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# Prey Specificity Of Thanasimus Dubius Between Latent And Intermediate Phases Of Southern Pine Beetle

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# PREY SPECIFICITY OF *THANASIMUS DUBIUS* BETWEEN LATENT AND INTERMEDIATE PHASES OF SOUTHERN PINE BEETLE

By

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This study investigates the pheromone preference of a bark beetle predator *Thanasimus dubius* between latent and intermediate phases of southern pine beetle. Two trap treatments were set up in each SPB phase. Standard Lindgren funnel traps were baited with either SPB lures or *Ips* lures. The number of *T. dubius* caught in each trap was recorded and data was analyzed using both the Mann Whitney U test and a two-way factorial ANOVA. *Thanasimus dubius* showed no variation in SPB pheromone preference but did show a slight increase in preference for *Ips* pheromones in intermediate phase areas. A protocol was developed to identify prey DNA within gut contents of *T. dubius* to understand prey preference in relation to pheromone preference. Primers were developed to amplify CO1 gene sequences from five different bark beetles. All primers were specific to their own DNA and able to detect at least 0.2 picograms of DNA.

# DEDICATION

I would like to dedicate this thesis to my husband, Michael Campbell who has supported me throughout the entire process. He offered his innovative ideas that were often times difficult to understand but there was always a method to his madness.

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# CHAPTER I

# LITERATURE REVIEW

This review of southern pine beetle (*Dendroctonus frontalis* Zimmerman) literature should yield some vital background information needed to understand the relationship between southern pine beetle and its main predator, the checkered clerid beetle (*Thanasimus dubius* Fabricius). Subtopics include; biology of the southern pine beetle and their associates, *T. dubius*, and molecular tools that can be useful for understanding the biology and behavior of *T. dubius* in relationship with bark beetles.

## Southern Pine Beetle

The southern pine beetle (SPB) (*Dendroctonus frontalis* Zimmerman), a native pest of the southern yellow pine tree species, has been known to decimate many timber stands in the Southeastern United States, as well as parts of Mexico and Central America (Hopkins, 1921; Thatcher et al., 1980). In 1985, SPB infestations contributed to record timber losses in Texas ($63,125,248) and in Louisiana ($143,623,486). Average yearly timber loss due to SPB was approximately $3.9 million per year between 1971 and 1996 in Mississippi (Price et al., 2006). During the last 20 years research has been conducted to understand the population dynamics and behavior of SPB in an effort to reduce losses to pine timber.

The southern pine beetle is capable of killing many healthy, vigorous trees when their population numbers are high (Hopkins, 1921). Loblolly (*Pinus taeda* L.) and shortleaf (*P. echinata* Mill) pines are most susceptible to SPB (Thatcher et al., 1980). However, SPB has been known to attack Virginia pine (*P. virginiana* Mill*)*, slash pine (*P. elliotti* Engelm), longleaf pine (*P. palustrus* Mill), pitch pine (*P. rigida* Mill) and eastern white pine (*P. strobus* L.) (Hopkins, 1921; Thatcher and Barry, 1982). When the first female beetle successfully enters a host tree, she releases the aggregation pheromone frontalin. This pheromone is the main attractant that draws other beetles to attack and overcome the tree's defense mechanisms (Vité and Francke, 1976). Once beetles have made their way into the host, they construct brood galleries in the phloem tissue of the tree. An immature southern pine beetle spends most of its life cycle inside the tree until it emerges as an adult in search of a new host and mate (Thatcher et al., 1980). Depending on temperature, it takes approximately 26 to 54 days to complete the egg to adult stage of the life cycle. The SPB may have as few as three generations, or as many as seven generations per year depending on population levels (Dixon and Osgood, 1961). When population numbers are low, SPB must rely on weakened or dying trees to maintain their population (Dixon and Payne, 1979b).

Traditionally, SPB populations could be placed into two categories; endemic and outbreak. Outbreak conditions consist of more than 1 multi-tree infestation per 1000 acres of susceptible host (Price et al., 1998). Endemic conditions have not been defined numerically, but until recently the term was applied to any population level less than outbreak. The term 'endemic' as a descriptor of SPB populations is now avoided due to confusion with the term relating to native organisms. Southern pine beetle populations

are now divided into three categories; latent, intermediate, and outbreak (Clarke, 2010). The latent phase is defined as areas with no infestations and no or few SPB caught in detection survey traps. Intermediate phase areas have a few small infestations, but no more than 1 multi tree infestation per 1000 acres of land (Clarke, 2010).

## Southern Pine Beetle Associates

Other bark beetles that are commonly associated with the SPB are the black turpentine beetle (*D. terebrans* Olivier*),* the four-spined engraver (*Ips avulsus* Eichoff*),*the five-spined engraver (*I. calligraphus* German)*,* and the six-spined engraver (*I. grandicollis* Eichoff*)* (Dixon and Payne, 1979c). These five species of bark beetles make up the southern pine bark beetle guild (Nebeker, 2004). All five species can be found occupying the same tree while stratified throughout different parts of the tree. This phenomenon is known as resource partitioning and allows different species of beetles to successfully utilize different portions of the same host tree (Dixon and Payne, 1979c). The black turpentine beetle, (BTB) is found mainly in the lowest portion of the tree bole (Lee and Smith, 1955; Godbee and Franklin, 1976). The *Ips* species are found throughout the upper portion of the bole and branches, yet there is still some overlap with SPB in the mid-bole area (Speers, 1971; Dixon and Payne, 1979c).

*Ips* beetles generally attack weakened, dying trees. Slash piles and trimmings from forestry activities can attract these beetles and allow populations to build (Mason, 1969; Shea, 1971). Natural disturbances such as drought, tornadoes, ice storms, and hurricanes can create a large amount of suitable habitat for these beetles to reproduce

(Kalkstein, 1976). Suitable habitat conditions allow *Ips* populations to build to a point that these beetles can mass attack and kill healthy pine trees (Hain and Mcclelland, 1979).

There are eleven species of native pine trees susceptible to one or more of the *Ips* species. Signs and/or symptoms of infestation include reddish brown needles, red boring dust and brood galleries beneath the bark. The shape of the brood galleries differs among the southern pine beetle guild and can be used to identify species (Tragardh, 1930). Small round emergence holes in the bark are also evidence of infestation (Goyer et al., 1981). Unlike SPB, the male *Ips* beetle initiates colonization and is the first to enter a tree (Vité and Pitman, 1968; Vité et al., 1972). Males bore into the tree and create a nuptial chamber which one to four females may visit for mating. After eggs are laid, it takes several days of warm weather for them to hatch. Larvae then tunnel in the phloem tissue until pupation occurs. After pupation, the young adults emerge from the host tree to search for a new host and mate (Speers, 1971; Connor and Wilkinson, 1983). See table 1.1 for a list of developmental rates for *Ips* species.

Table 1.1. Developmental rates of the southern pine bark beetle guild and *Thanasimus dubius*.

| Species | Egg | Larvae | Pupae | Adult | References |
|---|---|---|---|---|---|
| *Thanasimus dubius* | 7-28 days | 9-14 days | 15-33.5 days | ~100 days | (Nebeker and Purser, 1980) |
| *Dendroctonus frontalis* | 3-11 days | 7-13 days | 5-17 days | 6-14 days | (Gagne, 1980) |
| *Dendroctonus terebrans* | 10-14 days | | 10-14 days | | (Smith and Lee, 1957) |
| *Ips spp* | 3-10 days | 8-12 days | 10-14 days | | (Connor and Wilkinson, 1983) |

Fungi and mites are important associates of SPB due to their complex symbiotic relationship with SPB (Krokene et al., 1996; Klepzig, 2000). Southern pine beetles carry fungi in a crevice in their exoskeletons called a mycangium (Rumbold, 1931). As the beetle enters a host tree, the fungus is inoculated into the tree and begins growing, causing water and nutrient pathways to become clogged in the tree (Bramble and Holst, 1940). There are three main species of fungi that play a role in the SPB lifecycle: *Ophiostoma minus, Ceratocystiopsis ranaculosus*, and *Entomocorticium sp.* (Klepzig and Wilkens, 1997a).

*Ophiostoma minus* is the causal agent of 'blue stain' in the wood (Bridges and Moser, 1983). Initially, *O. minus* aids SPB in killing the host tree (Ross et al., 1992). However, once the fungus is established, SPB must compete with the fungus for the brood resources within the phloem of the tree (Klepzig, 2000). *Ophiostoma minus* has been shown to reduce the success of larval SPB development in the tree (Hetrick, 1949; Bridges and Perry, 1985; Klepzig, 2000). *Ophiostoma minus* has the ability to outcompete *C. ranaculosus,* yet one species of *Entomocorticium* can maintain resource space in the vicinity of *O. minus* (Klepzig and Wilkens, 1997b). *Ophiostoma minus* development is partially controlled by phoretic mites (Klepzig, 2000). These mites feed on the fungi inoculated by SPB and are carried from one host to another on the exterior of southern pine beetle's body(Bridges and Moser, 1983). Phoretic mites help to mediate the balance between SPB brood and fungus development by feeding on *O. minus* and *C. ranaculosus* (Klepzig and Wilkens, 1997a; Klepzig, 2000; Lombardero et al., 2000).

Bark beetles are associated with several natural enemies, most of them being arthropods. A number of insect predators and parasitoids have been identified as

predators of bark beetles. Goyer et al. (1981) recorded the most frequently encountered predators of bark beetles in Louisiana. These included *Corticeus* spp. (Coleoptera: Tenebrionidae), *Scoloposcelis mississippensis* (Hemiptera: Anthocoridae), *Aulonium ferrugineum* (Coleoptera: Colydiidae), and *Medetera bistriata* (Diptera: Dolichopodidae). *Corticeus glaber* has been studied more intensely that the other insect predators mentioned and is considered to be an important facultative predator of southern pine beetle (Smith and Goyer, 1982). There is little information regarding *S. mississippensis, A. ferrugineum,* and *M. bistriata* as bark beetle predators. Parasitoids have received much attention in the last decade. Bark beetle parasitoids have been shown to respond to bark beetle pheromones and host volatiles (Pettersson, 2000; Wang and Shi, 2001). Perhaps the most intensely studied predator of SPB is the checkered clerid beetle, *Thanasimus dubius* Fabricius (Franklin and Green, 1965).

### *Thanasimus dubius*

The checkered clerid beetle, (*Thanasimus dubius)*, is a natural enemy of SPB and other members of the southern pine bark beetle guild (Thatcher and Pickard, 1966). *Thanasimus dubius* is predatory during larval and adult stages, feeding on both bark beetle larvae and adults and relies on bark beetle pheromones to locate their prey (Vité and Williamson, 1970; Dixon and Payne, 1979a; Mizell et al., 1984a). Pheromones exploited by a predator are called kairomones (Brown, 1970). Researchers reported that *T. dubius* has a strong kairomonal predatory response to the SPB pheromone frontalin (Mizell et al., 1984a). Laboratory assays have shown *T. dubius* can reduce SPB's successful entry into a host by 50% (Thatcher and Pickard, 1966). For these reasons, *T.*

*dubius* is considered to be the main predator of SPB and a good source of biological control in nature (Berisford, 1980; Nebeker and Mizell, 1980).

Generally, *T. dubius* arrives shortly after southern pine beetles start colonizing a host tree (Vité and Williamson, 1970). They hunt for prey by running over the surface and inside the bark crevices. Once captured, *T. dubius* tears the prey open and feeds on the soft tissue inside the body (Frazier et al., 1981). As *T. dubius* run over the tree surface hunting prey, mating also occurs. After mating, females lay eggs in the phloem tissue of the tree (Mignot and Anderson, 1969). Once hatched, *T. dubius* larvae feed on bark beetle larvae and pupae under the bark. (Mizell and Nebeker, 1981c). Larval developmental rates average 12 days, but can take up to 22 days. Pupation occurs in the outer bark and takes about 15 to 21 days (Table 1) (Thatcher and Pickard, 1966). *Thanasimus dubius* has two to four generations per year, depending on temperature (Thatcher and Pickard, 1966). Each *T. dubius* adult can kill from 0 to 20 southern pine beetle adults/day (avg. 2.2) with total lifetime kills ranging from 30 to 336, depending on lifespan (Thatcher and Pickard, 1966).

