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Acoustic communication in the Mountain Pine Beetle, *Dendroctonus ponderosae* Hopk. (Coleoptera: Scolytidae): Characterization of signals and anatomy

By

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Abstract

Mountain Pine Beetles, (*Dendroctonus ponderosae* Hopkins employ sounds for intra- and inter-specific communication. Signals associated with three behavioural contexts were analyzed. Analysis of signals revealed air- and solid-borne components. Airborne signals were found to have an average intensity of 55.71 dB at 2 cm, while solid-borne signals showed an average velocity of 2.76 ± 0.93 mm/s at 1 cm. Airborne signals were broadband between 6.5 kHz – 83.3 kHz, with energy divided into up to 5 energy peaks. Comparisons between signal types revealed differences in the frequency distribution, and dominant frequencies. Solid-borne components of the signals showed frequencies between 4.6 kHz and 22.8 kHz. Examinations of gross anatomy revealed a putative candidate for tympanal ear present on the dorsal meta thoracic surface of the animal. Preliminary behavioral trials conducted, using manipulated natural signals in artificial settings, elicited an acoustic behavioral response.

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Chapter 1. Introduction

1.1 General Introduction

Bark beetles (Scolytidae: Curculionoidea) pose one of the largest insect threats to North American coniferous forests. Normally these native beetles target sick or dying trees. Under outbreak conditions, however, they will attack *en masse* to kill healthy trees (Ryker, 1988). The causes of recent outbreaks are the subject of much speculation. One hypothesis suggests that the recent advances in fire fighting techniques have led to a staggering increase in the number of ageing and drought stressed trees (Raffa, 1988; Williams and Liebhold, 2002). This increase in unhealthy trees has led to an increase in the number of parasitized host trees, causing explosive outbreaks that have decimated large stands of both healthy and dying trees. Increases in global temperature have also been implicated- as the normal winter temperatures climb ever higher, smaller proportions of beetle populations are being killed off by the cold. These outbreaks have a direct and immensely detrimental impact on the North American forestry and lumber industries (Patriquin et al., 2005).

Management of bark beetle populations relies heavily on an understanding of their life history, sensory ecology, survival and reproductive strategies. Research into bark beetle communication has focused primarily on their chemical ecology. Within Scolytidae, chemical cues are widely used for both intra- and inter- specific communication (Byers, 1983; Borden, 1989), such as terpene cues in host selection, and pheromone communication of warnings, sexual attraction and rivalry (Wood and Vité, 1961; Moeck et al., 1981; Wood, 1982). This mode of communication has been extensively studied, and has been widely exploited through the production of lures and

pheromone traps in an attempt to manage outbreaks (Byers, 1983). However, this biased focus into understanding chemical communication has not yet found the necessary level of success in bark beetle control strategies (Rudinsky and Michael, 1972; Raffa et al., 1993). Given that communication is vital to the survival of any organism, it is remarkable that, to date, only one mode of communication has been explored in detail, while less attention has been given to other modes of communication, such as the acoustic and vibratory communication in bark beetles.

Sound production in bark beetles has been widely documented in the scientific literature. Of the estimated 6000 extant species of the family Scolytidae, acoustic signaling has been shown to occur in most that have been examined (Ryker and Rudinsky, 1976). Although the function of the signals remains unclear, bark beetles have been shown to produce acoustic signals during, 1) male-male interactions (i.e. aggression); 2) male-female interactions (i.e. mate attraction, and courtship); and 3) response to stressors (i.e. predators) (Rudinsky and Michael, 1972).

Scolytidae produce sounds via a mechanism called 'stridulation' (Ryker and Rudinsky, 1976), which involves the rapid friction of two body structures, one containing the file (*pars stridens*) and the other the scraper (*plectrum*) (Barr, 1969). Acoustic signals are produced when the teeth of the file are struck individually and in sequence by the scraper, each tooth producing one single discrete sound (Barr, 1969). Scolytids have been shown to produce signals via three different stridulatory mechanisms: 1) elytro-tergal stridulation, 2) vertex-pronotal stridulation, and 3) gula-prosternal stridulation, depending on the species (Barr, 1969). Given the widespread distribution of acoustic signaling mechanisms, it is surprising that receptor organs have not been identified to date in any

Scolytidae species.

Acoustic sensation in insects serves two main functions, conspecific communication and predator detection (Yack, 2004). Insects sense acoustic stimuli in a variety of different ways; airborne signals are generally perceived via tympanal hearing organs (ears) and trichoid sensillae capable of detecting near-field sounds, while solid borne vibrational signals are generally perceived via subgenual organs (Yager, 1999; Yack, 2004). Tympanal hearing has evolved no less than 19 times within 8 insect orders, a number which will undoubtedly grow with future research (Yager, 1999; Yack and Dawson, 2008). Hearing has been widely studied in other large insect orders including Orthoptera, Lepidoptera, and Hemiptera. It is therefore puzzling that tympanal hearing within Coleoptera has been found in only two families to date, Scarabaidae and Cincindellidae (Yager and Spangler, 1995; Forrest et al., 1997). In order to determine whether an insect has a functional sense of acoustic reception three lines of evidence are typically pursued: 1) identification of a receptor system, 2) demonstration of a physiological response to a biologically relevant stimulus, and 3) the presence of an acoustically mediated behavioral response (Yack and Fullard,1993). The general purpose of this thesis is to explore the capacity of sound reception in the scolytid beetle *Dendroctonus ponderosae.*

1.2 The Mountain Pine Beetle – Life History in Brief

The mountain pine beetle, *D. ponderosae* Hopkins, is considered to be the single most destructive species of bark beetle in North America (Ryker, 1988; Patriquin et al., 2005). It is an endemic insect and as such, a natural pest component of the western coniferous forests. Current outbreak conditions in British Columbia are stated as being

the worst in recorded history (Patriquin et al., 2005). Unlike some of the other less harmful bark beetles, mountain pine beetles will attack living host trees. As the temperature begins to rise in early spring, adult females emerge, and actively seek out suitable hosts. Host localization takes place based on chemical cues given off by both the host tree, and any existing resident bark beetle females (Raffa et al., 1993). Once a host is located the female beetle tunnels into the bark of the tree while releasing an aggregating pheromone that attracts males, as well as other females, effectively escalating the attack (Rudinsky and Michael, 1974; Ryker, 1988). Singular attacks on the host tree might be met with stiff resistance, in the form of resin or 'pitch', while mass attacks over short periods of time coupled with fungal inoculation (brought in by the beetles) can cripple these defenses (Rudinsky, 1962). As males emerge they orient towards the female pheromone and join in the digging of galleries (Raffa et al., 1993). Once the male-female interaction has begun a second pheromone is released, the anti-aggregation or masking pheromone which signals to other beetles to stay away, a chemical 'No Vacancy' sign (Raffa et al., 1993). The pair will work together to construct a gallery under the bark of the host tree with bouts of digging being interrupted by bouts of courtship and mating (Rudinsky, 1962). The result is a narrow gallery, running parallel to the grain of the wood (Figures 1.1-1.2). As the gallery is being built the phloem is inoculated with blue stain fungus spores (two species of fungus: *Ceratocystis montia* Rumb., and *Europhium sp.*), held within the beetles' mycangia, and eggs are oviposited along the gallery walls. (Mycangia are specialized invaginations and/or specialized hairs present on the exterior of the animal, used in the collection and dissemination of fungal spores (Batra, 1963).) The eggs develop over a period of 3-11 days from oviposition. The larvae, once hatched,

feed on the cambial layer digging out tunnels perpendicular to the female gallery (Figure 1.2). After the fourth instar, the larva pupates, picking up fungal spores from the pupal chamber that it then carries off as adults. Tree pathology occurs due to the larvae feeding as well as the blue stain fungus, both of which completely block the phloem of the infested tree, causing the death of any tissues above the damage (Rudinsky, 1962).

Despite the obvious importance of stridulation in *Dendroctonus* life history, to date no research has been conducted that examines how bark beetles sense the acoustic nature of their surrounding environment, and no receptor organ has been identified. This leaves many questions unanswered: What is the mode of transmission, airborne or vibratory? What are the main characteristics of the different signal types? Do these animals possess an acoustic receptor organ, and if so, where?

Figure 1.1 Repertoire of sounds in *D. ponderosae*. **a**) Acoustic interactions begin with the female foundress already in her burrow, and the male approaching. The burrow entrance is blocked by a pile of wood shavings and frass loaded with pheromones which stimulate the male to produce sound. The male produces a long train of "attractant" (complex) chirps. Inset shows a close up of a 'pitch tube' or entry point through which the female has bored (photo: Leslie Chong, Simon Fraser University). Sap emanations are clearly visible as a defense mechanism from the tree. **b**) The female now becomes acoustically active producing simple chirps in response to the male (complex) chirps. It is at this point that she begins to release her anti-aggregation pheromone, effectively masking that section of tree and signaling her acceptance of the male. **c**) Prior to copulation the male emits more "attractant" (complex) chirps. He then attacks the female with vicious bouts of biting, jostling and pushing. Between these battles there are periods of silence during which the male and female cooperate to bore out the gallery and remove frass from the tunnel. **d**) Just prior to copulation the male produces a new "courtship" signal, an uninterrupted train of simple chirps coupled with gentle nudging; **e**) when exposed to an intruding male, resident male *D. ponderosae* beetles will defend their territories acoustically by producing short bouts of complex "territorial" chirps. **f**) Pinching the animals between thumb and index finger elicits the "stress" chirp in male beetles, which is characterized by a train of simple chirps. Parts **a-e**) redrawn from Ryker (1988). Acoustic signaling discussed in more detail in Chapter 2 on Signal Characterization.

