination of ethanol, S-fuscumol, and a blend of monoterpenes to bait felled spruce logs and promote infestation. Infested logs can then be placed into a -2ºC freezer to simulate overwintering conditions and if they are left in the freezer a minimum of 6 weeks, the beetles will develop into adulthood as normal when removed from the freezer and warmed up to approximately 20ºC. *Tetropium fuscum* can also be reared in lab colonies using adults that were either trapped live in the field or reared from infested wood using the same process as is used in the field, but without the need for baiting. Adults can be placed directly onto fresh cut spruce logs to mate, lay eggs, and allow infestation of the wood.

Tetropium fuscum is notable because it illustrates a poorly studied scenario in which an introduced species establishes but does not spread well. *Tetropium fuscum* has been occasionally detected in pheromone traps in a few outlier locations in New Brunswick – likely the result of infested wood transported by campers. Other introduced

insects that have gone on to be invasive possess traits commonly associated with invasiveness. Spongy moth, which is a voracious defoliator (Liu et al. 2023), has a generalist diet and can astoundingly feed on over 300 host plant species (Ponomarev et al. 2023). The spotted lanternfly, a relatively novel introduced species in North America, does not cause problems in its native China due to a parasitoid wasp that keeps populations under control (Broadley et al. 2023). Spotted lanternfly feeds on over 70 host plant species (Derstine et al. 2020), but also benefits from enemy escape; its preferred host of the tree of heaven makes it taste unpleasant to birds (Jones et al. 2022). Another introduced longhorn beetle, the Asian longhorned beetle (*Anoplophora glabripennis*), is a successful invader in part due to enemy escape (Sacco 2004). Spongy moth was first introduced to North America in 1869 (Mull and Spears 2022) and the spotted lanternfly was first detected in 2014 (Leach et al. 2019), both at quite different time points than the introduction of *T. fuscum*, making it more complicated to compare their spread with that of *T. fuscum*. Although Asian longhorned beetle was first detected in Canada in 2003 (Javal et al. 2019), a mere 4 years after the first detection of *T. fuscum*, we unfortunately cannot directly compare the spread and invasiveness of these two species either. This is because Asian longhorn has established populations and then been eradicated several times in North America (Javal et al. 2019), while *T. fuscum* has never been eradicated from its introduced range. *Tetropium fuscum* has been spreading slowly in North America (Figure 1.1) and, apart from these few sporadic detections in New Brunswick, has spread only about 150 km from its epicenter since its introduction circa 1990 (Rhainds et al. 2011, CFIA 2017). A contrasting case can be seen in another introduced beetle pest species, Emerald Ash Borer (*Agrilus planipennis)*. *Agrilus planipennis* was first

discovered in Detroit, Michigan (USA) in 2002 (Cappaert et al. 2005), but further investigation revealed it has likely been present in Detroit since the early to mid 1990s. As we have evidence that *T. fuscum* has been present in North America since approximately the same time (Sweeney et al. 2004), these two species are ideal for comparison of range expansion under different circumstances. *Agrilus planipennis* and *T. fuscum* are in stark contrast in terms of range expansion. Despite invading North America at approximately the same time, *A. planipennis* has spread more than 1000 km from its epicenter (Cooperative Emerald Ash Borer Project, 2021). While *A. planipennis* and *T. fuscum* share some similarities such as time of introduction into North America, springtime emergence (Juutinen 1955, Brown-Rytlewski and Wilson 2005), and larval phloem-feeding (Juutinen 1955), there are also differences that could help explain their contrasting performances in North America. Perhaps the most important difference between the two species though, is that unlike *A. planipennis*, *T. fuscum* has a native congener in its invasive range that shares the same host plants.

Figure 1.1: Slow North American spread of *T. fuscum* shown by areas of regulation in 1999 (A) and 2022 (B). *Agrilus planipennis* regulated area in 2002 (C) and 2022 (D) is shown for comparison.

Tetropium cinnamopterum Kirby (Coleoptera: Cerambycidae) is a congener to *T. fuscum*, native to northeastern North America and is very similar to *T. fuscum* in many ways. Its similarity in morphology is what caused *T. fuscum* to be mistaken for *T. cinnamopterum* when it was first collected in Nova Scotia (Canada) (Sweeney et al. 2004). Like *T. fuscum*, *T. cinnamopterum* also attacks spruce trees (*Picea* spp.) but it has a broader host range and attacks other species of conifer (Furniss and Carolin 1980). *T. cinnamopterum* tends to preferentially attack very weakened or moribund trees (Flaherty et al. 2011), contrasted with *T. fuscum*'s ability to attack apparently healthy trees (Smith and Humble 2000). *Tetropium cinnamopterum* emerges on average 2 weeks later than *T. fuscum*, but they both have a flight period of approximately late May to August (usually peaking in June) and there would be ample overlap in their flight periods, which would allow them to be active at the same time (Rhainds et al. 2011). Both species are active during the day and share the same male-produced mating and aggregation pheromone of *S*-fuscumol (Silk et al. 2007, Rhainds et al. 2010, Sweeney et al. 2010).

Hybridization and mating errors

Hybridization between an introduced species and a native congener can shape the establishment and spread of the introduced species (Hata et al. 2019). This phenomenon occurs across taxa, evidenced in birds (e.g. introduced and native mallards in Australia) (Simberloff 1996), mammals (e.g. the introduced red deer with the native sitka deer) (Long et al. 1998), fish (e.g. the introduced Westslope cutthroat trout with the native rainbow trout) (Weigel et al. 2003), amphibians (e.g. the introduced tiger salamander and the California tiger salamander) (Riley et al. 2003), and insects (e.g. the introduced

winter moth with the native Bruce spanworm) (Havill et al. 2017). Hybridization fosters genetic diversity by blending new gene combinations through introgression, which may impact the native species, the introduced species, or both (Harrison and Larson 2014). When introgression favours the introduced species, increased genetic diversity may boost adaptability to the new environment, aiding establishment and facilitating spread (Bugarella et al. 2019, Edelman and Mallet 2021). Conversely, this process can also introduce deleterious alleles, potentially reducing the fitness of the introduced species (Dagilis and Matute 2023). There is also some evidence to suggest that heterospecific mating and hybridization with a native congener can help the introduced species overcome Allee effects, an issue that often plagues founding populations (Mesgaran et al. 2016).

Hybrid offspring often exhibit intermediate traits, offering hybrids a competitive edge to exploit ecological niches unutilized by either parent species (Runemark et al. 2019). Such advantage can propel hybrid establishment and spread in the novel habitat, sometimes surpassing their parent species, complicating the establishment of the introduced species and challenging the stability of the native species (Wei et al. 2023). On the other hand, hybrids with reduced fitness or reproductive challenges, such as fertility or viability issues, may hinder the successful establishment and spread of the introduced species (Schumer et al. 2015, Dagilis et al. 2019). Genetic swamping, marked by overwhelming introgression, could erode unique genetic traits in affected populations, impacting the fitness of the native species and influencing overall ecological dynamics, thereby affecting the stability of both parental species (Todesco et al. 2016, Fu et al.

2022). By “stability”, we refer to the ecological and genetic equilibrium of the parents with the ecosystem. This encompasses scenarios ranging from the potential collapse of the two species into one hybrid population, to the partial replacement of the parent species by a hybrid swarm, and even to the extinction of one or the other species. Understanding these potential outcomes is pivotal for evaluating the ramifications of introduced-native species hybridization, profoundly shaping the establishment, spread, and evolutionary path of the introduced species in unfamiliar habitats.

The presence of an invasive species and a native congener in the same region do not, of course, guarantee hybridization. There are many possibilities, ranging from complete lack of interspecific mating to formation of a single panmixic population. Heterospecific matings, do not always result in fertile, viable hybrid offspring or introgression (Larson et al. 2019, Wei et al. 2023). Precopulatory mechanisms like using different host plants (Prager et al. 2014, Fiteni et al. 2022), geographical separation (Schwartz et al. 2010, Servedio 2016), and phenology (Ekrem and Kokko 2023) can prevent mating and fertilization from taking place. Some insects display peri-copulatory barriers to fertilization, but not to mating, such as genital lock and key mechanisms (Masly 2012, Langerhans et al. 2016), cryptic female mate choice (Eberhard 1997, Firman et al. 2017), and male sperm competition (Danielsson 1998, Simmons 2002). Pre- and peri-copulatory barriers to mating or fertilization typically prevent or diminish production of hybrid offspring. Post-copulatory barriers to hybridization can include things such as low survival of offspring, low fitness of offspring, sterility or decreased

fertility in offspring (Jiggins et al. 2001, Goldberg et al. 2005, López-Fernández and Bolnick 2007, Sonnleitner et al. 2013).

Heterospecific matings may not necessarily constitute a mating error, particularly in cases where hybrid offspring exhibit increased fitness. However, cases in which mating is prevented or leads to offspring of low fitness, but in which mating is still attempted, constitute mating errors and may be particularly interesting because they may lead to an impediment to population growth. Insects typically have a much shorter adult life span than vertebrates and this gives them a shorter window to find suitable mates and produce offspring. If a heterospecific mating produces no offspring or offspring of low fitness, then this is a waste of mating resources and is of particular consequence to insect species that live for a matter of days to weeks. Thus, regardless of the point at which limitations are placed on production or fitness of hybrid offspring, or the mechanisms through which they are imposed, the result may be curbed population growth for one or both hybridizing populations.

Experimental Design

Because of the similarities between *T. fuscum* and *T. cinnamopterum*, particularly traits associated with mate attraction and mating behaviour, we looked at whether mate choice errors on the part of *T. fuscum* could be contributing to its slow spread in North America. It is important for us to know if *T. fuscum* is mating with *T. cinnamopterum* in the field for several reasons. *Tetropium cinnamopterum* infests a broader range of trees than *T. fuscum* does, and *T. fuscum* is capable of infesting apparently healthier trees than

T. cinnamopterum. If there is introgression between these two species, the result may be hybrid individuals that display the most undesirable traits of each parental species and as a result are potentially more problematic for our forests than either parental species. On the other hand, if *T. fuscum* is mating with *T. cinnamopterum* and they are either producing sterile offspring or not producing any offspring at all, this is an utter waste of mating resources and could help explain why *T. fuscum* is spreading so slowly. The main goal of my thesis was to test the hypothesis "The slow North American spread of *T. fuscum* is due, at least in part, to mate choice errors with *T. cinnamopterum*." The following section outlines my experimental designs and how this work contributed to answering my main research hypothesis.

Chapter 2 evaluates whether *T. fuscum* and *T. cinnamopterum* males will make mating errors in a laboratory setting. The first experiment was a no-choice mating experiment in which one male and one female beetle were placed in a petri dish arena and given a set amount of time to commence mating. We had four treatments in all possible combinations of male and female *T. fuscum* and *T. cinnamopterum*. This experiment was designed to evaluate whether *Tetropium* males will make mating errors in a situation where the only other option would be not to mate at all. Species tend to be densest at the epicenter of their range and sparsest at the range edges (Sagarin and Gaines 2002; Sagarin et al. 2006), and thus this experiment mimics the conditions *T. fuscum* would encounter at the edges of its range. The second experiment was a choice mating experiment in which one male was given a female of each species and allowed a set

amount of time to "choose" a female to copulate with. There were two treatments - one for each species of male. This experiment was designed to determine whether *Tetropium* males make mating errors when they could engage in a conspecific mate pairing instead.

Results in a laboratory setting are not necessarily indicative of what the results would be in a natural setting (Calisi and Bentley 2009) and as such it is important to also determine whether *T. fuscum* males make mating errors in the field. It is extremely difficult to measure this directly for several reasons. *Tetropium* is cryptic in nature, blending in well with its environment, and *T. fuscum* and *T. cinnamopterum* cannot easily be distinguished in the field, making it very difficult to not only find mating pairs of *Tetropium* in the field but also to identify whether the pairing is heterospecific or conspecific. We also would have had to find a mating pair before they commenced mating and observe them for the duration of copulation. Due to these difficulties, we chose to take an indirect approach to determine whether *Tetropium* males make mating errors in the field. Chapter 3 outlines a SNP assay and a field survey designed to check for introgression in *Tetropium* populations in Nova Scotia and New Brunswick. Colleagues in Quebec (Canada) collected and mailed *T. cinnamopterum* specimens to me and colleagues in Norway, France and Italy collected and mailed *T. fuscum* specimens to me. We chose these locations for specimen collection because both areas are well outside the sympatric range of *T. fuscum* and *T. cinnamopterum* in North America. We extracted DNA from these samples and used ddRADseq (Aguirre et al. 2019; Sundan et al. 2019; Molina et al. 2023) to sequence portions of *T. fuscum* and *T. cinnamopterum* genomes. We had the sequencing work conducted by Génome Québec. Using bioinformatics

techniques, we used the returned sequence data to identify single-nucleotide polymorphisms (SNPs) that could be used as a diagnostic tool to distinguish *T. cinnamopterum* from *T. fuscum* and to identify hybrid individuals if they occur. We collected *Tetropium* specimens from five sites across Nova Scotia and New Brunswick and extracted genomic DNA from them to use for a field survey to look for introgression. We applied the SNPs we had elucidated to the field survey DNA to check for individuals displaying SNPs specific to each of the two *Tetropium* species.

The results of our mating behaviour experiments showed that under no-choice conditions, both *T. fuscum* and *T. cinnamopterum* males made mating errors. *Tetropium fuscum* males still made errors under choice mating conditions but *T. cinnamopterum* males had a strong preference for conspecific females under these conditions. *Tetropium cinnamopterum* females are significantly larger than *T. fuscum* females (Chapter 4), so we wanted to assess whether female body size was a driving factor in *Tetropium* male mate choice rather than species. We investigated body size as it relates to fecundity in *T. fuscum* females by assessing the effect of female body size on egg size and lifetime egg lay rate. We also measured time to successful copulation as it relates to female body size to determine if *Tetropium* males showed a preference for larger females.

This work serves to illuminate the intricate dynamics between the invasive *T. fuscum* and its native counterpart, *T. cinnamopterum*, providing insights applicable to similar species pairs. This study examines how mating errors and hybridization between an introduced species and a native congener influence the ability of the introduced

species to establish, adapt, evolve, and spread. Understanding these interactions not only sheds light on the immediate implications for *T. fuscum* and *T. cinnamopterum* but extends to broader implications for invasive species management. The insights gleaned from this research not only improve our predictive capacity concerning the behavior of invasive species in novel environments but also furnish valuable guidance to forest managers striving to alleviate the impacts of such species on ecosystems.

Author Contributions

Chapter 2: Mate choice errors may contribute to the slow spread of an invasive Eurasian longhorn beetle in North America (Coleoptera: Cerambycidae).

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Jennifer L. Anderson led conceptualization of the work, designed and conducted experiments, analyzed the data, produced first draft of manuscript, and made appropriate edits on advice of co-authors. Stephen B. Heard helped with experimental design, editing feedback on all drafts and consultation on writing/experimental process. Deepa S. Pureswaran helped with experimental design and editing feedback on all drafts. Jon Sweeney helped with experimental design and feedback on late-stage draft of manuscript.

Chapter 3: Introgression with a native congener could limit invasiveness in a non-native longhorned beetle.

This chapter will be submitted for publication with the following authorship list: Jennifer L. Anderson, Stephen B. Heard, Jason Addison, René Malenfant, and Deepa Pureswaran.

Jennifer L. Anderson led conceptualization of the survey and bioinformatics, conducted experiments, executed bioinformatics and other data analysis, produced first draft of manuscript, and made appropriate edits on advice of co-authors. Stephen B. Heard helped with experimental design, editing feedback on all drafts and consultation on writing/experimental process. Deepa S. Pureswaran helped with experimental design and editing feedback on all drafts. Jason Addison helped with experimental design and advising. René Malenfant helped with bioinformatics and data analysis.

Chapter 4: No evidence that body size and fecundity influence mating in an invasive longhorn beetle.

This chapter will be submitted for publication with the following authorship list: Jennifer L. Anderson, Stephen B. Heard, Jon Sweeney, and Deepa Pureswaran.

Jennifer L. Anderson conceptualized the study, conducted most experiments, analyzed all data, produced first draft of manuscript and made appropriate edits on advice of co-authors. Stephen B. Heard helped with experimental design, editing feedback on all drafts and consultation on writing/experimental process. Deepa S. Pureswaran helped with experimental design and editing feedback on all drafts. Jon Sweeney helped with experimental design and feedback on late-stage draft of manuscript and contributed data on lifetime egg lay from previously unpublished data.

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Chapter 2 – Mate choice errors may contribute to the slow spread of an invasive Eurasian longhorn beetle in North America (Coleoptera: Cerambycidae)[a]

Jennifer L. Anderson[1], Stephen B. Heard[1], Jon Sweeney[2], Deepa S. Pureswaran[2]

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[1] Department of Biology, University of New Brunswick, 10 Bailey Drive, Fredericton, New Brunswick, Canada, E3B 5A3

[2] Canadian Forest Service, Atlantic Forestry Center, 1350 Regent St, Fredericton, New Brunswick, Canada, E3C 2G6

Abstract

Tetropium fuscum (Coleoptera: Cerambycidae) is a Eurasian beetle that first became invasive to Nova Scotia, Canada around 1990. In the time since its introduction, *T. fuscum* has not spread more than 150 km from its point of introduction. In its invasive range, *T. fuscum* coexists with its congener *Tetropium cinnamopterum*. Although they are ecologically similar species, *T. fuscum* tends to infest healthier trees and has a smaller host range than *T. cinnamopterum*. If they successfully interbreed, this could lead to hybrid individuals that are more problematic than either parent species. On the other hand, if *T. fuscum* has the capacity to make mating errors in the field but is not producing hybrid offspring, then this waste of mating resources could help explain the slow spread of *T. fuscum* in North America. We conducted no-choice and choice mating experiments between *T. fuscum* and *T. cinnamopterum* males and females and determined that both *T. fuscum* and *T. cinnamopterum* males make mate-choice errors with heterospecific females in a laboratory setting. Our results suggest that mating errors may play a role in the slow spread of *T. fuscum* in North America.

Introduction

Invasive species are a threat to global biodiversity (Rhymer and Simberloff 1996; Vitousek et al. 1997) and those that successfully establish exploit resources such as food, shelter, and mates, thereby decreasing resources available to native species. Furthermore, depletion of food sources or removal of an important predator by an invasive species can have catastrophic ripple effects in an ecosystem.

The rate of invasion by introduced species has been steadily rising due to climate change, habitat modification, international trade (Findley and O'Rourke 2007) and globalisation in transport of unprocessed wood products (Haack 2006), allowing for accidental introductions (Allendorf et al. 2001; Sax et al. 2007; Kelly et al. 2010). Invasive species are now ubiquitous (Seebens et al. 2007) and cause significant ecological and economic impacts around the globe (Vitousek et al. 1996; Pimentel et al. 2000, 2005). Nearly half of the endangered species in the USA are threatened because of competition with and predation by invasive species (Stein and Flack 1996).

Several factors determine whether an introduced species will establish itself and become invasive in a novel habitat (Ehrlich 1989; Williamson 1996). Understanding factors that drive invasiveness could allow us to predict and prevent potential invaders and manage those already present (Pyšek and Richardson 2007). Traits that are predictors of invasiveness across taxa include high dispersal ability (Moyle 1986; O'Connor 1986; Kolar and Lodge 2001), high reproductive rates (Gallagher et al. 2014, Mathakutha 2019), high competitive ability (Newsome and Noble 1986; Moyle 1986; O'Connor

1986), propagule pressure (O'Connor 1986; Kolar and Lodge 2001), association with humans (Kolar and Lodge 2001; García-Berthou 2007; Mathakutha 2019), fast growth (Newsome and Noble 1986; Kolar and Lodge 2001), ability to tolerate and adapt to a broad range of conditions (Ehrlich 1986; Moyle 1986), large body size (Ehrlich 1989; Kolar and Lodge 2001; García-Berthou 2007) and a generalist diet (Ehrlich 1986). However, the specific combination of species traits that would allow a species to invade one habitat may not extend to the same species in another habitat or a different species in that same habitat (Lodge 1991).

Many species are accidentally introduced but don't establish and introduced species rarely experience population growth sufficient to gain pest status (Williamson 1996). Species that successfully establish but then undergo limited spread, such as the phloem-feeding longhorn beetle, *Tetropium fuscum* Fabricius (Coleoptera: Cerambycidae), are poorly understood and offer an interesting window on traits and ecological factors that determine invasiveness. We examine some factors that may be negatively impacting reproductive rate in *T. fuscum*, and thus impeding its ability to invade in North America.

Tetropium fuscum experienced initial success in establishment and population growth upon its invasion in North America (in or before 1990), but by 2010 it had spread only ~80 km from its point of entry in Halifax, Nova Scotia (Canada) (Rhainds et al. 2011). To date, it has only been identified in one small area in southeastern part of the neighbouring province of New Brunswick (CFIA 2017), an additional 70 km from its

point of introduction. *Tetropium fuscum* is a phloem-feeding longhorn beetle native to Western Europe and Northern Eurasia (Juutinen 1955), first discovered in mature red spruce trees (*Picea rubens* Sargent) in Point Pleasant Park, Halifax, NS, Canada in 1999 (Smith and Hurley 2000). However, collections in the Nova Scotia Museum of Natural History indicate that it had been present since at least 1990, having been misidentified as its native counterpart *T. cinnamopterum* Kirby (Sweeney et al. 2004). In its native range, *T. fuscum* attacks predominately stressed or moribund Norway spruce (*P. abies* (Linnaeus) Karsten) (Juutinen 1955), but in Nova Scotia it has been observed attacking apparently healthy red spruce, white spruce (*P. glauca* Moench (Voss)), black spruce (*P. mariana* (Miller) Britton, Sterns and Poggenburg), and Norway spruce (Smith and Humble 2000; Sweeney et al. 2001). *Tetropium fuscum* is unusual in that the invading population neither died out, nor saw rapid and successful expansion in North America. *Tetropium fuscum* has established a stable population in the Halifax area, but its expansion into other parts of North America has been extremely slow (Rhainds et al. 2011). *Tetropium fuscum*'s coexistence with the native congener *T. cinnamopterum* in the invaded range and their ecological similarities could result in Allee effects that contribute to its slow spread in North America. The two species share many similarities including phenology and preferred host plants. *Tetropium fuscum* and *T. cinnamopterum* both emerge in the spring beginning in May and their flight period lasts until late August (Juutinen 1955). Although *T. fuscum* emerges on average 2 weeks earlier than *T. cinnamopterum*, their flight periods overlap significantly (Rhainds et al. 2011). *Tetropium fuscum* is limited to trees in the genus *Picea* (spruces), while *T. cinnamopterum*'s somewhat broader host range includes *Picea* spp. among other conifers.

(Furniss and Carolin 1980), providing plenty of opportunity for interspecific encounters. Notably, they share their highly conserved male-produced pheromone component fuscumol, used in aggregation and mate-finding (Silk et al. 2007; Rhainds et al. 2010; Sweeney et al. 2010).

We hypothesised that *T. fuscum* males, where the two species co-occur, make mate choice errors by sometimes mating with *T. cinnamopterum* females rather than *T. fuscum* females. If so, then mating errors could be a factor decreasing population growth and slowing range expansion. Males of both *T. fuscum* and *T. cinnamopterum* emit S-fuscumol which synergizes attraction of males and females of both species when combined with host (spruce) volatiles (Sweeney et al. 2010). Thus, pheromone blends emitted by males of one species may attract females of both species, particularly if the male is emitting from a host tree shared by both species. This means females of both *Tetropium* species could land on trees where males of either species are emitting pheromones, setting the stage for mate choice errors. If *T. fuscum* males mate with *T. cinnamopterum* females but do not produce viable, fertile offspring, wasted mating resources would hinder population growth of *T. fuscum*. We tested whether mate choice errors occur in *T. fuscum* males, using 1) choice experiments reflecting mate encounters at the centre of the invaded range where both species are common and 2) no-choice experiments as might occur at range edges where *T. fuscum* will more frequently encounter *T. cinnamopterum*.

Materials and Methods

No-choice mating experiment

Source of beetles

We obtained *T. fuscum* from a laboratory colony reared at the Great Lakes Forestry Centre, in Sault Ste. Marie (Ontario, Canada). We placed them in a refrigerator at 5ºC, in a containment lab at the Atlantic Forestry Centre, Fredericton, New Brunswick until used in experiments.