Field studies have shown that *T. dubius* has a longer emergence time than the southern pine bark beetle guild due to the slower development rate (Dix and Franklin, 1977; Schroeder, 1999b). For this reason, it is a good management practice to leave dead or dying trees standing so all natural bark beetle predators can complete development and prey on any bark beetles that have already emerged.

*Thanasimus dubius* has also been found to feed on *Ips* spp., and ambrosia beetles (*Platypus flavicornis*) (Lawson and Morgan, 1992; Clarke and Menard, 2006). Mizell et al. (1984a) reported *T. dubius* showed a kairomonal response to the *Ips* pheromone

Ipsdienol. The study also concluded that *T. dubius* has the ability to respond to the host tree volatile alpha-pinene but not beta-pinene. Little information is available regarding synergistic effects between the pheromones and *T. dubius* behavior. *Dendroctonus terebrans* (BTB) may be a source of food for adult *T. dubius*, however there is no information suggesting *T. dubius* larvae can successfully develop on BTB brood (Reeve et al., 2009). Like SPB, BTB emits the pheromone frontalin and therefore, should not be ignored as a potential prey source for *T. dubius* (Payne et al., 1987).

There has been conflicting information as to how to describe *T. dubius*. Some researchers consider *T. dubius* a bark beetle specialist due to their feeding preference towards southern pine bark beetle guild (Riley and Goyer, 1986). Other researchers describe *T. dubius* as a generalist predator due to the predatory behavior on multiple prey species (Nebeker and Mizell, 1980; Mizell and Nebeker, 1982). Reeve et al. (2009) classify *T. dubius* as a generalist predator that specializes on SPB due to their strong preference for the SPB pheromone frontalin.

## Bark Beetle Pheromone Biology

All members of in the southern pine bark beetle guild produce and respond to their own pheromones, which may be aggregation or inhibition pheromones (Bedard et al., 1970). The aggregation of bark beetles consists of three phases: host location, colonization of a suitable host, and colonization of adjacent hosts (Renwick and Vité, 1972).

Bark beetles must first be able to locate and determine a suitable host. Researchers believe this is generally done by locating host terpenes (Renwick and Vité,

1972). Terpenes are chemicals given off by a tree and can indicate stress and weakness (Smith, 1966). Once a suitable host is located, bark beetles release aggregation pheromones that attract more beetles in an attempt to mass attack and overcome any defense mechanisms the tree may have (Vité and Pitman, 1968).

There are two recognized defense mechanisms for conifers, an induced hypersensitive response and a preformed resin system (Birgersson and Leufuen, 1988). The induced hypersensitive response targets bacterial and fungal invasion. When stimulated, the hypersensitive response causes rapid cell necrosis and a build-up of wound periderm which cuts off nutrients to the invading organism, thus not allowing it to grow (Nebeker et al., 1993). The preformed resin system allows trees to ooze sap or 'pitch out' foreign objects that penetrate the bark (Berryman, 1972). The preformed resin system is composed of oleoresin ducts within the xylem and phloem cells (Tisdale et al., 2003). Resin flow may be the most important attribute involved in tree resistance to bark beetle attack, and it is partly under genetic control (Nebeker et al., 1988; Roberds, 2003). Additionally, the resin contains monoterpenes such as limonene, alpha-pinene, beta-pinene, myrcene and delta-3-carene that have shown to be slightly toxic to adult bark beetles (Callaham, 1966; Coyne and Lott, 1976; Cates and Alexander, 1982).

The availability of oleoresin is dependent upon season and health of the tree (Smith, 1966; Christiansen et al., 1987). Healthy trees with a strong defensive system make colonization difficult for bark beetles (Hodges and Lorio, 1973; Birgersson, 1989). Pine trees considered to be healthy have a strong and abundant source of oleoresin that creates pressure for increased flow rate through the tree (Hodges et al., 1979). Aggregation pheromones do make colonization of healthy hosts possible for bark beetles

because mass beetle attacks can overwhelm the tree resin defense (Vité and Pitman, 1968). Once a host tree is successfully colonized and used as habitat for bark beetle brood, colonization of adjacent trees will be attempted (Flamm et al., 1993). The bark beetles use the same aggregation pheromones to colonize new host trees (Vité and Pitman, 1968).

Bark beetle pheromones can act as inhibition or aggregation pheromones (Bedard et al., 1970; Strom et al., 1999). Vité et al (1972) suggested there are two components of aggregation pheromones; contact pheromones, which are released after the beetle makes contact with a susceptible host, and frass pheromones, which are released after feeding on the host. The concentration of aggregation pheromones produced is largely dependent upon the contact pheromones (i.e. the number of beetles landing on the host) (Vité et al., 1972). Inhibition pheromones, also known as anti-aggregation pheromones, are often necessary later to avoid over-crowding and competition within a host (Birch and Wood, 1975; Byers and Wood, 1980).

SPB has a unique aggregation pheromone complex. Host monoterpenes with the addition of frontalin are attractive to nearby SPB and associated insects (Payne et al., 1978). Studies indicate that both male and female SPB are attracted to frontalin in equal numbers (Vité and Pitman, 1969; Hughes, 1976). Initially, researchers believed female SPB produced the only aggregation pheromone, and the males produced an anti-aggregation pheromone, endo-brevicomin (Dixon and Payne, 1979b). Sullivan et al. (2007) recently found evidence that the male beetles at the infestation site produce low quantities of the pheromone endo-brevicomin. At low concentrations, endo-brevicomin synergizes with frontalin and draws other SPB in the area to the host tree. However, at

high concentrations, endo-brevicomin disrupts attraction (i.e. anti-aggregation pheromone) (Sullivan et al., 2007). High concentrations of endo-brevicomin indicate the later stages of a mass attack and will direct beetles to infest surrounding trees in order to decrease intra-specific competition (Vité et al., 1985; Raffa, 2001; Sullivan et al., 2007; Moreno et al., 2008). Recently, endo-brevicomin has been used to trap SPB during intermediate and latent phases. Sullivan (2007) found that by using endo-brevicomin in low concentrations, SPB trap catches can be tripled when compared to the standard alpha-pinene and frontalin combination used by the U.S.D.A. Forest Service.

Compared to SPB, there has been little research conducted on *Ips* pheromones (Smith et al., 1993). Studies indicate that male *Ips* beetles initiate colonization and release the aggregation pheromones rather than females (Vité and Pitman, 1968). Ipsenol is the aggregation pheromone produced by *I. grandicollis* (Vité and Francke, 1976) and is also attractive to *I. avulsus* (Hedden et al., 1976). *Ips calligraphus* males produce ipsdienol, the main aggregation pheromone, while both the males and females produce *cis* and *trans*-verbenol upon a suitable host (Renwick and Vité, 1972). Ipsdienol is attractive to *I. avulsus* (Hedden et al., 1976) as well as other *Ips* species, and is attractive to several predators (Birch, 1978).

Miller et al. (2005) studied the attractiveness of the *Ips* pheromones, ipsenol, ipsdienol, and lanierone across the southeast and reported that *I. avulsus* is attracted to each of the three pheromones, *Ips grandicollis* is attracted to ipsenol and ipsdienol with or without lanierone and *Ips calligraphus* responded in low numbers to all traps baited with ipsdienol regardless of the other pheromones. Miller et al. (2005) concluded that the three pheromone blends work for most southeastern *Ips* species but are not sufficient for

catching *I. calligraphus* alone, and more pheromone studies need to be conducted to improve *I. calligraphus* trap catches.

Several studies have tried to gain better understanding of how the pheromone complexes affect each bark beetle species. Birch et al (1980) conducted a field test using cut *P. taeda* logs and forced various species of bark beetles to colonize the logs in an attempt to understand pheromone attraction between species. Their results showed that SPB was not attracted to logs occupied by *I. avulsus, I. grandicollis,* or *I. calligraphus*. *Ips avulsus* attraction was actually enhanced by the boring of male *I. grandicollis* and *I. calligraphus.* However, *I. calligraphus* was inhibited in areas where *I. avulsus* was present. Both *I. calligraphus* and SPB inhibited the response of *I. grandicollis*. In return, SPB attraction was inhibited by *I. grandicollis.*

These results suggest a highly interactive and complex environment in which bark beetle associates must partition their resources in order to maintain a high level of reproductive success (Birch et al., 1980). Billings (1985) concluded that high concentrations of host volatile inhibits *I. avulsus* attraction, yet enhances SPB and natural enemies. *Ips grandicollis* and *I. calligraphus* attraction seemed unaffected by the addition or removal of synthetic host volatile lures. Their results, along with those of Miller and Borden (2000), corroborated the hypothesis that host volatiles influence bark beetle pheromone attraction as well as other associated insects.

Many studies have shown bark beetle pheromones are exploited by predators. Depending on prey availability, *T. dubius* response to *Ips* pheromones may be strong or weak. During an outbreak of SPB, *T. dubius* will maintain a strong preference for the pheromone frontalin and very little preference for *Ips* pheromones (Billings and

Cameron, 1984). Research has shown in latent areas (areas with no detectible SPB outbreaks and very few or no beetles are captured in spring and fall trapping surveys), *T. dubius* shows an increase in preference for *Ips* pheromones (Billings and Cameron, 1984). However, it is still unclear how *T dubius* preference varies between intermediate phase areas of SPB versus the latent areas. This information can help us determine to what extent *T. dubius* is responsible for contributing to unprecedented declining SPB populations now being reported in many parts of the historic SPB range.

## PCR Analysis of Predatory Arthropod Gut Contents

Molecular analysis of predatory gut contents has the ability to determine how efficient a natural enemy complex is, as well as determine what species of prey is preferred. Field analysis of predation by generalist predators can be difficult to obtain, particularly for species that feed in cryptic habitats and ingest soft body parts that may not be recognizable post consumption (Symondson, 2002). For this reason, researchers have developed molecular approaches for the identification of prey remains in predator guts.

Zaidi et al (1999) reported the first successful application of DNA molecular markers (PCR techniques) to identify prey remains in predatory arthropod gut contents. In a lab setting, carabid beetles were allowed to consume up to 6 mosquitoes during a two hour period following a five day starvation period. Eight beetles were killed at 0, 2, 5, 10, and 20 hours post consumption. Successful PCR amplification of mosquito DNA was observed from 0 to 20 hours even if the beetle only consumed one mosquito. PCR proved

to be highly sensitive to small amounts of DNA and showed promise for use in field settings.

Agusti et al (2003) reported the first use of DNA based techniques such as PCR on field collected predators. Nucleotide sequences of the collembolan mitochondrial cytochrome oxidase 1 (CO1) genes were obtained and used to determine prey preference of spiders (Agusti et al., 2003).

Another study by Traugott et al. (2008) investigated the difference in sensitivity between singleplex and multiplex PCR. Carabid beetles were fed aphids that had been parasitized. After extracting DNA from the whole beetle, PCR was carried out to amplify parasitized prey DNA. They concluded that singleplex PCR can detect minute concentrations of DNA over longer periods of time than multiplex PCR.