Figure 1.1

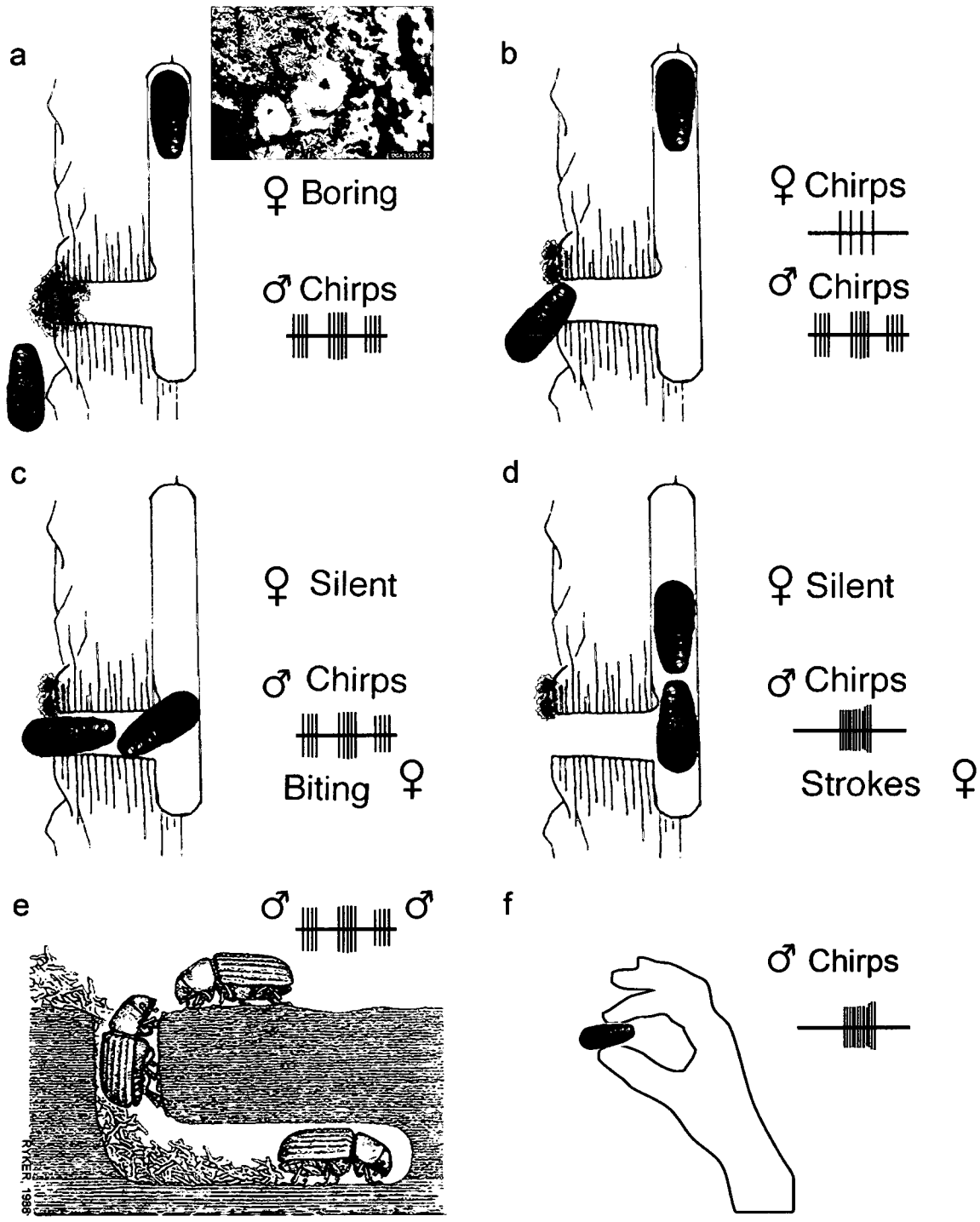
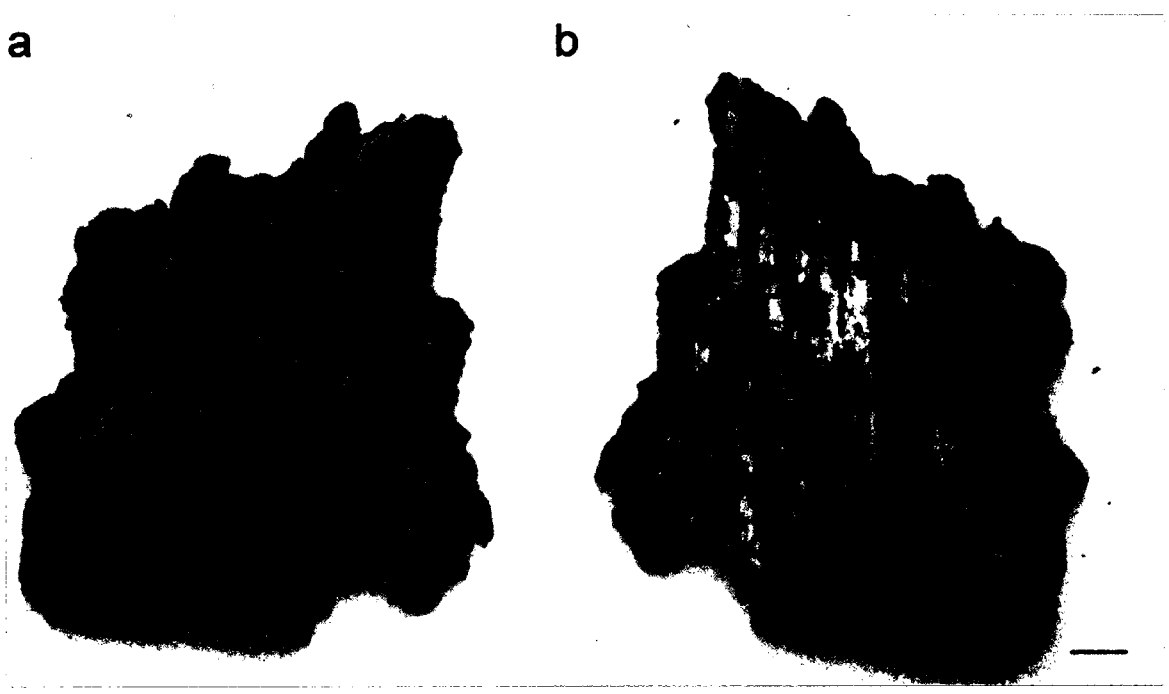

a
♀ Boring
♂ Chirps
b
♀ Chirps
♂ Chirps
c
♀ Silent
♂ Chirps
Biting ♀
d
♀ Silent
♂ Chirps
Strokes ♀
e
♂ ♂
f
♂ Chirps

Figure 1.2 Galleries of *D. ponderosae.* **a)** External view of bark of *Pinus ponderosae* colonized by *D. ponderosae*. Emergence holes can be seen indicated by the arrows. **b)** Inner surface of the same piece of bark seen in a). Female galleries (F) are formed by cooperative digging between the adult male and female *D. ponderosae* parallel to the grain of the tree. The larvae produce larval galleries (L). These are excavated perpendicular to the grain of the wood as they feed. The larvae ultimately kill the host tree by severing all the vascular tissue. The larvae pupate in the pupal chamber shown as the small depression surrounding the emergence borehole (B).

Figure 1.2



1.3 Objectives

The long term objective of this thesis is to identify putative acoustic receptor organs in bark beetles, using the species *D. ponderosae* as a model. I will work towards identifying receptor organs by focusing on three specific objectives: 1) characterize the airborne and vibration-borne components of the signals, 2) identify putative receptor organs morphologically, and 3) test the hypothesis that these receptors are functioning in acoustic reception, by using a behavioral bioassay.

Objective: Signal Characterization

Stridulation has been shown to play a role during various behavioural contexts. Acoustic signaling has been shown during the entire duration of the male-female courtship interaction (Rudinsky and Michael, 1974, Ryker, 1988) (Figures. 1.1-1.2). In addition to signaling during male-female interactions, bark beetles also produce three distinct acoustic signal types: 1) stress signals, produced by males when attacked by predators; 2) "territorial" chirps produced by males when attacked by other males (Rudinsky and Michael, 1974); and 3) female clicking, thought to be involved in spacing when forming galleries (Ryker, 1988).

Previous research on *Dendroctonus* signal characterization was conducted using analog tape, which provides a good level of sensitivity to the temporal structure of sounds. However, while that recording device can also provide a higher frequency bandwidth, such characteristics were not analyzed in any of the previous studies. Therefore, during my undergraduate thesis, I used a contemporary recording device (B&K Ultrasound sensitive microphone coupled to a solid state data recorder), which can

capture a broader spectrum of frequencies with high precision to repeat the original signal characterizations, primarily focusing on the airborne characteristics of the stress signal. My preliminary research suggested that bark beetle acoustic signals contained a significant amount of ultrasound, as well as suggesting the possibility of vibrational components (Fleming, 2007). Together, these two modes of communication would suggest that bark beetle communication is more complex (utilizing multiple modalities, and possibly multiple components within each modality) than previously thought, and suggests the existence of possible receptor organs capable to detecting airborne or substrate-borne vibrations. This study will test the hypothesis that *Dendroctonus ponderosae* employ acoustic signals (either airborne or vibrational) in conspecific communication by analyzing the uncharacterized signals in their acoustic repertoire.

Objective: Receptor Morphology

Insects sense the acoustic components of their surroundings using a variety of auditory sense organs, all of which can be characterized as mechanoreceptors (Yack, 2004). Insect acoustic receptor organs can be divided into four major classes including: trichoid sensillae, Johnston's organs, tympanal organs, and subgenual organs. Trichoid sensillae are hair like projections of the cuticle that are sensitive to the movement of air particles elicited by near-field sounds (Yack, 2004). Antennal or Johnston's organs, are mechanosensory receptor cells, usually located on the second antennal segment, capable of detecting minute movements of air particles in the insects direct surroundings as exemplified by the mosquito auditory sense (eg. the mosquito (*Toxorhyinchites brevipalpis*)) (Göpfert and Robert, 2001). Tympanal organs (ears) are capable of detecting far-field sounds up to distances exceeding 1.5 km (eg. the Bladder Grasshopper

(*Bullacris membracioides*)) (Van Staaden and Römer, 1997). Insect tympanal ears are generally associated with three distinct morphological traits including a thin membranous tympanum, associated trachea or air sac, and innervation capable of transducing the mechanical effect of the pressure waves into a synaptic signal (Hoy and Robert, 1996; Yack, 2004). Several potential organs involved in detection of substrate-borne vibrations include the subgenual organ, trichoid sensillae, and campaniform sensillae (Yack, 2004). However, the most widely known vibrational receptor is the subgenual organ, a chordotonal organ located in the tibiae of certain insects capable of detecting substrate borne vibrations up to 5 kHz (Hill, 2001; Virant-Doberlet and Cokl, 2004; Yack, 2004).

Given the widespread occurence of acoustic signals (Rudinsky and Michael, 1972), it is highly likely that bark beetles possess organs capable of detecting their rich repertoire of acoustic signals. Examination of the signal properties would suggest that two possible types of receptor organs may be present in bark beetles: either an airborne acoustic receptor (i.e. tympanum, or near field receptor), and/or a vibrational signal receptor (i.e. subgenual organ). At present there is no obvious structure that could be associated with sound reception. I will attempt to use the existing road map of coleopteran neuroanatomy (Holste, 1910), coupled with my own anatomical observations (morphological and neural), to identify putative receptor structures capable of supporting an acoustic receptor organ, as well as to identify the nerve branches which innervate these structures. Given what is known of insect audition, and of the prevalence of acoustics in bark beetle biology, it is interesting that there has been no research to date that investigates the acoustic sensory systems of the bark beetle.

Objective: Behavioural Playbacks

Two methods have been suggested for confirming the presence of an acoustic receptor organ: 1) record directly from living nerve branches, while exposing the animal to an acoustic stimulus, to assess the organism's capacity to hear these signals, and 2) use an acoustic signal as a behavioral bioassay to show that these signals are biologically relevant because they will elicit a response in the intended receiver (Yack, 2004). Such examinations will fulfill the last criterion for establishing hearing and communication in an invertebrate organism. Acoustic playbacks have been essential in establishing the more specific details of acoustic communication in insects. Within Coleoptera a permutation of an acoustic playback was instrumental in defining the presence of a hearing organ in Scarabaeidae. Forrest et al. (1995), used acoustic traps to elicit a startle response in wild scarab beetles. They achieved this by placing buckets with ultrasonic speakers in the field and emitting ultrasonic signals into the night sky, startling passing scarab beetles into the traps. This example was the first conclusive piece of evidence of acoustic reception in Coleoptera. In my study, playbacks will be achieved by playing back the biologically relevant acoustic stimuli characterized in Chapter 2. Chapter 4 will attempt to show the presence of an acoustic receptor organ by observing any behavioural changes occurring in response to a biologically relevant acoustic stimulus.

Chapter 2. Signal Characterization

2.1 Introduction

General Introduction on Sound Production

Organisms across various taxa have been shown to utilize a variety of mechanisms to produce signals through various substrates, and such remarkable diversity has enticed researchers to investigate its importance in animal behaviour. Sound production is widespread in insects, evolving several times throughout the class Insecta. Acoustic signaling has been shown to function during mate attraction, courtship, territoriality, aggression, prey detection, and predator avoidance (Gerhardt and Huber, 2002). Of the various mechanisms used to produce acoustic signals, stridulation is the most common method of sound production having been extensively studied in various insect orders, such as Orthoptera, Coleoptera, and Hymenoptera (Johnson and Triplehorn, 2004).