We obtained *T. cinnamopterum* from baited red spruce bolts. In April 2015, we haphazardly chose and felled 10 red spruce trees (*Picea rubens*) with a diameter at breast height of 25 cm at the Acadia Research Forest, Noonan (New Brunswick, Canada) (46º 0' 2.99" N, 66º 20' 32.72" W). We cut each bole into six 120 cm long logs and arranged them in pyramid-style decks (3 largest logs on the bottom, 2 on the second layer, and 1 on top) to favour infestation by *T. cinnamopterum*. We attached three lures including fuscumol, ethanol and a blend of monoterpenes, as outlined by Sweeney et al. (2010), to enhance attraction and increase the likelihood of infestation. In October 2015, we took the top three logs from each deck, cut each into four 30 cm long bolts and held outdoors in an open but covered storage shed at the Acadia Research Forest and exposed to ambient temperatures until late December. We brought bolts to the Atlantic Forestry Centre, Fredericton (New Brunswick, Canada), 40 at a time and reared them in sealed Plexiglas cages in a quarantine facility at 20-24ºC with constant dehumidification (as outlined in Dearborn et al. 2016) and a 16:8 photoperiod [L:D] to obtain live adult beetles. Once beetles began to emerge (4 weeks on average), we brushed the bolts down

twice per day - once in early morning and once in early afternoon - to ensure collection of beetles as close to emergence as possible. These bolts produced only *T. cinnamopterum*. We sexed the beetles and verified species upon collection and placed them immediately in the same fridge as *T. fuscum*. All beetles were individually placed in 1.5 mL microcentrifuge tubes and labelled with sex, species, and emergence date. We stored beetles in a 2ºC refrigerator upon collection. *Tetropium* are sexually mature upon their emergence (Juutinen 1955) and we did not retain any beetles for longer than 7 days to minimize effect of senescence on mating behaviour. Since beetles in the Spondylidinae subfamily are not known to feed as adults (Švácha and Lawrence 2014; Haack 2017; Monné et al. 2017), we did not provide the beetles with food. We did, however provide them with cotton soaked in water to keep them hydrated.

No-choice mating protocol

We checked beetles for vigour prior to use in matings by observing their behaviour. Beetles that we classified as "vigorous" had an energetic and lively appearance with their legs moving constantly whereas non-vigorous beetles moved in a sluggish manner and often displayed signs of fungal or mite infections. Some beetles lived longer than others and thus we held beetles for variable amounts of time; however, most beetles were used within 7 days of collection to ensure they were not more than a week old at the time they were used in mating trials. We presented beetles with potential mates, without choice, in a petri dish lined with moistened filter paper. We used four treatments: 1. *T. fuscum* male with *T. fuscum* female; 2. *T. fuscum* male × *T. cinnamopterum* female; 3. *T. cinnamopterum* male × *T. cinnamopterum* female; and 4. *T.*

cinnamopterum male × *T. fuscum* female (n = 85, 154, 132, and 91, respectively). We excluded any beetles with obvious deformities and attempted to match males and females by size as much as possible. After 30 minutes, we allowed any pairs that were engaged in successful copulation to continue to completion.

Mating behaviour

We define a mating attempt as an instance in which a male is observed trying to mount a female and orient their genitalia together. This behaviour includes the male positioning himself dorsally and slightly posterior to the female, extending his aedeagus, and attempting to connect it to the female's ovipore. Mating attempts are distinguished from instances when a male simply climbs over a female while walking around the petri dish. Successful mating attempts are when the male and female connect through the aedeagus and ovipositor. When this connection is made there is a visible transparent tube extending from the posterior end of one beetle to the posterior end of the other. Typically, during successful copulation, female *Tetropium* run around and drag the males behind them by their genitalia.

Statistical analysis

We compared five response variables across treatments: proportion of beetle pairs attempting to mate, proportion mating successfully, time until first mating attempt, time until successful mating, and time spent in copula.

As our no-choice mating experiment is essentially two independent no-choice mating experiments, one using *T. cinnamopterum* males and another using *T. fuscum* males, we ran some of the analyses for these two experiments separately. We chose to do this for proportion of males that attempted and proportion of males that succeeded because the comparisons we were interested in were treatment 1 (*T. cinnamopterum* male with *T. cinnamopterum* female) compared to treatment 2 (*T. cinnamopterum* male with *T. fuscum* female) as well as treatment 3 (*T. fuscum* male with *T. cinnamopterum* female) compared to treatment 4 (*T. fuscum* male with *T. fuscum* female). For each comparison we tested the prediction that the proportion of mating attempts would be greater with conspecifics than heterospecifics using a two-sided Fisher's Exact Test. We similarly tested a second prediction, that the proportion of pairs with successful matings would be greater with conspecifics than heterospecifics.

As both *T. fuscum* and *T. cinnamopterum* males respond behaviourally to contact pheromones present in female cuticular hydrocarbons, time until first mating and time until successful mating reflect events respectively before and after males contact females and gain information about their identity (Silk et al. 2011). We asked whether there were differences among treatments 1 to 4 in time until first mating attempt, which would reflect behaviour of *Tetropium* males before they obtain information about cuticular hydrocarbons. We did a Box-Cox transformation data for time until first mating attempt, using the R package bestNormalize (v 3.3.5 2021) (Peterson and Cavanaugh 2019) to determine the most effective transformation within the Box-Cox family. The best lambda values were 0.15 for time until first mating attempt and 0.22 for time until successful

mating attempt. We performed a two-way ANOVA on each response variable, using male species and female species as factors, to compare times among treatments of the Box-Cox-transformed data for variables. We used Tukey's HSD for pairwise comparisons of significant main effects.

A longer time until a successful mating attempt indicates that the male is less inclined to mate with the female they are interacting with. This longer time to success coupled with behaviour of *Tetropium* males after touching the females with their antennae prior to copulation indicate this reluctance is based on the female's cuticular hydrocarbon composition. Once a male had committed to mating with a particular female, we expected the time spent in copula to be the same whether with a heterospecific or conspecific female. We transformed our duration of copula data using a hyperbolic arcsine transformation based on recommendation of bestNormalize. We then tested this hypothesis by conducting a two-way ANOVA on the transformed data with male species and female species as factors. We performed all statistics in R using base R version 4.0.4 (2021).

Choice Mating Experiment

Source of beetles

In April 2016, we felled 6 red spruce trees (*Picea rubens*) with a mean diameter at breast height of 25 cm from each of 4 sites: Acadia (NB) (46º 0' 2.99" N, 66º 20' 32.72" W), Sandy Lake (NS) (44º 44' 42.67" N, 63º 40' 40.76" W), Antrim (NS) (44º 57' 59.80" N, 63º 22' 18.58" W), and Westchester (NS) (45º 36' 52.86" N, 63º 42' 25.59" W). We

also felled 2 additional trees of the same criteria from Acadia and transported them to a fifth site in Memramcook (NB) (46º 3' 8.06" N, 64º 34' 46.45" W). We arranged the trees into decks and baited them with pheromone as described for *T. cinnamopterum* in the no-choice mating experiment. In November 2016, we cut the top three pieces of wood from each deck into four 30 cm bolts and brought the bolts back to the Atlantic Forestry Center in Fredericton, New Brunswick. We cut up all six logs from the two Memramcook decks to increase the number of beetles we got from this site. We placed the bolts into a walk-in freezer at -2ºC in order to simulate winter conditions. We left the bolts in the freezer until January 2017, when we brought batches of bolts out of the freezer and warm them up in sealed Plexiglas cages in containment facilities at 20-24ºC with constant dehumidification (as outlined in Dearborn et al. 2016) and a 16:8 photoperiod [L:D] to allow the beetles to develop into adults. We collected and stored the beetles as for the no-choice mating experiment.

Choice mating protocol

We checked beetles for vigour prior to use in matings, as in the no-choice experiment. Most beetles were used within 10 days of collection. We had two treatments for this experiment: 1. *T. fuscum* male presented with *T. fuscum* female and *T. cinnamopterum* female; and 2. *T. cinnamopterum* male presented with the same choice (n = 42 and 30 respectively). We placed the females together and placed the male directly across a petri dish lined with moistened filter paper. We gave the males 30 minutes to begin copulating with one of the females. If at the end of the 30-minute time period, the male was in copula with one female, we removed the second female and left the mating

pair in the dish until completion of copulation. If, at the end of the 30-minute time period, the male was not in copula with a female, we removed all three beetles from the petri dish.

Statistical analysis

We compared four response variables across treatments: time until first mating attempt, species of female first touched by male, species of female that males first attempted to mate with, and species of female for successful matings.

As our choice mating experiment is essentially two independent choice mating experiments, one using *T. cinnamopterum* males and another using *T. fuscum* males, we ran some of the analyses for these two experiments separately. We chose to do this for species of first touch female and species of first female attempted because the comparisons that were meaningful to us were *T. cinnamopterum* males with conspecific females compared to heterospecific females as well as *T. fuscum* males with heterospecific females compared to conspecific females. For each experiment, we tested for preference of species of first-touch female using an Exact Binomial Test with p set at 0.5. In each case, we used a second Exact Binomial Test with p set at 0.5 to look at preference of species of female first attempted with. We did not do formal statistics on our time until successful mating in this experiment because of the clear-cut pattern for preference of conspecific females and the low sample size of heterospecific matings in both treatments.

We used the R package bestNormalize (v 3.3.5 2021) (Peterson and Cavanaugh 2019) to determine the most effective transformation for the data, leading us to do a logarithmic transformation. We performed a two-way ANOVA, using male species and heterospecific vs. conspecific females as factors, to compare times among treatments of the transformed data, followed by a Tukey's HSD for pairwise comparisons of significant main effects. We conducted all statistical analysis in R using base R version 4.0.4 (2021).

Results

No-choice mating experiment

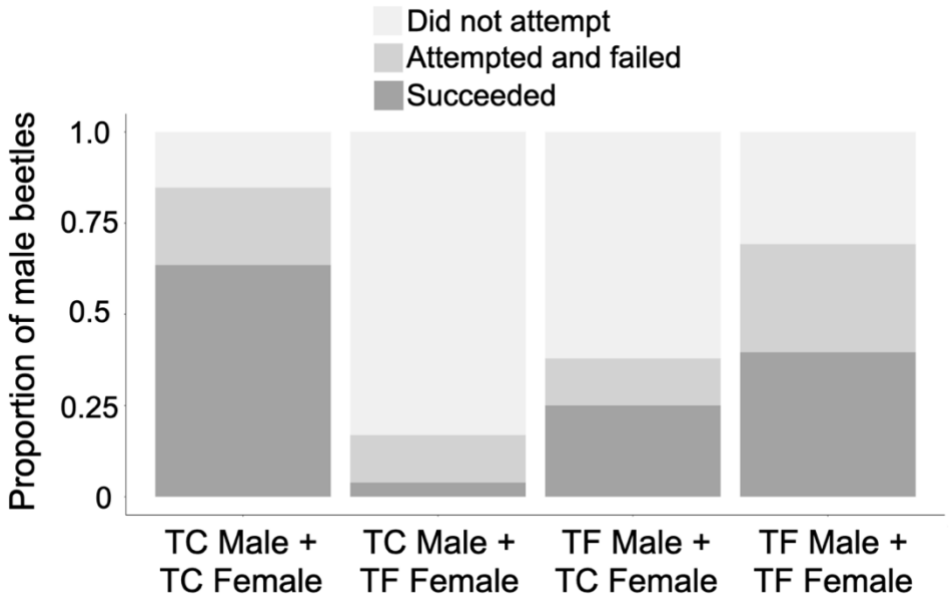

Figure 2.1: Proportion of *Tetropium fuscum* (TF) and *Tetropium cinnamopterum* (TC) males in a no-choice mating experiment that did not attempt to mate, attempted to mate but failed, and succeeded to mate (n = 85, 154, 132, 91)

Tetropium cinnamopterum males both attempted ($p < 2.20\times10^{-16}$; 95% CI [0.11, 0.24]) and succeeded ($p < 2.20\times10^{-16}$; 95% CI [0.01, 0.08]) significantly less with heterospecific females than with conspecific females. We saw the same pattern with *T. fuscum* male attempts ($p = 5.81\times10^{-6}$; 95% CI [0.30, 0.47]) and successes ($p = 0.02$; 95% CI [0.18, 0.33]) (Fig. 2.1).

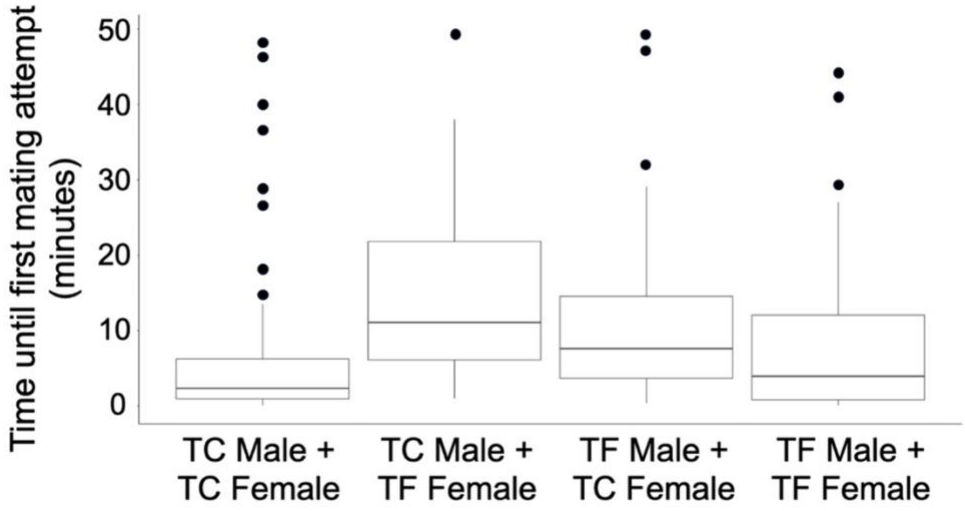

Figure 2.2: Time until first mating attempt by *Tetropium fuscum* (TF) and *Tetropium cinnamopterum* (TC) males in a no-choice mating experiment (n = 72, 26, 50, 63 respectively). Lines represent Q1-3, whiskers show +/- 1.5×IQR and dots represent outliers.

Neither male ($F_{1,203} = 0.83$; $p = 0.36$) nor female ($F_{1,203} = 0.58$; $p = 0.45$) species had a significant effect on time until first mating attempt (Fig. 2.2), but the interaction of the two was significant ($F_{1,203} = 29.77$; $p = 1.41\times10^{-7}$). Tukey's HSD analysis suggests that both *T. cinnamopterum* and *T. fuscum* males take significantly longer to attempt to mate with heterospecific females than conspecific females ($p = 3.03\times10^{-5}$, $p = 0.02$ respectively).

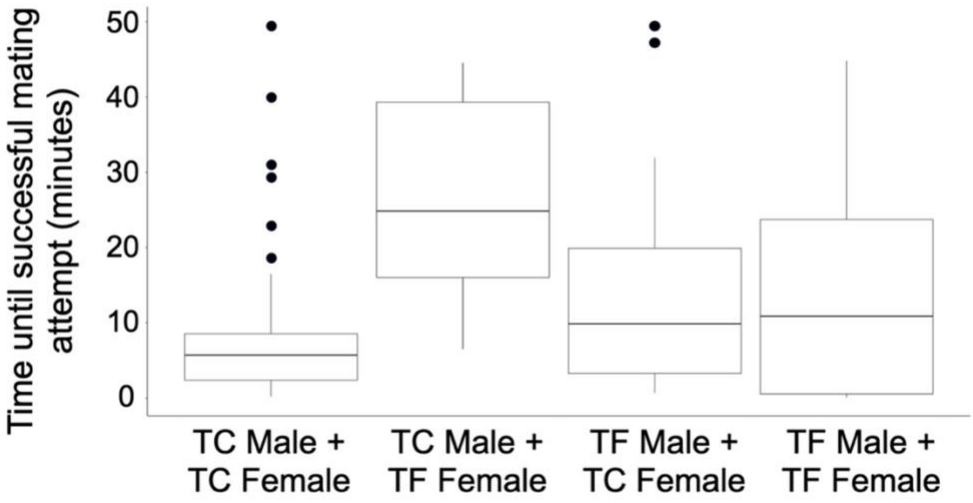

Figure 2.3: Time until successful mating attempt by *Tetropium fuscum* (TF) and *Tetropium cinnamopterum* (TC) males in a no-choice mating experiment (n = 54, 6, 33, 37 respectively). Lines represent Q1-3, whiskers show +/- 1.5×IQR and dots represent outliers.

Male species had no effect on time until successful mating attempt (Fig. 2.3; $F_{1,122} = 0.70$; $p = 0.40$), nor did female species ($F_{1,122} = 0.17$; $p = 0.68$), but the interaction of the two was significant ($F_{1,122} = 9.73$; $p = 2.27\times10^{-3}$). *Tetropium cinnamopterum* males took significantly longer to successfully mate with heterospecific females than conspecific (Tukey's HSD; $p = 0.02$), but *T. fuscum* males did not (Tukey's HSD; $p = 0.66$).

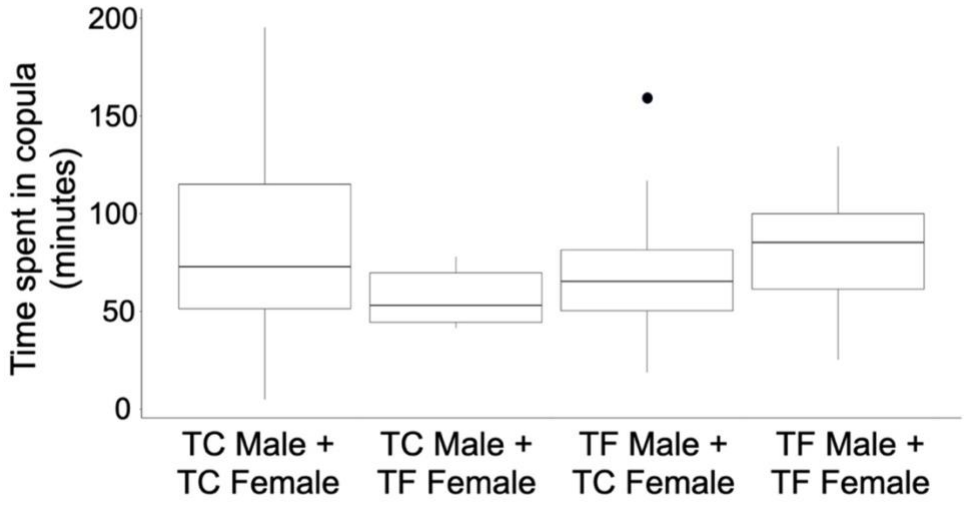

Figure 2.4: Time spent in copula by *Tetropium fuscum* (TF) and *Tetropium cinnamopterum* (TC) males in a no-choice mating experiment (n = 54, 6, 33, 37 respectively). Lines represent Q1-3, whiskers show +/- 1.5×IQR and dots represent outliers.

There was no effect of male species ($F_{1,122} = 0.29$; $p = 0.86$), female species ($F_{1,122} = 0.61$; $p = 0.44$), or the interaction of the two ($F_{1,122} = 3.49$; $p = 0.06$) on time spent in copula (Fig. 2.4).

Choice mating experiment

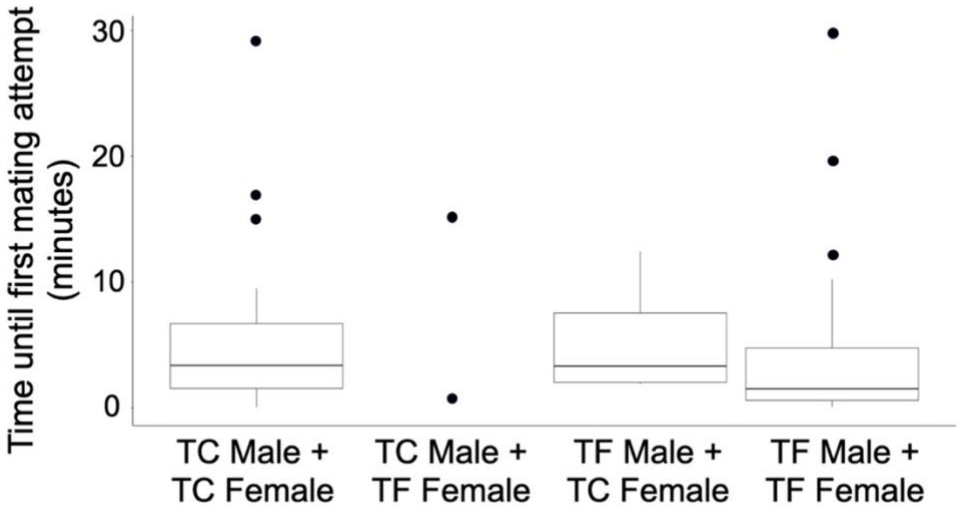

Figure 2.5: Time until first mating attempt by *Tetropium fuscum* (TF) and *Tetropium cinnamopterum* (TC) males in a choice mating experiment (n = 27, 2, 5, 28 respectively). Lines represent Q1-3, whiskers show +/- 1.5×IQR and dots represent outliers.

Species of male had no significant effect on time until first mating attempt ($F_{1,58}$ = 1.41; p = 0.24) (Fig. 2.5). Species of female also had no effect on time until first mating attempt for either *T. fuscum* or *T. cinnamopterum* males ($F_{1,58}$ = 0.66; p = 0.42) (Fig. 2.5).

Species of first touch female for *T. fuscum* males was 25 conspecific and 17 heterospecific. For *T. cinnamopterum* it was 13 conspecific and 17 heterospecific. Neither *T. cinnamopterum* nor *T. fuscum* males showed any significant preference for conspecific or heterospecific females at first touch ($p = 0.58$, 0.41 respectively).

Species of female for first mating attempt for *T. fuscum* males was 28 conspecific and 5 heterospecific. For *T. cinnamopterum* it was 27 conspecific and 2 heterospecific. Both *T. cinnamopterum* and *T. fuscum* males showed significant preference for conspecific over heterospecific females at first mating attempt ($p=1.62\times10^{-6}$, 5.65×10^{-6} respectively).

Of the 42 *T. fuscum* males used in the choice mating experiment, 12 successfully mated. Eleven of those 12 matings were conspecific. Of the 30 *T. cinnamopterum* males used in this experiment, 17 mated successfully and all 17 were conspecific. Even with the low sample size of the choice mating experiment, we saw a clear-cut pattern of both species of male preferring conspecific over heterospecific females, but we cannot reject rates of heterospecific choice as high as 19% (95% CI 0 - 19.5%) (Pezzullo, 2009).

Discussion

We saw evidence of interspecific mating by both *T. fuscum* and *T. cinnamopterum* males in the no-choice experiment (Fig 2.1). *Tetropium cinnamopterum* males both attempted and succeeded significantly less with heterospecific females than with conspecific females, but the rates of heterospecific attempts and successes were both considerable. *Tetropium fuscum* males tended to both attempt and successfully mate with heterospecific females less frequently than with conspecific females, and the difference was statistically significant. While our results indicate that *T. fuscum* males prefer conspecific females, they tend to attempt and succeed with heterospecific females more frequently than do *T. cinnamopterum* males. This may suggest that *T. fuscum* males are less able to discriminate between *T. fuscum* and *T. cinnamopterum* females, or that they have a lower specificity for conspecific females than *T. cinnamopterum* males. Under choice mating conditions, *T. fuscum* and *T. cinnamopterum* males both made first mating attempts in the same mean amount of time, regardless of whether that attempt was on a heterospecific female or a conspecific female. We considered that perhaps males would simply mate with the first female they contacted in the petri dish, but in fact first touch female species did not adhere to any significant pattern and both species of males preferentially made their first mating attempt on conspecific females over heterospecific females. This indicates that males are "choosing" conspecific females over heterospecific females in most – but not all – cases.

Even though both *T. fuscum* and *T. cinnamopterum* males took significantly longer to attempt mating with a heterospecific female than a conspecific one under no-

choice mating conditions, they still mated quite rapidly with heterospecific females (Fig. 2.2). We observed the same trend in time until successful mating (Fig. 2.3). In both cases, we often observed males touching females with their antennae prior to attempting to copulate. *Tetropium fuscum* and *T. cinnamopterum* males respond to sex pheromones present in the cuticular hydrocarbons on the surface of females (Silk et al. 2011), and thus it is likely that *Tetropium* spp. males need to touch females in order to determine their suitability as a mate. Further analysis of correlation between touching a female and initiation of copulation would be necessary to confirm this.

Although *T. fuscum* males were less likely both to attempt mating and to successfully copulate with heterospecific females than conspecific females under no-choice mating experiment conditions, many of them did eventually attempt to mate with heterospecific females, if given enough time. This suggests *T. fuscum* males may become less choosy the longer they go without locating a mate, a situation that may be most common at range edges. Furthermore, the fact that we saw 1 out of 12 *T. fuscum* males under choice mating conditions mating heterospecifically even in the presence of a conspecific female indicates that *Tetropium* can make mating errors even when they have ample opportunity not to do so. Invasive species populations are often the densest at the epicenter of invasion and become more sparsely distributed closer to the range edge (Udvardy 1969, Sagarin and Gaines 2002, Sagarin et al. 2006, Mlynarek et al. 2017). Thus, conditions at the edge of the *T. fuscum* invasive zone would mimic those of a no-choice mating experiment: the population density at the edge of the *T. fuscum* range in Nova Scotia would be sparse, and *T. fuscum* males would encounter *T. cinnamopterum*

females far more frequently than *T. fuscum* females. They may therefore make mate choice errors if enough time elapses without locating a suitable, conspecific, mate. Furthermore, if those matings do not produce fertile offspring, this would reinforce the defined range edge between *T. fuscum* and *T. cinnamopterum* in Nova Scotia. Although we didn't observe any heterospecific matings by *T. cinnamopterum* in the choice experiment, this may simply reflect the small sample size.

Both *T. fuscum* and *T. cinnamopterum* males spent the same mean amount of time in copula with heterospecific females as with conspecific females. This suggests that *Tetropium* males determine the suitability of a mate based on the precopulatory act of touching the cuticular hydrocarbons of the female. If the barrier to copulation were something pericopulatory, like a genital lock-and-key mechanism, we would expect to see prematurely terminated copulation in heterospecific pairs.