Muilenburg et al. (2008) used the same basic technique to confirm predation of red oak borer eggs (*Enaphalodes rufulus* Haldeman) by several different ant species. However, Muilenburg amplified a portion of the ant's 16S RNA gene rather than using mitochondrial genes (Muilenburg et al., 2008). The lab study showed that *E. rufulus* DNA persisted in the ant's gut for at least 24 hours, and the field study demonstrated that ant predation of *Enaphalodes rufulus* eggs does occur. Various DNA based techniques such as PCR are becoming increasingly useful for field researchers to understand trophic interactions.

*Thanasimus dubius* is known as a generalist predator on bark beetles with a preference for the SPB pheromone, frontalin (Reeve, 1997). However, there is little evidence regarding how preferential these predators remain to SPB once they land on a tree infested with multiple bark beetle species. Through the use of PCR and DNA

analysis of the gut contents, we can gain a better understanding of predatory preference and the impact *T. dubius* has on the southern pine bark beetle guild. PCR will also allow researchers to determine how pheromone preference of *T. dubius* relates to prey preference, a concept that currently has little information and understanding.

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# CHAPTER II

# PHEROMONE RESPONSE OF *THANASIMUS DUBIUS* BETWEEN LATENT AND INTERMEDIATE SOUTHERN PINE BEETLE PHASES


## Abstract

The checkered clerid beetle, (*Thanasimus dubius* Fabricius) is considered a specialist predator of the southern pine beetle, (*Dendroctonus frontalis* Zimmerman), because of a preference for southern pine beetle pheromones. However, *T. dubius* will feed opportunistically on *Ips* engraver beetles and other insects. Past research indicates that *T. dubius*, as well as other coleopteran predators, have exhibited learning capabilities that might mediate switching behavior between multiple prey species, depending on the relative abundance of prey species. This study examines *T. dubius* preference for various bark beetle pheromones between areas with SPB populations available and areas that have no apparent SPB populations. We hypothesized that *T. dubius* predatory behavior might change depending on the relative abundance of southern pine beetle and *Ips* beetles. A pheromone trapping study was set up in two areas: 1) Latent areas where SPB populations are absent; and 2) intermediate areas where SPB populations are moderate. Two treatments consisted of Lindgren funnel traps baited with either SPB pheromone or *Ips* pheromone lures. Four traps of each treatment were placed in two separate national forests in Texas, Louisiana, and Mississippi, and one national forest in Alabama (for a

total of 56 traps). Traps contents were collected weekly for a duration of four weeks. The interaction between lure type (treatment) and region (latent vs. intermediate) did not significantly affect the number of *T. dubius* captured per trap per day, indicating no evidence of switching behavior.

## Introduction

The southern pine beetle (SPB), *Dendroctonus frontalis* Zimmerman, is an aggressive pest of pine trees in the southeast U.S (Hopkins, 1921). Its hosts consist mainly of shortleaf pine, (*Pinus echinata* Mill), loblolly pine, (*P. taeda* L.), and Virginia pine, (*P. virginiana* Mill) (Payne, 1980). SPB is considered to be aggressive due to their ability to mass attack and kill healthy pine trees (Hopkins, 1921). The beetles communicate by chemical messages called pheromones (Payne, 1974). The main pheromone that causes the beetle to aggregate is called frontalin and is produced by the female beetles only (Hughes, 1976).

An infestation begins in the spring or fall when a female SPB, known as the pioneer beetle, successfully locates and infests a host tree (Payne, 1974). She then releases the pheromone frontalin, wich attracts more male and female SPB to the host tree (Coster et al., 1977). Males produce another pheromone, endo-brevicomin which synergizes the aggregation effect on frontalin. However, as more male beetles populate the host tree, the concentration of endo-brevicomin gets higher and begins to deter SPB from that host tree, sending them to an adjacent host tree (Sullivan et al., 2007). If suitable host material is available, the next generation of beetles will emerge and infest more trees (Lorio and Hodges, 1974). As overlapping generations of beetles occur, a

noticeable spot head will display the direction the infestation is spreading. (Fitzgerald et al., 1994).

The U.S.D.A. Forest Service monitors SPB (*Dendroctonus frontalis* Zimmerman, SPB) populations each spring and fall by placing Lindgren funnel traps baited with pheromone lures in each state in southeastern U.S. (Billings, 1994). Over the past decade, SPB populations in Arkansas, Mississippi, Louisiana, Texas, and Alabama have shown a declining trend (Billings, 2010). In Texas, Louisiana, and Arkansas nearly zero SPB have been caught in monitoring traps in at least 4 years, and no SPB outbreaks have been recorded in the last 10 years (Billings, 2009). However, although there have been few SPB infestations in Mississippi and Alabama, funnel traps still catch SPB in low numbers. These SPB population levels can be categorized into three different phases; latent, intermediate and outbreak (Clarke, 2009). The areas in which SPB populations are below detectable levels are referred to as latent phase areas. SPB populations in Texas, Louisiana, and Arkansas are currently consistent with the definition of latent phase due to no captures of SPB in monitoring traps and the total lack of recent infestations. The intermediate phase areas are regions where SPB have been caught in traps but no outbreaks have been recorded. Currently, areas of intermediate SPB population phase are located in Mississippi, Alabama, Georgia, and most other southern states east of the Mississippi River. The outbreak phase is characterized by extremely high SPB population levels and active infestations that result in tree mortality. Small infestations can occur in intermediate phase areas however; they are not considered outbreaks until there is at least one multi tree infestation per 1000 acres of land (Billings, 1979).

While SPB have continued to be undetectable in many areas, *Ips* populations remain strong. Excessive drought conditions and hurricanes are partially responsible for the increase *Ips* activity (Negrón et al., 2009). Wind disturbance can increase *Ips* activity by damaging roots and branches (Fredericksen et al., 1995). Drought and other stressors decrease pine tree resistance mechanisms, thus making them more susceptible to bark beetle attack (Paine et al., 1985).

Historically, SPB populations have been cyclical (every 6-9 years) in nature (Price and Doggett, 1978). It is unclear why SPB populations have declined over the past decade. Silvicultural practices, such as commercial thinning and proper site planting, are more common in modern forestry and have possibly contributed to the observed decline (Vité, 1972; Belanger, 1980; 1984). For example, landowners can now take advantage of cost share programs that help make timber management possible for the small landowner (Mayfield et al., 2006). These practices will result in more vigorous stands of trees, making them more resistant to attack (Brown et al., 1987). However there is also little beetle activity in pine stands within national forests that rarely get thinned or managed. Therefore, timber management is probably not a main cause of SPB decline.

Bark beetle predators are another possible reason for the decline in SPB populations. The predator *Thanasimus dubius* has a voracious appetite and utilizes bark beetle pheromones to locate its prey (Billings and Cameron, 1984). Several studies have shown that *T. dubius* displays a strong preference for the SPB pheromone frontalin, yet they also have the ability to exploit *Ips* pheromone components to locate prey sources (Mizell et al., 1984a). It is possible that these predators play a role in maintaining low SPB population levels due to their strong preference for frontalin (Reeve, 1997).

Switching behavior has been proposed in *T. dubius* (Murdoch and Oaten, 1975; Reeve et al., 2009). Switching behavior is defined as a density dependent behavior and implies that a predator will disproportionally prefer the most abundant prey (Murdoch, 1969a). Such behavior is thought to stabilize prey populations by allowing the prey species with the lowest population to recover (Murdoch and Oaten, 1975). This theory predicts that when SPB are at low populations, *T. dubius* should prefer pheromones of the next most abundant prey, such as *Ips* bark beetles. Murdoch and Oaten (1975) showed evidence that prey preference may vary greatly across individuals of the same predator species. This indicates that even though an individual predator may display switching behavior, it is not an indication that the entire predator population is switching. Rather, the individual could be display associative learning capabilities that result in switching behavior (Murdoch, 1969b; Hassell, 1978). These concepts are underdeveloped in *T. dubius* behavior and association with prey.

Two studies have looked at *T. dubius* behavior change between outbreak and latent phases (Billings and Cameron, 1984; Reeve et al., 2009). Billings and Cameron (1984) demonstrated a slight increase in preference for *Ips* pheromones between an SPB outbreak site and an endemic site. Reeve et al. (2009) found that *T. dubius* was attracted to traps baited with frontalin in areas where SPB have never occurred. Their study implied that *T. dubius* are hard-wired to seek prey when frontalin is encountered. More research needs to be conducted to clear up the gaps in *T. dubius* responses to various pheromone complexes. Statistical analysis of baited trap catches in latent versus intermediate phases can demonstrate an increase or change in preference for *Ips* pheromones.

The objective of the present study was to determine if *T. dubius* populations in regions with no detectable levels of SPB populations (latent phase areas) display any change in preference for SPB and *Ips* pheromones when compared to *T. dubius* populations in regions with moderate SPB populations (intermediate phase areas). We hypothesized that *T. dubius* would exhibit different preferences for SPB and *Ips* pheromones depending on the prevalence of SPB in a given region.

## Materials and Methods

The Tombigbee National Forest (NF) and Holly Springs NF, both in Mississippi, and the Talladega NF in Alabama served as the areas in an intermediate SPB phase. Since Texas and Louisiana's SPB populations are considered to be in the latent phase (Billings, 2010), the Winn and Catahoula Ranger Districts (Kisatchie NF) in Louisiana and the Sabine and Angelina NF served as the latent phase treatment areas for this study. During the fall of 2009 and spring 2010, standard 12 unit Lindgren funnel traps baited with either *Ips* or SPB lures were placed in each NF or ranger district, totaling 56 traps each season.

Intermediate phase SPB traps were placed in hardwood stands adjoining a pine stand in an attempt to reduce the conditions that would favor a potential SPB outbreak. *Ips* traps were placed in mature loblolly pine stands. Traps were hung from tree branches approximately seven to eight feet above ground in arbitrary locations throughout study sites. All traps were spaced at least 400 meters apart. Insects were collected from each trap on a weekly basis from November 3 to November 25, 2009 and May 11 to June 1, 2010. Specimens were preserved by freezing until identification could be completed. Numbers of identified specimens were counted and recorded for data analysis.

Two trap treatments were used in each location. Treatment 1 consisted of traps baited with components of the *Ips* pheromone complex; lanierone (2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one), racemic ipsenol (2-methyl-6-methylene-7-octen-4-ol), racemic ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol), and a host volatile. The host volatile was an ultra high release (UHR) alpha and beta pinene combination; 70:30 blend of 50% ee (1R,5R)-2,6,6-Trimethyl bicyclo[3.1.1]hept-2-ene : 90% ee (1S)-6,6-Dimethyl-2-methylenebicyclo[3.1.1]heptanes. All three *Ips* lures and the host volatile lure were placed in combination onto each trap. Treatment 2 consisted of traps baited with components of the SPB pheromone complex; racemic frontalin (1,5-dimethyl-6,8-dioxabicyclo[3.2.1] octane), racemic endo-brevicomin (endo-7-Ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane), and the same UHR alpha/beta pinene blend as above. The endo-brevicomin lure was placed 3 m from the trap in an attempt to lower the concentration of the lure, making it an aggregation pheromone rather than anti-aggregation pheromone (Sullivan et al., 2007). See Table 2.1 for elution rates of all pheromones used.

Table 2.1. *Ips* and SPB Lures and their associated elution rates

| ***Ips* lures** | Ipsenol | Ipsdienol | Lanierone | Host Lure |
|---|---|---|---|---|
| | 500 µg/day | 250 µg/day | 25 µg/day | 1-3 g/day |
| **SPB Lures** | Frontalin | Endo-brevicomin | Host Lure | |
| | 6-8 mg/day | 100 µg/day | 1-3 g/day | |

Due to miscommunication, the protocol for the fall 2009 trapping season varied between the latent and intermediate phases. Latent phase SPB treatments were placed in pine stands rather than hardwood stands. Also the *Ips* treatments in the latent phase traps did not contain a host volatile lure. This difference made it necessary to analyze the 2009 season data separate from the 2010 season.