Stridulation involves the rubbing of two specialized body parts. Usually one part will contain the "file" or *plectrum*, which is a series of teeth or ridges, while the other will contain the more simplified *pars stridens* or "scraper" structure, both of which are highly sclerotized structures, and are scraped across each other to produce vibrations, which can transmit acoustic signals through both air and substrate (Gerhardt and Huber, 2002). For example, in katydids (Tettigoniidae), acoustic signals are produced by rubbing the *pars stridens* located on one wing against the plectrum on the opposing wing (Morris and Pipher, 1972). Stridulation dominates as the most abundant form of sound production within the order Coleoptera (Lyal and King, 1996).

Curculionoidea is the largest of the superfamilies of Coleoptera with an estimated 60,000 described species, and another 500,000 yet to be described (Johnson and Triplehorn, 2004). Stridulation has been shown to occur in at least half of the described species within this superfamily (Lyal and King, 1996) and is well documented in the family, Scolytidae, first noted by Hopkins in 1909. Scolytidae, or the bark beetles, are one of the most economically important subfamilies, responsible for millions of dollars worth of losses in timber and product each year (Patriquin et al., 2005). Today, this method of communication is believed to be present in most of the 6000 extant species (Barr, 1969; Ryker and Rudinsky, 1976; Wood, 1982; Lyal and King, 1996), exhibiting a great deal of variability both across species and sexes, in associated organs, behaviours and display methods (Barr, 1969).

Acoustic Communication in *Dendroctonus ponderosae*

Three types of stridulatory organs are known to occur within the family Scolytidae: Gula-Prosternal type, Vertex-Pronotal type, and Elytra-Abdominal tergites type (Barr, 1969). Species within the genus *Dendroctonus*, utilize the elytra-abdominal stridulatory mechanism for sound production (Barr, 1969). A detailed summary of the literature of sound production in the genus *Dendroctonus* can be seen in Table 2.1. This table outlines the species differences with respect to number of toothstrikes in the different species, as well as the differences between the signals produced by different sexes.

Denctroctonus ponderosae, like other *Dendroctonus* species, stridulate using the elytra-abdominal mechanism. *Dendroctonus* species employ complex acoustic signals during interactions with both con- and hetero-specifics (Hopkins, 1909; Barr, 1969;

Safranyik and Wilson, 2006). Male *D. ponderosae* produce acoustic signals when the plectrum (a sclerotized portion of the posterior margin of the 7th abdominal segment), is scraped against the *pars stridens* (a series of teeth located on the underside of his left elytron) (Figure 2.1) (Hopkins, 1909; Chapman, 1955; Lyon, 1958; Barr, 1969). The sounds generated by males have been categorized as: stress (a simple signal produced when the animal is exposed to a noxious stimulus), attractant (a complex signal employed by the male as he approaches and engages the female), courtship (a simple signal typically seen to follow complex attractant stridulation during mating), and rivalry/aggressive (a complex signal produced when two males are in direct competition) (Michael and Rudinsky 1972; Rudinsky and Michael, 1974; Ryker and Yandell, 1983; Yandell, 1984). Females, however, lack elaborate stridulatory structures, but nevertheless have been reported to produce simple chirps when defending a burrow or during initial interactions with males, and short clicks while forming egg galleries (Rudinsky and Michael 1973; Ryker and Rudinsky, 1976; Rudinsky, *et al.*, 1976). Although previous studies have provided valuable information on the general context and temporal characteristics of signaling, they did not examine the spectral qualities, modes of transmission, or receptor mechanisms used, leaving many questions yet to be answered.

In a preliminary study during my undergraduae honours project, I recorded and characterized the airborne component of the stress signals produced by the male Mountain Pine Beetle. The results indicated that stress signals were very broadband with much of the acoustic energy falling within the ultrasonic range (Fleming, 2007). Therefore, the purpose of this study was to determine the characteristics of the repertoire of acoustic and vibratory signals of the bark beetle *Dendroctonus ponderosa*, and

determine how these signals are being transmitted under natural conditions. Such studies describing, in detail, the characteristics of the signals produced could enable us to further hypothesize about the biophysical and environmental constraints that have shaped the evolution of bark beetle communication system, and provide us with insights into possible receptor mechanisms that can detect and process such signals.

Table 2.1 Summary of sound production within the genus *Dendroctonus*.

Genus and Species	Stridulating Sex	Stridulation Type	Stress	Male-Male
			Toothstrikes/per chirp	
			Stress (Simple)	Complex/Simple
D. brevicomis (= *D. barberi*)	m, f	e-t *	25-58 (Rudinsky and Michael, 1974)	19-56 (Rudinsky and Michael, 19
D. micans	m		noted but no values (Barr, 1969; Ryker, 1988; Lyal and King, 1996)	noted but no values (Barr, 1969 Ryker, 1988; Lyal and King, 199
D. pseudotsugae	m, f	e-t *	52-67 (Michael and Rudinsky, 1972)	26-58 (Rudinsky and Ryker, 197
D. terebrans	m		noted but no values (Barr, 1969; Ryker, 1988; Lyal and King, 1996)	noted but no values (Barr, 1969 Ryker, 1988; Lyal and King, 199
D. valens	m, f	e-t *	"	"
D. simplex	m, f		"	"
D. punctatus	m		"	"
D. frontalis	m, f	e-t *	13-58 (Rudinsky and Michael, 1974)	11-21 (Rudinsky and Michael, 19
D. parallelocolis	m		noted but no values (Barr, 1969; Ryker, 1988; Lyal and King, 1996)	noted but no values (Barr, 1969 Ryker, 1988; Lyal and King, 199
D. murrayanae	m		"	"
D. jeffreyi	m		"	"
D. adjunctus (= *D. convexifrons)*	m	e-t	"	"
D. ponderosae (= *D. monticolae*)	m, f	e-t *	25-38 (Michael and Rudinsky, 1972) 19.6-25.7 (Yandell, 1984)* 27.2±1.0 (Ryker and Rudinsky, 1976)	23.6-31.6 (Yandell, 1984)* 35.0±0.9 (Ryker and Rudinsky, 19
D. aztecus	m		noted but no values (Barr, 1969; Ryker, 1988; Lyal and King, 1996)	noted but no values (Barr, 1969 Ryker, 1988; Lyal and King, 199
D. obesus (= D. engelmanni*)*	m		"	"
D. ruffipennis	m, f	e-t *	10-25 (Rudinsky and Michael, 1974)	8-23 (Rudinsky and Michael, 197

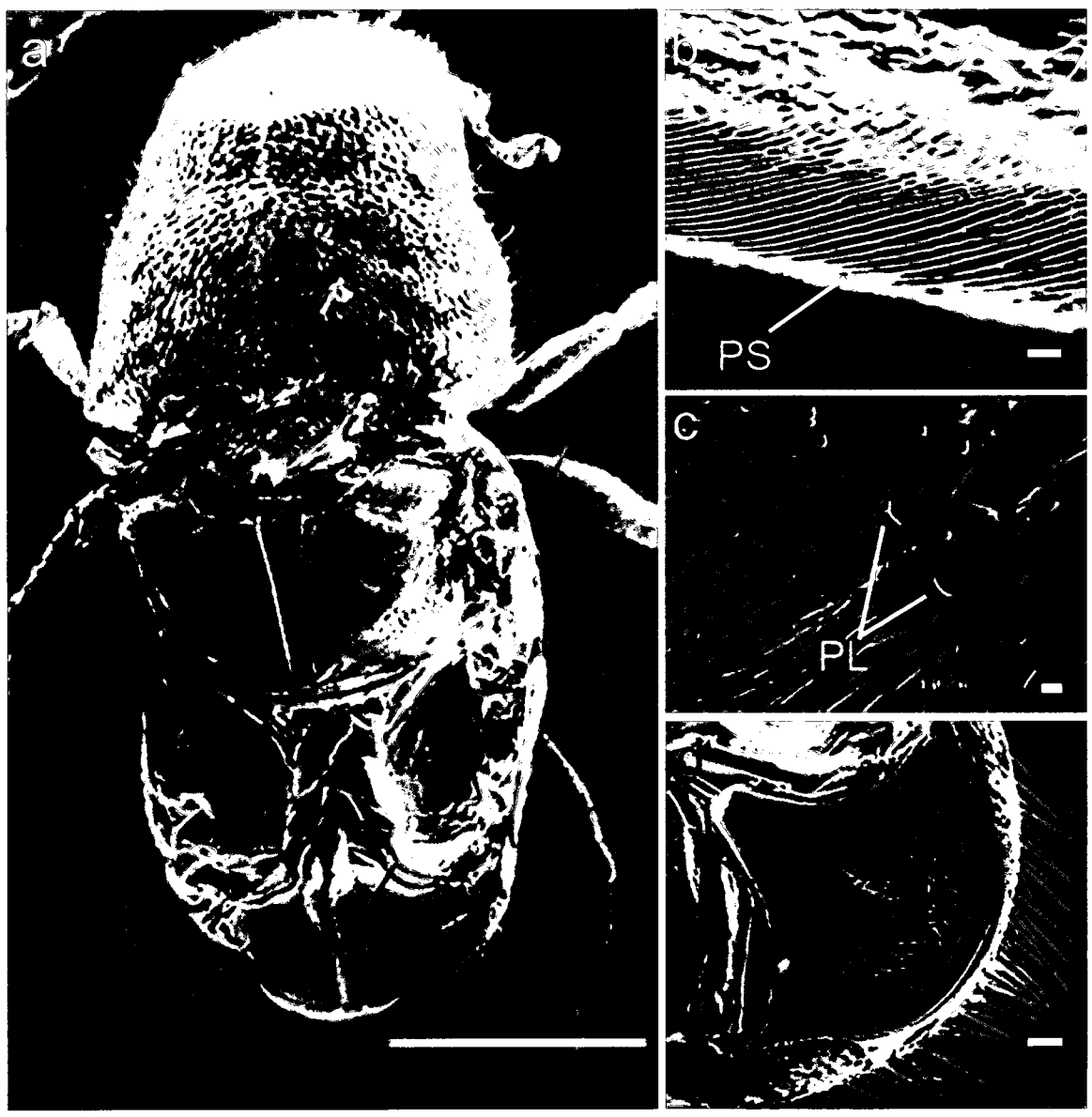
* indicates where a female has been shown to produce multi-impulse chirps (e-t= elytro tergal stridulation)
† Complex signals refers to all signals present within a male female interaction having two or more components
‡ Simple signals refers to all signals present within a male female interaction having one chirp element

Male-Female		Female	
Toothstrikes/per chirp			
Complex†	Simple‡	Click	Chirp
15-48 (Rudinsky and Michael, 1974)	N/A	present (Ryker, 1988)	present
noted but no values (Barr, 1969; Ryker, 1988; Lyal and King, 1996)	noted but no values (Barr, 1969; Ryker, 1988; Lyal and King, 1996)	N/A	N/A
32-67 (Rudinsky and Ryker, 1976)	17-61 (Rudinsky and Ryker, 1976)	present (Ryker, 1988)	not noted to be present (Ryker, 1988)
noted but no values (Barr, 1969; Ryker, 1988; Lyal and King, 1996)	noted but no values (Barr, 1969; Ryker, 1988; Lyal and King, 1996)	N/A	N/A
"	"	not present (Ryker, 1988)	several different types of multipulse chirps noted in this species (Ryker, 1988)
"	"	N/A	
26-60 (Rudinsky and Michael, 1974)		not present (Ryker, 1988)	present (Ryker, 1988)
noted but no values (Barr, 1969; Ryker, 1988; Lyal and King, 1996)	noted but no values (Barr, 1969; Ryker, 1988; Lyal and King, 1996)	N/A	N/A
"	"	N/A	N/A
"	"	N/A	N/A
"	"	N/A	N/A
26-61 (Michael and Rudinsky, 1972) 33.8-34.8 (Yandell, 1984)* 41.0±1.2 (Ryker and Rudinsky, 1976)	26.6±1.1 (Ryker and Rudinsky, 1976)	present (Ryker, 1988)	present (Ryker, 1988) 6.4-8.6 (Yandell, 1984)* 8.1±0.4 (Ryker and Rudinsky, 1976)
noted but no values (Barr, 1969; Ryker, 1988; Lyal and King, 1996)	noted but no values (Barr, 1969; Ryker, 1988; Lyal and King, 1996)	N/A	N/A
"	"	N/A	N/A
13-28 (Rudinsky and Michael, 1974)	N/A	present	present

Figure 2.1 Scanning electron micrographs of the sound producing structures in *D. ponderosae*.

a) Dorsal view of a male *D. ponderosae* with elytra and meta-thoracic wings removed exposing underlying structures. The arrow indicates location of the *plectrum* (PL) (scale bar = 1mm). **b)** Close up of the inner surface of the right elytron of a male, arrow indicates the *pars stridens* (PS) (scale bar = 10 μm). **c)** Detailed high-magnification view of the plectrum on a male beetle, plectrum appears as 2 hardened sclerotized tubercles projecting from the apex of the 7th abdominal tergite. **d)** detail of the 7th abdominal tergite of a female *D. ponderosae*, note the absence of the plectrum (scale bars **c-d** = 10μm).