Our matings were all conducted in petri dish arenas and thus may not fully capture insect behaviour in nature. This is, however, the closest approximation to nature we can provide – a common constraint, especially for work with invasive species. Furthermore, laboratory matings are commonly used to model arthropod mating behaviour for a wide range of taxa including beetles (Nilsson et al 2002, Kumano et al. 2010, Rutledge and Keena 2012), moths (Jiménez-Pérez and Wang 2003, Bento et al. 2006), bed bugs (Reinhardt et al 2009), predatory bugs (Bonte et al 2011), and wolf spiders (Vaccaro et al 2010). Additionally, we used colony-bred *T. fuscum* in our no-choice mating experiment due to the lack of availability of wild insects. Although this

can result in a population that is very distinct from the wild population, the use of colony insects in mating experiments is common practice (Jiménez-Pérez and Wang 2003, Kumano et al 2010, Rutledge and Keena 2012). Acquiring mature insects in the wild for mating experiments (e.g., Vaccaro et al 2010) means there is no certainty in whether an individual has mated or not. Moreover, wild-caught organisms must then be tested in environments to which they have not been acclimated, which may cause differences in behaviour due to stress associated with capture and transport. Some researchers capture wild organisms and use them to establish a colony (Bento et al 2006, Bonte et al. 2011). This seems to be the best practice because having few generations minimizes the deviation from the natural population but having the colony in the lab allows researchers to isolate organisms early and ascertain their mating status - as we did in the choice mating experiment to alleviate these potential confounding issues.

In Nova Scotia, the population density of *T. fuscum* is highest at the epicenter, decreasing outward (Heustis et al. 2017, Anderson unpublished data). It is therefore probable that at the edges of the invasive range of *T. fuscum* in Nova Scotia, *T. fuscum* males are more likely to encounter *T. cinnamopterum* females than *T. fuscum* females. Furthermore, although we see that *Tetropium* males are unlikely to make mating errors when they have an equal chance of mating with a conspecific female as a heterospecific female, we do see that when they are not given a choice, both *T. fuscum* and *T. cinnamopterum* males will make mating errors. Thus, at the edge of their range, *T. fuscum* males may be making mating errors. In the absence of fertile hybrid offspring,

this could reinforce the edge of their range, and prevent the population from spreading into surrounding areas.

There are many factors that could prevent an introduced species from successfully establishing and becoming invasive in a novel habitat. For *T. fuscum* in North America, *Alle effects*, a phenomenon where the per-capita growth rate or individual fitness of a species increases with its population density, especially at low densities, may be a primary factor in its slow spread. Influenced by social interactions, mate finding, and predator avoidance, these effects become important when population size falls below a certain threshold. They emphasize the necessity of maintaining minimum population sizes for species survival, especially in less populated areas, playing a key role in the dynamics of endangered and invasive species, as well as in population genetics. Allee effects, often intensified by small population sizes or low densities, can create a feedback loop where difficulties in finding mates further inhibit population growth. This highlights unique risks for species, not only from external threats like predation or habitat loss but also from challenges inherent in sustaining adequate population sizes. In conservation biology, addressing these effects is critical, particularly for species nearing critical population thresholds (Courchamp et al. 1999; Stephens et al. 1999; Barron et al. 2020; Surendran et al. 2020). The inability to find suitable mates, a key element of Allee effects, limits the spatial spread of species (Rhainds et al. 2015), even after a stable population is established (Keitt et al. 2001). Mate choice errors at the range edges of invaded zones, such as *T. fuscum* males choosing *T. cinnamopterum* females due to their

abundance, may force males into mating errors or not mating at all. This can lead to sterile, non-viable offspring, or no offspring, further hindering population growth.

Of course, Allee effects are not the only mechanism that could be behind the slow range expansion of *T. fuscum*. Darwin's naturalization hypothesis suggests that when a species invades an area where a congener is established, they will be less likely to successfully establish due to a high degree of relatedness and thus higher competition for resources (Darwin 1859; Jiang et al. 2010; but see: Ricciardi and Mottiar 2006; Park and Potter 2013; Sol et al. 2021). *Tetropium fuscum* may be in direct competition for resources with *T. cinnamopterum* and is exploited by some of the same species of parasitoids that use *T. cinnamopterum* as their primary host (Flaherty et al. 2011). *Tetropium fuscum* has been in Nova Scotia for over 30 years and the population persisted, and largely displaced the native *T. cinnamopterum* in the invaded zone (Dearborn et al. 2016). *Tetropium cinnamopterum* infests a broad range of conifers and is limited to weakened or moribund trees, and *T. fuscum* is only known to infest *Picea* spp. but can infest apparently healthy trees. This overlap in host trees means that *T. fuscum* and *T. cinnamopterum* could be in direct competition for phloem to rear their young. Furthermore, the similarity in pheromone produced by the males of these two species could result in shared predators and parasitoids that are attracted to the pheromone of the two species. Pinned range edges in a species' geographical range can result from many things including strong competition with another species (Heller and Gates 1971; Bull and Possingham 1995; Case and Taper 2000) and Allee effects. Thus, the pinned range edge that we observe in the established population of *T. fuscum* in Halifax could result

from a combination of Allee effects from low population density of *T. fuscum* at the range edge, as well as direct competition with *T. cinnamopterum*.

There are several possible consequences to heterospecific matings between *T. fuscum* and *T. cinnamopterum*. If there are no viable offspring, it would be an utter waste of reproductive resources for *T. fuscum* males to mate with *T. cinnamopterum* females. Copulation by *Tetropium* spp. can take several hours to complete (Fig. 2.4), and these beetles only live for 1-4 weeks on average, depending on temperature (Juutinen 1955). Thus, the time it takes to locate and copulate with a female is a non-negligible proportion of the entire lifespan of a *Tetropium* male. Conversely, if these two species can produce fertile hybrid offspring, this could pose its own set of challenges to forest managers. Hybrid offspring may exhibit traits intermediate to their parents (Roe et al 2014, Patterson et al 2017), hybrid breakdown (McQuillan et al 2018, Pâques 2019) or hybrid vigour (Shao et al. 2019; Kumar et al. 2020). We are currently measuring outcomes of hybrid matings between *T. fuscum* and *T. cinnamopterum* in order to investigate this possibility (Chapter 3).

From a forest management perspective, *T. fuscum* is not spreading rapidly and destructively as other invasive forest pests such as emerald ash borer (*Agrilus planipennis* Fairmaire). *Agrilus planipennis* invaded North America in 2002, has already killed hundreds of millions of ash trees (*Fraxinus* spp.) in the USA alone (Herms and McCullough 2014), and is now spreading in eastern Canada. It is estimated to cost tens of billions of dollars in mitigation efforts (Kovacs et al. 2010). However, our results do not

mean that we should ignore the potential for future *T. fuscum* spread. Many invasive species experience a "lag phase" in which their population size and range do not rapidly increase at the beginning of the invasion (Mack 1981) while the population evolves to be better adapted to the novel environment, or until environment changes allow the species to spread (Crooks and Soulé 1999). It is important to continue survey and monitoring of *T. fuscum* populations in North America, so that we are not caught off guard should a sudden increase in population size or emergence of introgressed individuals become problematic.

We have demonstrated that *T. fuscum* and *T. cinnamopterum* males make mate choice errors in the lab and present a logical case that this may also happen in the field. This likely plays a role in impeding the North American spread of *T. fuscum*. Future work should focus on determining whether these mating errors have the capacity to produce offspring. Although very few morphologically ambiguous *Tetropium* specimens have been identified in eastern Canada, morphology is not a reliable predictor of introgression (Rhymer et al. 1994) and hybrids may exhibit the phenotype of one parent. *Tetropium fuscum* attacks more vigourous trees than *T. cinnamopterum* (Smith and Humble 2000), although *T. cinnamopterum* can attack a broader range of conifers in North America than *T. fuscum* can (Furniss and Carolin 1980).

Hybrid individuals produced by crossing *T. fuscum* and *T. cinnamopterum* could display both traits and thus be more threatening to our forests than either parental species. There are similar concerns in other invasive insects. For instance, *Operophtera brumata*

Linnaeus is invasive to Northeastern North America and coexists with its native congener *O. bruceata* Hulst (Elkinton et al. 2010; Simmons et al. 2014). As seen in *Tetropium*, sex pheromones are highly conserved across the genus, and the sex pheromone blend of *O. brumata* females attracts both *O. brumata* and *O. bruceata* males (Khrimian et al. 2010). Unsurprisingly, *O. bruceata* and *O. brumata* are known to hybridize (Elkinton et al. 2010), and the hybrids are fertile (Havill et al. 2017). Hybridization between an invasive and its native congener as is seen in the *Operophtera* system could facilitate invasive species like *T. fuscum* to invade more successfully by alleviating the Allee effects often seen in small founder populations of invasive species (Elkinton et al. 2014). Furthermore, the intermediate traits exhibited by hybrids could confer an invasive and evolutionary advantage to the hybrid offspring (Havill et al. 2017), and thus the hybrids may pose a greater risk to the ecosystem than either parental species. It is therefore important to determine whether these two species are capable of hybridization and if these potential hybrid individuals from *T. fuscum* and crosses are fertile and display hybrid vigour.

If mating errors are indeed an important mechanism behind the relatively slow North American spread of *T. fuscum*, there are implications beyond the slow *Tetropium* invasion. While some invasive species establish without any close relatives sharing their new habitat, many others, like *Tetropium*, invade alongside native congeners. Adding mate-choice errors to the list of reasons this matters advances our understanding of why some introductions spread catastrophically, while others fade quietly away.

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Chapter 3 – Introgression with a native congener could limit invasiveness in a non-native longhorned beetle

Jennifer L. Anderson[1*], Stephen B. Heard[1], Jason Addison[1], Deepa S. Pureswaran[2]

*Corresponding author

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[1] Department of Biology, University of New Brunswick, 10 Bailey Drive, Fredericton, New Brunswick, Canada, E3B 5A3

[2] Canadian Forest Service, Atlantic Forestry Center, 1350 Regent St, Fredericton, New Brunswick, Canada, E3C 2G6

Abstract

Establishment and spread of introduced species in novel habitats are influenced by factors such as genetic variation, environmental adaptability, and interactions with native species. *Tetropium fuscum*, an introduced longhorn beetle from Northern Europe, has experienced slow spatial spread since its arrival in North America in 1990. Despite its establishment and dominance over native species like *T. cinnamopterum* within a sympatric zone in Nova Scotia and New Brunswick (Canada), *T. fuscum* has only spread approximately 150 km over a span of nearly three decades. Previous research revealed mating errors between *T. fuscum and T. cinnamopterum* in a laboratory setting. Hybridization between invasive and native species has been documented across various taxa and can result in a myriad of outcomes including speciation, hybrid vigour, and inbreeding depression. Hybrid vigour can result in hybrid offspring outcompeting their parents, thereby depressing population growth of the parental species. On the other hand, inbreeding depression or wasted mating efforts without producing offspring could impede population growth. We aimed to determine whether heterospecific mating between *T. fuscum* and *T. cinnamopterum* leads to introgression in natural settings, potentially explaining the halted spread of *T. fuscum*. We performed de novo assembly of a reduced-representation genome for *Tetropium* sp. We then used this generated genome to create a species discrimination assay to detect introgression between these species. We provide evidence of five hybrid individuals out of 362 individuals examined, within the sympatric zone of *T. fuscum* and *T. cinnamopterum,* suggesting that introgression in the hybrid zone might be slowing the spread of the introduced species. Furthermore, we discovered two morphologically deceptive individuals, suggesting potential cryptic presence of *T. fuscum*

that can go undetected. Definitive identification of *Tetropium* individuals through genetic methods is required in future efforts to track the spread of this invasive species.

Introduction

Many factors influence the likelihood that an introduced species will establish and thrive in a novel habitat, including but not limited to adaptability to a wide range of environmental conditions (Kolar and Lodge 2001; Lee 2002; Moles et al. 2012), competitive ability against native species (Shea and Chesson 2002; Vilà et al. 2011), escape from natural enemies (Liu and Stiling 2006; Parker et al. 2013), escape from disease (Torchin et al. 2003; Parker et al. 2013), similarity between the novel ecosystem and the originating ecosystem (Sax et al. 2013; Early and Sax 2014), and genetic variation (Sakai et al. 2001; Dlugosch and Parker 2008). Many introduced species fail to establish in the novel habitat (Kolar and Lodge 2001; Simberloff 2009; Blackburn et al. 2011), but those that do establish may thrive and spread well (Sakai et al. 2001; Blackburn et al. 2011). Another outcome is establishment of an introduced species without either thriving or becoming problematic (Kolar and Lodge 2001; Simberloff 2009; Blackburn 2011).

One aspect of species establishment is the potential for hybridization between an invader and its native congener. Such hybridizations have been documented in many taxa including plants (Riesberg and Carney 1998; Ellstrand and Schierenbeck 2000; Hovick and Whitney 2014), amphibians (Fitzpatrick and Shaffer 2007; Fitzpatrick et al. 2010), reptiles (Engeman et al. 2011; Bock et al. 2021), fish (Epifanio and Nielsen 2000; Crispo et al. 2011), birds (Li et al. 2010), mammals (Hubbard et al. 1992, Jenks and Wayne 1992) and insects (Elkinton et al. 2010, Havill et al. 2017, Andersen et al. 2019). The consequences of such hybridizations can range from speciation (Lowe and Abbott 2004,

Seehausen et al. 2008, Abbott et al. 2013), increased weediness in plants (Ellstrand & Schierenbeck 2000), population decline (Harrison and Larson 2014) or extinction (Rhymer and Simberloff 1996; Todesco et al. 2016), hybrid vigour (Chen 2010, Schnable and Springer 2013), and outbreeding depression (Rhymer and Simberloff 1996; Frankham et al. 2011). Fertile hybrids can lead to species extinction, with parental species replaced with a hybridized population due to genetic swamping (Rhymer and Simberloff 1996; Seehausen 2004). Since hybrids only produce hybrid offspring, introgressed individuals in the population may quickly outnumber the pure species, producing a hybrid swarm (Ellstrand and Schierenbeck 2000, Epifanio and Phillip 2000) and this can result in loss of the distinct genomes of the parental species (Allendorf and Leary 1988). This effect is exacerbated if either parental species already has low population numbers (Dowling and Secor 1997). In the case of infertile hybrids, the result can be Allee effects which lead to population decline (Levin et al. 1996; Schlaepfer et al. 2002). Even in the absence of Allee effects, production of non-viable or non-fertile offspring through hybridization squanders mating resources (Rhymer and Simberloff 1996; Burke and Arnold 2001; Todesco et al. 2016).

Hybrid offspring derived from genetically diverse populations can exhibit higher invasive potential than either parent population alone due to their enhanced genetic capacity to adapt to novel environments (Arnold 1997, Ellstrand and Schierenbeck 2000, Seehausen 2004, Mallet 2007, Dlugosch and Parker 2008, van Kleunen et al. 2010, Abbott et al. 2013). This increased adaptability, or hybrid vigour, arises from the introduction of new combinations of alleles into the population, potentially providing

these individuals with greater fitness across various environments (Harrison 1993, Whitham et al. 2006, Chen 2010, Schnable and Springer 2013). Hybrid species may exhibit traits that make them more resistant to control measures that are effective against their parent species. For example, they may be more resistant to certain pesticides (Carrière et al. 2010) or less susceptible to parasitoids that target their parent species (Brodeur and Boivin 2004).

Aside from the possibility of hybridization and mating errors, the presence of a native congener can directly impact an introduced species' ability to spread and establish. Darwin's naturalization hypothesis suggests that an introduced species is less likely to establish and spread successfully in a habitat where closely related species are present (Darwin 1859; Strauss et al. 2006; Jiang et al. 2010). This disadvantage comes from competition for resources as well as the presence of parasitoids, predators and diseases that affect close relatives and could easily switch hosts to impede the establishment of the introduced species (Strauss et al. 2006; Jiang et al. 2010). Hybridization between an invader and a native congener can further complicate these processes through hybrid vigour or hybrid disadvantage in offspring (Ellstrand and Schierenbeck 2000; Hovick and Whitney 2014), introgression of advantageous genes from one species to another (Rhymer and Simberloff 1996; Ellstrand and Schierenbeck 2000), and genetic swamping (Rhymer and Simberloff 1996; Seehausen 2006; Todesco et al. 2016).

Taxonomic confusion due to blurring of genetic and phenotypic boundaries between species may make it difficult for researchers to accurately identify and track

invasive species, which can hinder efforts to control the species and assess its impacts (Mallet 2005; Seehausen et al. 2008; Harrison and Larson 2014). An introduced species can sometimes go undetected for years if it closely resembles an established native species. *Tetropium fuscum* Fabricius (Coleoptera: Cerambycidae) is a longhorn beetle native to Northern Europe and Western Eurasia (Juutinen 1955) that was accidentally introduced in North America around 1990 (Smith and Hurley 2000). It was first discovered in Point Pleasant Park in Halifax, Nova Scotia (Canada) an area already inhabited by its native congener *T. cinnamopterum* Kirby (Coleoptera: Cerambycidae) (Sweeney et al. 2004). Although these two species are now sympatric in the invaded region in Nova Scotia and New Brunswick (Canada), their natural ranges do not overlap and thus we treated them as reproductively isolated, separate species for our analyses.

Anderson et al. (2022) showed that *T. fuscum* and *T. cinnamopterum* make mating errors with one another in a laboratory setting., We investigated whether these potential mating errors in the field resulted in introgression, between sympatric populations of the two species, that could slow the spatial spread of *T. fuscum* in North America (Sweeney et al. 2006; Rhainds et al. 2010). Following its establishment in Halifax (Nova Scotia, Canada) around 1990, *T. fuscum* spread approximately 80 km from its point of introduction over a span of approximately 20 years (Rhainds et al. 2011). One isolated detection - in the southeastern region of the adjacent province of New Brunswick (Canada) - an additional 70 km from its last point of detection (CFIA 2017) - indicates that as of 2017 *T. fuscum* had not spread more than 150 km from its point of introduction. This contrasts with many other introduced insect species. For example, the emerald ash

borer (*Agrilus planipennis* Fairemaire) (Coleoptera: Buprestidae) was first discovered in North America in 2002 but had likely been present for five to ten years before its discovery (Scarr et al. 2002; Cappaert et al. 2005). It has caused widespread damage to ash trees both in Canada and the USA and has spread more than 1000 km from its epicenter of invasion (Cooperative Emerald Ash Borer Project, 2021). *Agrilus planipennis* and *T. fuscum* are both introduced wood boring beetles in eastern North America (Smith and Hurley 2000; Poland and McCullough 2006) but their difference in success in their introduced ranges could be attributed to *T. fuscum* having a native congener that feeds on the same host plants, whereas *A. planipennis* does not (Herms and McCullough 2014). Despite its limited spread, T. *fuscum* has largely displaced the native *T. cinnamopterum* (Dearborn et al. 2016) within the sympatric zone, but the underlying mechanisms of its slow spread remain elusive.

We hypothesized that mate choice errors could contribute to the slow spread of *T. fuscum* in North America in one of two ways. If *T. fuscum* and *T. cinnamopterum* are not hybridizing in the field, given laboratory evidence that *T. fuscum* males are potentially making mating errors in the field, *T. fuscum* could be largely wasting mating resources to locate and mate with heterospecific females that do not produce viable offspring. On the other hand, hybridization between *T. fuscum* and *T. cinnamopterum* could contribute to either halted spread of *T. fuscum* or spread of *T. fuscum* going undetected. Hybrid depression may produce offspring poorly suited to adapt to the novel environment in northeastern North America and result in nonviable or infertile offspring (Barton and Hewitt 1985; Jiggins et al. 2001). This poor adaptation potential may then contribute to

the slow spread of the *T. fuscum* population in Nova Scotia and New Brunswick. Phenotypic presentation of hybrid offspring is variable, with offspring sometimes presenting morphologically as a combination of parental species or with the phenotype of one parent species dominating in the hybrid offspring (Barton and Hewitt 1985; Arnold 1997; Seehausen 2004). This could confound monitoring efforts to trap and morphologically identify *T. fuscum* to track its spread.

We tested the hypothesis that heterospecific matings between *T. fuscum* and *T. cinnamopterum* produce hybrid offspring in the field. Detecting introgression between *T. fuscum* and *T. cinnamopterum* in the sympatric zone was complicated by the lack of a published genome for either species at the time of our study. To address this, we generated a reduced-representation genome for these species, a process which involves sequencing a subset of the complete set of genomic DNA (gDNA) found in an organism. This approach was chosen over full genomic sequencing for its efficiency and cost-effectiveness, especially since our study's objectives did not necessitate sequencing the entire genome. By focusing on specific genomic regions of interest, particularly those involved in species discrimination and potential hybridization events, we aimed to understand the slow spread of *T. fuscum* in North America.

We used Double Digest Restriction-site Associated DNA Sequencing (ddRADseq), a technique that selectively sequences genomic fragments flanked by specific restriction enzyme sites. This is achieved by cutting the DNA with two different restriction enzymes and then sequencing the resulting fragments. This technique is

particularly useful for identifying Single Nucleotide Polymorphisms (SNPs), the most common type of genetic variation and a powerful marker for studying genetic differences between populations, tracing inheritance, and understanding genetic predispositions to certain traits or diseases. Using NGS Illumina ddRADseq and *de novo* assembly, we created a species discrimination assay using SNPs to assay introgression between *T. fuscum* and *T. cinnamopterum* in the sympatric zone.

In addition to genetic analysis, we also examined morphology of the beetles to determine how their genetic composition may affect their phenotypic characteristics. It is important to know if hybrid individuals are present in the sympatric zone as they may affect forest management strategies. Conversely, the absence of introgression and the potential for wasted mating efforts could help explain the limited spread of *T. fuscum* in North America.

Materials and Methods

Library Construction

Source of Beetles

We chose sites outside the sympatric zone of *T. cinnamopterum* and *T. fuscum* to collect beetles for genetic sequencing to identify species distinguishing SNPs. We obtained beetles for library construction from Lindgren funnel traps baited with fuscumol, ethanol, and a blend of monoterpenes. Our *T. cinnamopterum* specimens came from three field sites outside Québec City (Québec, Canada) (46° 56' 55.9104" N, 71° 29' 59.01" W; 47° 46' 0.12" N, 70° 39' 27" W; 47° 17' 58.92" N, 71° 11' 38.04" W), and we obtained *T. fuscum* from field sites in Norway (59°38'24.4"N 10°46'54.6"E), Italy (45º50’15’’N 11º28’54’’E), and France (44º51’03.8’’N 2º06’24.5’’E). Our collaborators shipped collected beetles to us in 24% ethanol. Upon receiving the beetles, we carefully rinsed them in distilled water to remove all traces of ethanol to avoid ethanol related issues in the genomic DNA (gDNA) extraction process, before storing them in clean 1.5 ml microcentrifuge tubes at 2ºC.

ddRADseq

We used Qiagen DNeasy Blood and Tissue Kits (Qiagen, Redwood City, CA) to perform our genomic DNA (gDNA) extractions. We crushed each individual beetle in a 1.5 ml microcentrifuge tube using a pestle to ensure we were able to extract enough gDNA. We checked the extraction products for quality by running 5µl of gDNA mixed with 2 µl of NEB Purple Dye 6x on a 1% agarose gel with SybrSafe. We used lambda (λ) DNA (molecular weight: 31.5×10^6 daltons) as a positive control. We omitted samples

that had more than a single band, indicating the DNA had been sheared during processing. We also removed any samples that had a faint band, indicating that concentration of the extracted DNA was too low for us to use. Our final selection parameter was that the single, high-density band was similar in molecular weight to lambda (λ) DNA to increase likelihood that it was only gDNA present in our sample. We checked gDNA concentration using a Qubit dsDNA HS Kit (Thermo Fisher Scientific Inc., Waltham, MA), and then diluted samples to a final volume of 40 µl at a final concentration of 10 ng/µl.

To obtain ddRAD-seq data we followed the general protocol described by Poland et al (2012). We selected the restriction enzymes MluCI (5'-AATT-3'; NEB) and Sau96I (5'-GGNCC-3'; NEB) and attached our adapters (shown in Appendix A) following Addison and Kim (2018) (see Appendix B for more detail). For each gDNA sample to be digested, we mixed 10 µl of our prepared 10 ng/µl gDNA with 2 µl 10x Cutsmart Buffer, 1 µl each of restriction enzymes Sau96I and MluCI, and 11 µl of nuclease-free water. We incubated our samples for digestion at 37ºC for 60 minutes. After the 60-minute digestion period, we placed our samples on ice until ligation.

We ligated our digested gDNA products with 4 sets of multiplex identifier (MID) barcode Y-adapters (Table 3A.1, Appendix A), designed to attach to MluCI cut sites, and 82 adapters with unique MID tags (Table 3A.2, Appendix A) designed to attach to Sau96I cut sites. We cleaned our ligation products using a QIAquick PCR purification kit (Qiagen, Redwood City, CA). We then ran our purified PCR products on a 1.5% agarose

gel and using a UV light, extracted individual bands between 200 bp and 500 bp in length. We resuspended our extracted gel products in Qiagen Buffer EB to a final volume of 40 µl and sent them to Génome Québec (Québec, Canada) for Illumina sequencing using HiSeq 2500PE 125bp.