Raw data from each trap were standardized by converting to the number of insect species caught /trap/day (Billings, 1994). Data analysis was performed using the nonparametric Mann Whitney-U test and a 2x2 Factorial Analysis of Variance in SPSS (IBM, 2010) with a significance level of 0.05.

## Results

Shapiro-Wilk test for normality revealed that the fall 2009 *T. dubius* trap catch data was not normally distributed ($p<0.0001$). For this reason, the non-parametric Mann Whitney-U test was used to rank the means of trap catches between phases, seasons and trap type. A non-parametric test does not make assumptions about the probability distribution of the variables, nor does it require random samples. The Shapiro-Wilk test for normality showed that the spring 2010 data was normally distributed ($p=0.140$), and therefore the use of a 2x2 factorial analysis of variance (ANOVA) was appropriate.

### Non-parametric

A significant difference ($p=0.011$) between the years was observed when trap type and phase were pooled together. The total number of *T. dubius* caught for each year decreased 71% from 2009 to 2010 across all locations (Fig. 2.1).

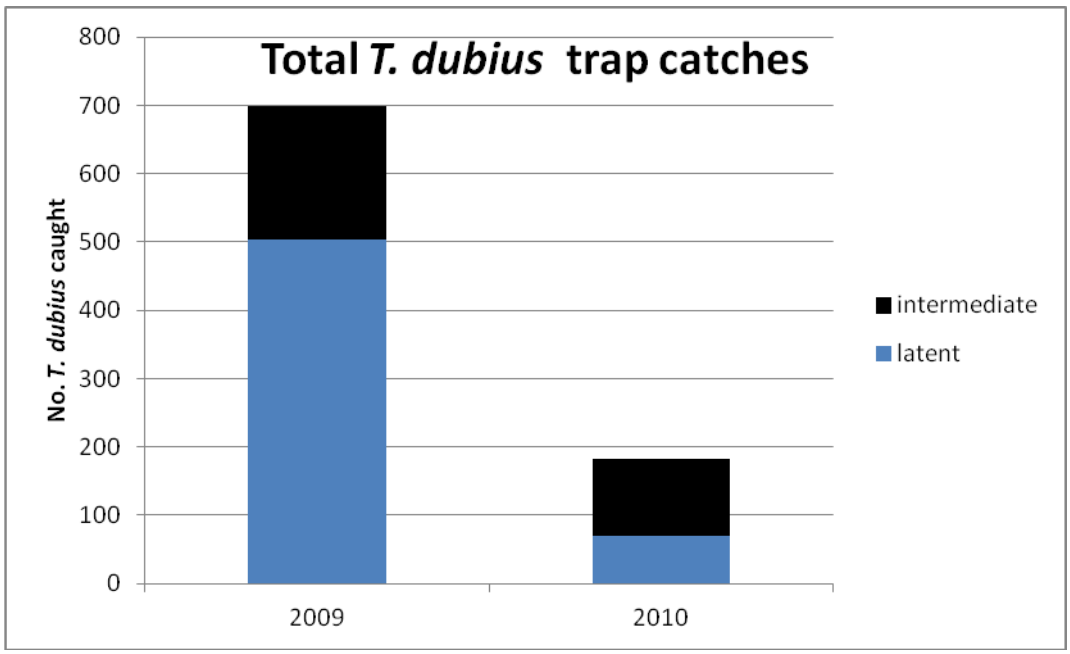


Figure 2.1. A comparison of the total *T. dubius* trap catches by year and phase. The figure shows a higher *T. dubius* population in 2009 than 2010 for both phases.

**Fall 2009**

Mann Whitney-U test determined that intermediate phase *Ips* traps caught a significantly higher number of *T. dubius* per trap per day than the latent phase traps, $U = 6.50$, $p < 0.0001$. On average, *Ips* baited traps in the intermediate phase caught twice as many *T. dubius* as *Ips* baited traps in the latent phase (Fig. 2.2 and 2.3). SPB baited traps in the latent phase caught almost 3.5 times more *T. dubius* than the SPB traps in the intermediate phase. This resulted in a significant difference between *T. dubius* response to SPB traps in each phase, $U = 164.0$, $p = .002$ (Fig. 2.3).

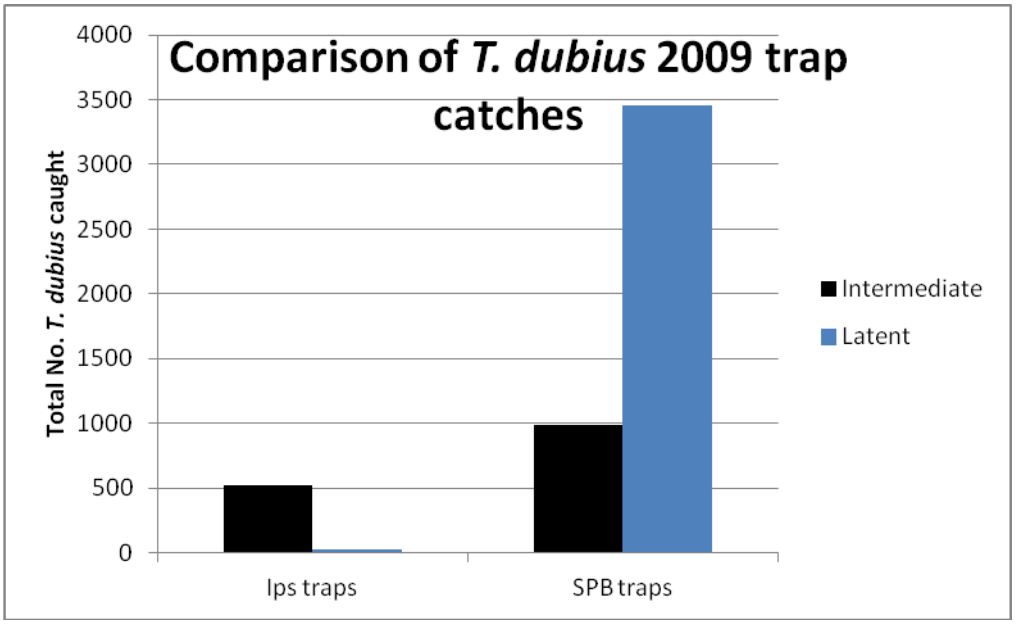


Figure 2.2. This graph displays the total number of *T. dubius* caught in *Ips* and SPB traps in each phase for 2009.Intermediate phase *Ips* traps caught more *T. dubius* than latent phase *Ips* traps. However the latent phase SPB traps caught more *T. dubius* than either SPB or *Ips* traps in the intermediate phase.

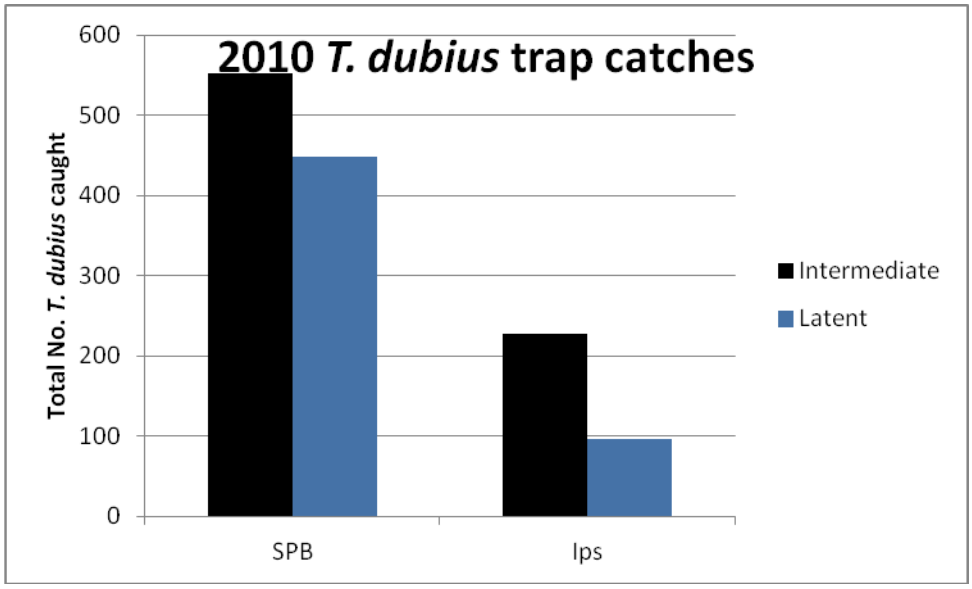


Figure 2.3. This graph shows the total number of *T. dubius* caught in *Ips* and SPB traps in each phase for 2010. The intermediate phase caught more *T. dubius* than latent phase in both trap types.

**Spring 2010**

For the spring 2010 trapping season, intermediate phase traps baited with *Ips* lures caught significantly more *T. dubius* /trap/day than latent phase *Ips* traps $U = 3.00$, $p < 0.001$. For SPB baited traps, there was no significant difference in the median *T. dubius* caught per trap per day between the two phases $U = 79.0$, $p = 0.430$. Latent phase SPB traps caught a total of 449 *T. dubius* while Intermediate SPB traps caught 552, however, this difference was not significantly different (Fig. 2.3).

**Factorial ANOVA**

A 2x2 factorial analysis of variance was conducted on the 2010 data only. 2009 data was not used due to the deviations from protocol between the two phases. Analysis of variance results correlated with the non-parametric results. Intermediate phase traps baited with *Ips* pheromones caught significantly more *T. dubius* than latent phase traps $F(1,26)=29.529$, $MSe=1.243$, $p<0.0001$. The mean number of *T. dubius* caught per trap per day in the intermediate phase was 2.7, while the latent phase was 0.39. The intermediate phase SPB trap treatment caught 50% more *T. dubius* than the latent phase SPB treatment. Due to high variability, this result was not significant $F(1,26)=1.842$, $MS_e=24.427$, $p=0.186$. With trap types pooled, the 2x2 ANOVA revealed a main effect of phase, $F(1,52)=6.349$, $MS_e=12.835$, $p=0.015$ and a main effect of treatment, $F(1,52)=14.985$, $p<0.0001$. However; the ANOVA revealed no interaction of treatment and phase, $F(1,52)=0.016$, $p=0.898$. We hypothesized that the effect of *Ips* versus SPB treatment is the same for latent phase areas as it is for intermediate phase areas. There

was insufficient evidence to conclude that the effect of trap treatment is different for the intermediate and latent phase areas.

Since *T. dubius* responded stronger to *Ips* pheromones in the intermediate phase, an ANOVA was conducted to see how *I. avulsus* and *I. grandicollis* populations varied between the two phases. There were no significant differences between the number of *I. avulsus* trapped in latent and intermediate phases of SPB, $F(1,26)=0.810$, $MS_e=3269.098$, $p=0.377$. The intermediate phase did catch significantly more *I. grandicollis* than the latent phase $F(1,26)=19.285$, $MS_e=58.179$, $p<0.0001$.

## Discussion

Overall, our results are consistent with the existing literature. The effect of trap treatment was not different between the two phases. *Thanasimus dubius* responded more strongly to SPB pheromones than *Ips* pheromones in both phases (Vité and Williamson, 1970; Dixon and Payne, 1980; Billings and Cameron, 1984; Mizell et al., 1984a).