Figure 2.1



2.2 Methods

Animals

Dendroctonus ponderosae Hopkins were reared from naturally infested bolts of ponderosa pine (*Pinus ponderosa*) obtained from Doug Linton, (Pacific Forestry Division, of NRCAN) collected from a region just south of Merrit, BC, Canada (49°52'31.51"N, 120°53'34.09"W). Bolts were stored in an insect housing facility at Carleton University, in sealed 15 gal. Sterlite containers at 3-5°C and brought to room temperature (20°C) as the beetles were required for experiments. The sex of the animals was initially determined by lightly squeezing individuals and noting the presence or absence of audible chirps, a trait only found in males (Chapman, 1955; McCambridge, 1962). Following experimentation animals were preserved using Chauthani and Callahan (C&C) fixative (Chauthani and Callahan, 1966), and stored for later anatomical dissections. Gender was subsequently confirmed by observing the dimorphism of the 7th abdominal tergite (Lyon, 1958; Barr, 1969). Following all trials logs were autoclaved (Amsco Renaissance, 3021 Gravity Steam Sterilizer, Steris Corporation, Mentor, Ohio) and subsequently incinerated. Voucher specimens of test animals were fixed using C&C fixative (Chauthani and Callahan, 1966), these were kept refrigerated in the lab at Carleton University.

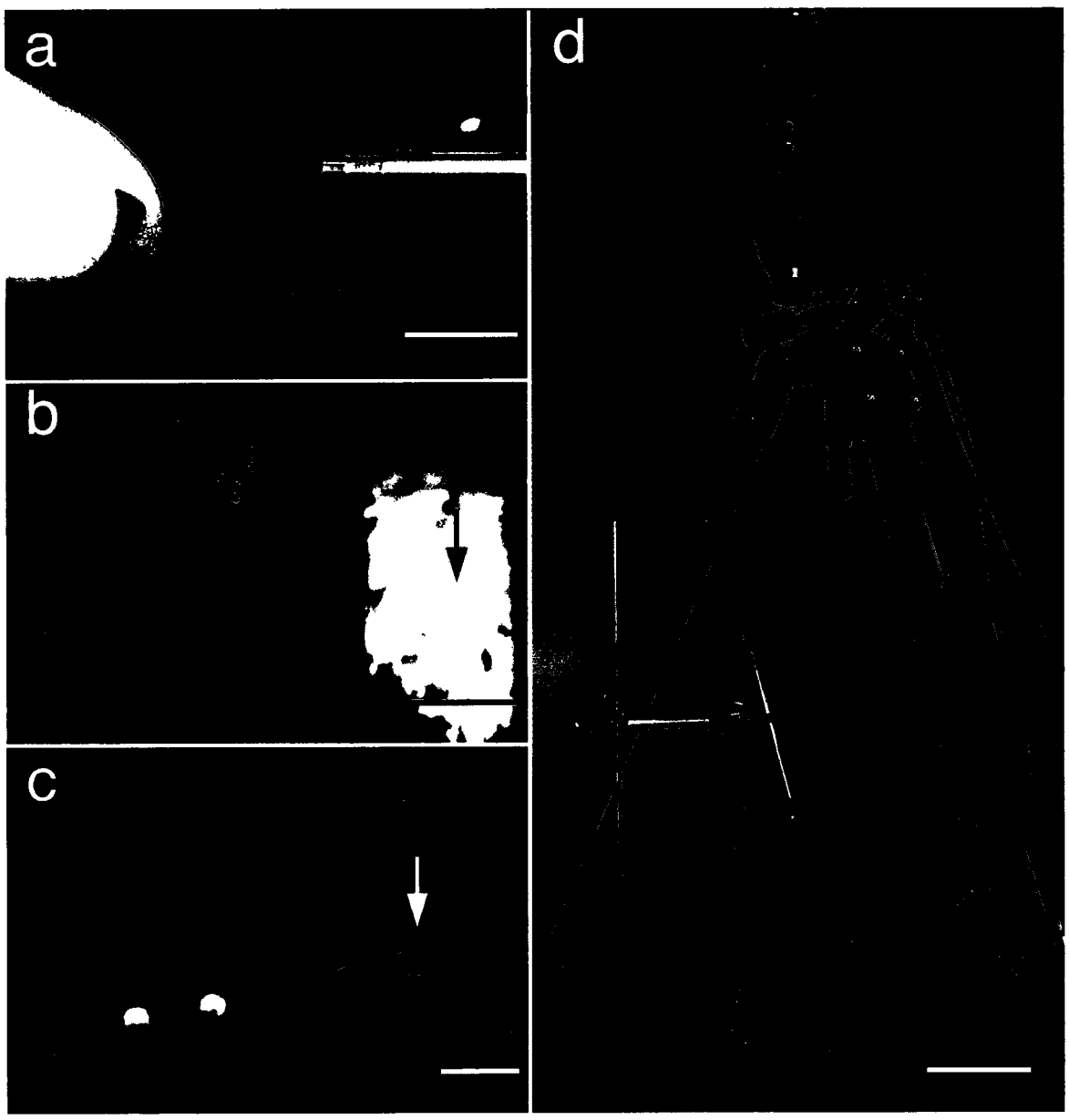
Recording Preparations

Signals were recorded during three different conditions: 1) stress in the male beetles; 2) male-female interaction; and 3) male-male interaction as defined by previous

authors (Ryker and Rudinsky, 1976; Ryker, 1988) (Figure 2.2 a-c). Assessments of both the air-borne and solid-borne components were made for the latter two conditions. All measurements were taken in a walk-in type sound isolated anechoic chamber (Model C-14A MR, Eckel Industries of Canada, Morrisburg, Ontario; 2.4m X 2.4m X 2.4m) at room temperature 22 ± 2.0˚C.

Figure 2.2 Methods for recording acoustic signals from male *D. ponderosae.* **a)** Induction of the stress sound by pinching animal between thumb and index finger, at a distance of 1cm from the microphone (scale bar = 0.5 cm). **b)** Male-female interaction, in this image the female is seen tunneling into a bolt of red pine (*Pinus resinosa*), while the male is approaching. The arrow indicates the reflective disc placed on the surface for recording vibrations (scale bar = 0.5 cm). **c)** A small arena was cut down to the phloem layer and used for male-male interaction. Arrow indicates the cage for the interacting males, two reflective target discs can be seen at 2 and 3 cm (scale bar = 1 cm). **d)** Detail of entire setup showing laser Doppler vibrometer, B&K microphone, and a bolt of red pine (*P. resinosa*) (scale bar = 10 cm).

Figure 2.2



Stress Sounds Set-up

Adult male beetles were collected from the original inoculated log. Ten individuals were induced to signal by grasping the animal between the thumb and index finger (Figure 2.2 a), and lightly pinching the pronotum and head while avoiding the elytra (to diminish any muffling or frequency deviations). Recordings continued until the animals were released.

Since stress signals were produced by pinching a handheld beetle, vibrational signals were not recorded for these trials. Airborne sounds were recorded using a Brüel and Kjær ¼" condenser microphone (Model 4939, Nærum, Denmark) with grid off, placed at distances of 1, 2, 4, and 8 cm from the beetle. Sounds were amplified with a Brüel & Kjær Nexus conditioning amplifier (type 2690, Nærum, Denmark) recorded onto a Fostex solid state data recorder (FR-2 Field Memory Recorder, Fostex, Boonton, NJ) at a sampling rate of 192 kHz, and subsequently analyzed for their spectral and intensity characteristics (see below).

Male-female Interactions Set-up

Recordings of interactions between males and females were conducted by inoculating freshly cut bolts of red pine (*P. resinosa*) with newly emerged females. Female beetles were placed near pre-drilled boreholes (~2mm) and then secured in place for 24 hours using empty gel capsules placed over the borehole. Once a burrow was established (as noted by the accumulation of frass directly above the borehole), the gel capsule was removed and a newly emerged male was placed on the surface of the log at a

distance of 1 cm from the gallery entrance (Figure 2.2 b). The interaction was recorded from this time until signaling stopped, using an ultrasound sensitive microphone, laser vibrometer, and video camera.

Airborne sounds were recorded using the same B&K microphone setup as described above, placed 1 cm away from the gallery entrance. For laser recordings a small portion of the cortex of the log was cut away to reveal the vascular tissue 1 cm away from the female burrow. A laser target disc was then placed directly onto the phloem layer. Vibrational signals were recorded using laser Doppler vibrometry (PDV-100, Polytec Inc., Ann Arbor, MI, USA) at the most sensitive setting (Velocity 22 mm/s; high pass filter off; Low Pass Filter 20 kHz). Laser signals were recorded directly onto a Marantz solid state data recorder (PMD 671, D&M Professional, Itasca, IL) at a sampling rate of 44.1 kHz. All behaviours and corresponding events were monitored using a Sony high definition MiniDV camcorder (HDR-HC7, Sony, Tokyo, Japan) with a second microphone (ECM-MS908C, Sony, Tokyo, Japan) adjacent to the setup connected to the microphone jack.

Male-male Interactions Set-up

Male-male encounters were achieved by cutting a narrow arena (0.5 X 1 cm) out of the cortex of the log as described above, ensuring that encounters were staged on the phloem layer (Figure 2.2 c). Beetles were secured within the arena by placing a section of fiberglass mesh directly above the arena. Directly adjacent to the arena a larger section of the cortex (1 cm X 3cm) was cut away for recording purposes, this area remained uncovered. Beetles were placed into the arena one at a time. The first male was introduced into the arena and when all signaling stopped (a consequence of being

handled) the other male was added. Vibrational signals were recorded from laser target discs placed in the uncovered portion of the arena at 1, 2, and 3 cm from the interacting beetles, while airborne signals were recorded with the B&K microphone suspended at a distance of 1 cm above the arena. Sound and vibration recordings were conducted as described above for male-female recordings.