Bioinformatics

In the absence of a published *Tetropium* sp. genome, we generated a reduced-representation reference genome using *de novo* assembly of fragments cut by our two restriction enzymes (MluCI and Sau96I). We used steps 1 to 5 of the pipeline GBS-SNP-CROP (v. 4.1) (Melo et al. 2016), single end (SE) version of the code, to trim Illumina adaptors and barcodes from our individual reads, demultiplex the reads, and generate our reduced-representation genome using a consensus of sequences from our *T. fuscum* and *T. cinnamopterum* specimens. We performed these first 5 steps on the cedar server of ACENET, through the Digital Research Alliance of Canada. We left most of the parameters for these steps set to the default by the original authors except for two key changes. We altered step one to include the cut sites for our restriction enzymes MluCI and Sau96I (^AATT and ^GNCC, respectively). For step four, we set the -db parameter to 1 in the VSEARCH deduplication process to ensure the entire dataset was treated as a single block, optimizing the efficiency of deduplication for our specific dataset size and complexity. We chose this approach to maintain simplicity and computational efficiency in handling the genomic data from the two closely related species. We aligned our reads to the reduced-representation genome using the mem command in BWA (v. 0.7.17-r1188) (Li and Durbin 2009), then converted the SAM file to a sorted BAM file using the view and sort commands, respectively, in SAMtools (v. 1.17) (Danecek et al. 2021). We

called our SNPs using Freebayes (v.1.3.6) (Garrison and Marth 2012), with a quality cut-off of 30 for mapping quality and 20 for base quality. We further filtered our SNPs using parameters of max-missing 0.70 and minDP 8 and converted the resulting file to PLINK format using VCFtools (v. 0.1.16) (Danecek et al. 2011). We ranked our SNPs in order of sequence quality using Tres (v. 0.5.35) (Kavatiokis et al. 2015) and selected SNPs that had a Pairwise Wright's F_{ST} value of >0.90. An F_{ST} value of 1 would indicate that a SNP is completely species discriminating, while a value of 0 would indicate no differentiation. We chose 0.90 as our cut off because there was a steep drop off in F_{ST} value of SNPs below this threshold and we wanted to use SNPs that were as species discriminating as possible for our assay. When more than one SNP above our quality threshold was located on the same read, we selected the SNP of highest quality ranking (Table 3B.1, Appendix C), based on F_{ST} score, and discarded the other(s) to avoid redundancy due to genetic linkage.

Field Survey in the introduced range

Source of beetles

We chose five sites across Nova Scotia and New Brunswick (Canada) in a transect to examine the pattern of potential introgression ranging from sites heavily infested with *T. fuscum* (Sandy Lake and Antrim), moving towards sites less infested or not infested at all with *T. fuscum* (Memramcook and Acadia). Our sites were at the following locations (Figure 3.1): Sandy Lake (NS) (44°44'42.67"N, 63°40'40.76"W), Antrim (NS) (44°57'59.80"N, 63°22'18.58"W), Westchester (NS) (45°36'52.86"N,

63°42'25.59"W), Memramcook (NB) (46°3'8.06"N, 64°34'46.45"W), and Acadia (NB) (46°0'2.99"N, 66°20'32.72"W).

In April 2016, at all sites except Memramcook, we felled six haphazardly chosen red spruce trees (*Picea rubens* Sarg) with a mean diameter breast height of 25 cm. We cut each tree into 6 pieces, approximately 120 cm in length, and arranged them into pyramid-style decks. We increased likelihood of infestation by attaching three lures: fuscumol, ethanol, and a blend of monoterpenes (Sweeney et al. 2010). At Memramcook, we did not fell trees as this site was on privately owned land; instead, we transported two trees cut from our Acadia site and placed them in the same baited deck configuration as at our other sites.

We allowed the decks to remain in the field to increase level of infestation until November 2016, at which point we retrieved them and brought them to the Atlantic Forestry Center in Fredericton, New Brunswick (Canada) for storage. We cut each log into 30 cm sections for transport and upon arrival in Fredericton, we placed these sections into a -2º freezer. We left the bolts in the freezer until January 2017 to mimic overwintering conditions and to allow adequate time for development to occur. When we retrieved the bolts from the freezer, we placed them into Plexiglas cages for adult emergence. We set up cages in a containment lab with a temperature of 20-24ºC, constant dehumidification (as outlined in Dearborn et al. 2016), and a 16:8 photoperiod. Upon emergence, starting approximately 4 weeks after setup, we collected individuals by brushing down bolts twice daily and placed them in 2.5 mL microcentrifuge tubes

labelled with species, sex, date of collection, and bolt ID. We used morphology of the pronotum to identify beetles to species; *Tetropium cinnamopterum* has a dimpled pronotum while the pronotum of *T. fuscum* is rugose (Figure 3.2) (Yanega 1996; Smith and Hurley 2000). We placed individuals in a -2ºC freezer inside the containment lab to cull them before DNA extraction.

Figure 3.1: Transect of five field sites in Nova Scotia and New Brunswick (Canada) where we trapped *Tetropium* sp. beetles to conduct our field survey, testing for introgression between *Tetropium fuscum* and *Tetropium cinnamopterum*.

a.

b.

Figure 3.2: Morphology of the pronotum in *Tetropium* is used as the distinguishing feature to identify species. *Tetropium cinnamopterum* characteristically has a dimpled pronotum, displayed on an individual trapped in our Quebec site (A). *Tetropium fuscum* displays a rugose pronotum, as seen in an individual trapped in Italy (B).

SNP Assay on Field Survey Beetles

We extracted whole genomic DNA from 1500 beetles using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Redwood City, CA). Due to the presence of additional SNPs in the flanking sequences of most of our SNPs of interest, we opted to use wobbles - a mixture of oligos containing all possible variant complements at each SNP site - in our primer design to accommodate these additional SNPs in the primer docking sequences. We designed our primers using the PrimerQuest Tool (IDT Technologies) using a cut-off of no more than 3 wobbles per primer to minimize mismatch during annealing. With this cut-off, we were able to design primers (Table 3C.2, Appendix C) for 25 of our 36 loci. We added CS1 (5'-ACACTGACGACATGGTTCT-3') and CS2 (5'-TACGGTAGCAGAGACTTGGTCT-3') universal sequences to our primers to incorporate the sequences into our PCR products as outlined in U'ren and Arnold (2022). We used PrimerPooler (Brown et al. 2017) (ΔG = 57ºC, [$MgCl_2$] = 1.5mM, [dNTP] = 200µM) to assess our primer sequences and determine which primers could be pooled for multiplex PCR amplification with minimal primer dimer formation. As PrimerPooler suggested two pools of 11 SNPs each and a third pool of 3 SNPs, we opted to continue our analysis with 22 SNPs to minimize the number of PCR pools. We then ran a Principal Component Analysis to ensure that our 22 SNPs of interest were able to discriminate our two species by using them on genetic data of individuals from our Québec and Europe sites collected during our initial library construction step. While many modern studies analyze much larger sets of SNP loci, we did not need to map linkage groups, conduct GWAS, or make other kinds of inferences that rely on wide genome coverage. Instead, we sought only to use SNPs to distinguish parental and hybrid beetles, a purpose for

which we expected a dozen or two loci to be ample – and we show below that it was (Figure 3.3).

We performed a power analysis to determine the appropriate sample size of individual beetles to examine to detect hybrid individuals if they comprise at least 0.5% of the total *Tetropium* population within the sympatric zone. We chose a cut-off of 0.5% because hybridization below this level would be considered an extremely rare event and of minimal concern to forest managers.

$$P = 1 - ((1 - \alpha)^n)$$

P = probability of detecting hybrids with sample size n

α = proportion of *Tetropium* population that is made up of hybrid individuals

n = sample size

$$P = 1 - ((1 - 0.005)^{672})$$

$$P = 0.966$$

Our power analysis indicated that with a sample size of 672 beetles, we had a 96.6% chance of detecting hybrid individuals in the *Tetropium* population if they comprised at least 0.5% of the population.

We performed multiplex PCR using two primer pools on DNA (Table 3C.3, Appendix C) samples from 672 individuals to a final volume of 25 µl using a Qiagen Multiplex PCR kit with Q solution. We combined our two DNA pools for each individual and visualized our PCR products on a 2% agarose gel with SybrSafe stain. We used a Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA) to check PCR product concentration.

We incorporated unique MID barcodes into the pooled PCR products for each individual and sequenced the final amplicon library using Illumina MiSeq PE75 sequencing technology (Génome Québec).

Bioinformatics and Population Genetics

We trimmed Illumina adaptors and barcodes from our sequence data using the BBDuk function in BBTools (v. 39.01) (Bushnell 2014), aligned our trimmed reads to the reduced-representation genome we previously generated and converted our fastq files to bam format using BWA (Li and Durbin 2009). We then merged our individual bam files using SAMtools (v1.17) (Danecek et al. 2021). We filtered our data for mapping quality and base quality using Freebayes (v.1.3.6) (Garrison and Marth 2012), with a quality cut-off of 30 and 20 respectively.

We indexed our merged bam file and merged it with data from our library construction to include genetic data from individuals from all sites using SAMtools

(v1.17) (Danecek et al. 2021). We filtered our merged data based on missing data in individuals and missing data in our loci with BCFtools (v1.17) (Danecek et al. 2021) using cut-offs of 0.8, indicating no more than 80% missing data allowed, for both parameters. We generated a VCF file containing only our loci of interest using VCFtools (v0.1.16) (Danecek et al. 2011). We then converted our VCF file to STRUCTURE format using PLINK (v1.90b5) (Purcell et al. 2007). We used STRUCTURE (v2.3.4) (Pritchard et al. 2000) to assign our individuals to the *T. cinnamopterum* or *T. fuscum* populations (K=2), using the popinfo option with migprior set to 0.01 to account for migration in the population. We chose a cut-off of 0.80 for distinguishing admixed individuals from pure species, classifying any individual with more than 0.80 assignment to one species as that species. We chose this threshold of 0.80 assignment to one species to be able to detect potential presence of F2 or backcrossed individuals in our assay.

We calculated 95% confidence intervals for incidence of hybrid individuals at Antrim, Sandy Lake, and Westchester and for incidence of cryptic individuals at Sandy Lake using a binomial CI calculator (Pezzullo 2009). We defined cryptic individuals as beetles whose morphology indicated they were *T. fuscum* or *T. cinnamopterum* but whose genetic composition was assigned more than 80% to the other species.

Results

Table 3.1: Sequential reduction in the number of SNPs, from initial discovery to the final selection, through various steps of quality filtering, primer design, and pooling determination.

Filtering Step	Program	SNPs left
Initial discovery	Freebayes	3777
Quality Ranking	TRES	60
Same-read elimination	-	45
Primer design	Manual	25
Multiplex PCR Pooling	PrimerPooler	22
Mapping quality	Freebayes	20

We began with a substantial output from our initial reduced-representation genome sequencing: 201,812,798 reads from Illumina RADseq and and additional 4,995,148 reads from MiSeq sequencing of field survey individuals. Despite the initial discovery of 3777 SNPs using Freebayes, subsequent filtering stages were crucial to identify reliably species discriminating SNPs. After rigorous quality ranking with TRES and elimination of same-read SNPs, we narrowed down to 60 high-quality SNPs.

Further refinement involved selecting SNPs based on primer design and multiplex PCR pooling. The manual primer design process and the use of PrimerPooler led to a reduction in the number of usable SNPs, primarily due to the complexity of flanking sequences and the practical considerations of PCR pooling. Eventually, we selected 22 SNPs for further analysis. However, the final sequencing of field survey samples prompted us to eliminate two more SNPs due to their low read quality, leaving us with 20 SNPs (Table 3.1)

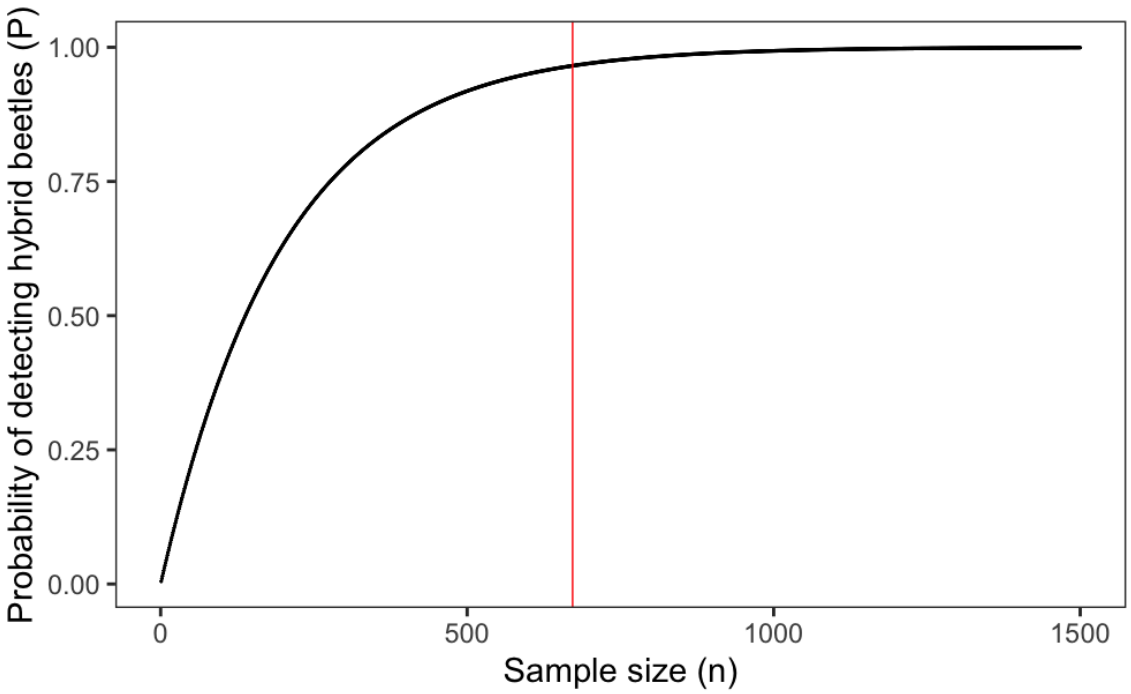

Figure 3.3: Outcome of power analysis, which was instrumental in choosing the appropriate sample size of beetles to sequence to detect introgression in *Tetropium* populations in New Brunswick and Nova Scotia (Canada). The selected sample size of 672, indicated by the red line, was based on this analysis.

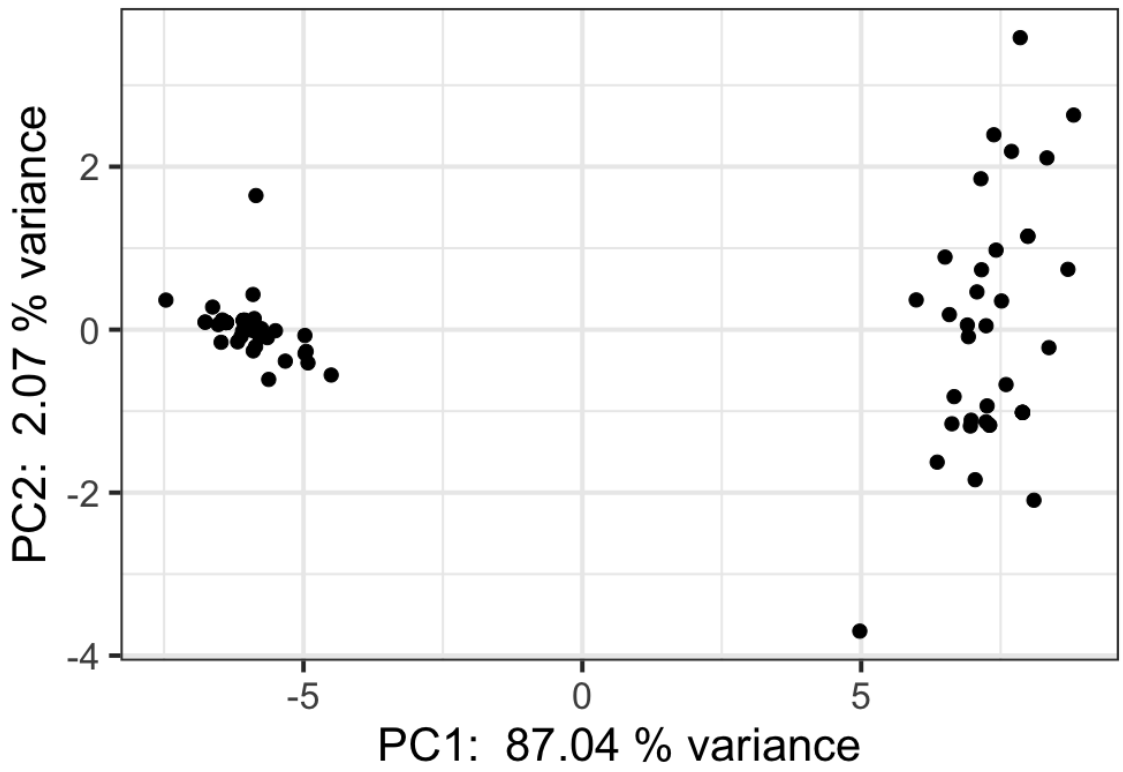

Figure 3.4: The results of a PCA conducted on genetic data using the remaining 22 SNPs to demonstrate strong species discrimination between *T. cinnamopterum* and *T. fuscum*. This clear separation along the primary axis affirmed the effectiveness of the chosen SNPs in species identification. *Tetropium cinnamopterum* individuals, collected in Québec, are represented by the left cluster, while *T. fuscum* individuals from Europe are represented by the right cluster.

We found that our 22 SNPs of interest that we used for multiplex PCR amplification of our field survey beetles strongly discriminated between the *T. fuscum* and *T. cinnamopterum* individuals used to generate our SNP assay. This high degree of species discrimination gave us confidence in using the limited number of SNPs that we

were left with after our filtering and quality control step in our species-discriminating SNP assay.

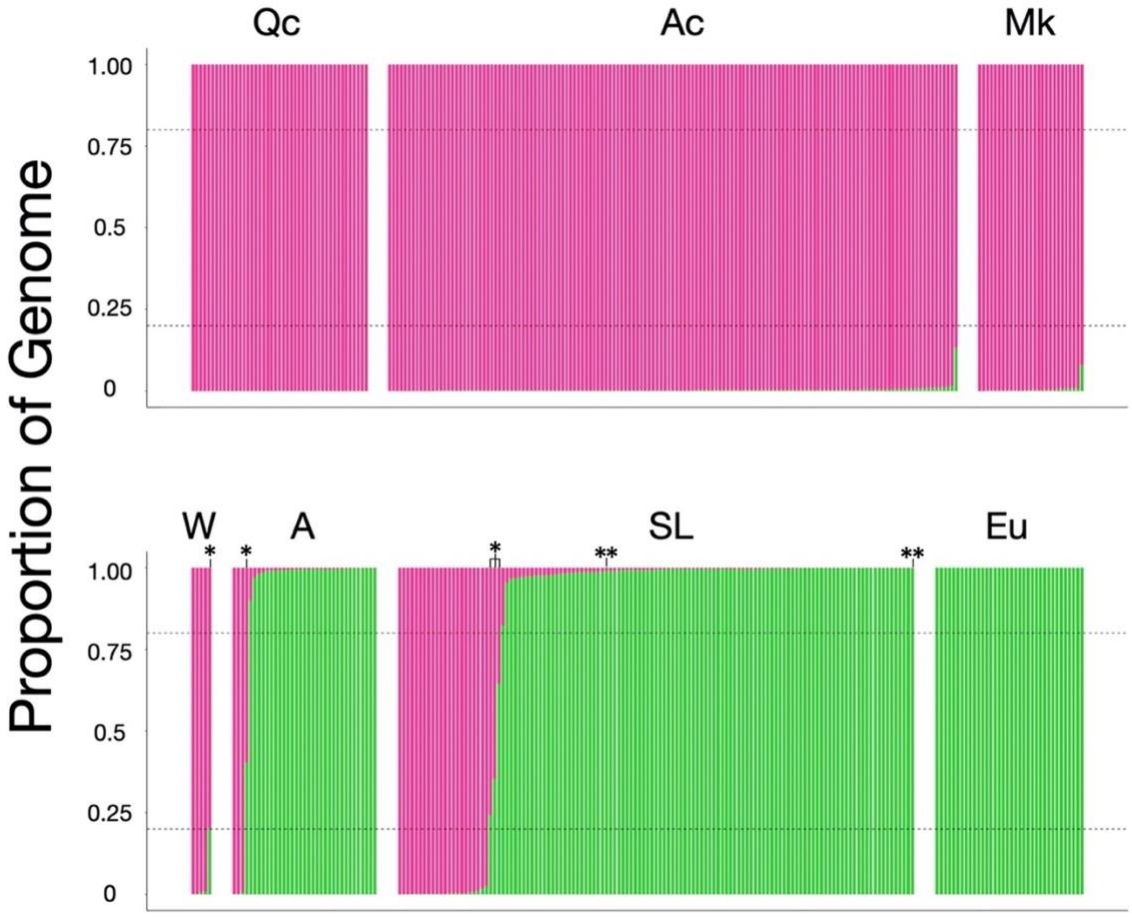

Figure 3.5: Structure plot of *Tetropium* sp. beetles trapped at our 2 library construction sites – Quebec (Qc) and Europe (Eu) (n= 45 and 36) and our 5 field survey sites – Acadia (Ac), Memramcook (M), Westchester (W), Antrim (A), and Sandy Lake (SL) (n = 145, 27, 5, 35, 125 respectively) showing the proportion of the genome for each individual that is assigned to *T. cinnamopterum* (pink) or *T. fuscum* (green) (k=2). Dotted black lines denote the limits for assignment as a purebred or hybrid individual (0.2 and 0.8). Hybrid individuals are denoted by * and cryptic individuals are denoted by **.

We identified hybrid individuals at three of the five surveyed sites. Specifically, we found one hybrid at Westchester, three at Sandy Lake, and one at Antrim. However, none of the hybrids detected were F1 individuals, which would typically show a 50% genetic contribution from each parent species. The limited number of specimens at Westchester precludes robust conclusions regarding hybridization levels there, but at Antrim and Sandy Lake, hybridization rates are estimated to range from 0.1% to 15% and 0.5% to 7%, respectively, as detailed in Table 3.3. Additionally, at Sandy Lake—proximal to the invasion's epicenter—we discovered two cryptic individuals morphologically identified as *T. cinnamopterum* but genetically classified as *T. fuscum*. Figure 3.5 and Table 3.2 illustrate the varied genetic assignments to the parental species and provide a statistical viewpoint on the prevalence of hybrid and cryptic individuals. Table 3.3 outlines the 95% confidence intervals for the true level of hybridization at the sites where we detected hybrids.

Table 3.2: Beetles that were identified as having less than 80% assignment to the either species or less than 80% assignment to the species they present as morphologically (denoted by *) (*T. cinnamopterum* = TC, *T. fuscum* = TF). Beetle ID denotes which species the individual was identified with upon collection.

Beetle ID	**Site**	**Proportion of genome assigned to TF**	**Proportion of genome assigned to TC**
TC-1299	Westchester	0.201	0.799
TC-1011	Sandy Lake	0.241	0.759
TC-525	Sandy Lake	0.351	0.649
TC-1279*	Sandy Lake	1	0
TC-1280*	Sandy Lake	0.992	0.008
TF-1250	Sandy Lake	0.356	0.644
TF-887	Antrim	0.402	0.598

Table 3.3: Results of an exact binomial test to determine the 95% confidence intervals for incidence of hybrids and cryptic individuals at sites where they were detected.

Detection of Hybrid Individuals				
Site	**Total Individuals**	**Hybrid**	**Proportion Hybrid**	**95% CI**
Antrim	35	1	0.029	0.001-0.149
Sandy Lake	125	2	0.024	0.005-0.069
Westchester	5	1	0.200	0.005-.0716
Detection of Cryptic Individuals				
Site	**Total Individuals**	**Cryptic**	**Proportion Cryptic**	**95% CI**
Sandy Lake	125	2	0.016	0.002-0.057

a. 

b. 

Figure 3.6: Individuals trapped at our Sandy Lake site that display the characteristic dimpling on the pronotum that is characteristic of *T. cinnamopterum* but were identified as having a genetic composition of 99.9% (a) and 99.6% (b) consistent with the *T. fuscum* genome.

In Figure 3.6, we present two intriguing cases from the Sandy Lake site, where individual beetles exhibited profound discrepancies between their morphological features and genetic profiles. Morphologically, these beetles possessed the characteristic dimpling typically associated with purebred *T. cinnamopterum*, as seen in Figure 3.2a, leading to their initial morphological classification as such. However, genetic analyses using SNP assays and structure analysis showed a contrasting picture, identifying them as over 99% *T. fuscum* genetically. These findings highlight the challenges in species identification within the field and demonstrate the critical need for incorporating both morphological and genetic data to accurately determine species lineage.