There was a significant difference between trapping seasons that could be due to several variables. First, the number of *T. dubius* captured in fall 2009 was _% higher than spring 2010 trapping season. The U.S.D.A. Forest Service monitoring traps have displayed a similar decline in *T. dubius* populations across the entire southern region (Billings, 2009). Both the traps for this research project, as well as other U.S.D.A. Forest Service traps caught more *T. dubius* during the fall season than the spring season. Across the entire south, the U.S.D.A. Forest Service reported a 17% decline in the average number of *T. dubius* caught in 2010 from 2009 (Billings, 2010). On average, monitoring traps in Texas caught 11.6 *T. dubius* per trap per day, the highest count of all states in the

south (Billings, 2010). This study showed a mean number of 4 *T. dubius* per trap per day, lower than the U.S.D.A. Forest Service traps. This difference could be due to the fact that the traps were placed out later in the spring, possibly toward the end of peak bark beetle flight period, which *T. dubius* has been shown to follow (Mizell, 1980; Reeve et al., 1980). The spring flight or dispersal period for bark beetles is generally between March through May (Gara, 1967). A standard protocol for spring trapping season is to place the traps out when the dogwood trees begin to bloom (Billings, 1994).

The most concerning source of variability was the change in protocol between the two seasons. Fall 2009 latent phase *Ips* traps did not contain a host volatile, to which *T. dubius* will respond (Dixon and Payne, 1980; Mizell et al., 1984b). Without the host volatile, *T. dubius* were less likely to be attracted to the traps baited with *Ips* lures. Also during this trapping season latent phase SPB traps were placed in pine stands rather than hardwood stands. The intermediate traps in hardwood stands could have possibly caught fewer *T. dubius* than the latent phase traps. It is difficult to discern which variables were responsible for the significant difference in trap catches between the seasons, but it is likely a combination of all variables mentioned played some role.

**Fall 2009**

There were significant differences in *T. dubius* catches for both trap types between intermediate and latent phase fall 2009 traps. SPB traps in latent phase areas caught significantly ($p=0.002$) more *T. dubius* than the intermediate traps. This is likely due to a much higher *T. dubius* population in the latent phase areas than intermediate.

The Texas Forest Service reported very high populations of *T. dubius* until 2010 (Billings, 2010).

In 2009 the intermediate phase *Ips* traps caught significantly ($p>0.0001$) more *T. dubius* than the latent phase *Ips* traps. This difference could be due to the fact that latent phase traps did not contain a host volatile, which *T. dubius* are known to show a slight response to (Mizell et al., 1984a; Costa and Reeve, 2011). Intermediate phase traps baited with *Ips* treatment caught approximately five *T. dubius* per trap per day compared to latent phase traps which caught zero *T. dubius* during a four week period. This significant result could be due to the addition or removal of the host volatile. More research is needed to determine if *T. dubius* have more than a 'slight' response to host volatiles.

**Spring 2010**

During spring of 2010, both the Mann Whitney-U test and ANOVA revealed no significant differences the numbers of *T. dubius* caught between latent and intermediate phases for traps baited with SPB lures, $U = 79.0$, $p = 0.430$, $F(1,26)=1.842$, $MS_e=24.427$, $p=0.186$. *Thanasimus dubius* demonstrated a strong association with SPB pheromones regardless of phase. These results are similar to research by Reeve et al. (2009) who also saw a strong association with SPB pheromones across a large geographical range. The spring 2010 results did not correspond with fall 2009 results. In the fall 2009 season, recall that the latent phase SPB traps caught significantly higher numbers of *T. dubius* than the intermediate phase, most likely from the extremely high *T. dubius* populations in Texas. The change in result between the seasons was probably due to inconsistent trapping methods between the phases.

It is interesting to note that intermediate *Ips* traps again caught significantly more *T. dubius* than latent phase *Ips* traps. We observed a significantly stronger interaction between *T. dubius* and *Ips* treatments in the intermediate phase than latent phase of SPB. A laboratory wind tunnel experiment was used to study *T. dubius* response to various bark beetle pheromones, in which *T. dubius* has showed a response to ipsenol, a pheromone released by *I. grandicollis* (Costa and Reeve, 2011). Costa and Reeve (2011) saw no significant differences between *T. dubius* interaction with ipsenol versus frontalin in the upwind flight assay. Intermediate phase traps baited with *Ips* components caught significantly more *I. grandicollis* than the latent phase, which could indicate a higher *I. grandicollis* population. A higher *I. grandicollis* population may have triggered an increased response from *T. dubius* to ipsdienol, located on the *Ips* treatment. However, *I. grandicollis* mean trap catches were not higher than *D. frontalis* mean trap catches. Mean trap catch for *D. frontalis* was 76.67/trap/day while *I. grandicollis* was 17.434/trap/day. These results do not support the assumption that *T. dubius* preys preferentially on the most abundant host or has a switching behavior. Based on our results, *T. dubius* has the potential to play a large role in the collapse of and the lack in rebuild of SPB populations in latent phase areas due to their strong preference for the pheromone frontalin.

This study showed a different trend from previous literature (Billings and Reeve 1984). A significant increase in *T. dubius* attraction to *Ips* pheromones was expected in the latent region due to the apparent absence of SPB. If switching behavior was occurring, *T. dubius* should have responded more strongly to *Ips* pheromones since that particular prey source is much more prevalent than SPB. It is also possible that the behavior switch had already occurred prior to this research. Since Billings have Reeve

observed a switch in behavior from outbreak conditions to latent, then they may be no more behavior switching between latent and intermediate phase areas.

*Thanasimus dubius* pheromone preference is most likely a method of finding an abundant source of prey rather than finding the most appealing type. Laboratory studies have found that *T. dubius* prefers small prey, such as *I. avulsus* and feed readily on both *I. grandicollis* and *D. frontalis* (Mizell and Nebeker, 1981b; 1982). More research needs to be conducted as to how pheromones relate to prey preference in order to link these two concepts together.

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# CHAPTER III

# PCR ANALYSIS OF GUT CONTENTS IN CHECKERED CLERID BEETLES (*THANASIMUS DUBIUS* F.)


## Abstract

The checkered clerid beetle, *Thanasimus dubius* (Fabricius), is a bark beetle predator that displays a preference for frontalin, a major pheromone component of the southern pine beetle (SPB), *Dendroctonus frontalis* Zimmerman. Researchers have assumed that *T. dubius* preference for frontalin equates to a feeding preference for the SPB over other closely related bark beetle prey species. However, little is known about whether *T. dubius* preferentially feeds on SPB over other bark beetle species when all are present in the same tree. In this study, we developed a molecular technique that utilizes polymerase chain reaction (PCR) to aid in determining if *T. dubius* maintains a feeding preference for SPB in the presence of multiple bark beetle prey species. Primers have been designed and optimized for *T. dubius* and common prey members of the southern pine bark beetle guild (*D. frontalis, D. terebrans, Ips avulsus, I. grandicollis,* and *I. calligraphus*) to separate DNA markers for each species. Subsequent PCR analysis of *T. dubius* beetle gut contents will allow researchers to determine feeding preference when the predator is presented with a choice between multiple bark beetle species in varying

degrees of abundance. Completion of this PCR protocol will allow future tests in the field for predatory preference of *T. dubius* among the five southern pine bark beetles.

## Introduction

Conventional methods of identifying arthropod prey species from predator gut contents consist of visual identification of prey remains and waste products (Dennison and Hodkinson, 1983). Utilizing gut dissections of insect predators to understand predator-prey relationships can be difficult when attempting to determine prey species (Feller et al., 1985 ). Some prey remains may be too small or masticated to identify even microscopically. Molecular methods such as polymerase chain reaction (PCR) are useful for identifying microscopic prey fragments from the gut or feces of animals (Symondson, 2002). PCR uses short deoxynucleotides called primers that bind to a portion of the subject's DNA, which along with a thermostable DNA polymerase, allows for the amplification of a defined region of DNA (Saiki et al., 1988). After PCR amplification, the resulting products can be electrophoresed through an agarose gel and the DNA band(s) viewed under ultra-violet light (Simon-Reuss et al., 1964; King et al., 2008).

Selective primers can be used to determine specific prey interactions (Jarman et al., 2004). When this method is utilized in field studies, primers must be highly specific due to the diversity of prey in field settings (Juen and Traugott, 2005). Specific primers are needed to prevent cross-amplification of the incorrect template, thus creating false positive results (Juen et al., 2003). False-negative results can also be obtained due to PCR inhibitory substances in the DNA extract such as residual ethanol, plant derived phenol compounds and various polysaccharides (Monteiro et al., 1997; Juen and Traugott, 2006).

Optimization and extra purification steps may be necessary to obtain high quality DNA that does not result in false negatives. Another potential disadvantage to using this technique is that DNA degrades within the insect gut over a period of time due to the enzymatic processes in the gut (Hoogendoorn and Heimpel, 2001). DNA that is degraded may not be detected by primers during PCR (Symondson, 2002). Despite these disadvantages, PCR analysis allows research entomologists to further their identification and understanding of complex arthropod interactions (Symondson, 2002; Juen and Traugott, 2005).

One intensely studied predator-prey interaction is that of the checkered clerid beetle (*Thanasimus dubius* Fabricius) with multiple bark beetle species, in particular, the southern pine beetle (SPB), (*Dendroctonus frontalins* Zimmerman) (Riley and Goyer, 1986). *Thanasimus dubius* is known to be predaceous on multiple bark beetle species. However, it is considered to primarily target the SPB due to its preference for the SPB pheromone, frontalin (Vité and Williamson, 1970; Reeve et al., 2009). The predator will also feed on other members of the southern pine bark beetle guild such as *Ips avulsus* Eichoff*, I. grandicollis* Eichoff*, I. calligraphus* German and *D. terebrans* Olivier. The five members of the southern pine bark beetle guild are important pests on pine trees in the southern U.S., and all species utilize their own pheromones to locate and infest host trees. Most studies have focused on *T. dubius* preference for various bark beetle pheromones (Billings and Cameron, 1984; Mizell et al., 1984b; Erbilgin, 2000). However, it remains unclear if *T. dubius* maintains a strong feeding preference for SPB once it lands on a tree with multiple prey species present, or if *T. dubius* will feed opportunistically on any bark beetle which it encounters.

If *T. dubius* preference for SPB pheromones does indeed translate into a feeding preference for SPB in trees infested with multiple bark beetle species, *T. dubius* impacts on SPB populations may be more dramatic than previously realized. Additionally, *T. dubius* feeding preference for SPB when presented with a choice between multiple bark beetle species could partially explain recent unprecedentedly low populations of SPB throughout the southeastern U.S. Many have hypothesized that *T. dubius* plays an important role in regulating SPB populations during outbreak conditions (Thatcher and Pickard, 1966; Vité and Williamson, 1970; Frazier et al., 1981; Reeve, 1997). However, field studies have not been able to quantify how significant the impact of *T. dubius* is on SPB, especially during low SPB populations (Moore, 1972a; Turnbow, 1976; Nebeker and Mizell, 1980).

Lab studies have shown that *T. dubius* will feed readily on *Ips avulsus, I. grandicollis,* and *I. calligraphus* (Riley and Goyer, 1988; Lawson and Morgan, 1992; Reeve, 2003). Laboratory based behavioral bioassays provide interesting insights into predatory behavior in an artificial setting: however, behaviors observed in a laboratory setting are not always a realistic portrayal of predatory behavior in nature (Mizell and Nebeker, 1982; Reeve et al., 1995). To physically observe *T. dubius* while feeding in nature is difficult due to the size and mobility of the beetle. Given that *T. dubius* feeds on the soft tissue inside bark beetles, there are no identifiable remains such as bark beetle elytra inside their gut. Because of this, researchers have had a difficult time quantifying the impact *T. dubius* has on bark beetles in field settings. This lack of knowledge of *T. dubius* behavior is one reason some researchers do not consider *T. dubius* to be a significant source of biological control for SPB (Nebeker and Mizell, 1980; Berryman,

1982). It is questionable if *T. dubius* remains strictly preferential to feeding on SPB in the presence of other bark beetle species.

The overall objective of this study was to develop primers using published gene sequences that specifically detect DNA of members of the southern pine bark beetle guild within the gut contents of *T. dubius*. This method will allow researchers to more accurately estimate the impact this predator has on the southern pine bark beetle guild.