Signal Analysis

Airborne and vibrational signals were analyzed using Raven Bioacoustics Research Program (Cornell Laboratory of Ornithology, Ithaca, NY, USA). The bottom 800 Hz were filtered from the airborne signals due to the presence of a very strong noise profile. Since the airborne sounds contained such high frequencies it was justified that there was no signal present in the bottom 800 Hz. In the case of vibrational signals the noise component was more pronounced so the bottom 400 Hz were filtered, additional filtering was achieved by applying a spectral subtraction of the noise profile using Wavepad acoustic program v 3.20 (NCH Swift Sound, Bruce, ACT, Australia). In both cases filtering was applied in order to better visualize the signals but not for spectral analysis.

Temporal characteristics of all signal types investigated in this study have been previously reported for airborne sounds (Michael and Rudinsky, 1972; Rudinsky and Michael, 1973; Rudinsky and Michael, 1974). Therefore measurements of temporal structure of signals were limited to noting the chirp duration and structure so that it could be confirmed that these signals were comparable to those made under similar conditions in previous studies. Within this study a chirp was defined as "the shortest unitary rhythm

element of a sound emission that can be readily distinguished as such by the unaided human ear", after Broughton (1963). Chirps have been further categorized as simple or complex types (Ryker, 1988). A simple chirp was defined as a series of regularly spaced toothstrikes as part of only one contiguous subunit. Complex chirps had two or more subunits, broken up by brief periods of silence (up to 3 ms in duration) (Ryker and Rudinsky, 1976).

Spectral analysis was performed on each chirp component for each signal type for both airborne and vibratory signals. Power spectra were produced using a 512-point Fast Fourier Transform (FFT) Hanning window. For each chirp (simple chirps), or each chirp component (complex chirps) the dominant peak, as well as the first, second and subsequent peaks (up to 5) were identified for the first 5 signals during an interaction. A Kruskal-Wallis non-parametric analysis of variance was conducted comparing the distribution of frequency peaks across the three different conditions, for both the simple and complex chirp types. If significance was found this was followed by a Wilcoxon test as a post-hoc analysis. The results of this analysis were presented on Figures 2.13 and 2.14, as letters indicating where a significant difference was observed. A non-parametric analysis was chosen due to the fact that the data points did not follow a normal distribution.

Sound Pressure Level of Airborne Sounds

Sound production was induced in adult male beetles at 3 different recording distances (1, 2, and 4 cm). Recordings were made using a Brüel & Kjær 1/4 inch microphone type 4939 with grid off, and amplitudes measured as voltages on an

oscilloscope (THS720A, Tektronix, Richardson, TX, U.S.A.). The corresponding peak-to-peak voltages (mV) were recorded at the varying distances. It was determined that at a distance of 2 cm sound levels would be most accurate as well as biologically relevant. Calibration involved producing an intensity series of continuous pure tones centered at the mean peak frequency of chirps (~10 kHz) generated with a Waveform Generator (50MS/s, Tabor Electronics, Tel Hanan, Israel) and broadcast through a tweeter (Horn Tweeter, GT-1016, response 3.5-40 kHz). Decibel measurements of the pure tones were taken using an Integrating Sound Level meter (Type 2239, Brüel & Kjær, Nærum, Denmark) placed at the same distance from the sound source as the microphone. The peak-to-peak voltage, and dB SPL were recorded for the pure tone over 5 amplitudes. A log-log plot was prepared by plotting the peak-to-peak voltages against the dB SPL for the pure tone, yielding a straight line. Signal intensity values for the stress signals were determined by acquiring the equation of the line described by the calibration plot and applying this function to the voltages collected from the signals.

Calibration of Vibrational Signal Velocity

Velocity measurements of vibration signals were determined by recording rivalry signals at distances of 2 and 4 cm from the closest male using the laser vibrometry set up described above. Calibration signals were created by using an electrodynamic mini-shaker (Type 4811, B&K, Nærum, Denmark), driven by a calibrated power amplifier, (Type 2718, B&K, Nærum, Denmark) and a Waveform Generator (50MS/s, Tabor Electronics, Tel Hanan, Israel). A pure tone of known velocity output was generated by the mini-shaker (1 V = 5 mm/s) (PDV-100 Manual, Polytec Inc., Ann Arbor, MI, USA), and recorded onto the data recorder using the same levels used to record the beetle

vibration signals. By cross multiplying the average relative amplitude of the signals as output by Raven bioacoustics program with the average relative amplitude of the 1 volt peak to peak calibration signal the average velocity of the signal was calibrated. This was chosen over other methods in that it allowed for a greater sample size without having to modify the signal prior to calibration.

2.4 Results

Stress Sounds

Upon 'attack', males generated a train of chirps that typically continued until animals were released (Figures 2.3, 2.4). In 8 of the 10 trials simple chirps were the dominant form (Figure 2.3). Two of the 10 animals sampled showed mostly complex chirps (Figure 2.4). Simple chirps were shorter in duration (26.6 ms ± 10.7 ms, n= 240) than complex chirps (91.6 ± 12.4 ms, n = 60).

The first 5 simple chirps from 8 individuals were analyzed, giving a total of 40 chirps. Simple chirps had up to 4 frequency peaks (F1- F4), which occurred between 8.9 and 74.93 kHz (Table 2.2) (n=40) (Figure 2.3). Dominant frequency was defined as the frequency with the highest relative intensity. In 76% of these simple chirps the dominant frequency occurred in the sonic range (below 18 kHz), with the remaining 24% in the ultrasound range. Stress chirps were predominantly of the simple type with an average dominant frequency of 8.91 ± 2.10 kHz (n=40).

The first 5 complex chirps from 2 individuals were analyzed, giving a total of 10 chirps. Complex chirps had from 2 to 5 components (mean 2.8 ± 0.6 components) (Figure 2.4). The frequency distribution observed in each of the components of the complex chirps fell within a similar range to those described for the simple chirps. For each

component, the proportion of samples where the dominant peak was in the sonic or ultrasonic range was also examined. Components 1, 2, and 3 showed dominance below 18 kHz in 45.4, 54.5 and 90.0% of analyzed signals respectively. Complex chirps, when present, showed an average dominant frequency of 16.55 ± 14.60 kHz (n=10) for component 1, 9.34 ± 2.17 (n=10) kHz for component 2 and 8.51 ± 2.60 (n=10) for component 3.

Sound intensities of the signals measured at 2 and 4 cm were 55.71 and 47.14 dB SPL respectively. Peak relative intensity was measured at 1 cm, 4 cm, 8 cm, and 10 cm. Relative decay was calculated over this range of distances where it was shown to decay by 63%, 73%, and 99% respectively (Figure 2.5).

Figure 2.3 Simple stress chirps. **a)** Oscillogram illustrating a series of 14 chirps filtered in Raven to remove the bottom 800 Hz of noise. **b)** Four chirps from (**a**) (black circles) shown at an expanded time scale, with a corresponding spectrogram. **c)** A single chirp from (**b**) (black circle), showing individual tooth strikes. **d)** Power spectrum of the chirp presented in part (**c**).

Figure 2.3

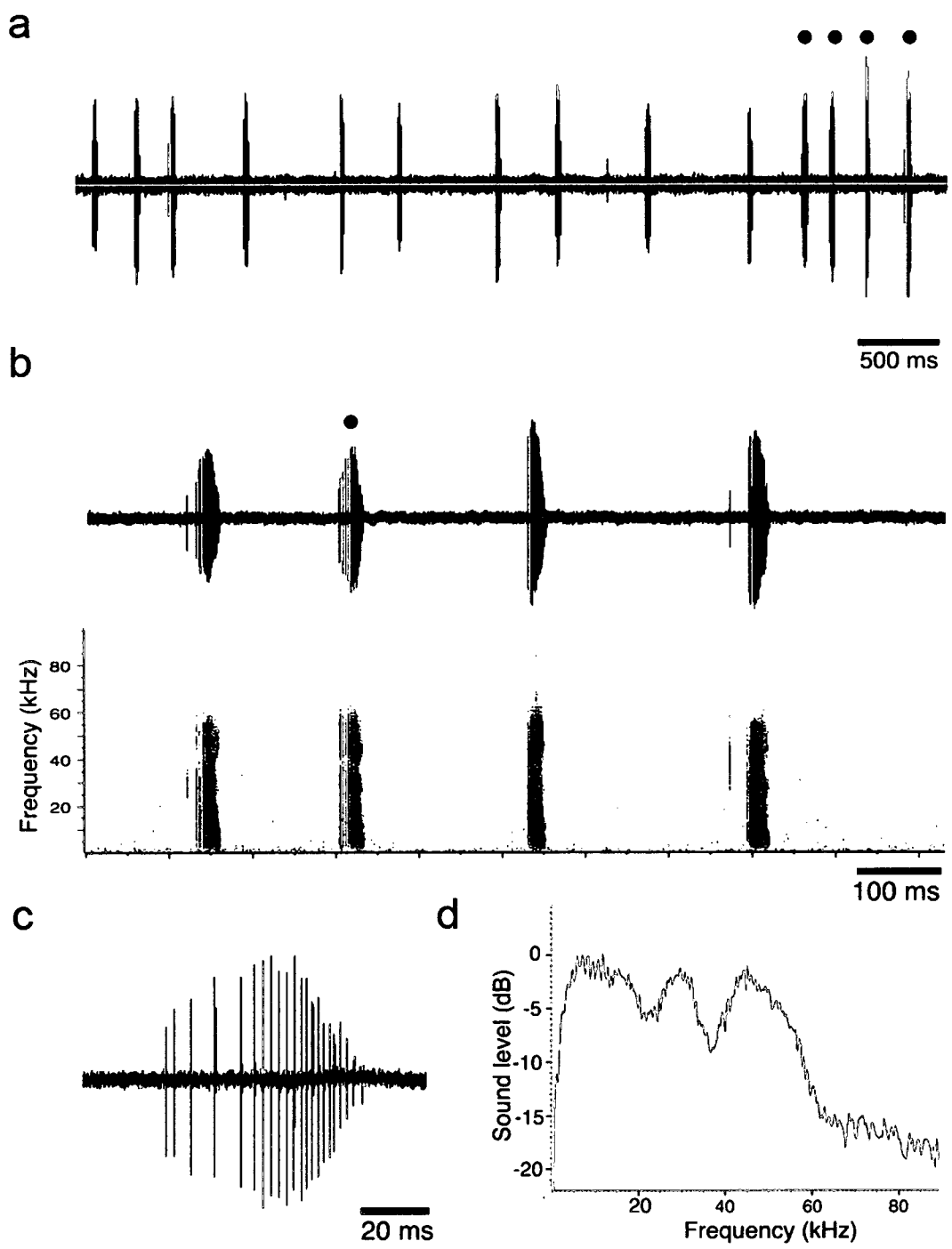

a
500 ms
b
Frequency (kHz)
80
60
40
20
100 ms
c
20 ms
d
Sound level (dB)
0
-5
-10
-15
-20
20
40
60
80
Frequency (kHz)

Figure 2.4 Complex stress chirps. **a)** Oscillogram illustrating a series of chirps filtered in Raven to remove the bottom 800 Hz of noise. **b)** Four chirps from the train in (**a**) (black circles) shown at an expanded time scale, with a corresponding spectrogram. **c)** A single chirp from (**b**) (black circle), showing the individual subunits of the complex chirp. d) Spectra of the 3 chirp components shown in (**c**)