Discussion

Our study offers significant insights into invasion ecology and rate of spread of invasive species using interactions between *T. fuscum* and *T. cinnamopterum* in the field. Our data provides evidence of admixture between these species, although hybridization seems rare (detected in 5 out of 362 individuals surveyed). We found evidence of admixture at three of our five field survey sites – Sandy Lake, Westchester, and Antrim - all sites located in Nova Scotia (Canada), although low sample size at Westchester may not give an accurate depiction of hybrid levels. Moreover, we found two individuals morphologically identified as *T. cinnamopterum* at our Sandy Lake site that genetically aligned with *T. fuscum*, suggesting potential cryptic *T. fuscum* presence in the sympatric zone. Discovering both hybrids and cryptic individuals in the field implies active gene flow between the two species, suggesting that heterospecific mating occurs in nature. Cryptic hybridization can mask the true extent of interspecies interactions, as some individuals may morphologically resemble one species, like *T. cinnamopterum*, while genetically aligning with another, such *as T. fuscum* (Figure 3.6). These cryptic *T. fuscum* individuals most likely result from past hybridization events where, over generations, repeated backcrossing with purebred *T. fuscum* would diminish the genetic influence of *T. cinnamopterum*, but offspring might still retain alleles that allow them to present morphologically as the latter.

Our initial hypothesis was that low *T. fuscum* population density might lead to Allee effects at the range edge, where limited population growth could occur due to mate-finding failure. However, our findings, especially at Sandy Lake near the invasion

epicenter, indicate a more complex scenario. If hybridization was only possible between purebred *T. fuscum* and *T. cinnamopterum*, we would have expected only to detect F1 hybrid individuals with genetic assignment of approximately 50% each of *T. fuscum* and *T. cinnamopterum*. All the hybrid individuals we detected showed varying levels of assignment to the two parental species, and none were 50/50. This observed level of introgression suggests that F1 hybrid offspring are viable and fertile, either mating with each other or through backcrosses with pure species.

Despite detecting hybrids, the overall low level of observed hybridization suggests either that such mating events are rare, or that hybrid offspring are less fit than purebreds. If cross-species matings yield fewer offspring compared to matings within the species, these wasted reproductive efforts might be a key factor to the slow spatial expansion of *T. fuscum*. Moreover, if hybrid backcrosses to *T. fuscum* are more successful than those to *T. cinnamopterum*, at the range edge, purebred *T. fuscum* and the hybrid individuals may mostly be encountering *T. cinnamopterum* individuals. This would produce Allee effects leading to lower reproduction rates and limited spread of the population.

Considering the evidence of hybrid and cryptic individuals in the sympatric zone, relying solely on morphology for species identification in the sympatric zone is not a 100% reliable method. Hybrids and cryptic individuals may have different reproductive rates, lifespans, or disease susceptibilities compared to purebreds. Monitoring and management strategies thus may need revision, with an emphasis on molecular methods

to ensure accurate specimen identification. It would be prudent for forest managers to trap for *Tetropium* outside the known North American range of *T. fuscum* and conduct genetic analysis. Delving into the genetic basis of cryptic individuals' morphological traits can unravel the processes determining their appearance.

Although we have evidence of hybrids that are viable as adults, their fitness relative to their parents and the investigation of their reproductive success, and ecological roles compared to purebreds is a promising research direction. In its native range, *T. fuscum* infests primarily Norway spruce (*Picea abies* Karsten), whereas in its invaded range it infests red spruce (*P. rubens*), black spruce (*P. mariana* Mill), white spruce (*P. glauca* Moench), as well as Norway spruce, and can infest apparently healthy trees (Sweeney et al. 2004). *Tetropium cinnamopterum* has a broader host range, comprising *Picea* spp. as well as other conifers (Furniss and Carolin 1980). Offspring that have the broader host range of *T. cinnamopterum* and the ability to infest apparently healthy trees like *T. fuscum* could be more dangerous to the health of the forest than either parental species. However, the current low economic impact and slow spread of *T. fuscum* compared to other species such as the emerald ash borer, *Agrilus planipennis*, suggests that introgression might have hindered rather than improved the invasion potential of this species. Future work focusing on assessing the fitness and fertility of F1 hybrids in crosses with other F1 individuals, backcrosses with purebred individuals and other combinations may provide insight into the dynamics underlying these hybridization events and implications for future spread, competition, and invasiveness.

Not all invasive species that establish in novel habitats might be as successful or noticeable as the ones that get extensively researched. This may result in a bias of believing that established introduced species are more successful or damaging, when they are simply more studied due to the impact they have on human activity. This raises the question of whether *T. fuscum*'s behavior is typical of invasive species or if it's an exception that we've managed to discern. There are numerous examples in the literature of introduced species that have established but not become problematic or particularly invasive. Mealybug ladybirds (*Cryptolaemus montrouzieri* Mulsant) originate from Australia and have been introduced throughout the world as a biological control agent. Although they consume a broad range of hosts, they prefer hemipterans and do not pose a serious threat to humans or vegetation (Kairo et al. 2013). Box tree moth (*Cydalima perspectalis* Walker) is native to East Asia and has been introduced to Europe (Leuthardt et al. 2013). Although it is a pest for box trees (Székely et al. 2011), its preferred temperature range may not allow it to become highly destructive (Nacambo et al. 2014). Research that largely focuses on visible, widespread invasive species that damage food or fibre might skew our understanding of species invasions. In the case of invasive species that establish but remain relatively unnoticed due to modest population sizes or confined distribution areas, their minimal ecological or economic impacts could allow them to escape rigorous scientific inquiry. The challenge lies in detecting and understanding these 'silent' invaders to develop a complete picture of invasion dynamics.

The interactions between *T. fuscum* and *T. cinnamopterum* in the sympatric zone are likely intricate. It appears that the presence of a congener could be limiting the

invasion potential of *T. fuscum,* whose spatial spread about three decades after introduction remains limited, through introgression or the presence of natural enemies. The presence of cryptic individuals and hybrids points to a complex mix of genetics, ecology, and evolution, significantly affecting conservation, taxonomy, and our grasp on species interactions during biological invasions. These findings not only reshape our understanding of species interactions during biological invasions, but also underscore a need to revaluate conservation strategies, ensuring the preservation of biodiversity in the face of complex ecological dynamics.

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Appendices

Appendix A – Library Preparation

This appendix outlines adapter sequences affixed to DNA fragments from individuals (Table 3A.1), as well as the barcode sequences we attached to our individual DNA sequences during library construction (Table 3A.2). The adapters are used in Illumina sequence to affix the fragments to the sequencing plate. The barcodes are to allow for pooling of DNA from all individuals during library construction. The barcodes are used to later assign individual sequences to the individuals they belong to.

Table 3A.1: Y-adapters used in *Tetropium* sp. genotyping-by-sequencing library preparation

Adapter name	Sequence (5' to 3')
5bp_Y_adapter	GCATG
5bp_Y_adapter_RC	CATGC
6bp_Y_adapter	ATGCAT
7bp_Y_adapter	CGTTGCA
7bp_Y_adapter_RC	TGCAACG
8bp_Y_adapter	GCATGTCC
8bp_Y_adapter_RC	GGACATGC

Table 3A.2: Barcodes (5' to 3') used to tag individual *Tetropium* sp. gDNA samples for Illumina sequencing in GBS library construction.

Barcode	**Barcode sequence**	**Individual barcoded**	**Site of origin**
barcode_01	AAGTGA	TC	Québec
barcode_02	AATCG	TC	Québec
barcode_03	ACAGA	TC	Québec
barcode_04	ACCA	TC	Québec
barcode_05	AGAATGA	TC	Québec
barcode_06	AGGAG	TC	Québec
barcode_07	AGTCAAGA	TC	Québec
barcode_08	AGTGTTAA	TC	Québec
barcode_09	AGTTAAT	TC	Québec
barcode_10	ATCATACCT	TC	Québec
barcode_11	ATGG	TC	Québec
barcode_12	ATGTTCAAT	TC	Québec

barcode_13	ATTACA	TC	Québec
barcode_14	CACGACCA	TC	Québec
barcode_15	CAGGCCACT	TC	Québec
barcode_16	CAGGCG	TC	Québec
barcode_17	CCACCA	TC	Québec
barcode_18	CCACTGG	TC	Québec
barcode_19	CCATCCACT	TC	Québec
barcode_20	CCTG	TC	Québec
barcode_21	CGACG	TC	Québec
barcode_22	CGATGCGT	TC	Québec
barcode_23	CGCTCA	TC	Québec
barcode_24	CTCACT	TC	Québec
barcode_25	CTCGTCGT	TC	Québec
barcode_26	CTTCCTCT	TC	Québec
barcode_27	GACAG	TC	Québec
barcode_28	GACATCCA	TC	Québec

barcode_29	GACTCGG	TC	Québec
barcode_30	GAGCGAA	TC	Québec
barcode_31	GATGA	TC	Québec
barcode_32	GCGCCACT	TC	Québec
barcode_33	GCTAACA	TC	Québec
barcode_34	GGAG	TC	Québec
barcode_35	GGCCGA	TC	Québec
barcode_36	GTATA	TC	Québec
barcode_37	GTGCACCA	TC	Québec
barcode_38	TATGGA	TC	Québec
barcode_39	TATTCCACT	TC	Québec
barcode_40	TCAT	TC	Québec
barcode_41	TCCGCA	TC	Québec
barcode_42	TCTCA	TC	Québec
barcode_43	TGCGAGA	TC	Québec
barcode_44	TGCTGAA	TC	Québec

barcode_45	TGGC	TC	Québec
barcode_49	TGACGCCA	TF	Italy
barcode_50	CAGATA	TF	Italy
barcode_51	GAAGTG	TF	Italy
barcode_52	TAGCGGAT	TF	Italy
barcode_53	TATTCGCAT	TF	Italy
barcode_54	ATAGAT	TF	Italy
barcode_55	CCGAACA	TF	Italy
barcode_56	GGAAGACAT	TF	Italy
barcode_57	GGCTTA	TF	Italy
barcode_58	AACGCACATT	TF	Italy
barcode_59	GAGCGACAT	TF	Italy
barcode_60	CCTTGCCATT	TF	Italy
barcode_61	GGTATA	TF	Italy
barcode_62	TCTTGG	TF	Italy
barcode_63	GGTGT	TF	Italy

barcode_64	GGATA	TF	Italy
barcode_69	TTCGTT	TF	Italy
barcode_70	ATATAA	TF	Italy
barcode_71	TGGCAACAGA	TF	Italy
barcode_72	CTCGTCG	TF	Italy
barcode_73	GCCTACCT	TF	Italy
barcode_74	CACCA	TF	Italy
barcode_75	AATTAG	TF	Italy
barcode_76	GGAACGA	TF	France
barcode_77	ACAACT	TF	France
barcode_78	ACTGCT	TF	France
barcode_79	CGTGGACAGT	TF	France
barcode_81	TGCTT	TF	France
barcode_82	GCAAGCCAT	TF	France
barcode_83	CGCACCAATT	TF	France
barcode_84	CTCGCGG	TF	France

barcode_85	AACTGG	TF	France
barcode_86	ATGAGCAA	TF	France
barcode_87	CTTGA	TF	France
barcode_88	GCGTCCT	TF	France
barcode_89	ACCAGGA	TF	Norway
barcode_90	CCACTCA	TF	Norway

Appendix B – Genome by Sequencing Library Preparation Protocol (Kim and Addison 2018)

This is a protocol intended for the Addison lab (headed by Dr. Jason Addison) at the University of New Brunswick (Fredericton, New Brunswick, Canada) written by Dr. Jin Hong Kim and Dr. Jason Addison (reproduced here with permission from Dr. Addison). This protocol has not been published but has been used for several research projects since it was written and provides details of the process we used for library construction.

Supplemental Recipe for Genotyping-By-Sequencing (GBS) Library Construction

I. Materials

- DNeasy Blood & Tissue DNA Extraction Kit (Cat. no. 69506, QIAGEN; 250 rxn)

- RNase A (Cat. no. 19101, QIAGEN; 250 rxn, 10 µl per sample)

- Lambda DNA (Cat. no. N3011S; 500 µl at the concentration of 500 ng/µl)

- SYBR™ Safe DNA Gel Stain (Cat. no. S33102, Invitrogen; 400 µl).

- Qubit™ dsDNA BR (Broad Range) Assay Kit (Cat. no. Q32853, Invitrogen; 500 rxn)

- Qubit™ assay 0.5ml tube (Cat. no. Q32856, Invitrogen; 500 tubes)

- Qubit™ dsDNA HS (High Sensitive) Assay Kit (Cat. no. Q32854, Invitrogen; 500 rxn)

- 100 ng of template genomic DNA adjusted at the concentration of 10 ng/µl.

- Restriction enzyme A: Sau96I (Cat. no. R0165S, NEB; 200 rxn, 5 Unit (1 µl) per sample).

- Restriction enzyme B: MluCI (Cat. no. R0538S, NEB; 100 rxn, 10 Unit (1 µl) per sample).

- Nuclease-free H_2O (Cat. no. 11-05-01-04, IDT)

- Sau96I-cut site (Forwardly Barcoded) adapters to index individual samples.

- MlucI-cut site (Y-adapters) adapters to index a sub-population of samples, not for individual samples.

- Universal Forward and Reverse primers for Illumina sequencing platform

- T4 Ligase (Cat. no. M0202L, NEB; 250 rxn, 400 Unit (1 µl) per sample).

- ATP (Co-enzyme) (Cat. no. P0756S, NEB; 1 mM per sample)

- QIAquick PCR purification Kit (Cat. no. 28104, QIAGEN; 50 rxn)

- NEBNext® Ultra™ II Q5® Master Mix (Cat. no. M0544S, NEB; 50 rxn)

- QIAquick Gel Extraction Kit (Cat. no. 28704, QIAGEN; 50 rxn)

II. Methods

DNA extraction

1. Carefully read and follow the manufacturer's protocol. No modification required. Normally use 10 µl of RNase A. At the end, elute the same column twice with 50 µl of

EB buffer (final volume 100 µl). Don't forget preparing negative control per extraction assay.

2. Prepare two aliquots of lambda DNA diluting at the concentration of 50 ng/µl (1/10 dilution) and 10 ng/µl (1/50 dilution) in a total volume of ≥100 µl to use as a positive control in gDNA-checking electrophoresis and GBS library construction, respectively.

3. For indirect quality and quantity check, load 5 µl of gDNA on 1% agarose gel. Load 5 µl of 50 ng/µl lambda DNA as a positive control. Compare your gDNA and lambda DNA. If your gDNA bands are too faint, increase loading volume up to 10 µl. Also, load 5 µl of negative control from DNA extraction.

4. Select best quantity and quality gDNA samples based on gel image. Use 5 µl of selected gDNA samples to measure their concentrations using Qubit dsDNA BR Kit.

5. Prepare an aliquot of selected gDNA samples diluting at the concentration of 10 ng/µl in a total volume of ≥40 µl. Use 8-strip PCR tubes for the aliquot preparation. For sure, measure their concentration using Qubit dsDNA HS Kit.

Digestion

1. Carefully read the instructions of Sau96I and MluCI, and check their activating/inactivating temperature and time. Turn thermocycler on, and preview digestion program (may be 'Sau96I').

2. Thaw 10x Cutsmart buffer at room temperature (RT). Keep it on ice.

3. Dispense 10 µl of individual gDNA aliquots (100 ng per sample) to 8-strip PCR tubes. Loosely close caps. Leave them at RT. Don't need to keep it on ice.

4. Prepare enzyme mixture with ≥5% extra volume (Table 1). Don't forget gently pipetting up and down at least more than 20 times for full homogenization. Include a positive control (10 µl of 10 ng/µl lambda DNA) and two negative controls; 1) from gDNA extraction and 2) this digestion step.

Table 1. Digestion protocol.

No.	Components	Concentration of Stock	Concentration of working solution	Concentration per reaction	Volume per reaction (µl)
1	10x Cutsmart buffer	10x	NA	1x	2
2	Sau96I	5U/ul	NA	5U	1
3	MluCI	10U/ul	NA	10U	1
4	Nuclease-free H2O	NA	NA	NA	11

5	Template gDNA	Various	~10 ng/µl	5 ng/µl (100 ng in 20 µl)	10
	Total				20

5. Dispense 10 µl of enzyme mixture to individual tubes. Vortex for 1 sec, and spin down.

6. Check bubbles at the bottom of individual tubes. If there are bubbles, spin down again.

7. When digestion is finished, place tubes on rack pre-chilled on ice for ligation.

Ligation

1. Prepare adapters one day before the digestion day.

1) Turn thermocycler on, and check adapter-making program (may be 'adapter')

1) Completely thaw stock of top and bottom oligonucleotides of individual adapters at RT.

2) Vortex individual tubes at straight and upside down for 2 to 3 sec. Spin down. Arrange top and bottom oligonucleotides in parallel on the same rack not to be confused.

3) Prepare 8-strip PCR tubes. See Table 2 for detail.

4) For Sau96I-cut site adapters (Forwardly barcoded adapters), dispense 40 µl of nuclease-free H_2O to individual tubes. Open and carefully arrange caps of top and bottom

oligonucleotides. Add 5 µl of top oligonucleotide and 5 µl of bottom oligonucleotide of individual barcodes to tube (Now total volume is 50 µl). Close caps of stock top and bottom oligonucleotides as soon as finishing combine not to be confused, then keep them on ice until completing to prepare all barcodes.

Table 2. Protocol of Sau96I-cut site or MluCI-cut site adapter preparation.

No.	Components	Concentration of stock (µM)	Concentration of working solution	Concentration per reaction (µM)	Volume per reaction (µl)
1	Top oligonucleotide	100	NA	10	5
2	Bottom oligonucleotide	100	NA	10	5
3	Nuclease-free H_2O	NA	NA	NA	40
	Total				50

5) Prepare MluCI-cut site adapters (Y-adapters) following the same way to make Sau96I-cut site adapters.

6) See table 3 for detail. Vortex individual strip tubes for 1 sec, and spin down. Place tubes on the block of thermocycler checking any bubbles at the bottom of individual tubes. Double check if the program is set with a touch-down starting from 95°C and decreasing -1°C per minute to 25°C (total 70 min). Recommended to leave tubes at 4°C in thermocycler overnight for stable annealing.

Table 3. Adapter-making program

No.	Temperature (°C)	Time (minute)	Note
1	95	5 min	Initial denaturation
2	95 > > >25	-1°C per minute	Annealing
3	4	Overnight	Stabilization

2. Adapter mixture preparation

1) Vortex both adapters for 2 to 3 sec at straight and upside down, and spin down. Place them at RT to prepare *adapter mixture in 8-strip PCR tubes.

→ Sau96I-cut site adapter + MlucI-cut site adapter at the ratio of 1 (0.01 μM) to 100 (1 μM) in a total volume of ≥50 μl (Table 4)

2) Take two steps of 1/10 serial dilution only for Sau96I-cut site adapters,

i.e., 10 µM → 1 µM → 0.1 µM. In every dilution, vortex as soon as done to mix, and wait for ≥10 min. Vortex again and spin down, then go to next dilution.

(CAUTION! This is a serial dilution. So, you use the final dilution moving forward)

3) Don't need to dilute Y-dapters. Combine both adapters per individual barcode following the protocol in Table 4.

4) As soon as finishing making adapter mixture, place them on ice.

→ HEREAFTER ALL STEPS ON ICE !!!

Table 4. Protocol to make adapter mixture.

No.	Adapter	Concentration of working solution (µM)	Concentration per adapter mixture (µM)	Volume per reaction (µl)
1	Sau96I-cut site adapter	*0.1	0.01	5
2	MluCI-cut site adapter	10	1	5
3	Nuclease-free H_2O	NA	NA	40
	Total			50

*After three step dilution

3. Adapter ligation

1) Prechill racks on ice. Turn thermocycler on, and display ligation program (may be 'LIG22').

2) Completely thaw 10x Cutsmart buffer and ATP at RT. As soon as thawed, keep them on ice.

3) For beetles, don't need to do again adapter calibration. Just use the last concentration of adapter at the ratio of 1 (0.1 μM of forward barcoded adapter) : 100 (10 μM of Y-adapter).

4) Place digestion reactions in the pre-chilled rack on ice.

5) Prepare ligation mixture following the protocol in Table 5. Use half volume of 10x Cutsmart buffer because digestion reactions already contain the same 10x Cutsmart buffer. Once done combining all components, except adapter mixtures and digested DNA, completely homogenize ligation mixture by gently pipetting up and down more than 20 times not making bubbles.

Table 5. Ligation mixture protocol

No.	Components	Concentration of stock	Concentration of working solution	Concentration per reaction	Volume per reaction (µl)
1	10x Cutsmart buffer	10x	NA	0.5x	2
2	ATP	10mM	NA	1mM	4
3	Adapter mixture	10 µM[1]	0.01µM[2]/1µM[3]	0.1µM/10µM	10
4	T4 ligase	400U/µl	NA	400U	1
5	Nuclease-free H_2O	NA	NA	NA	3
6	Digested DNA	5ng/µl	NA	2.5ng/µl (100ng in 40 µl)	20

7					40

[1]Before dilution of Sau96I-cut site adapter, [2]Sau96I-cut site adapter after 3-step dilution, [3]MluCI-cut site adapters.

6) Individually dispense 10 µl of ADAPTER MIXTURE to digested DNA tubes. Then, dispense 10 µl of LIGATION MIXTURE to digested DNA tubes. As soon as done adding ligation mixture, vortex for 1 sec, and spin down. Place them back on ice, and bring to thermocycler. Put them on block of thermocycler carefully checking bubbles at the bottom of tubes. If you find any bubbles, spin down again. Run ligation program. Recommended to leave them at 4^{o}C for overnight after 2 h running is done. If you don't want to wait, go to next step purifying ligates.

Clean-up of ligates and PCR

1. Clean-up of ligates: Carefully read the instruction of QIAquick PCR purification kit. No need modification, except the first and last step.

1) At the first step of purification, transfer 10 µl of individual ligates from maximum 24 samples to a new 1.5 ml microcentrifuge tube, e.g., if combining 10 µl of ligates from 24 samples, a total volume is 240 µl. So, you should add 1,200 µl of PB buffer (5 x 240 µl), and final volume is 1,440 µl. Vortex for 3 sec at strait and upside down. Make sure the mixture is completely homogenized. Volume capacity of a column is up to 700 µl. So, you can't filter all mixture at once in a column. Therefore, you should divide the mixture into 3 x 480 µl, i.e., spin a same column three times.

2) At the last step (elusion), recommended to resuspend a column with 40 µl EB buffer twice, so total volume will be 80 µl per 24 samples.

2. PCR (See the protocol in Table 6): You will send sequencing-ready-libraries of which the concentration is adjusted at 75 ng/µl in 50 µl total volume (May have been changed, so please carefully read the instruction of sample submission for Massive Parallel Sequencing in McGill Genome Centre). In a case of 48 samples, at least 8 PCRs are required to reach to the concentration (even after gel extraction). So, I normally have prepared 4 PCRs per 24 samples when I wanted to sequence them on 1 of 8 Illumina sequencing lanes in a flow cell. Therefore, use 15 µl of purified ligates per PCR, i.e., 60 µl (4 PCRs x 15 µl) of the 80 µl for actual library construction. Separately purify a PCR that used 15 µl of 20 µl of remaining purified ligates, and measure its quantity using Qubit dsDNA BR Kit. This will enable you to assume quantity of other 4 PCRs before gel extraction.

Table 6. PCR protocol.

No.	Components	Concentration of stock	Concentration of working solution	Concentration per reaction	Volume per reaction (µl)
1	2x PCR master mix[1]	2x	NA	1x	25
2	Forward primer[2]	100 µM	10 µM	0.2 µM	5
3	Reverse primer[3]	100 µM	10 µM	0.2 µM	5
4	Pooled and purified ligates[4]				15
	Total				50

[1]NEBNext® Ultra™ II Q5® Master Mix (Cat. no. M0544S, NEB), [2,3]Universal sequencing primer, [4]If you want to know their concentration, increase the eluting volume of EB buffer from 80 µl to 85 µl at the end of purification and then use 5 µl to measure the concentration of ligates with Qubit™ dsDNA HS Assay Kit (It used to be around 7 or 8 ng/µl in sea urchin ligates)

Clean-up of PCR products and gel extraction

1. Carefully read and follow the merchandiser's protocol (QIAquick PCR Purification Kit) to purify and concentrate the 8 PCRs. As the purification of ligate products, no modification required, except the first and last steps.

1) at the first step, use a single column twice, e.g., combine 50 µl of 4 of 8 PCRs to a new 1.5 ml microcentrifuge tube (total volume is 200 µl). Add 1,000 µl (5 x 200 µl) PB buffer to the combined 4 PCR mixture. Vortex for 2 to 3 sec at straight and upside down. Repeat vortexing the tube until the PCRs and PB buffer are completely homogenized. Don't spin down! Transfer half (600 µl) of the mixture to a column twice, and spin down. Transfer the remaining 600 µl to the same column and spin down.

2) at the last step, need to minimize elusion volume for gel extraction. Resuspend a column with 20 µl of EB buffer twice (final volume is 40 µl per column).

2. Carefully read and follow the merchandiser's protocol (QIAquick Gel Extraction Kit). As the purifications using QIAquick PCR Purfication Kit, no modification required, except the first and last steps.

1) Remove old 0.5x TBE buffer in electrophoresis tank. Rinse the tank with distilled water. Be careful not to damage thin wires at the bottom of tank during rinsing. Clean all other components such as comb and tray. Completely dry all electrophoresis parts. Use new and fresh 0.5x TBE buffer per assay of gel extraction. Prepare 1.5% agarose gel.