## Methods

### Primer design

Species-specific primers were designed for *D. frontalis, D. terebrans, I. avulsus, I. grandicollis, I. calligraphus* and *T. dubius* using the Primer3 program (Rozen and Skaletsky, 2000). Mitochondrial cytochrome oxidase I (COI) sequences were obtained for each species from National Center for Biotechnology Information (Benson et al., 2006). Primers for *T. dubius* were selected from haplotype 3 and 21 gene sequences as *T. dubius* in the southeast region are usually of these haplotypes (Schrey et al., 2005). All primers developed were 19 to 23 nucleotides in length and had a GC content between 40 and 57%. The NETPRIMER program (Premier Biosoft International, Palo Alto, CA, USA) was used to test for primer-dimer activity. Primers were custom synthesized from Eurofins MWG Operon (Huntsville, AL). Primers were resuspended at 200µM in 1 M Tris-HCl (pH 7.75) and diluted to a concentration of 2 µM prior to use. See table 3.1 for species specific-primer sequences.

Table 3.1. Primer sets developed for each species from mitochondrial CO1 gene sequences published on National Center for Biotechnology. Each primer set with specifically detect the target species DNA for future gut content analysis.

| Species | Forward Primer 5` to 3` | Reverse Primer 5` to 3` |
|---|---|---|
| *Thanasimus dubius* | ctgctcctaaaattgaagatacacc | gagcccctgatatagcattcc |
| *Dendroctonus frontalis* | gaaggttcttcagtagattgtgc | taattgcacctgctaatactgg |
| *Ips avulsus* | gactcttggggtttgtagtttga | agatttgtgctccatgaaagg |
| *Ips grandicollis* | ccactatttacaggacttacac | catcagggtaatctgaataacg |
| *Ips calligraphus* | gcttacttggtttcgtagtatg | gcaataatagcaaagactgc |
| *Dendroctonus terebrans* | gagcctatttcacatctgc | ggataatcagagtaacgacg |

**Sample preparation and PCR**

All beetles used for this study were trapped live using baited Lindgren funnel traps on the John Starr Memorial Forest in Oktibbeha County, Mississippi. *Thanasimus dubius* were held at room temperature and starved for a total of 24 hours prior to killing to eliminate any potential bark beetle DNA in the gut. All beetles were killed and stored in 100% ethanol at -80˚C until DNA isolation was performed. Whole beetles were ground up after a quick freeze in liquid nitrogen. DNA isolation was carried out using the DNeasy tissue protocol as outlined by the manufacturer (Qiagen, Valencia, CA). DNA samples were stored at -80˚C after isolation.

PCR amplifications were set up in 10 µl reactions with each reaction containing 200 µM dNTPs, 0.5 µM of each primer, 1X GC buffer, 2 µM $MgCl_2$, 0.2 units of Phusion

High Fidelity polymerase (Finnzymes) and 1-300 ng of DNA, depending on the species of beetle. See Table 2 for the range of DNA concentration for each beetle species.

Amplifications were performed in a MyCycler thermocycler (Bio-Rad) with an initial denaturation of 98º C for 30 seconds, 30 cycles of 98º C for 7 seconds, the calculated Tm for 15 seconds, 72º C for 18 seconds, and a final extension period of 72º C for 7 minutes.

Table 3.2. Average DNA concentrations of four individuals obtained by a Nano-drop for each whole beetle species after DNA extraction.

| Species | Average DNA concentration |
|---|---|
| *Thanasimus dubius* | 333-368 ng/µl |
| *Ips avulsus* | 18-37 ng/µl |
| *Ips grandicollis* | 35-59 ng/µl |
| *Ips calligraphus* | 90-129 ng/µl |
| *Dendroctonus frontalis* | 25-64 ng/µl |
| *Dendroctonus terebrans* | 228-398 ng/µl |

**Primer specificity and sensitivity**

Individual species primer sets were tested for specificity against target species, host species, and other beetle species. Specificity tests were conducted on each primer set to ensure the absence of cross species amplification and false positives. At least two

individuals of each species were tested in each PCR. All PCRs included DNA of the target species for a positive control and nano-pure water served as the negative control.

DNA from each beetle was serially diluted in $H_20$ and sensitivity tests were conducted at 1:10, 1:100, 1:1,000, 1:10,000 and 1:100,000 dilutions or until the primers failed to amplify DNA. DNA samples from four individuals of each species were standardized to the same DNA concentration and then serially diluted.

**Gut content PCR**

Sixteen live *T. dubius* caught in lindgren funnel traps were starved for a minimum of 24 hours. Each *T. dubius* was allowed to feed on one live *D. frontalis*. Four total *T. dubius* were killed in pre-chilled 100% ethanol at approximately one and two hours post consumption. Pre-chilled ethanol immediately chills the beetle to prevent regurgitation. Beetles were then placed in storage at -80ºC.

Prior to DNA extraction, *T. dubius* were slowly thawed from -80ºC to approximately 4 ºC over a period of four days. This slow thaw period helped minimize DNA damage and allowed the ethanol to push any water out of the beetles. Elytra, wings, head, and legs were cut off and the remaining thorax and abdomen was placed in 1.5 ml tube. Samples were prepared and DNA was extracted as described above.

After extraction the purified DNA was stored at -80ºC. PCR amplification of the gut contents was carried out as described above. Two positive controls were included for all experiments. The *T. dubius* primer set served as the first positive control to ensure that the DNA isolation protocol was successful. The second positive control included DNA

from a *D. frontalis* beetle to ensure the master mix for the *D. frontalis* primer set was accurate.

### Agarose Gel Electrophoresis

Electrophoresis was used to view PCR on a 2% agarose gel. Electrophoresis was conducted at 110 volts for approximately one hour in 10mM sodium boric acid buffer. A UVP TM-36 transilluminator (302 nm lamps) was used to produce images from GelRed (Biotium) stained gels. A copy stand supported a Canon Powershot G6 camera over the light source. The camera was coupled with both a B+W 090 filter and a B+W 486 UV IR Cut filter to exclude unwanted wavelengths from the digital camera's sensor. The complete apparatus was located in a dedicated darkroom.

## Results

### Primer specificity

Primers were tested for specificity among all southern pine bark beetle species as well as other beetles such as ambrosia and *Hylastes* spp. Most primer pairs tested were specific: they amplified only the target DNA and produced a PCR product of the expected size. The only exception was the primer set for *D. terebrans*, which also amplified *D. frontalis* DNA. It is possible that with additional modifications the nonspecific bands can be eliminated. Because of the target specificity of these primers, multiplex PCR may be possible in the future. Several multiplex test reactions were carried out; however time constraints limited the optimization of these reactions. Table

3.3 shows the expected fragment sizes amplified for each primer set. The data presented in Figures 3.1 through 3.4 demonstrates the specificity of each primer set.

Table 3.3. A list of the expected PCR product size and optimal annealing temperature for each species specific primer set.

| Species primer sets | Amplicon Size (base pairs) | Annealing Temperature |
|---|---|---|
| *Thanasimus dubius* | 222 bp | 62.4 ºC |
| *Ips avulsus* | 156 bp | 64.0 ºC |
| *Ips grandicollis* | 145 bp | 59.9 ºC |
| *Ips calligraphus* | 331 bp | 59.0 ºC |
| *Dendroctonus frontalis* | 205 bp | 63.0 ºC |
| *Dendroctonus terebrans* | 426 bp | 59.3 ºC |

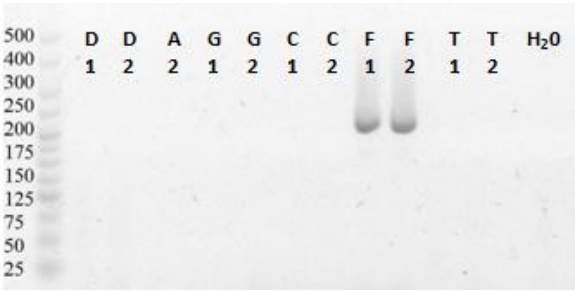


Figure 3.1 Specificity test for *Dendroctonus frontalis* primers show only amplification of target DNA. Each species of DNA is labeled: A= *Ips avulsus*, D= *Thanasimus dubius*, G= *Ips grandicollis,* C= *Ips calligraphus*, F= *Dendroctonus frontalis*, T= *Dendroctonus terebrans*, $H_2O$= negative control

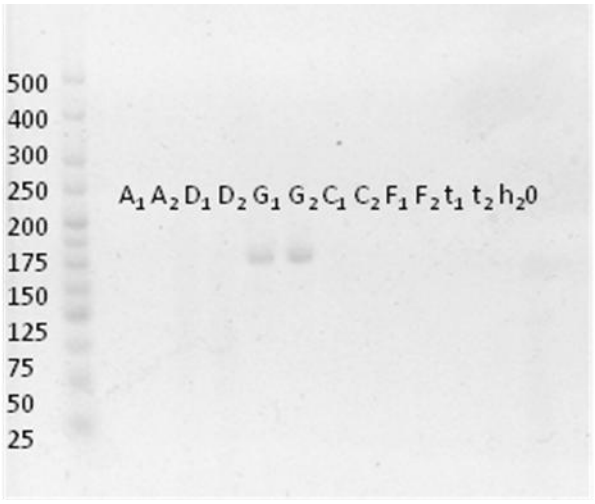


Figure 3.2. Specificity test for *Ips grandicollis* primers show only amplification of target DNA. Each species of DNA is labeled: A= *Ips avulsus*, D= *Thanasimus dubius*, G= *Ips grandicollis,* C= *Ips calligraphus*, F= *Dendroctonus frontalis*, T= *Dendroctonus terebrans*, $H_2O$= negative control

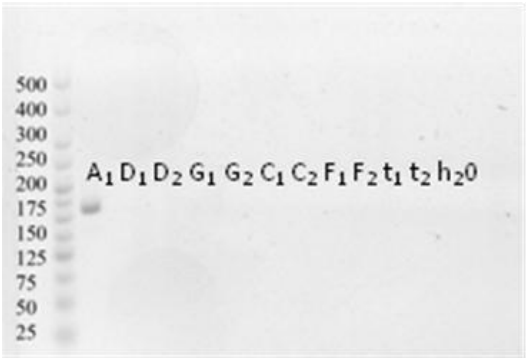


Figure 3.3. Specificity test for *Ips avulsus* primers show only amplification of target DNA. Each species of DNA is labeled: A= *Ips avulsus*, D= *Thanasimus dubius*, G= *Ips grandicollis,* C= *Ips calligraphus*, F= *Dendroctonus frontalis*, T= *Dendroctonus terebrans*, $H_2O$= negative control

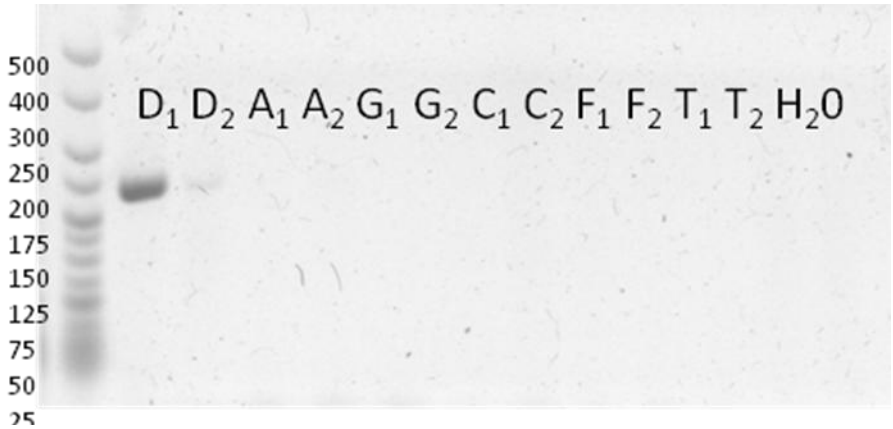


Figure 3.4. Specificity test for *Thanasimus dubius* primers show only amplification of target DNA. Each species of DNA is labeled: A= *Ips avulsus*, D= *Thanasimus dubius*, G= *Ips grandicollis,* C= *Ips calligraphus*, F= *Dendroctonus frontalis*, T= *Dendroctonus terebrans*, $H_2O$= negative control