Figure 2.4

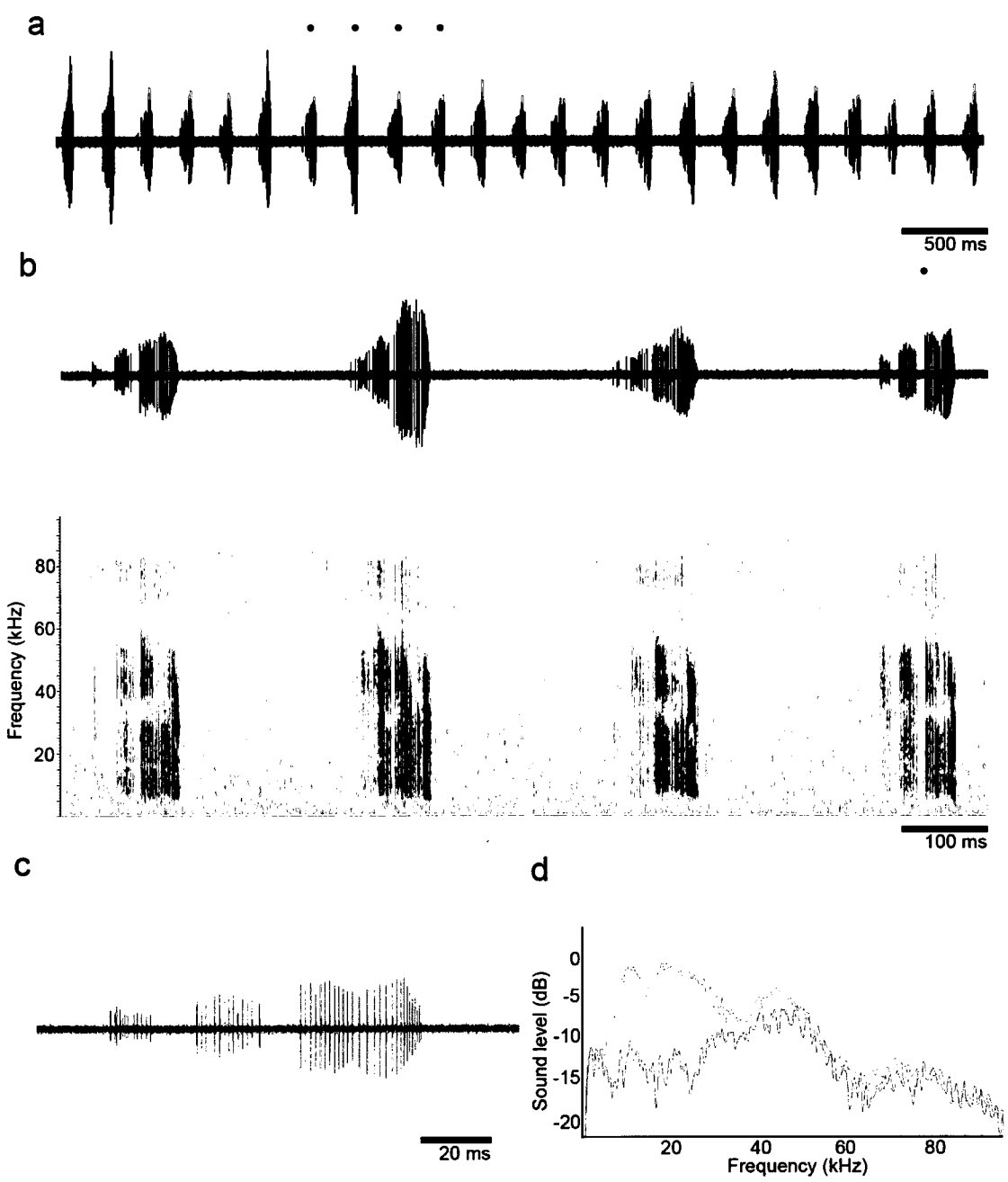

a
500 ms
b
Frequency (kHz)
80
60
40
20
100 ms
c
20 ms
d
Sound level (dB)
0
-5
-10
-15
-20
20
40
60
80
Frequency (kHz)

Table 2.2: Summary of temporal and spectral statistics of airborne sounds in *D. ponderosae*

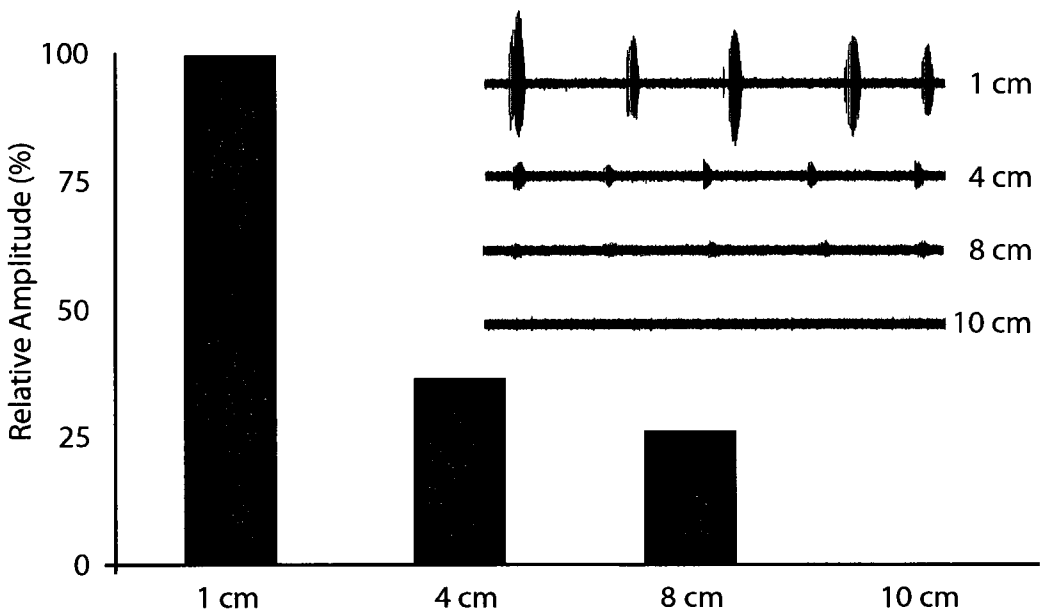
Context	Type	% of total	Mean duration + SD (ms) (241)*	Mean Comp. +SD	Component 1 Mean Frequency + SD (kHz) (n)				
					F1	F2	F3	F4	F5
Stress	Simple	93.3	26.66 ± 10.73	1	8.91 ± 2.10 (50)	27.71 ± 5.42 (50)	56.00 ± 15.91 (50)	74.93 ± 6.16 (45)	83.34 ± 0.13 (15)
	Complex	6.7	91.64 ± 12.41	2.90 ± 0.56	16.55 ± 14.60 (10)	47.73 ± 5.34 (10)	77.06 ± 22.49 (10)		
Male-female			(300)						
	Simple	31.2	44.00 ± 25.12	1	6.50 ± 2.33 (45)	24.18 ± 8.20 (45)	47.66 ± 14.86 (45)	64.91 ± 12.50 (40)	75.04 ± 2.48 (15)
	Complex	68.8	125.11 ± 40.52	2.38 ± 0.89	7.88 ± 4.07 (50)	28.08 ± 12.29 (50)	47.93 ± 12.66 (40)	71.81 ± 0.27 (10)	
Male-male			(287)						
	Simple	75.9	30.06 ± 17.00	1	9.36 ± 3.38 (50)	24.01 ± 7.91 (45)	45.48 ± 13.28 (45)	59.78 ± 7.60 (40)	70.47 ± 5.14 (30)
	Complex	24.1	92.26 ± 26.15	2.49 ± .50	12.50 ± 4.36 (35)	31.41 ± 6.63 (35)	46.88 ± 11.45 (35)	58.63 ± 11.09 (20)	75.38 ± 1.00 (5)

* numbers in brackets represent number of individual signals sampled, for the spectral characteristics an n of 5 signals per animal were sampled

Component 2 Mean Frequency + SD (kHz) (n)					Component 3 Mean Frequency + SD (kHz) (n)				
F1	**F2**	**F3**	**F4**	**F5**	**F1**	**F2**	**F3**	**F4**	**F5**
9.34 ± 2.17 (10)	27.50 ± 1.88 (10)	44.63 ± 1.06 (10)	78.34 ± 1.54 (10)		8.51 ± 2.60 (10)	25.60 ± 0.69 (10)	42.23 ± 0.95 (10)	78.60 ± 2.76 (10)	
7.81 ± 3.90 (50)	22.10 ± 9.22 (50)	40.47 ± 13.38 (50)	58.13 ± 13.16 (45)	75.00 ± 7.50 (20)	7.42 ± 3.23 (20)	23.13 ± 12.04 (20)	39.43 ± 14.95 (20)	58.19 ± 14.48 (20)	
10.23 ± 2.57 (35)	25.10 ± 5.13 (35)	43.70 ± 6.91 (30)	62.87 ± 5.97 (25)	81.44 ± 10.52 (10)	11.17 ± 3.45 (15)	27.81 ± 3.53 (15)	48.50 ± 11.50 (15)		

Figure 2.5 Mean percent intensity decay for airborne stress signals over distance (1, 4, 8, and 10 cm from source) (n = 5 samples per distance). Inset oscillograms show representative traces at each distance.

Figure 2.5


100
75
50
25
0
Relative Amplitude (%)
1 cm
4 cm
8 cm
10 cm
1 cm
4 cm
8 cm
10 cm

Male-Female Interactions

Males placed in the vicinity of a female borehole, were shown to produce a steady train of chirps (Figures 2.6 - 2.8) while digging into the female frass (Figure 2.2 b). Figure 2.6 shows a detailed account of a typical trial. After the male is placed on the log he immediately begins his approach to the gallery entrance, producing complex chirps as he nears the borehole. After the initial bout of complex chirps the male becomes silent at which point he is in the burrow and should now begin his physical courtship of biting and pushing (Ryker, 1988). The cycle of complex chirping and silence is repeated once more before the signaling turns to simple chirping. Measurements were taken observing the change in signal structure over time. Analysis of signal type over time was accomplished by dividing trials into 1 minute segments and then comparing the proportion of simple to complex signals. Figure 2.9 shows that as time progressed the proportion of simple signals within the trial grew as the proportion of complex signals declined. Temporal measurements for signal analysis were taken from the first 30 chirps generated by the male from the beginning of an encounter, which are considered to be 'attractant' chirps based on earlier reports (only the first 30 chirps occurring within the first minute of the encounter were sampled) (Ryker, 1988). In some instances the male produced stress sounds while being placed. However in every case the male stress sounds ceased once the animal was released. As the male searched for the borehole he remained silent until coming into contact with the female's frass, at which point he began his attractant chirp (Ryker, 1988). In some instances throughout the trials female chirps were also observed (Figure 2.7). Presumed female chirps were distinguished from male simple chirps by their shorter duration, decreased number of toothsrikes and longer interval between

toothstrikes (Ryker and Rudinsky, 1976). Female chirps were also much lower in relative intensity than those of the male, since during the entire encounter females were within the burrow, while the males were on the surface of the bolt (Figures. 2.1 d, 2.6). In contrast to the trend seen with stress chirps, complex chirps were predominant (70.3%) during the first minute of male-female interactions (Figures 2.7, 2.9; Table 2.2). While all 10 animals displayed complex chirps, 9 out of 10 animals also produced simple chirps at some point throughout the first minute analyzed, as well as later through the trial.

Spectral characteristics of airborne sounds generated by males were measured for the first 5 simple and 5 complex chirps from 10 individuals occurring within the first minute of the encounter. Simple chirps recorded at 1 cm from the microphone typically had between 3 and 5 peak frequencies (F1, F2, F3, F4 and F5), which occurred at 6.50, 24.18, 47.66, 64.91 kHz, and 75.03 kHz respectively (Table 2.2). In 64% of simple chirps analyzed the dominant frequency occurred at the F1 position within the range of 4.5-5.5 kHz (Figure 2.7), although in a few instances dominant frequencies were observed as high as 44.5 kHz. Spectral characteristics were not assessed for female airborne signals, since the females were always in the gallery, and therefore attenuated by the male blocking the hole.