2) Add fresh 8 µl of 6x DNA loading dye to each purified PCR products. Vortex for 1 sec and spin down for 1 sec. Now total volume should be 48 µl per tube. Load 24 µl of 48 µl of PCR-dye mixture on a well of 1.5% agarose gel.

3) After finishing electrophoresis, cut bands between 200 bp and 500 bp on Blue light. Completely melt a piece of cut gel at 56°C in an incubator. As the purifications with QIAquick PCR Purification Kit, use a column to purify 2 x melted gel solution.

4) At the end of gel extraction, recommended to resuspend one column with 20 µl of EB buffer twice (total volume is 40 µl). Use a 1.5 ml microcentrifuge tube for two columns. Now final volume is 80 µl per 8 PCRs. Use 5 µl to quantify with Qubit™ dsDNA BR Kit. Make it sure that its concentration is ≥ 70 ng/µl. Use the other 5 µl for electrophoresis on 1.5% agarose gel to check if unspecific bands under 200bp were well removed by gel cutting.

III. Shipping

1. Call to Air Liquid and ask them when dry ice is available to purchase. Get a dry ice striker from Chris. Prepare Styrofoam and size-matching carton boxes. Check the address of Massive Parallel Sequencing facility in McGill Genome Sequencing Centre.

2. At the shipping day or just a day before shipping, transfer a library from a 1.5 ml microcentrifuge tube to a well of recommended Eppendorf 96-well plate (to prevent volume loss from evaporation). Completely seal the plate with recommended sealing tape (Should be Thermo Fisher Scientific one). Place the plate in a used or new cyrostorage box. Fix it not to move during shipping. Recommended to fill extra spaces with proper size pieces of bubble wrap. Keep it at -20°C freezer until packing in dry ice is done.

3. At the shipping day morning, get dry ice. Download a template excel file from Nanuq system, and completely fill submission forms. Upload the excel file to the system, and

electronically submit the sequencing request form. At the end of electronic submission, you can print the form out. Bring the documents to your PI (Nanuq account holder) and get his/her signature on the last page of the documents. Enclose them in proper size of plastic bag, and place it on the top of Styrofoam box. Send a copy of the submission form with GBS libraries to the McGill Genome Center.

Appendix C – Field Survey

Table 3C.1: SNPs used for species discrimination with their estimated minor allele frequencies (MAF) and minor allele counts (MAC) in *T. cinnamopterum* (TC) and *T. fuscum* (TF) populations taken from plink output using --freq command. Minor allele is depicted as allele most frequently seen in entirety of *Tetropium* spp. population, comprising both species.

			Tetropium fuscum		*Tetropium cinnamopterum*	
SNP ID	**Minor Allele**	**Major Allele**	**MAF**	**MAC**	**MAF**	**MAC**
MRG-1859	C	A	0.973	72	0	0
MRG-7328	T	A	1	74	0	0
MRG-10568	A	G	0.973	72	0	0
MRG-27970	G	A	1	72	0	0
MRG-110964	T	C	0	0	0.5	38
MRG-131117	A	G	0	0	0.5	45

MRG-142444	G	A	1	74	0	0
MRG-148863	C	T	0	0	0.5222	47
MRG-194034	G	T	0	0	0.8649	64
MRG-311133	G	A	1	72	0	0
MRG-334504	A	G	0.5556	30	0	0
MRG-515972	G	A	0	0	0.6222	56
MRG-727381	C	A	0	0	0.5161	32
MRG-878189	G	A	0	0	0.5	45
MRG-1676594	T	C	1	72	0	0
MRG-1733142	A	G	0	0	0.5	43

MRG-2028876	G	A	0	0	0.4878	40
MRG-2248377	G	C	0	0	0.5	39
MRG-2353694	T	C	0	0	0.4872	38
MRG-2773845	A	G	0	0	0.5244	43
MRG-5382535	T	G	0	0	0.9375	75
MRG-5687800	G	A	0.54	27	0	0
MRG-6007860	C	T	1	70	0	0
MRG-42674281	T	A	0.5	33	0	0
MRG-43600353	C	T	0.5	34	0	0

Table 3C.2: Locus sequences with corresponding primers designed for amplification of each site. SNPs are shown by IUPAC codes and SNPs of interest are underlined. Loci denoted with an * were not used in final analysis.

Locus ID	**Locus sequence (5' to 3')**	**Forward primer (5' to 3')**	**Reverse primer (5' to 3')**
MRG-1859	GGCTTCGATTCTGCTGTCGTCGTGTTCGTATCGACTTCTTTTCCTCTTCG**[M]**TGGAGGTTTCGAGTTGAATGCAAGGGCGAAGGGGTATCGCTGTAAGAACC	ATTCTGCTGTCGTCGTGTTC	GGTTCTTACAGCGATACCCC
MRG-7328	GTCCCCCCTGGYCTCCGACTAGTCCAGAGAGTCCCTCCAGCCTCG**[W]**TACTCAAYGACCACTCCCCACGTCCGCGGGGTAAGTGGTCATTGAGGTCG	GGYCTCCGACTAGTCCAGA	TCAATGACCACTTACCCCGC
MRG-10568	GTCCCAGTTGGTAGCCCTTGGGTTTCTTGAGRCAGTTATAGTCTT**[R]**ACGTGAGTTGCCTCTACGTTGAATAGGATATGTCTATGGTCAGACATGG	CCTTGGGTTTCTTGAGRCAG	TCCTATTCAACGTAGAGGCAAC
MRG-27970	GGCCGGCTATCCACCGAKACACTTAAGCG**[R]**CGACCATGGAAAGGTG	GCTATCCACCGAKACACTTAAGC	GTGACARCAACCACCTTTCC

	GTTGYTGTCACAAGGGAACAAGTTGTCG		
MRG-110964	GGCCATCYTTACGGRTGAACAGAKGGCTAGCKYTCAACMCYCCGM**[Y]**TTCYAGMGCATCAGCCTCRACCCTCYAYGRGTCRAGTTAACCRGRGTTC	TCWTTACGGRTGAACAGAKGGC	RTWGAGGGTYGAGGCTGATG
MRG-131117	GTCCTTTCCACCACTGTTCTTTTGARATGATTTY**[R]**GATGGRYTTAAACCTCGAGAAAGATAATCTGCTGGATTGYCGCTTGAACR	CCTTTCCACCACTGTTCTTTTGA	YGTTCAAGCGRCAATCCAGC
MRG-142444	GCCCTCTCTACTGCTCTCTTCTGT**[R]**CATTMGCAAGTCTGCTCCTCATTGCAACAGGAAACCCCTATTATCCATAA	GCCCTCTCTACTGCTCTCTTC	GGGTTTCCTGTTGCAATGAGG
MRG-148863	GTCCTGAMGAGGGCTGATCACTYGAAGGTCTGA**[Y]**RTCGATGGMAAGTCGTCTYTTTCGTCTAYATTTACYGRTTATGTARATG	GAGGGCTGATCACTYGAAGGT	CGAAARAGACGACTTKCCATC
MRG-194034	AGTCCAACAGTTTTGGTTCGCATACCTGGCTGGGCTGCRCTACTGTGTTC**[K]**GTCTCAGCTCTGCYKTGGTGGCGCTGCGAAATACGGGAAGCCGTGCTGCT	GCTGGGCTGCRCTACTGT	CGTATTTCGCAGCGCCAC

MRG-311133	GGCCTTGTTACATCGAATAAKCAA CYAYTATTTTCCCACCTAYG**[R]**CT TAGTGAACCGCRTACYTCATGATA ATGGGAGYCAGTTCACCTCCAAGC	GGCCTTGTT ACATCGAAT AAKCA	TGGAGGTG AACTGRCT CCCA
MRG-334504	GGCCTTGCCACCAYGTTTTCTGAY TGATTAACCTATC**[R]**GGTGATAAA YCGCGAGATAAKGCATCTGCTGGA TTATMCTCAGATTTKAC	GCCTTGCCA CCAYGTTTT CT	TGCMTTAT CTCGCGRT TTATCA
*MRG-515972	GACCTCTCATTCCCCACAACRGAC TCAATATCCCCCGGAGC**[R]**TAGGG CAACACCGGTGTCTGATCTATAYC GTACGAGTGCCGTAGRTGRTA	ACCTCTCAT TCCCCACAA CR	CGGCACTC GTACGRTA TAGAT
MRG-727381	GTCCTRMGGCTTGACATCCTCYG**[M]**ACGRAGRATRGATGGAAGYCCT GCTTTGCCAGATCCTCTGCKATYT CRGC	GTCCTRMG GCTTGACAT CCTCY	GGATCTGG CAAAGCA GGRCT
MRG-878189	ACACCTTTAAATGCGGTTYTACCY AGKTCGAYCYTGAACYGACYAAY GAG**[R]**AGGCAGAATAAAKCGYYC TATTTCATYATRYCTTCGAGCTGC AAC	ACACCTTTA AATGCGGTT YTACC	ATAGSRCG MTTTATTC TGCC

MRG-1676594	GTCCCTYTMGCTTACTGTCTTTTCCCTTCACYATTGCTGAC**[<u>Y</u>]**CYYATGCTCYATTATARGAYTCCCGGTAATATAKTAGTCTACGATGTTCT	TMGCTTACTGTCTTTTCCCTTCA	CCGGGASTCYTATAATRGAGCA
MRG-1733142	GCCCCAGYMATCCATTGGGATG**[<u>R</u>]**AAMACTTTYRCCTTCTCTCAGTGGAYTGRMAAATGTCGATAGACTTGCTG	GCCCCAGYMATCCATTGGG	TTKYCAWTCCACTGAGAGAAGGY
MRG-2028876	GCCCTCTGKYAGTRGAATGCTGCAACYCKT**[<u>R</u>]**CCTCTCGTATRRAACYCCTACACCYTCGTTCTKGYTTYAACAGYTCAY	TCTGKWAGTRGAATGCTGCA	CMAGAACGARGGTGTAGGRGT
MRG-2248377	TGTGAGGAGCATGCCCYMTCCGTYAACCMCAGGAAGACGGARAYGGTACT**[<u>S</u>]**TTCYCAAGGAAAAGAAAACTTGAGGCAATGAGAGCATCCGTTCT	GTGAGGAGCATGCCCYMTCC	TGCTCTCATTGCCTCAAGTTTTC
*MRG-2353694	GACCCAKAAYYGARCCCTGCGGTACTCCCGATTTTG**[<u>Y</u>]**YTYTRTGYTGTAGTYCTTGGTGACTTTTATTTCTCTTTGTCTGAAATAAC	GACCCAKAASWGARCCCTGC	AAAGTCACCAAGRACTACARCA

MRG-2773845	GGCCGACAYAMATGAGTCAATAA TARTGCACYAGATGGCGCTGG**[R]**T GRCCATAATGTCYRGTGCTYGATC GTTTARACGKTACTAAGTCYACTT C	AATARTGCA CYAGATGG CGCTG	AAACGATC SAGCACYR GACA
MRG-5382535	GACCTTCCGYYRCTKGYCTCAGTT AYGMYGACCCAYAGYYCGRARCT TCT**[K]**ATGCTGGACGCAGTTCYGA AYKCACYAARATATCYGAAGGYA TAACTTTT	GSCTCAGTT ASGMYGAC CC	MRTTCRGA ACTGCGTC CA
MRG-5687800	GCCCTGACCACCATAGTCTTGAKT CGATCAA**[R]**TMAGATGCACTCATA CCTCGCGAAACGAGATCAGCGGG ATTATCGCACGA	GACCACCAT AGTCTTGAK TCGA	TTTCGCGA GGTATGAG TGCA
MRG-6007860	GACCCRTTGAGCTYTTGATAGRTG CAGA**[Y]**GKGGCARYTAARCTGTAC ACGGGGAGAAGAGAGATTMTAAA GTYTGGCTT	GACCCRTTG AGCYCTTGA TAGR	TCTCTTCT CCCCGTGT ACAGY
*MRG-42674281	GCCCCACTGACAAGGCAGKAGCT GATAAGAG**[W]**TGCGACAGAAGAA TGGCAAAATRTACCTGTCYARACC ATTGATGACTT	CCCACTGAC AAGGCAGK AG	GTCATCAA TGGTRTYG ACAGGT

MRG-43600353	RACATTTRYCCRAYTTTCAGTTGGYCGTAAATAKTTCCRARCATGAATCA[**Y**]CKGGATTCTCGCCTGCTTYCTTGAATTCGTTGC	TCAGTTGGSCGTAAATAKTTCCR	GAATTCAAGRAAGCAGGCGAGA

Table 3C.3: Pools of forward (FOR) and reverse (REV) primers used for multiplex PCR amplification of *Tetropium* sp. whole genomic DNA samples, as suggested by PrimerPooler.

Suggested Pool 1	
Locus ID	**Primer pair (5' to 3')**
MRG-1859	**FOR:** ATTCTGCTGTCGTCGTGTTC
	REV: GGTTCTTACAGCGATACCCC
MRG-7328	**FOR:** GGYCTCCGACTAGTCCAGA
	REV: TCAATGACCACTTACCCCGC
MRG-131117	**FOR:** CCTTTCCACCACTGTTCTTTTGA
	REV: YGTTCAAGCGRCAATCCAGC
MRG-142444	**FOR:** GCCCTCTCTACTGCTCTCTTC
	REV: GGGTTTCCTGTTGCAATGAGG

MRG-311133	**FOR:** GGCCTTGTTACATCGAATAAKCA
	REV: TGGAGGTGAACTGRCTCCCA
MRG-334504	**FOR:** GCCTTGCCACCAYGTTTTCT
	REV: TGCMTTATCTCGCGRTTTATCA
MRG-878189	**FOR:** ACACCTTTAAATGCGGTTYTACC
	REV: ATAGSRCGMTTTATTCTGCC
MRG-1733142	**FOR:** GCCCCAGYMATCCATTGGG
	REV: TTKYCAWTCCACTGAGAGAAGGY
MRG-5382535	**FOR:** GSCTCAGTTASGMYGACCC
	REV: MRTTCRGAACTGCGTCCA
MRG-5687800	**FOR:** GACCACCATAGTCTTGAKTCGA
	REV: TTTCGCGAGGTATGAGTGCA
MRG-6007860	**FOR:** GACCCRTTGAGCYCTTGATAGR
	REV: TCTCTTCTCCCCGTGTACAGY
Suggested Pool 2	
Locus ID	**Primer pair (5' to 3')**

MRG-10568	**FOR:** CCTTGGGTTTCTTGAGRCAG
	REV: TCCTATTCAACGTAGAGGCAAC
MRG-27970	**FOR:** GCTATCCACCGAKACACTTAAGC
	REV: GTGACARCAACCACCTTTCC
MRG-110964	**FOR:** TCWTTACGGRTGAACAGAKGGC
	REV: RTWGAGGGTYGAGGCTGATG
MRG-148863	**FOR:** GAGGGCTGATCACTYGAAGGT
	REV: CGAAARAGACGACTTKCCATC
MRG-194034	**FOR:** GCTGGGCTGCRCTACTGT
	REV: CGTATTTCGCAGCGCCAC
MRG-727381	**FOR:** GTCCTRMGGCTTGACATCCTCY
	REV: GGATCTGGCAAAGCAGGRCT
MRG-1676594	**FOR:** TMGCTTACTGTCTTTTCCCTTCA
	REV: CCGGGASTCYTATAATRGAGCA
MRG-2028876	**FOR:** TCTGKWAGTRGAATGCTGCA
	REV: CMAGAACGARGGTGTAGGRGT

MRG-2248377	**FOR:** GTGAGGAGCATGCCCYMTCC
	REV: TGCTCTCATTGCCTCAAGTTTTC
MRG-2773845	**FOR:** AATARTGCACYAGATGGCGCTG
	REV: AAACGATCSAGCACYRGACA
MRG-43600353	**FOR:** TCAGTTGGSCGTAAATAKTTCCR
	REV: GAATTCAAGRAAGCAGGCGAGA
Suggested Pool 3	
Locus ID	**Primer pair (5' to 3')**
MRG-515972	**FOR:** ACCTCTCATTCCCCACAACR
	REV: CGGCACTCGTACGRTATAGAT
MRG-2353694	**FOR:** GACCCAKAASWGARCCCTGC
	REV: AAAGTCACCAAGRACTACARCA
MRG-42674250	**FOR:** CCCACTGACAAGGCAGKAG
	REV: GTCATCAATGGTRTYGACAGGT

Chapter 4 – No evidence that body size and fecundity influence mating in an invasive longhorn beetle

Jennifer Anderson[1*], Stephen B. Heard[1], Jon Sweeney[2] and Deepa S. Pureswaran[2]

*Corresponding author

Key Words: fecundity, invasion biology, mate-choice, mating behaviour

[1]Department of Biology, University of New Brunswick, Fredericton, NB, Canada

[2]Canadian Forest Service, Atlantic Forestry Centre, 1350, Regent Street, Fredericton, NB, Canada

Abstract

Mating success of adult insects is of utmost importance to fitness. Male mate-choice preferences are therefore often driven by female traits that result in highest fertilization and offspring production for males. *Tetropium fuscum,* a wood-boring longhorn beetle native to western Europe and northern Eurasia, was introduced to eastern North America circa 1990. Since its arrival, its territorial expansion has been relatively modest, not venturing beyond a 150 km radius from the epicentre of its invasion. We investigated female *T. fuscum* egg production in relation to body size and fecundity, as well as the effect of female body size on male mating behavior, to determine whether female body size drives male mate choice. We predicted that larger female *Tetropium* spp. would produce more/larger eggs, and that male *Tetropium* spp. would more readily mate with larger females. Although we found that larger females had higher lifetime egg lay, we did not find any preference by either *T. fuscum* or *T. cinnamopterum* males for larger females, suggesting body size is not the main driving factor for mate choice in these insects.

Introduction

Mating success is crucial for adult insects as it determines their ability to produce viable offspring and pass on their genes to the next generation. Optimal decision-making about mate choice is particularly important for insects that do not feed as adults, such as cerambycid beetles belonging to the subfamily Spondylidinae (Švácha and Lawrence 2014; Haack 2017; Monné et al. 2017). Such insects invest all their energy and resources in locating suitable mates and reproducing, and it is imperative that they find a mate quickly, particularly due to their short breeding window. However, it is equally crucial that males choose female partners that are fecund and capable of producing viable offspring, thereby maximizing the fitness of their offspring. Such a preference for fecund females would have significant implications for the reproductive success of both male and female beetles and ultimately influence the survival of the species.

Tetropium fuscum Fabricius (Coleoptera: Cerambycidae, Subfamily: Spondylidinae) is a wood-boring longhorn beetle that is native to western Europe and northern Eurasia (Juutinen 1955) but has been introduced to eastern North America. It was first discovered attacking mature red spruce trees (*Picea rubens* Sarg.) in Point Pleasant Park in Halifax, Nova Scotia (Canada) in 1999 (Smith and Hurley 2000) but vouchers in the insect collections at the Nova Scotia Museum of Natural History indicate that it has likely been in North America since at least 1990 (Sweeney et al. 2004). *Tetropium fuscum* is unusual in that unlike many successfully invasive wood-boring beetles, its range has not expanded more than 150 km beyond its original area of introduction (Rhainds et al. 2011).

Male mate-choice preferences are typically driven by female traits that lead to the highest fertilisation rates and maximum offspring output (Bonduriansky 2001). This prioritisation of reproductive success is evident, for example, in male curculionids, in which mating with larger, more fecund females resulted in higher reproductive success (Harari et al. 1999). An example of these dynamics may be observed between *Tetropium fuscum* and its native congener *T. cinnamopterum*. These two species are prone to making mating errors with one another in laboratory conditions (Anderson et al. 2022). Contributing to these errors, they share significant similarities including the same male-produced sex-aggregation pheromone (Silk et al. 2007; Rhainds et al. 2010; Sweeney et al. 2010), similar host plants (Juutinen 1955; Furniss and Carolin 1980), and overlapping emergence times and flight periods (Juutinen 1955; Rhainds et al. 2011).

What mate-choice cues might *Tetropium* males use? Body size is positively correlated with fecundity in many insect species (Holland et al. 2005), including in cerambycids (Naves et al. 2006) with larger females producing more eggs per ovariole, possessing more ovarioles, or having higher rate oviposition rates (Togashi and Togashi 2017; Togashi and Yamashita 2017; Nime et al. 2019; Togashi et al. 2020). Elytron length is strongly positively correlated with other body size measurements in beetles such as mass and pronotum width (Chown et al. 1998; Smith et al. 2000), and thus is a simple metric for comparing body size of longhorn beetles to one another.

Tetropium cinnamopterum females are significantly larger than *T. fuscum* females, possibly indicating higher fecundity. Female body size is typically a rough metric for comparing fecundity within a species. Although *T. fuscum* and *T. cinnamopterum* are not the same species, we took their similarity in mating behaviour and their conserved mating pheromone to treat them as if they are a single species or a single species complex. We examined relationships among female *T. fuscum* body size, fecundity from measurements of egg size and abundance, and lifetime egg lay rate. We also considered the effect of female body size (measured by elytra length) on the time until first mating attempt by males and time until successful mating. The aim was to ascertain whether potential female fecundity influenced male mate choice decisions. Our hypothesis was that larger female *Tetropium* spp. would produce more or larger eggs, irrespective of species, and that male *Tetropium* spp. would show a preference for mating with larger females.

Materials and Methods

Source of Beetles for Mating Experiments

We haphazardly chose and felled 6 red spruce trees in April 2018 at each of two sites - Sandy Lake in Halifax, Nova Scotia (Canada) (44º44'42.67"N, 63º40'40.76"W) and Acadia Research Forest in Noonan, New Brunswick (Canada) (46º0'2.99"N, 66º20'32.72"W). Trees had a mean diameter at breast height of 25 cm. We cut each tree into six pieces, 120 cm in length each, and arranged them in a pyramid-style stack with thickest pieces on the bottom of the stack to encourage infestation by *Tetropium*. We baited the decks with ethanol, fuscumol and a blend of monoterpenes as described in Sweeney et al. (2010) to attract egg-laying females to the felled trees. We left the decks in the field for the duration of the *Tetropium* flight period, until October 2018 when we returned to harvest them. We cut the logs into approximately 30 cm pieces and transported them securely to a containment facility at the Atlantic Forestry Centre (AFC) in Fredericton, New Brunswick. We placed the cut bolts in a -2ºC freezer to mimic an overwintering period until January 2019 when we retrieved them. We placed the frozen bolts in secure Plexiglas cages in a 20–24ºC containment lab under constant dehumidification (as outlined in Dearborn et al. 2016) with a photoperiod of 16:8 [L:D] to mimic the arrival of spring, allowing the beetle larvae to warm up and develop into adults. Adults emerged starting 3 weeks and continuing until approximately 12 weeks after being removed from the freezer. We collected the emerged beetles early each morning to reduce the risk that they were not able to mate before collection. Upon collection, we recorded the sex, species, collection date, and site of collection for each beetle. We measured the right elytron of each female as outlined above. In total, we

collected and measured 344 females - 183 *T. cinnamopterum* and 161 *T. fuscum*. We then stored the beetles in 1.5 mL microcentrifuge tubes with moistened cotton in a 3–4ºC fridge to increase longevity. We attempted to count number and size of eggs in a selection of females from our felled trees, but as our specimens had been frozen pending egg counting, the eggs were not of high enough quality to examine. For this reason, although the beetles reared from these bolts were used in mating experiments, we assessed the relationship between beetle size and egg size and abundance in lab colony-reared beetles.

Egg Production

We obtained 22 *T. fuscum* females from a laboratory colony at the Great Lakes Forestry Center (Sault Ste. Marie, Ontario, Canada), measured their elytral length, and dissected them to look at egg production as it relates to body size. We measured the right elytron of each female from the point where the base of the pronotum meets the anterior end of the elytron to the distal end of the elytron, using a digital caliper to the nearest 0.01mm. We counted the number of eggs per female and measured the size of each egg along its longest axis, from the farthest point on each end, to the nearest 0.01mm. We took these measurements using microscope images and the image processing software FIJI (Schindelin et al. 2012). We compared measurements taken through FIJI against caliper measurements and the difference was negligible.

Lifetime Egg Lay

The data in this section were collected and provided by Jon D. Sweeney, a research scientist at the Canadian Forest Service, Atlantic Forestry Center, Fredericton NB. We reared adult beetles from sections of logs (1.2 or 2.4 m long, 20–35 cm diam) that had been freshly cut from red spruce trees and exposed to *T. fuscum* populations in the field near Halifax, Nova Scotia, from May to December in each of 2000, 2001, 2003 and 2005. We cut the logs into bolts about 30 cm long and stored them indoors at -2° C in the containment facility at the Atlantic Forestry Center (AFC) (Fredericton, New Brunswick, Canada) until set up to rear out *Tetropium* adults using methods described under source of beetles for mating experiments. We identified adults to species, sexed and numbered them, and measured the elytra of each female as for the colony beetles above. We stored the beetles at 3–4°C an average (± SE) of 1.94 (± 0.41) days before setting them up in the 20–24ºC containment lab to determine potential fecundity. We housed females one per cage in plastic cages (8 cm × 8 cm × 10 cm) ventilated on both ends with screen mesh and provided a fresh piece of spruce bark with sapwood attached (about 6 cm × 10 cm × 1.5 cm in size) on which to oviposit and a vial of 10% sugar water with a cotton wick. We replaced the piece of spruce bark with a fresh piece every 1 or 2 days until the female died. We dissected the bark pieces under a stereomicroscope to count the number of eggs laid under bark scales and in bark crevices. We also counted any eggs the female may have laid on the floor of the cage. We dissected the females after they died to count the number of mature eggs still within the ovaries and thus obtain an estimate of potential fecundity of each female over her adult life. For 25 of 35 females tested, we also placed a male *T. fuscum* in the cage; 21 of these 25 females had sperm in

the spermatheca indicating that mating had taken place. The remaining females were not provided with a male and had no sperm in the spermatheca.