Figure 3.5. Specificity test for *Dendroctonus terebrans* primers show only amplification of target DNA. Each species of DNA is labeled: A= *Ips avulsus*, D= *Thanasimus dubius*, G= *Ips grandicollis,* C= *Ips calligraphus*, F= *Dendroctonus frontalis*, T= *Dendroctonus terebrans*, $H_2O$= negative control

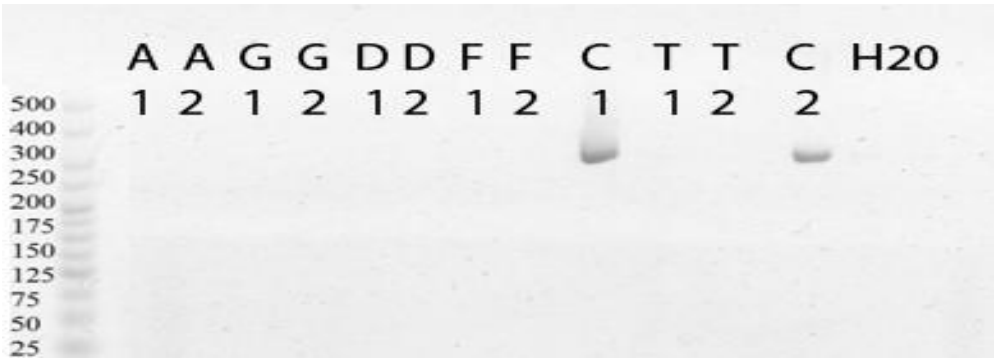


Figure 3.6. Specificity test for *Ips calligraphus* primers show only amplification of target DNA. Each species of DNA is labeled: A= *Ips avulsus*, D= *Thanasimus dubius*, G= *Ips grandicollis,* C= *Ips calligraphus*, F= *Dendroctonus frontalis*, T= *Dendroctonus terebrans*, $H_2O$= negative control

**Primer sensitivity**

After quantifying DNA (Table 3.2) using a NanoDrop 1000 spectrophotometer (ThermoScientific), for each species of beetle DNA was standardized and then serially diluted in water in increments of ten. All primers failed to amplify DNA at 1:100,000 ratio of DNA:water (Fig 3.7). The lowest detectible concentration of DNA for the *T. dubius* primers was approximately 0.03 ng/µl. All bark beetle primers were sensitive enough to detect approximately 0.001 ng/µl of DNA. Table 3.2 displays the average concentration of DNA per species of beetle, based on extractions from four individuals.

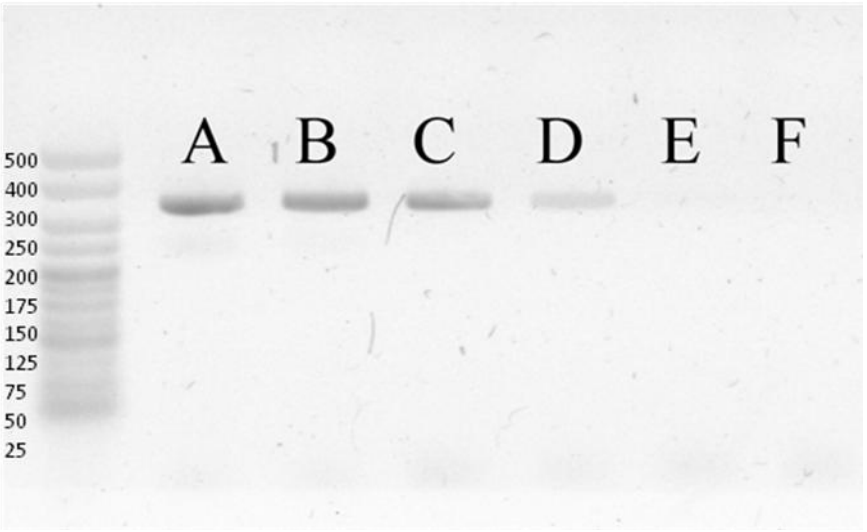


Figure 3.7. Serial dilution sensitivity result for *Ips grandicollis* primer set reveals lowest detection of DNA at approximately 0.001 ng/ul. A= 1:10, B=1:100, C=1:1,000, D=1:10,000, E=1:100,000, F=$H_20$

Four gut content assays were completed on *T. dubius* after having fed on one *D. frontalis* beetle. Two *T. dubius* were killed one hour post consumption and the other two were killed at two hours post consumption. *Dendroctonus frontalis* DNA was successfully amplified from all four *T. dubius* gut contents (Fig 3.8).

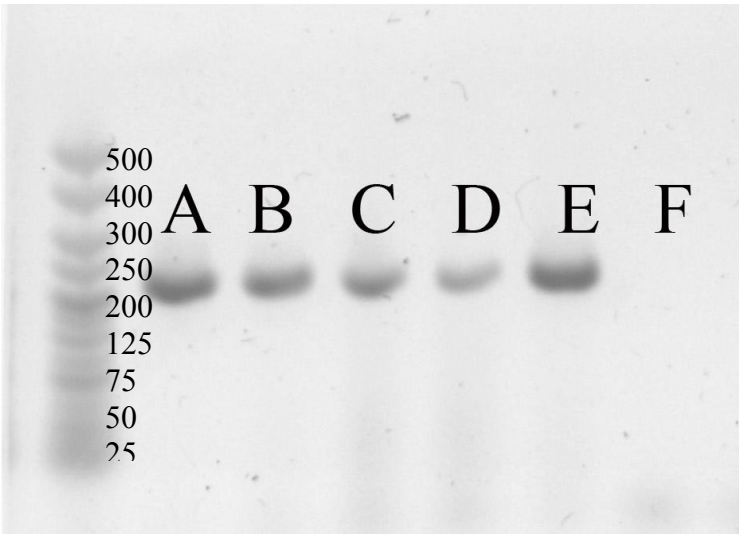


Figure 3.8. PCR of *T. dubius* gut contents after consumption of *D. frontalis.* A= *D. frontalis* DNA positive control, B and C= *D. frontalis* DNA in gut of *T. dubius* 1 hour after consumption, D and E= *D. frontalis* DNA in gut of *T. dubius* 2 hours post consumption. F= *T. dubius* gut after 24 hr starvtion.

## Discussion

Understanding the dynamics of predator-prey relationships is important to managing pest species correctly (Schroeder, 1999a; Nebeker, 2004). In many predator-prey interactions, pest populations are limited by predators, effectively minimizing host disturbance (Hassell, 1978). Some researchers speculate the build-up of *T. dubius* populations that feed on SPB significantly reduce the pest population during an outbreak (Stephen et al., 1989; Reeve and Turchin, 1993).

Other bark beetles such as *Ips* can build to very high population numbers and decline just as quickly. Researchers attribute host suitability and environmental conditions to these *Ips* population cycles rather than placing much emphasis on natural enemies like *T. dubius*. With learning capabilities and the possibility of switching

behavior, *T. dubius* behavior and prey preference needs to be better understood to determine the impact this predator has on bark beetle populations.

The prey preference of *T. dubius* in field settings is poorly understood. PCR allows researchers to know what prey has been eaten without physically seeing the predator feed. The interaction of bark beetles and predators such as *T. dubius* is an integral part of SPB population dynamics. A key component to studying this interaction is an understanding of *T. dubius* predatory behavior.

It must be noted that, at the present time, the application of this PCR protocol is limited without a working *D. terebrans* primer set. *Dendroctonus terebrans* is a close relative to *D. frontalis* and both produce and respond to the pheromone frontalin, a pheromone that *T. dubius* exploits (Mizell et al., 1984b; Phillips et al., 1989). To design a specific primer set for *D. terebrans* a larger CO1 sequence may be necessary. The bark beetle species each had approximately 1,000 base pair sequence available while *T. dubius* only had a 464 base pair sequence available. With larger sequences, it may be possible to select a region with more sequence variability thereby allowing for the selection of primers with greater species specificity. An option to overcome the unspecific *D. terebrans* primer set is to use a comparison of the specific *D. frontalis* primer set next to the *D. terebrans* primer set to determine if the DNA picked up by the *D. terebrans* primers is indeed *D. terebrans* or *D. frontalis.* A positive result from the *D. frontalis* primers would indicate *D. frontalis* DNA while a negative result from these primers would indicate *D. terebrans* DNA.

Metabolic rate or detection rate of DNA within the *T. dubius* gut was only carried out to two hours. This protocol needs to be carried out longer to the point where DNA

can no longer be amplified from gut contents due to either digestion or DNA degradation. Lab studies have shown *T. dubius* consume approximately 0-20 *D. frontalis* in a 24 hour period (Thatcher and Pickard, 1966). Our results indicate that bark beetle DNA within *T. dubius* gut contents is viable up to two hours. Juen and Traugott (2005) found they could detect 100% of prey DNA within gut contents of soil dwelling carabidae larvae 8 hours post consumption. Additionally, they could detect 50% of prey DNA 20 hours after consumption. Chen et al. (2000) detected aphid DNA within chrysopid and coccinellid predator gut contents up to approximately 8 and 16 hours post consumption. These studies indicate a need to carry the detection rate of DNA within *T. dubius* gut contents to longer time periods. Determining DNA half life or decay rate in gut contents is necessary to get an indication of the relative importance of positive results from a predator (Greenstone and Shufran, 2003).

The sensitivity of the bark beetles primers tested in singleplex reactions indicate that small amounts of bark beetle DNA within predator's gut contents should yield successful results. Primer sensitivity from previous studies yielded similar results. Traugott et al. (2006) developed primers that had a lower detection limit of 0.0005 ng/ul in singleplex PCR , and 0.002 ng/ul in multiplex PCR. Admassu et al. (2006) could detect target prey DNA in the presence of predator DNA at a concentration of 0.015 ng/ul. The primer set for *T. dubius* were a lower sensitivity (0.03 ng/ul) than the bark beetle primers (0.001 ng/ul). This does not cause much concern since *T. dubius* DNA will be abundant in PCR reactions. High bark beetle primer sensitivity is necessary because of the small quantity of bark beetle DNA in *T. dubius* gut contents. The results of our sensitivity analysis are comparable to that obtained by other researchers.

All primers were tested to ensure no off-target species amplification. With the exception of *D. terebrans,* all primer sets were highly specific when tested against other species of bark and wood boring beetles such as, *Xyloborus glabratus* (Eichhoff), *Xylosandrus crassiusculus* (Motschulsky), *Xyloborus atratus* (Eichhoff), and two *Hylastes* spp.

**Multiplex PCR**

All the primers developed in this study have the potential for incorporation into multiplex reactions to save time. Multiplex reactions utilize multiple primer sets in one PCR reaction to display several PCR products of varying sizes (Chamberlain et al., 1988). See appendix B for multiplex reactions that were tested. In general, multiplex reactions show slightly lower sensitivity than singleplex reactions (Juen and Traugott, 2006; Traugott et al., 2006). We attempted multiplex PCR in 2, 3, and 4-plex reactions. *Ips avulsus, D. frontalis,* and *T. dubius* primers were successfully optimized for multiplex reactions (Appendix B). *Ips calligraphus* primers conflicted with *T. dubius* primers in multiplex reactions by displaying extraneous bands. However, in singleplex reactions there were no extraneous bands from the *I. calligraphus* primer set. It is imperative to incorporate the *T. dubius* primer set into each multiplex as a positive internal control. Difficulty multiplexing the reactions primarily arose from the limited gene sequences available. Each bark beetle gene sequence was approximately 1,000 base pairs long while the gene sequence for *T. dubius* was only 464 base pairs long. Larger sequences will allow better primers to be designed, that can potentially be used in multiplex PCR. The use of multiplex PCR on the gut contents of field collected samples can increase the

probability of false negative results due to increased inhibition of PCR. Juen and Traugott (2006) suggest employing a separate PCR on negative samples to confirm there are no false negative results.