Male complex chirps had from 2 to 5 components (mean 2.3 ± 1.2 components) and typically had between 3 and 5 frequency peaks per component (F1, F2, F3, F4 and F5) (Table 2.2). Distribution of peak frequencies in the complex chirps showed the dominant frequency occurring below 18 kHz in 54.84, 64.58 and 72.41% of signals for component 1,2, and 3 respectively. While it can be said that complex "attractant" chirps showed dominance mainly in the sonic range, it should be noted, that on average, there

was a difference in the distribution of peak frequencies across the different components, with component 1 having the largest concentration of dominant peaks occurring in the ultrasonic range (Table 2.2).

Vibration signals were recorded on the phloem layer at a distance of ~1 cm from the interacting individuals. Generally the first component of the airborne signal was lost. Since this component had on average, the highest dominant frequencies, the loss of this component could be due to attenuation of the higher frequencies in the wood (Figure 2.8). Simple chirps typically had between 3 and 4 peaks occurring at 4.92, 8.71, 12.39 kHz, and 15.74 kHz, respectively, with dominant peaks occurring in the range of 5-6 kHz (Table 2.3). Complex chirps had up to 3 components, with dominant frequencies for each component occurring in the range of 4.5-5.5 kHz. Of the multiple components which made up a complex signal, the second component was the most intense (Figure 2.8) (Table 2.3). Velocity measurements were not calibrated on these signals, but were comparable in relative amplitude to those recorded for male-male signals, which were calibrated (see next section).

Figure 2.6 One male-female trial showing sequence of events occurring in a typical trial. Two minute sequence of male-female signaling showing bouts of chirping interrupted with bouts of silence. After first minute complex signaling gives way to interspersed simple signals and bouts silence. **1**) Male is placed on log at 1 cm from female borehole. **2**) Male approaches female borehole and begins chirping. **3**) Male enters female borehole and continues to produce complex chirps interspersed with bouts of silence. This trial ends in silence after 2 minutes of chirping (scale bar = 2s). Inset: **i**) Blow up of one complex "attractant" chirp occurring at 26.82 s. **ii**) Blow up of one simple presumed "courtship" chirp occurring at 1:17:72 s (Scale bars = 20 ms).

Figure 2.6

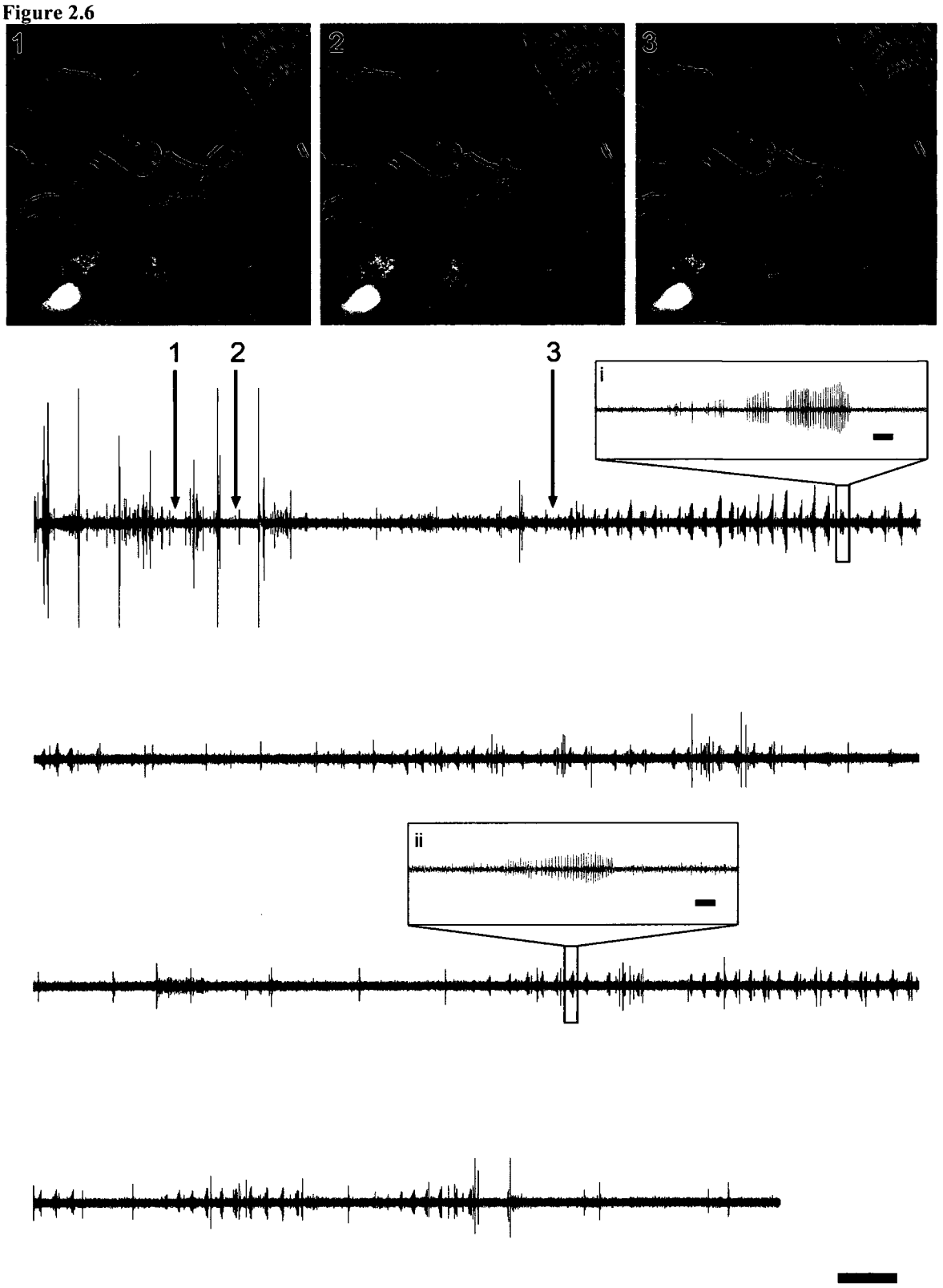


Figure 2.7 Airborne complex male-female chirps. **a**) Oscillogram illustrating a series of chirps filtered in Raven to remove the bottom 800 Hz of noise (asterisk marks presumed female chirps). **b**) Five chirps from the train in (**a**) (black circles), shown at an expanded time scale, and with a corresponding spectrogram. **c**) A single chirp from (**b**) (black circle), showing the individual subunits of the complex chirp. **d**) Spectra of the 3 chirp components shown in (**c**).

Figure 2.7

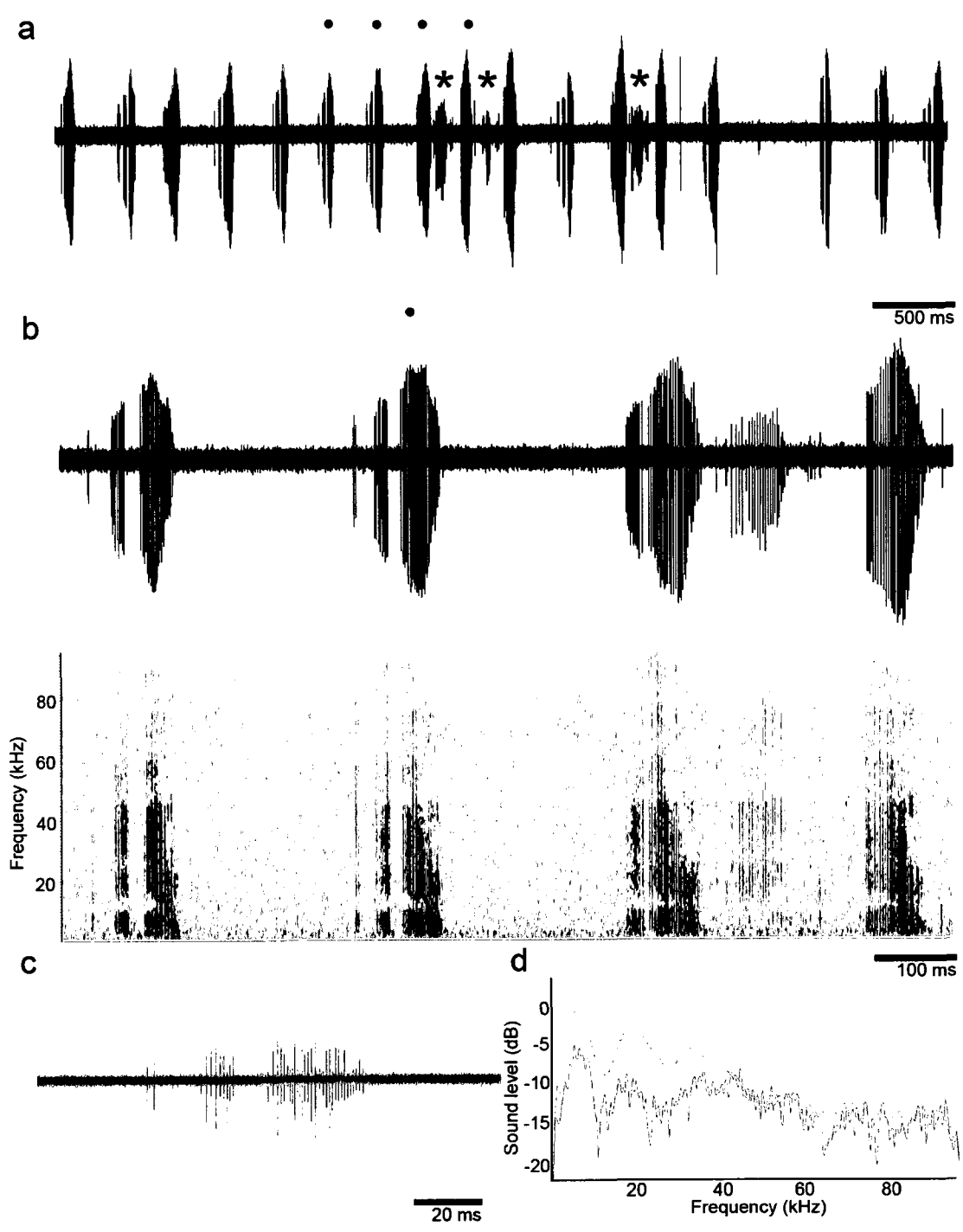

a
b
500 ms
Frequency (kHz)
80
60
40
20
c
20 ms
d
100 ms
Sound level (dB)
0
-5
-10
-15
-20
20
40
60
80
Frequency (kHz)

Figure 2.8 Vibratory component of male-female chirps. **a**) Oscillogram illustrating the vibratory component of the same series of chirps as in 2.8, with the bottom 400 Hz filtered in Raven. **b**) Four chirps from the train in (**a**) (black circles), shown at an expanded time scale, and with a corresponding spectrogram. **c**) A single chirp from (**b**) (black circle), showing the individual subunits of the complex chirp. **d**) Spectra of the 3 chirp components shown in (**c**).