Mating Experiment Setup

We checked beetles for vigour before setting them up for mating but as their longevity was not consistent, we kept them until they either died or were seemingly sluggish and close to death. We considered a beetle to be vigorous if it displayed strong frequent movement of the legs and it was able to run around easily. In each mating trial, we placed a single male and a single female beetle in a petri dish arena lined with moistened filter paper. We had 4 treatments: 1. *T. cinnamopterum* male with a *T. cinnamopterum* female; 2. *T. cinnamopterum* male with a *T. fuscum female;* 3. *T. fuscum* male with a *T. cinnamopterum* female; and 4. *T. fuscum* male with a *T. fuscum* female (n = 55, 15, 37, 27). Each pair was given 50 minutes to commence mating. At the end of the 50 minutes, we set aside any pairs that were in copula to complete copulation. We removed beetles that were not in copula and did not attempt to use them in subsequent matings. Where applicable, we recorded time to first mating attempt, total number of mating attempts, and time to success.

Statistics

We tested for a difference in female body size between *T. fuscum* and *T. cinnamopterum* females using an unpaired t-test using the R package ggpubr (v 0.4.0) (Kassambara 2021)

We looked for differences in female elytra length using female species, male species, and success of mating as factors, through a three-factor ANOVA in base R (v. 4.1.1) (R Core Team 2021). We checked for significant two- and three-way interactions; finding none (all $p>0.09$), we then ran an analysis including only the main effects.

We compared average egg length (mm), number of eggs inside a female, and total eggs laid to elytra length (mm) of *T. fuscum* females using the regression function in base R (v. 4.1.1) (R Core Team 2021) to perform linear regressions. For each of the three datasets we performed regressions on, we assessed the assumption of normality through a Q-Q plot of the regression residuals. In each case, the residuals closely followed the line of theoretical normal distribution, suggesting that the assumption of normality was met.

We tested for correlation between female elytra length and time until first mating attempt using a Pearson's correlation test in base R (v. 4.1.1) (R Core Team 2021)

Results

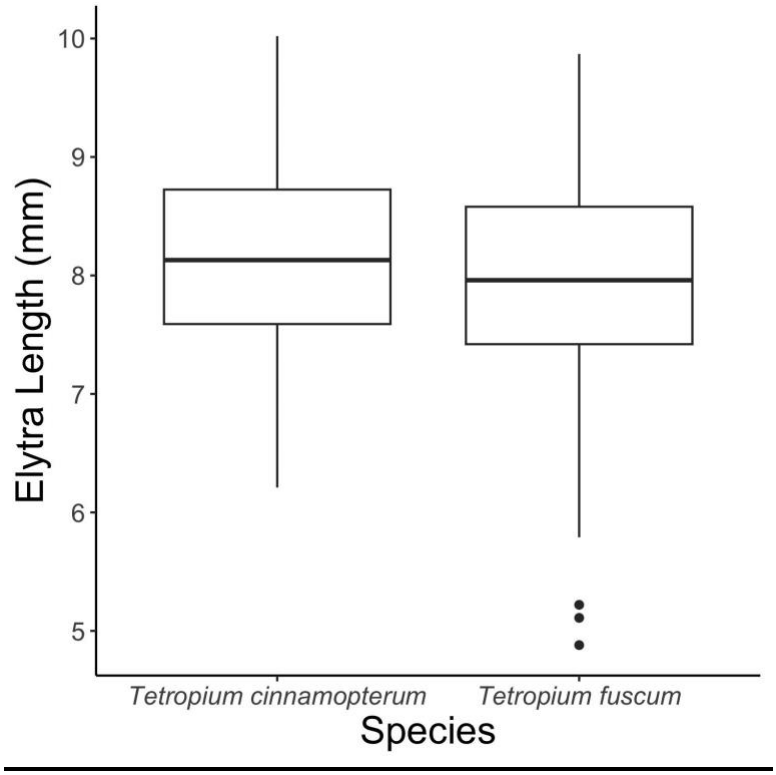

Figure 4.1: Measurement of female elytra length (mm) for *T. cinnamopterum* and *T. fuscum* females (n = 183, 161) shows that *T. cinnamopterum* females were significantly larger on average than *T. fuscum* females. Dots denote outliers, lines represent Q1-3, and whiskers show +/- 1.5 × IQR.

T. cinnamopterum females were on average larger than *T. fuscum* females (Figure 4.1) (mean 8.13 vs. 7.94 mm) ($p = 0.03$, $t = 2.16$, $df = 324$).

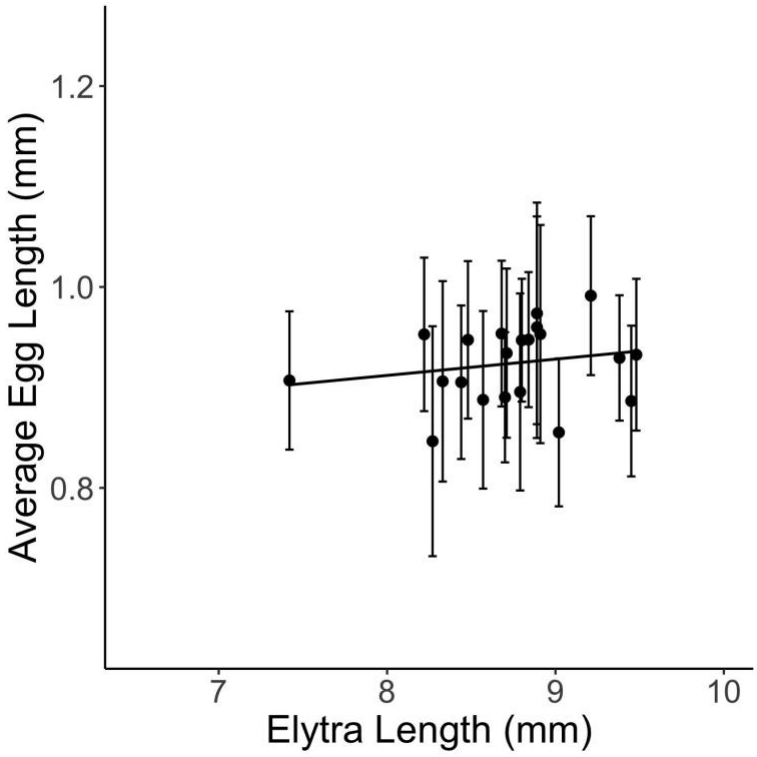

Figure 4.2: Average egg length of mature eggs removed from dissected *Tetropium fuscum* females (n = 22) has no significant relationship with elytra length of the female. Error bars represent +/- SD.

Elytron length did not significantly predict egg size in *T. fuscum* females (Figure 4.2) ($R^2 = 0.04$; df = 1, 19; p = 0.38).

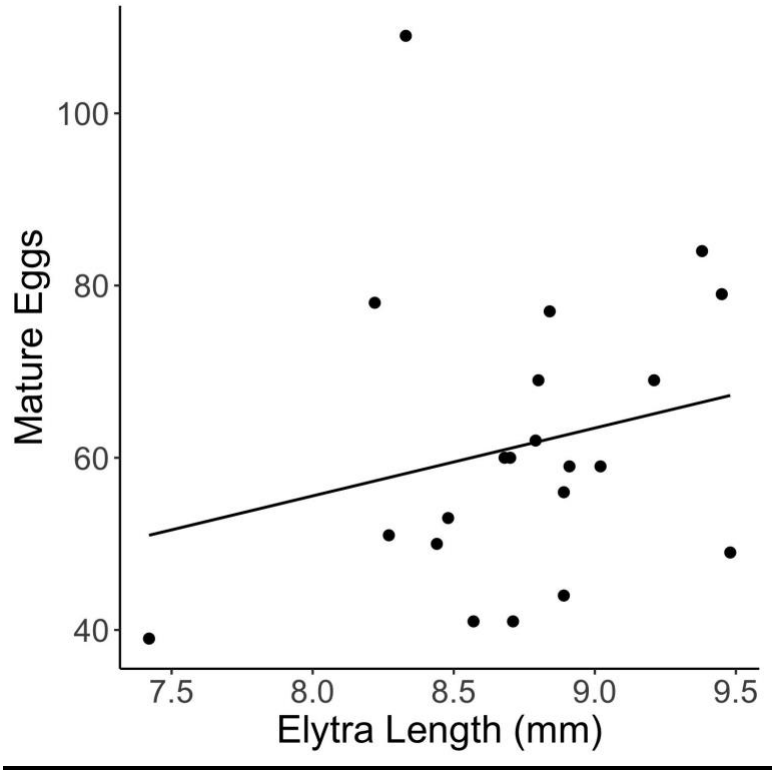

Figure 4.3: Number of mature eggs inside an adult *Tetropium fuscum* female (n = 22) has no significant relationship with elytron length

Elytra length in dissected female *T. fuscum* was not a significant predictor of number of mature eggs within the female (Figure 4.3) ($R^2 = 0.05$; df = 1, 19; p = 0.34).

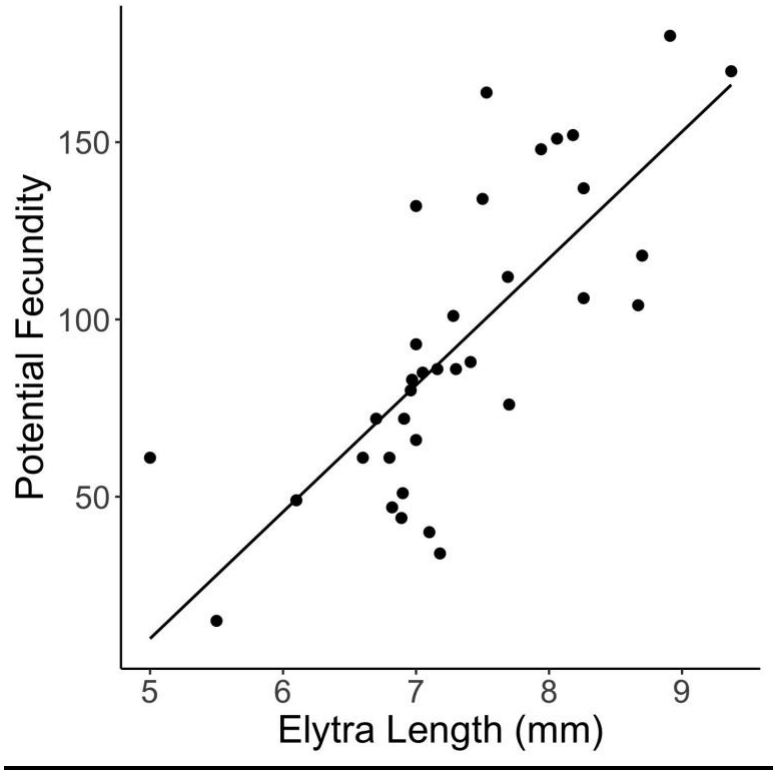

Figure 4.4: Potential fecundity of *Tetropium fuscum* females (n = 35), as measured by total egg lay, has a strong positive relationship with elytron length.

Tetropium fuscum females with longer elytra laid significantly more eggs over their lifetime (Figure 4.4) ($R^2 = 0.57$; df = 1, 33; $p = 1.67 \times 10^{-7}$).

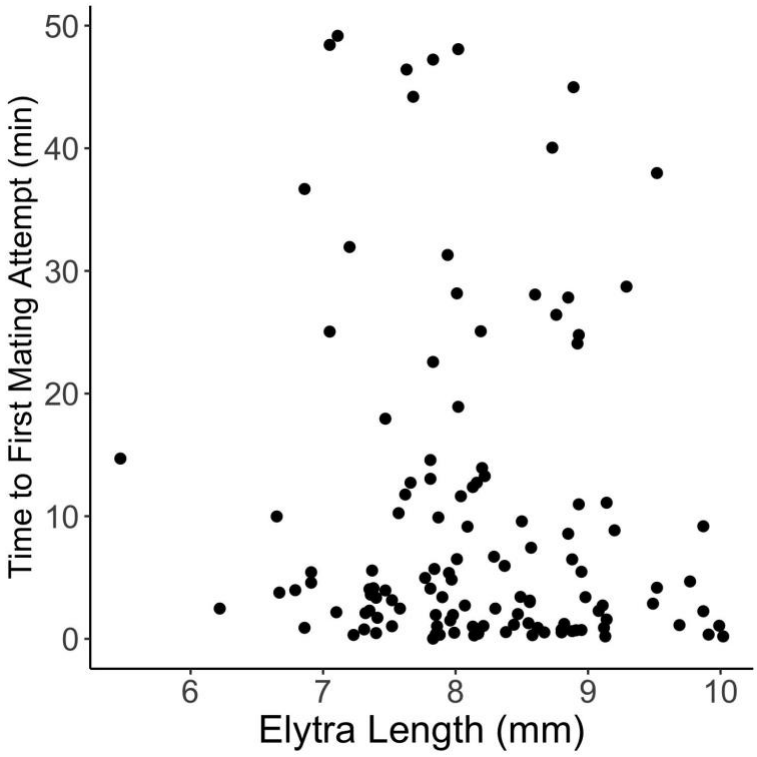

Figure 4.5: Time until first mating attempt between all combinations of *T. cinnamopterum* and *T. fuscum* males and females show no correlation with female body size (as measured by elytra length) (n = 125).

There was no correlation between female body size and time until first mating attempt in *T. cinnamopterum* and *T. fuscum* pairings, Pearson's correlation analysis (n = 125, r = -0.11, p = 0.22).

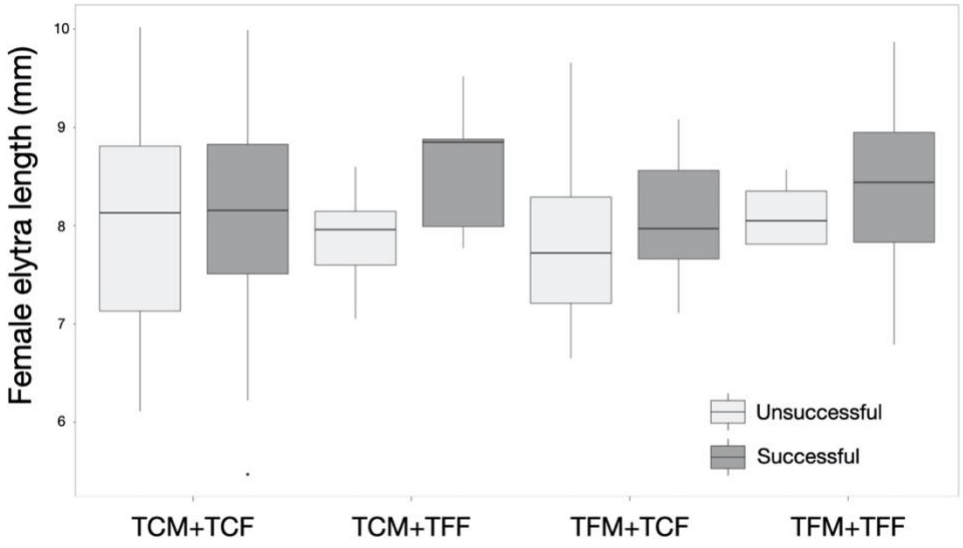

Figure 4.6: Neither *T. cinnamopterum* males (TCM) nor *T. fuscum* males (TFM) show a preference for larger females in their successful vs. unsuccessful mating with either *T. cinnamopterum* females (TCF) or *T. fuscum* females (TFF). Dots denote outliers, lines represent Q1-3, and whiskers show +/- 1.5 × IQR.

We saw no mating preferences related to size in either male *T. fuscum* or *T. cinnamopterum* (Figure 4.6). Three-way ANOVA comparing female body size as measured by elytra length in successful vs. unsuccessful matings in each of four mating combinations of *T. cinnamopterum* and *T. fuscum* males and females showed no significant difference between treatments (Table 4.1).

Table 4.1: Three-way ANOVA comparing female body size as measured by elytra length in successful vs. unsuccessful matings in each of four mating combinations of *T. cinnamopterum* and *T. fuscum* males and females.

Effect	**DF**	**SS**	**F value**	**p value**
Male species	1	0.02	0.02	0.88
Female species	1	0.87	1.22	0.27
Success	1	2.05	2.88	0.09
Residuals	126	89.86		

Discussion

Body size of a female partner seems to have very little influence over whether a male *Tetropium* will "choose" to mate with a particular female. Although *T. cinnamopterum* females are on average larger than *T. fuscum* females and larger females have a higher average lifetime lay rate, this increase in body size and fecundity does not make *T. cinnamopterum* females more attractive than *T. fuscum* females to male *T. fuscum*. *Tetropium cinnamopterum* males do mate more readily with *T. cinnamopterum* females than with *T. fuscum* females under choice mating conditions (Anderson et al. 2022). However, within *T. cinnamopterum*-*T. cinnamopterum* matings, the females with which male *T. cinnamopterum* successfully mated were not significantly larger than females they did not mate with (Figure 4.3), suggesting that their preference for a female is not related to female body size. The lack of correlation of body size with time to first mating attempt (Figure 4.2) may indicate that males need to touch the female in order to determine her acceptability as a mate. Many males did not attempt to mate with their provided female after physical contact. In no-choice mating conditions, *Tetropium* males make mating attempts on first contact more frequently when paired with conspecific females than with heterospecific females (supplemental material; Silk et al. 2011). This further suggests that physical contact plays a role in a male's decision to copulate with a female, likely through cuticular hydrocarbons that are present on the surface of female *Tetropium* (Silk et al. 2011), as is the case in many other cerambycids (Lacey et al. 2008; Ibeas et al. 2009; Yasui 2009).

There are many other factors besides fecundity that can influence mate choice; among these is female nutrition. In some species of beetle, the female's egg production and egg lay rate are tied to availability and quality of food sources upon emergence (Domek and Johnson 1991; Hopkins and Ekbom 1999; Richardson et al. 2019), but *Tetropium* is not known to feed in adulthood (Juutinen 1955; LeMay et al. 2010; Anderson et al. 2022) and thus the female's capacity to produce eggs as a function of body size is determined at the adult molt.

Although many insect groups have evolved a male preference for larger, more fecund females (Brown 1990, Harari et al. 1999, Bonduriansky 2001, Hunt and Simmons 2002, Edward and Chapman 2011), *Tetropium* males have seemingly not evolved this trait. This is surprising because selection should strongly favour response to accurate signals of mate quality, when these exist. Mating with larger females is usually advantageous because they produce significantly larger eggs (Fox and Csezak 2000), leading to larger larval size and survival rate and potentially other benefits (reviewed in Saeki et al. 2005). Honek (1993) reviewed the relationship between female body size and fecundity in 57 insect species and found many positive relationships between body size and fecundity both through dissection to examine number of stored eggs and through lifetime lay rate (Calvo and Molina 2005, Saeki et al. 2005).

Although size and number of eggs at time of dissection was not tied to female body size, we did find that larger *T. fuscum* females laid significantly more eggs than smaller females over their lifetime (Figure 4.4). Perhaps larger females are more able to

produce eggs over their lifetime through presence of more ovarioles, or higher egg production rates of their ovarioles. To investigate these hypotheses, we would need to conduct further experiments to look specifically at ovarioles in female *Tetropium*. Our work cannot identify why *Tetropium* males have not evolved the apparently optimal preference for larger females, but it seems clear that the lack of such a preference will reduce individual male fitness and likely reproductive success at the population level. Perhaps surprisingly, it is not uncommon for insects to make choices that deviate from what we would deem optimal. For example, Heard (1994) showed suboptimal oviposition-site selection by a mosquito; Grundwald et al. (2006) documented male fruit flies choosing to mate with females that were less genetically compatible even when presented with more compatible options; and Raine et al. (2007) showed that bumblebees do not always choose the most rewarding flower when presented with multiple options.

Mate choice between *T. cinnamopterum* and *T. fuscum* might depend on species-specific chemical cues rather than female size. Female *Tetropium* have cuticular hydrocarbons that vary more between the two species than the volatile male-produced pheromone (Silk et al. 2011). These cuticular hydrocarbons may be a more accurate index of female condition and fecundity than body size, but we would need to carry out further experiments to investigate this idea. *Tetropium fuscum* males make mating errors more often than *T. cinnamopterum* males in choice-mating experiment conditions (Anderson et al. 2022) and this may indicate that *T. fuscum* males are less able to distinguish between *T. cinnamopterum* and *T. fuscum* females or that they are less discriminating when choosing a mate.

In many insects, older insects are less choosy about mating partners (Gray 1999, Moore and Moore 2001, Anjos-Duarte et al. 2011, Kelly 2018). This pattern of choosing suboptimal mating partners to mitigate the risks of not mating at all may be observed in the *T. fuscum* population within its sympatric zone with *T. cinnamopterum*. For most species, the population density is highest at the center of the range and decreases moving out toward the range edges (Udvardy and Papp 1969, Sagarin and Gaines 2002, Sagarin et al. 2006, Mlynarek et al. 2017). At the edge of the *T. fuscum* range, Allee effects may occur wherein *T. fuscum* males are far more likely to find *T. cinnamopterum* females as potential mates – and thus, chemical cues common to females of both species may influence mating attempts more so than female size. Although the blend of cuticular hydrocarbons differs between females *T. fuscum* and *T. cinnamopterum,* they share a common compound, *S*-11-methyl-heptacosane, that, by itself, elicits copulatory attempts by male *T. fuscum* when applied to a solvent rinsed female at a female-equivalent dosage (Silk et al. 2011). When conspecific females are scarce, *T. fuscum* males that are choosy regarding species-specific cuticular hydrocarbons or female size may risk not mating at all, which is of course a much worse outcome.

The absence of a correlation between female fecundity and male mate choice in *Tetropium* challenges the notion that mate selection is solely determined by body size. Successful mating in beetles involves a complex interplay of factors, including male-produced olfactory cues (Fedina and Lewis 2007), male body size (Hanks et al. 1995; Harvey and Gange 2006), male age (Jones and Elgar 2004), parasite infestation (Demuth

et al. 2012), and nutrition (Haq et al. 2010; Romano et al. 2016). Understanding the significance of these factors provides valuable insights into beetle mating behavior and enables the development of pest control strategies like mating disruption. In contrast to the common influence of fecundity, body size, and egg production on insect mate choice, our research reveals that male mate choice in *Tetropium* spp. relies on species-specific cues rather than fecundity alone. However, if *T. fuscum* males struggle to distinguish between *T. cinnamopterum* and *T. fuscum* females in sympatric areas, this system may become compromised, leading to an increased occurrence of mating errors. The potential risk of not mating at all may outweigh the importance of being selective in their choice of mates for *T. fuscum* males. These findings emphasize the need for further investigation into the specific mechanisms governing insect mating behavior, including the impact of population density, mate availability, and species-specific chemical cues on mate choice and reproductive success.

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Supplemental Material: Mating preference of *Tetropium* sp. males upon first contact

Experimental Methods

We gleaned data not published in Anderson et al. 2022 to illustrate a pattern of *Tetropium* sp. males mating more frequently on first contact with conspecific females than with heterospecific females, under no-choice mating conditions. In this experiment, we presented one male with one female in a petri dish arena and gave them 50 minutes to commence mating. Our treatments were as follows: 1. *T. cinnamopterum* male × *T. cinnamopterum* female; 2. *T. cinnamopterum* male x *T. fuscum* female; 3. *T. fuscum* male x *T. cinnamopterum* female; 4. *T. fuscum* male x *T. fuscum* female. We analysed only successful pairings from this experiment. For a more detailed description of methods, see Anderson et al. 2022.

Statistical Analysis

The no-choice mating experiments conducted in Anderson et al. 2022, although grouped as a single experiment, were two separate no-choice mating experiments performed in parallel. The first involved *T. cinnamopterum* males presented with either a conspecific or heterospecific female and the second involved the same conditions but instead with *T. fuscum* males. For this reason, we analysed the two experiments separately.

For each set of conditions, we tested the prediction that a male beetle would attempt to mate more frequently on first contact with a conspecific female than with a heterospecific female using a two-sided Fisher's Exact Test.