PCR analysis of gut contents in insects has been a successful tool for understanding predator-prey relationships and the population dynamics of several insect species (Zaidi et al., 1999; Hoogendoorn and Heimpel, 2001; Symondson, 2002; Agusti et al., 2003; Juen and Traugott, 2005; Muilenburg et al., 2008). When prey contents within a predator's guts are unidentifiable, PCR is a powerful tool to overcome this obstacle. The results of this study indicate that PCR has the ability to increase the knowledge about *T. dubius'* prey preference in the field where multiple prey species are available. The five species of the southern pine bark beetle guild are considered to be the main source of prey for *T. dubius* and are a good starting point for studying predatory behavior. However, *T. dubius* has been observed feeding on ambrosia beetles and once on a *Sirex* species (Clarke and Menard, 2006). In laboratory settings, *T. dubius* have been reared successfully on the cow pea weevil *(Callosobruchus maculates*) (Mizell and Nebeker, 1982). This evidence suggests that *T. dubius* is a generalist predator; therefore, molecular markers for other insect species or genera should be developed in order to gain a better understanding of *T. dubius'* behavior in field settings. Future research also needs to investigate the relationship between the species of bark beetle *T. dubius* feeds on versus the lure type they are attracted to, and compare this relationship across different population phases of SPB. Once these concepts are understood, researchers can have more confidence in attributing the cause of bark beetle population cycles to predators or other environmental factors..

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# CHAPTER IV

# CONCLUSIONS

## Pheromone preference between SPB population phases

In this study, we concluded that *T. dubius* F. did not display switching behavior between latent and intermediate southern pine beetle population phases. *Thanasimus dubius* maintained a strong preference for southern pine beetle (SPB), *D. frontalis* Zimmerman pheromones regardless of no apparent SPB prey available in latent phase areas. We observed an increased interaction to *Ips* bark beetle pheromones in the intermediate phase SPB areas. This result was unexpected and may be partially due to trap location, high *Ips* populations, or some other variable that was not accounted for.

## PCR analysis of *Thanasimus dubius* gut contents

Species specific primers were optimized in singleplex reactions. Due to limited availability of gene sequences and time, multiplex PCR reactions were not optimized for all species specific primers that were developed in this study. Singleplex reactions successfully amplified 100% of *D. frontalis* Zimmerman DNA, two hours after consumption by *T. dubius.* DNA half-life within *T. dubius* gut contents will need to be addressed before using this PCR protocol.

## Overall significance of these two studies

Past research has shown that weather patterns such as drought have played a major role in southern pine beetle population dynamics. More recent research indicates that weather patterns, such as drought, do not significantly impact SPB as it does other species in the southern pine bark beetle guild (Turchin et al., 1991). Turchin et al (1991) suggests that SPB population dynamics display a delayed density dependent response, but the complex driving factors behind this response are still poorly understood, although we do know that host resistance, natural enemies, and competition from other bark beetles can affect SPB populations (Reeve et al., 1995). Host resistance in conjunction with climactic conditions are given most of the credit for impacting bark beetle populations (Reeve et al., 1995).

Our focus was on the natural enemies of SPB and other bark beetles, namely, *T. dubius*. Multiple lab studies indicate that *T. dubius* as adults are an important source of mortality of bark beetles (Mizell and Nebeker, 1981a; b; Reeve, 1997; Reeve, 2003). Further, *T. dubius* larvae have been shown to have a significant impact on bark beetle brood (Moore, 1972a; Linit and Stephen, 1983). Yet there is insufficient evidence to understand how *T. dubius* affect bark beetle populations in natural settings.

In the past, researchers assumed that *T. dubius* pheromone preference indicates the species of beetle they prefer to feed on. As a generalist predator, *T. dubius* prey preference cannot be determined by pheromone preference alone. Nebeker and Mizell (1980) discussed several behavior issues that are important in quantifying the impact of *T. dubius* on SPB. Two key factors that determine prey consumption by *T. dubius* is host selection and the level of hunger (Nebeker and Mizell, 1980). Host selection is primarily

determined by bark beetle pheromones. The pheromones utilized cause *T. dubius* to arrest their flight and seek prey. The degree of hunger plays a major role in the preference of prey, or lack thereof. Mizell and Nebeker (1982) observed no preference for three prey species (*D. frontalis, I. avulsus,* and the cow pea weevil, *Callosbruchus maculatus* (Fabricius)) when starved for various lengths of time. Yet when satiated, *T. dubius* displayed a preference for *I. avulsus* and the assumption was made that *T. dubius* preferred smaller sized prey (Mizell and Nebeker, 1982). It is not clear if these laboratory assays portray actual behavior in field settings.

Our research only investigated the predator-prey relationship of *T. dubius* as adults. *Thanasimus dubius* as predaceous larvae have potential for significant impact on bark beetle populations. Moore (1972b) attributed 24% of mortality of SPB to predators and parasitoids, half of which was caused by *T. dubius* larvae. Linit and Stephen (1983) observed a similar mortality impact on SPB (26%) that was caused by *T. dubius* larvae. Within tree predatory behavior needs to be studied more in depth to gain a full understanding of how strong *T. dubius* impact on bark beetles is, especially in trees containing multiple bark beetle species. The PCR protocol presented in Chapter 3 may work equally well on *T. dubius* as larvae. However, it must be noted that this protocol was developed only on adult beetles and needs further tests with larvae to ensure accurate results.

Reeve et al. (1995) stated that more detailed estimates of the mortality inflicted on SPB and other bark beetles across a range of prey densities is needed to understand the total impact *T. dubius* has on bark beetle population dynamics. It was also stated that more knowledge is need on the numerical response of bark beetle predation by *T. dubius*

larvae within natural tree settings. This knowledge can be obtained through the use of PCR on gut contents of *T. dubius*, and when coupled with pheromone preference, a more thorough analysis of predatory behavior can be determined. I propose that PCR may be the key to linking pheromone and prey preference together. PCR will enable researchers to gain a greater understanding of *T. dubius* predatory behavior, both as larvae and as adults. In return, researchers will be able to fill the necessary gaps in the predator-prey relationship and the effects *T. dubius* has on bark beetle population dynamics.

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# APPENDIX A

# ANOVA TABLES FROM SPRING 2010 DATA

***T. dubius* in *Ips* Traps**

**Tests of Between-Subjects Effects**

Dependent Variable:Trap SUM d

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 36.693[a] | 1 | 36.693 | 29.529 | .000 |
| Intercept | 65.537 | 1 | 65.537 | 52.740 | .000 |
| Phase | 36.693 | 1 | 36.693 | 29.529 | .000 |
| Error | 32.309 | 26 | 1.243 | | |
| Total | 122.366 | 28 | | | |
| Corrected Total | 69.002 | 27 | | | |

a. R Squared = .532 (Adjusted R Squared = .514)

***T. dubius* SPB Traps**

**Tests of Between-Subjects Effects**

Dependent Variable:Trap SUM clerid

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 45.001[a] | 1 | 45.001 | 1.842 | .186 |
| Intercept | 767.724 | 1 | 767.724 | 31.430 | .000 |
| Phase | 45.001 | 1 | 45.001 | 1.842 | .186 |
| Error | 635.097 | 26 | 24.427 | | |
| Total | 1410.541 | 28 | | | |
| Corrected Total | 680.097 | 27 | | | |

a. R Squared = .066 (Adjusted R Squared = .030)

***T. dubius* in *Ips* and SPB Traps Combined**

**Tests of Between-Subjects Effects**

Dependent Variable:Trap SUM clerid

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 276.166[a] | 3 | 92.055 | 7.172 | .000 |
| Intercept | 640.939 | 1 | 640.939 | 49.938 | .000 |
| Phase | 81.482 | 1 | 81.482 | 6.349 | .015 |
| Traptype | 192.322 | 1 | 192.322 | 14.985 | .000 |
| Phase * Traptype | .212 | 1 | .212 | .016 | .898 |
| Error | 667.405 | 52 | 12.835 | | |
| Total | 1532.906 | 56 | | | |
| Corrected Total | 943.571 | 55 | | | |

a. R Squared = .293 (Adjusted R Squared = .252)

***Ips avulsus***

**Tests of Between-Subjects Effects**

Dependent Variable:Trap SUM A

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 2646.564[a] | 1 | 2646.564 | .810 | .377 |
| Intercept | 101311.154 | 1 | 101311.154 | 30.991 | .000 |
| Phase | 2646.564 | 1 | 2646.564 | .810 | .377 |
| Error | 84996.551 | 26 | 3269.098 | | |
| Total | 195895.971 | 28 | | | |
| Corrected Total | 87643.116 | 27 | | | |

a. R Squared = .030 (Adjusted R Squared = -.007)

*Ips grandicollis*

**Tests of Between-Subjects Effects**

Dependent Variable:Trap SUM G

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 1122.012[a] | 1 | 1122.012 | 19.285 | .000 |
| Intercept | 3614.298 | 1 | 3614.298 | 62.123 | .000 |
| Phase | 1122.012 | 1 | 1122.012 | 19.285 | .000 |
| Error | 1512.667 | 26 | 58.179 | | |
| Total | 6935.000 | 28 | | | |
| Corrected Total | 2634.679 | 27 | | | |

a. R Squared = .426 (Adjusted R Squared = .404)

# APPENDIX B

# MULTIPLEX REACTIONS

**Multiplex #1**

Multiplex PCR reaction 1 consisted of *Ips avulsus, Dendroctonus frontalis,* and *Thanasimus dubius* primers. These amplifications were set up in 10 µl reactions with each reaction containing 200 µM dNTPs, 2.20 µM of each *Ips avulsus* primer, 1.8 µM of each *Dendroctonus frontalis* primer, 2.00 µM of each *Thanasimus dubius* primer, 1X GC buffer, 2 µM $MgCl_2$, 0.2 units of Phusion High Fidelity polymerase (Finnzymes) and 1-300 ng of DNA, depending on the species of beetle.

Thermocycler conditions consisted of an initial denaturation of 98º C for 30 seconds, 30 cycles of 98º C for 7 seconds, the calculated Tm for 15 seconds, 72º C for 18 seconds, and a final extension period of 72º C for 7 minutes.. The optimal annealing temperature was found to be approximately 55º C.

This reaction was successfully optimized and tested for specificity. All primers were specific when tested against each off target bark beetle species. Sensitivity analyses were not tested.

**Multiplex #2**

Multiplex PCR reaction 2 consisted of *Ips grandicollis, Ips calligraphus, Dendroctonus terebrans,* and *Thanasimus dubius* primers. These amplifications were set up in 10 µl reactions with each reaction containing 200 µM dNTPs, 1.70 µM of each *Ips grandicollis* primer, 1.30 µM of each *Ips calligraphus* primer, 2.10 µM of each *Dendroctonus terebrans* primer, 1.70 µM of each *Thanasimus dubius* primer, 1X GC buffer, 2 µM $MgCl_2$, 0.2 units of Phusion High Fidelity polymerase (Finnzymes) and the

same amount of DNA as above. These reactions were also ran using the same thermocycler conditions and annealing temperature as above. *Dendroctonus terebrans* primers were insufficient in visibility and unspecific when tested for specificity. This multiplex reaction could be optimized with new *D. terebrans* primers.