Results

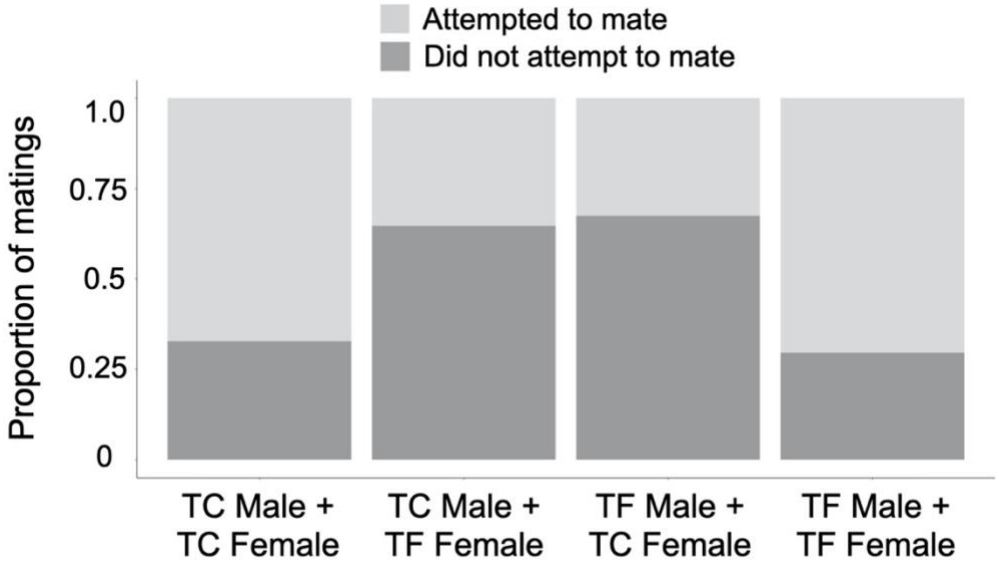

Figure 4S.1: Proportion of *Tetropium fuscum* (TF) and *Tetropium cinnamopterum* (TC) males from successful matings under no-choice mating conditions that did and did not attempt to mate on first contact when presented with either a conspecific or a heterospecific female (n = 55, 17, 43, 44).

Tetropium cinnamopterum and *Tetropium fuscum* males both attempted to mate on first contact significantly less with heterospecific females than with conspecific females (Two-sided Fisher's Exact: $p = 0.025$ and $p = 5.6 \times 10^{-4}$)

Chapter 5 – General Discussion

The slow spatial spread in North America of the invasive longhorned wood borer, *Tetropium fuscum* Fabricius (Coleoptera: Cerambycidae), and how its spread may be impacted by interactions with its native counterpart, *Tetropium cinnamopterum* Kirby (Coleoptera: Cerambycidae), are the focus of this thesis. Their mating behaviors, potential hybridization, and implications on forest ecology and management are pivotal not only in deciphering the dynamics of these species but also in enhancing our understanding of invasive species interactions in a broader context. Understanding the intricate and complex mechanisms that drive insect behavior, specifically in the context of mate choice, is crucial for developing strategies to control pest species and manage populations.

Our laboratory experiments established the foundational mating behaviors of *T. fuscum* and *T. cinnamopterum* (Chapter 2). Both no-choice and choice mating scenarios offer insights into the proclivity of these beetles to make mating errors. We found that *T. fuscum* and *T. cinnamopterum* males both made mating errors under no-choice mating conditions. However, under choice mating conditions, while *T. fuscum* males still made mating errors, *T. cinnamopterum* males had a stronger preference for mating with conspecific females. The finding that *T. fuscum* males may err in mate selection even when presented with a conspecific option underscores the complexities of their reproductive behavior and poses intriguing questions about evolutionary drives, particularly in areas where both species coexist. *Tetropium fuscum* has been established

in North America since approximately 1990 (Smith and Hurley 2000) and within its invasive range, has effectively displaced the native *T. cinnamopterum* (Dearborn et al. 2016). The case of *T. fuscum* and *T. cinnamopterum* parallels, in many ways, that of the invasive winter moth (*Operophtera brumata* L.) and its native congener the Bruce spanworm (*Operophtera bruceata* Hulst), which coexist in northeastern North America. Within the established range of *O. brumata*, the native *O. bruceata* is scarce (Elkinton et al. 2014) showing the same pattern of displacement of a native congener by an introduced species observed in *T. fuscum* and *T. cinnamopterum*.

Our SNP assay and field survey results (Chapter 3) provided a novel lens through which to view the real-world interactions of these beetles. Through development of this assay, we were able to identify and successfully amplify species discriminating single-nucleotide polymorphisms (SNPs) at 22 loci. In our field survey, we found hybrid individuals in three of our five field sites and cryptic individuals at the site closest to the epicenter of invasion. These findings add another layer to the complexities of these interactions. We detected only low levels of hybridization at two of our sites (2.9% at Antrim, 2.4% at Sandy Lake) – but a higher rate at Westchester (20%) - and none of the individuals we detected were F1 hybrids. Because of the low sample size at our Westchester site (1 hybrid out of 5 individuals), we cannot interpret the apparently higher rate of hybridization at this site. The degree of genetic assignment we observed in our hybrid individuals suggests that the F1 offspring from heterospecific matings between *T. fuscum* and *T. cinnamopterum* are viable and fertile. If F1 hybrids were not able to reproduce we would have expected only to find hybrid individuals that were genetically

assigned approximately 50% to each parental species but we found individuals with varying degrees of genetic composition. This indicates F1 hybrids can mate either with other hybrids, through backcrosses with purebred individuals, or through some combination thereof. The low numbers of hybrid individuals we observed may suggest that heterospecific matings are uncommon or that hybrid offspring are not as fit as their parents. Over time, however, presence of hybrids in the population may lead to increased levels of introgression between these species in the sympatric zone.

Like *T. fuscum* and *T. cinnamopterum*, *O. brumata* and *O. bruceata* use the same pheromone attractant and have been shown to occasionally hybridize (Elkinton et al. 2014). *Operophtera bruceata* possesses a greater resistance to colder temperatures and there is concern that hybrids displaying heterosis could exhibit this trait combined with the outbreaking capabilities of *O. brumata*. Similarly, hybrid offspring of *T. fuscum* and *T. cinnamopterum* may acquire *T. fuscum*'s ability to attack apparently healthier trees coupled with *T. cinnamopterum*'s broader host range. Conversely, if the hybrids display inbreeding depression, their low fitness could lead to population decline not only of the invasive *T. fuscum* but also the native *T. cinnamopterum* population within the sympatric zone (Rhymer and Simberloff 1996; Allendorf et al. 2001). Havill et al. (2016) found that hybrid offspring of *O. brumata* and *O. bruceata* can backcross with either parental species but that backcrosses with the invasive *O. brumata* provided significantly greater viability in the offspring. This may be the case with *T. fuscum* and *T. cinnamopterum* as well. If backcrosses to *T. fuscum* provide greater survival rate and/or fertility of offspring, repeated backcrosses to purebred *T. fuscum* may produce cryptic individuals like those

we detected at our Sandy Lake site (Nova Scotia, Canada) (Chapter 3). The detection of cryptic individuals with the morphology of *T. cinnamopterum* but the genetic composition of *T. fuscum* indicates that backcrosses may be asymmetrical. Investigation into the dynamics behind these interactions could provide greater insight into the underlying mechanisms of the limited spatial distribution of *T. fuscum* in North America. Understanding the viability and fertility of the various types of hybrid individuals may allow us to make better predictions about how these two species will interact and impact the ecosystem in the future.

We investigated preference of male *Tetropium* for more fecund females to explain mate choice errors and why *T. fuscum* males are more prone to making errors than *T. cinnamopterum* males (Chapter 4). Although *T. cinnamopterum* females have a significantly larger body size and egg laying capacity than *T. fuscum* females, this increase in size and fecundity does not appear to influence mate choice by *Tetropium* males. This suggests female body size is not the driving force behind mate choice in these species. Rather, they may rely on other species-specific cues for mate selection. Silk et al. (2011) demonstrated that female *Tetropium* possess cuticular hydrocarbons which are instrumental for mate recognition by *Tetropium* males. Considering *T. fuscum*'s seemingly greater proclivity to mate with heterospecific females, they might either struggle to differentiate the cuticular hydrocarbons of *T. fuscum* and *T. cinnamopterum* females or they might simply be less selective. This prompts further investigations into why *T. fuscum* males appear less discerning in their mate choices compared to *T. cinnamopterum* males.

There are many factors that determine the likelihood of an individual species to establish and become invasive in a novel habitat (Ehrlich 1986; Williamson 1996). An introduced species must survive and reproduce in the new habitat, which depends on factors like temperature, moisture, soil conditions and availability of resources like food, shelter and mates (Williamson and Fitter 1996; Lee 2002; Holt and Keitt 2004). It must also have a good ability to compete with native species for these resources (O'Connor 1986; Newsome and Noble 1986; Moyle 1986); this is determined by things like size and reproductive capacity (Peters 1983; Schmitt et al. 1995; Kingsolver and Pfennig 2004). Reproductive capacity is directly tied to availability of mates, size and number of broods per year, and the viability and fertility of the offspring (Reznick 1985; Clutton-Brock 1988; Kokko and Rankin 2006). *Tetropium fuscum*, which originates from western Europe and northern Eurasia (Juutinen 1955), finds climates similar to its native habitat in the maritime provinces of Canada (Figure 5.1), effectively outcompeting and displacing the native *T. cinnamopterum* during its establishment (Dearborn et al. 2016). It is less discriminating in its mate choice than *T. cinnamopterum* (Chapter 2) which could either lead to increased reproductive output due to more mating opportunities or decreased reproductive output due to wasted mating efforts. In Canada, the primary food source of *T. fuscum* is red spruce (*Picea rubens* Sargent), although in its invasive range it has been known to attack white spruce (*P. glauca* Moench (Voss)), black spruce (*P. mariana* (Miller) Britton, Sterns and Poggenburg), and Norway spruce (Smith and Humble 2000; Sweeney et al. 2001) – the latter being its preferred host in its native range (Juutinen 1955). *Tetropium fuscum* has also been recorded in both blue spruce (*P.*

pungens Engelm.) and Sitka spruce (*P. sitchensis* (Bong.) Carr.) in Europe, as well as in non-spruce species including the European silver fir (*Abies alba* Mill.), Baltic pine (*Pinus sylvestris* L.), and larches (*Larix* spp.) (Sweeney et al. 2004). The distribution of red spruce in Canada is concentrated in the southeast (Figure 5.2), with sparse establishment into northeastern United States. Although the limited range of red spruce in North America could place a limit on the ability of *T. fuscum* to invade in its introduced range, *T. fuscum* could also switch hosts and invade via other *Picea* spp. or other non-spruce conifers. A likely determining factor in its ability to expand its introduced range is the condition of the *Picea* spp. trees in the adjacent areas. While *T. fuscum* has been observed attacking apparently healthy spruce in Nova Scotia (Smith and Humble 2000; Sweeney et al. 2001), it only infests stressed or moribund trees in its native range in Europe (Juutinen 1955). If spruce forests are mainly healthy, suitable hosts will be harder to find and reproductive success lower but if conditions generate a lot of stressed spruce from things like several years of defoliation by spruce budworm, drought, or wind damage, then we may see faster and greater range expansion.

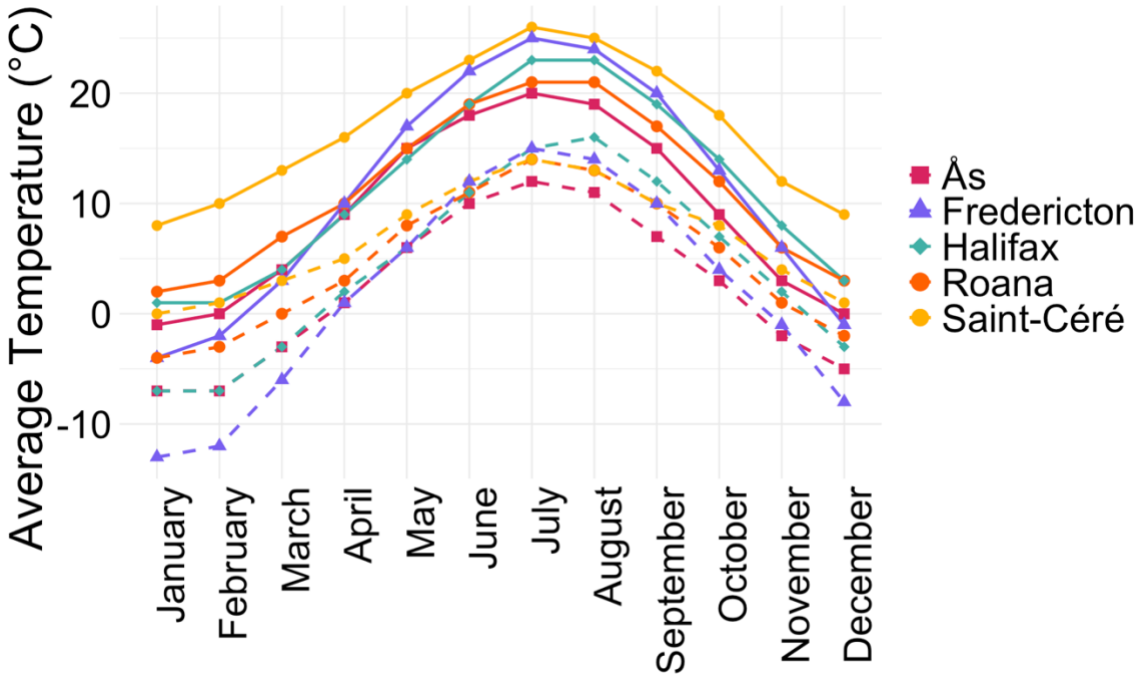

Figure 5.1: Average yearly high (solid lines) and low (dashed lines) temperatures (ºC) by month for Ås (Norway), Fredericton (Canada), Halifax (Canada), Roana (Italy), and Saint-Céré (France). Fredericton is outside of the invasive zone of *Tetropium fuscum* in the neighbouring province of New Brunswick while Halifax is in the center of the invasion zone in Nova Scotia. The other cities are places near field sites where we collected European *T. fuscum* for development of our SNP assay. © *WeatherSpark.com*

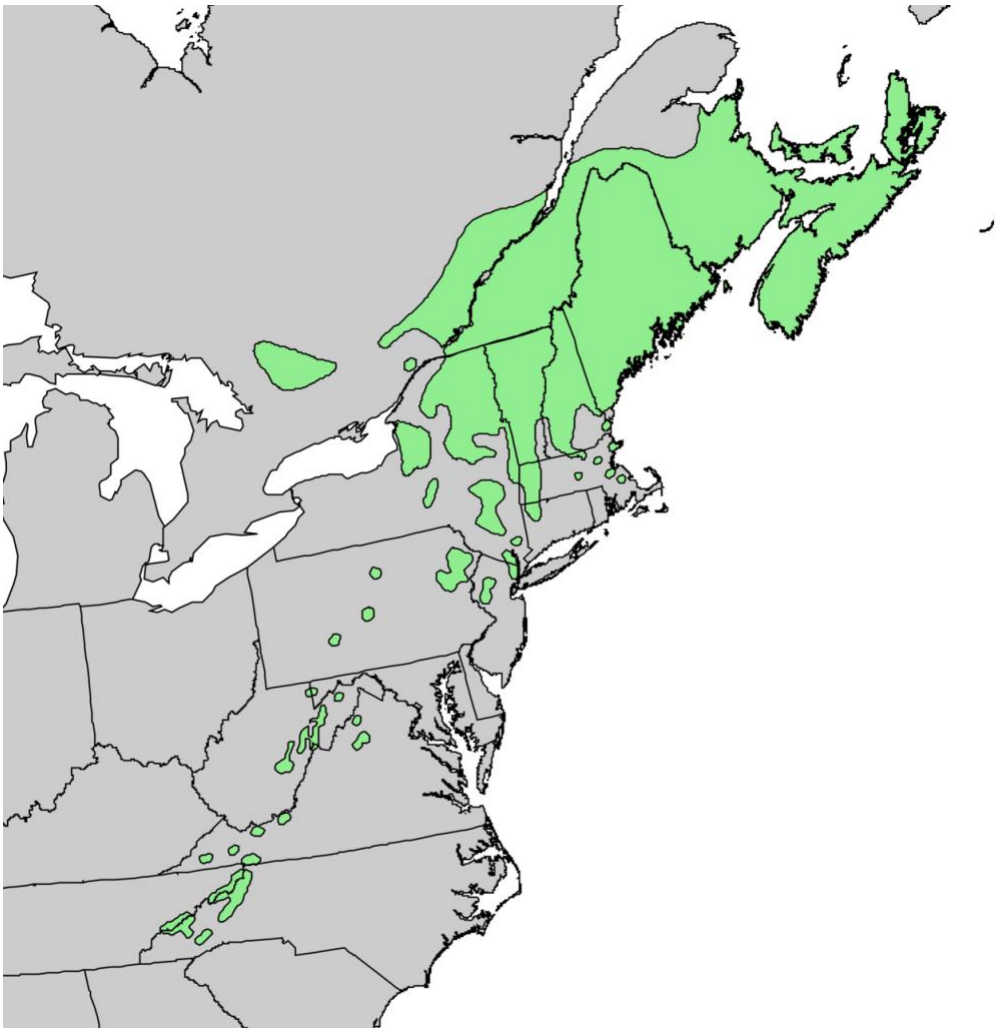

Figure 5.2: North American distribution of red spruce (*Picea rubens*), the preferred host of *T. fuscum* in its invasive range. Source: Wikimedia commons (Public Domain, Elbert E. Little Jr.).

An introduced species will have higher survival and thus reproduction rate if it is able to escape predation and parasitization (Keane and Crawley 2002; Mlynarek et al. 2017). Coming into a habitat where a closely related native is already established, like in the case of *T. fuscum*, means presence of predators and parasitoids that could also make a host switch and begin attacking the introduced species (Parker and Hay 2005; Strauss et al. 2006; Hawkes 2007). There are at least two species of parasitoid wasps that are native to northeastern North America and are known to attack both *T. fuscum* and *T. cinnamopterum*: *Wroughtonia occidentalis* Cresson (Hymenoptera: Braconidae) and *Rhimphoctona macrocephala* Provancher (Hymenoptera: Ichneumonidae) (Sweeney et al. 2005; Flaherty et al. 2011). Their presence and ability to exploit *T. fuscum* as a host may contribute to the suppression of *T. fuscum*'s spatial spread.

Tetropium fuscum possesses the correct combination of traits necessary to establish in northeastern North America and our research enriches our understanding of the underlying reasons for its slow spatial spread. Species are generally densest at the epicenter of the population and become progressively sparser moving toward the edges of the range (Sagarin and Gaines 2002, Sagarin et al. 2006, Mlynarek et al. 2017). At the edge of *T. fuscum*'s range, males may face Allee effects, often encountering predominantly *T. cinnamopterum* females. This raises the question of whether these males make a cost-benefit decision on whether the selection of a suboptimal mate outweighs the risk of not finding any mate at all. Elkinton et al. (2014) suggests that Allee effects at the edges of the established range of *O. brumata* may be mitigated rather than exacerbated by the presence of *O. bruceata*. However, given the low levels of

admixture observed in the sympatric zone, heterospecific matings in the field might either be infrequent or not consistently result in viable fertile offspring. This challenges the idea that *T. fuscum*'s range is restricted solely due to unsuccessful heterospecific mating at its edges and raises questions regarding the reproductive success of hybrids and the role of introgression in the spread of introduced species.

The slow spatial spread of *T. fuscum* may not be tied solely to the presence of *T. cinnamopterum*. Human intervention is an important aspect of discouraging spatial spread in a variety of ways that are not directly related to the biology of the invader or ecology of the novel habitat. Eradication and mitigation efforts like spraying pesticides or removal of infested trees can help dampen the effects of the novel species (Liebhold et al. 2005; Sharov and Liebhold 1998). Introduction of species-specific parasitoids can also help control the population and has been employed as a control agent against many introduced and pest species. For example, the parasitoid *Cyzenis albicans* (Diptera: Tachinidae) was introduced in the Pacific Northwest to control winter moth (*O. brumata*) outbreaks (Elkinton et al. 2014). Placing strict guidelines such as encouraging the public not to move firewood or placing a moratorium on removal of *Picea* spp. (spruces) from Nova Scotia, as was done in the case of *T. fuscum* (CFIA 2017), may have helped curb the spread of this species into other regions of North America.

Many other factors can result in limited spatial spread of introduced species like limited host range, lack of suitable habitat, and climate (With 2002; Pearson and Dawson 2003). Low genetic diversity may open the population up to disease and environmental

stress, thereby hindering the spread (Dlugosch and Parker 2008). It is also worth considering that many introduced species go through a latent growth period or "lag phase" in their population growth when they arrive in a novel habitat (Crooks 2005). For example, upon its initial introduction in Massachusetts (USA) the spongy moth (*Lymantria dispar*) spread slowly over 5 decades before becoming a serious pest (Liebhold 1992; Elkinton 2014). This delay in population growth can be a result of several factors including initial low density, adjustment period to a new environment, competition with native species, Allee effects, and genetic bottlenecks (Simberloff and Gibbons 2004). Over time, introduced species may overcome these obstacles as their population grows and evolutionary changes allow for new allelic combinations that make the population more resilient and able to adapt (Dlugosch and Parker 2008). The slow spatial growth of *T. fuscum* in North America could be due to environmental factors, monitoring and control measures or natural barriers. However, just because *T. fuscum* is showing slow spatial growth now doesn't mean this will always be the case. There's a risk that, like other invasive species, once it gets past its latent growth period, its population and spatial distribution could increase rapidly. Therefore, continuous monitoring and early intervention are crucial to manage and potentially mitigate its impact in North America.

Despite the current slow spatial spread of *T. fuscum* in North America, it is vital to understand the dynamics that are affecting its spread and its potential to spread in the future. Species that successfully become invasive in one habitat are more likely to establish in other places where they are introduced (Lawton 1990; Daehler and Strong

1993; Williamson and Fitter 1996). Furthermore, the most effective method of predicting how an invasive species will act in a novel habitat is to look at previous invasion events by the same species (Liebhold and Tobin 2008; Elkinton 2014). Understanding the mating behaviour of *T. fuscum* and *T. cinnamopterum* could shed light on the evolutionary trajectories of invasive and native species, especially in situations where they coexist. This knowledge can be applied to the study of other invasive-native species pairs, helping ecologists predict and manage potential problems before they escalate. While this research delved into many aspects of *T. fuscum* and *T. cinnamopterum* interactions, several avenues remain unexplored. Future research should further investigate the ecological impact of potential hybrids through determination of their viability and reproductive dynamics with other hybrids and pure-bred individuals. It could also better elucidate the factors influencing mate selection such as determining how mates are selected by *Tetropium* males, which could give us insight into the potential growth of the species and inform the development of better control strategies.

The interactions between *T. fuscum* and *T. cinnamopterum* are multifaceted and holds considerable ecological significance. In this study, we have identified possible factors that may contribute to the gradual expansion of *T. fuscum* in North America, though the actual mechanism is likely a complex interplay of multiple elements. Our research marks the beginning of a deeper investigation into these complexities, contributing to improved forest management techniques and providing insights into the broader context of invasive species dynamics. As global ecosystems face the challenges

of invasive species, studies such as ours will play a crucial role in enhancing our understanding and shaping our responses.

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Curriculum Vitae

Candidate's Full Name: Jennifer Anderson

Universities Attended: University of New Brunswick, B.Sc. 2015, PhD 2024

Publications:

Anderson JL, Heard SB, Sweeney J, Pureswaran D. 2022. Mate choice errors may contribute to the slow spread of an invasive Eurasian longhorn beetle in North America. NeoBiota 71:71-89.

Conference Presentations:

Oral Presentations

Acadian Entomological Society Meeting 2016
"Hybridization between the native Longhorn Beetle, *Tetropium cinnamopterum*, and the invasive Brown Spruce Longhorn Beetle, *T. fuscum*"
Authors: Jennifer L. Anderson, Deepa Pureswaran, Stephen B. Heard

Canadian Society of Ecology and Evolution Meeting 2017
"Could mating errors be slowing the spread of the invasive Brown Spruce Longhorn Beetle?"
Authors: Jennifer L. Anderson, Deepa Pureswaran, Stephen B. Heard

Canadian Society of Ecology and Evolution Meeting 2019
"Promiscuity with a native congener may be hindering the North American spread of the European spruce borer *Tetropium fuscum*"
Authors: Jennifer L. Anderson, Deepa Pureswaran, Stephen B. Heard, Jon Sweeney

International Union of Forest Research Organizations Meeting 2019
"The role of sexual blunders in the slow North American spread of the invasive Brown Spruce Longhorn Beetle"
Authors: Jennifer L. Anderson, Deepa Pureswaran, Stephen B. Heard

Canadian Society of Ecology and Evolution Meeting 2021 (online)
"Testing for introgression between native and invasive longhorn beetles."
Authors: Jennifer L. Anderson, Deepa Pureswaran, Stephen B. Heard, Jon Sweeney

Entomological Society of Canada Meeting 2022
"Mate Choice Errors in an Invasive Longhorn Beetle"
Authors: Jennifer L. Anderson, Deepa Pureswaran, Stephen B. Heard, Jon Sweeney

Canadian Spray Efficacy Research Group Meeting 2023
"Mate choice errors and the slow North American spread of an invasive Eurasian longhorn beetle."
Authors: Jennifer L. Anderson, Deepa Pureswaran, Stephen B. Heard, Jon Sweeney

Poster Presentations

European Congress of Entomology Meeting 2018
"Sexual Blunders and the slow North American spread of a European *Tetropium* beetle."
Authors: Jennifer L. Anderson, Deepa Pureswaran, Stephen B. Heard, Jon Sweeney