Figure 2.8

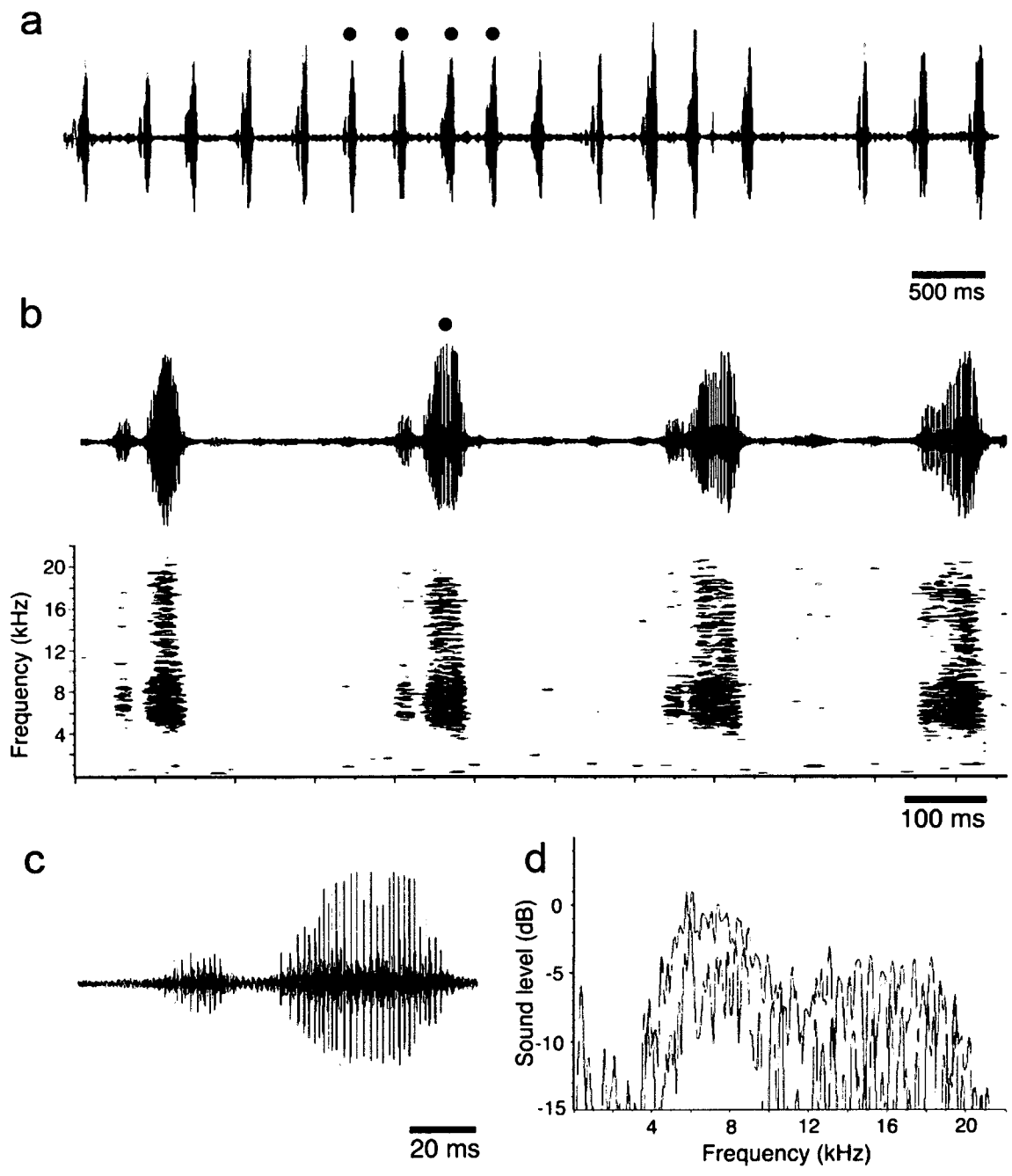

a
500 ms
b
Frequency (kHz)
20
16
12
8
4
100 ms
c
20 ms
d
Sound level (dB)
0
-5
-10
-15
4
8
12
16
20
Frequency (kHz)

Figure 2.9 Histogram showing the percentage of signal type (simple vs. complex) over time. Bars indicate the percentage of chirps within a 1 minute window of trial time. It can be seen that as time progresses the male-female interaction goes from predominantly complex signaling to simple signaling. Signals were sampled from 5 male-female trials.

Figure 2.9

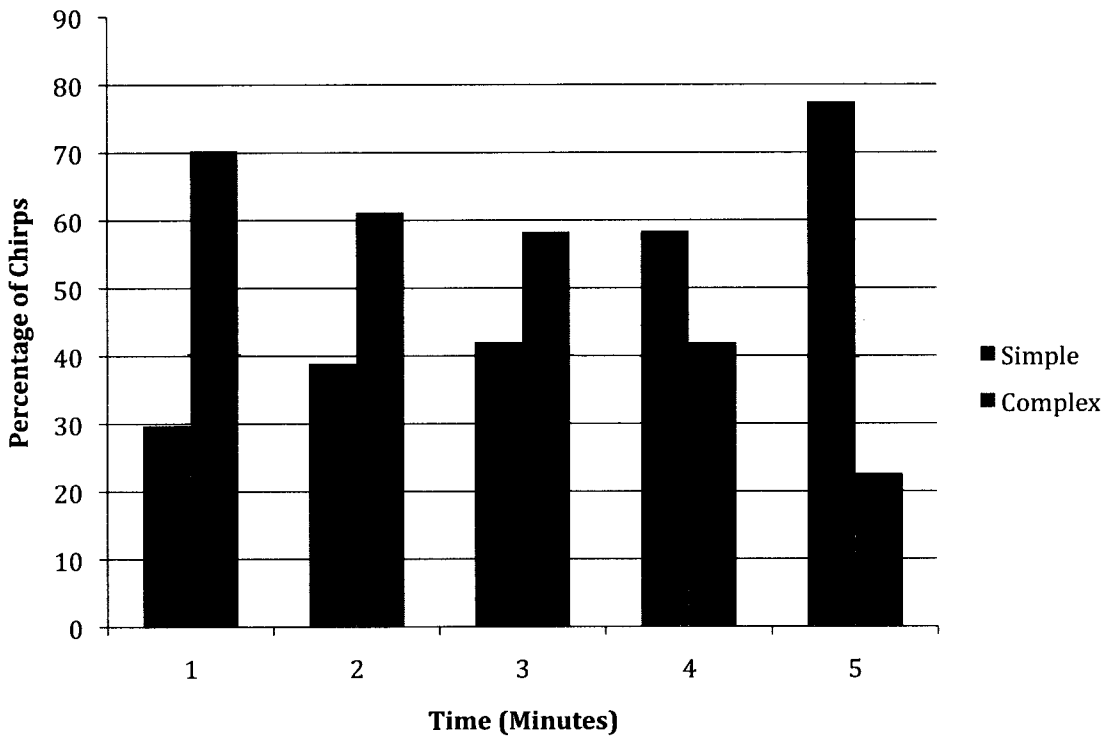

90
80
70
60
50
40
30
20
10
0
Percentage of Chirps
1
2
3
4
5
Time (Minutes)
Simple
Complex

Table 2.3: Summary of temporal and spectral statistics of vibrational signal components in *D. ponderosae.*

Context		Component 1 Mean Frequency +SD (kHz) (n)				Component 2 Mean Frequency +SD (kHz) (n)			
		F1	**F2**	**F3**	**F4**	**F1**	**F2**	**F3**	**F4**
Male-female	Simple	4.92 ± 1.02 (5)	8.71 ± 1.17 (5)	12.39 ± 1.51 (5)	15.74 ± 2.83 (4)				
	Complex	4.61 ± 1.21 (5)	8.13 ± 1.73 (5)	11.98 ± 2.10 (5)	14.29 ± 2.89 (5)	4.50 ± 0.96 (5)	8.23 ± 1.44 (5)	12.42 ± 1.21 (5)	15.89± 2.28 (5)
Male-male	Simple	6.75 ± 2.51 (5)	11.93 ± 3.12 (5)	17.80 ± 2.18 (5)	22.28 ± 6.97 (2)				
	Complex	7.10 ± 1.92 (5)	12.95 ± 2.85 (5)	17.97 ± 3.90 (5)	20.20 ± 2.98 (5)	6.35 ± 1.83 (5)	11.18 ± 3.42 (5)	17.08 ± 3.26 (5)	19.73± 1.60 (5)

* number in brackets refers to total number of signals analyzed 5 signals per individual to a maximum of 5 individuals per condition

Male-Male Interactions

When two males were constrained in a small arena, they both began to stridulate (Figures 2.10, 2.11). Since both animals were placed in the arena with their stridulatory apparatus intact, and assuming that both animals were producing sonic signals, it was not possible to distinguish between the two individuals. Therefore, temporal characteristics were made for the first 30 consecutive chirps from 10 interactions without specifying which individual produced them. The similarity of the signals also made it difficult to establish any calling rhythm quantitatively, however qualitative observation of some trials revealed signals of 2 different amplitudes which may be one male responding to the calls of the other (Figure 2.10). In 9 out of 10 trials the majority of chirps were simple (Table 2.2), and in 7 of the 10 trials also displayed complex chirps.

Spectral characteristics of airborne signals were measured for the first 5 simple and 5 complex chirps from 10 male-male interactions. Simple chirps recorded at 1 cm from the microphone had up to 5 peak frequencies occurring at 9.36, 24.01, 45.58, 59.78, and 70.47 kHz respectively (Figure2.10) (Table 2.2). In the case of male-male simple chirps the dominant frequency peaks occurred between 3 frequency ranges, 38% dominance occurring within the range of 34-40 kHz, 34% between 4 and 12 kHz, and 18% within the 15-21 kHz range, with the remainder of the signals not being assigned to any category. When the proportions of dominant frequencies were examined, male-male simple chirps were shown to have only 39% dominance below 18 kHz (Figure 2.13). Most of the peak frequency energy was present in the ultrasonic range for this signal type.

Complex chirps had from 2 to 5 components (mean 2.5 ± 0.5 components), with

the dominant frequencies being between 37-38 kHz for component 1, 35-37 for component 2, and, 27-28 kHz for component 3 (when present) (Figure 2.10) (Table 2.2). Distribution of peak frequencies in the complex chirps showed the dominant frequency occurring below 18 kHz in 12.96, and 7.69 % of signals for component 1, and 2 respectively. Qualitatively it was observed that a much higher proportion of chirps in the male-male interactions showed dominance in the ultrasonic range of frequencies than for the other two signal types (Figure 2.14).

Vibration signals were recorded on the phloem layer at distances of 1, 2 and 3 cm from the interacting individuals (Figure 2.11). The temporal structure was different between the airborne and vibrational components of the signals because some of the components were lost (due to attenuation) (Figure 2.9 and 2.10). Quantitative descriptions of the male-male interactions can be seen on Table 2.3. The peak frequency of simple chirps was between 8-9 kHz. In complex chirps, both the first and second component showed similar dominant frequency ranges of 8-11 kHz for component 1, and 8-10 kHz for component 2 (Table 2.3). Analysis of the amplitude of the components revealed that the second component was on average the most intense of all components. Velocity measurements calibrated at 1 cm away from the signal source were found to produce a range of velocities from 1.86 – 3.71 mm/s, with an average velocity of 2.76 ± 0.93 mm/s (n=10 chirps per animal over 5 animals). Signal intensity was observed to decay 30% from distance of 2 to 3 cm, but was still distinguishable after filtering (Figure 2.12).

Figure 2.10 Airborne male-male *D. ponderosae* chirps. **a**) Oscillogram illustrating a series of chirps showing both simple and complex filtered in Raven to remove the bottom 800 Hz of noise. **b**) Five chirps from (**a**) (black circles) shown at an expanded time scale, with corresponding spectrogram. **c**) A single chirp from (**b**) (black circle), showing individual tooth strikes (note the complex structure). **d**) Power spectrum of the time expanded chirp presented in part (**c**).

Figure 2.10

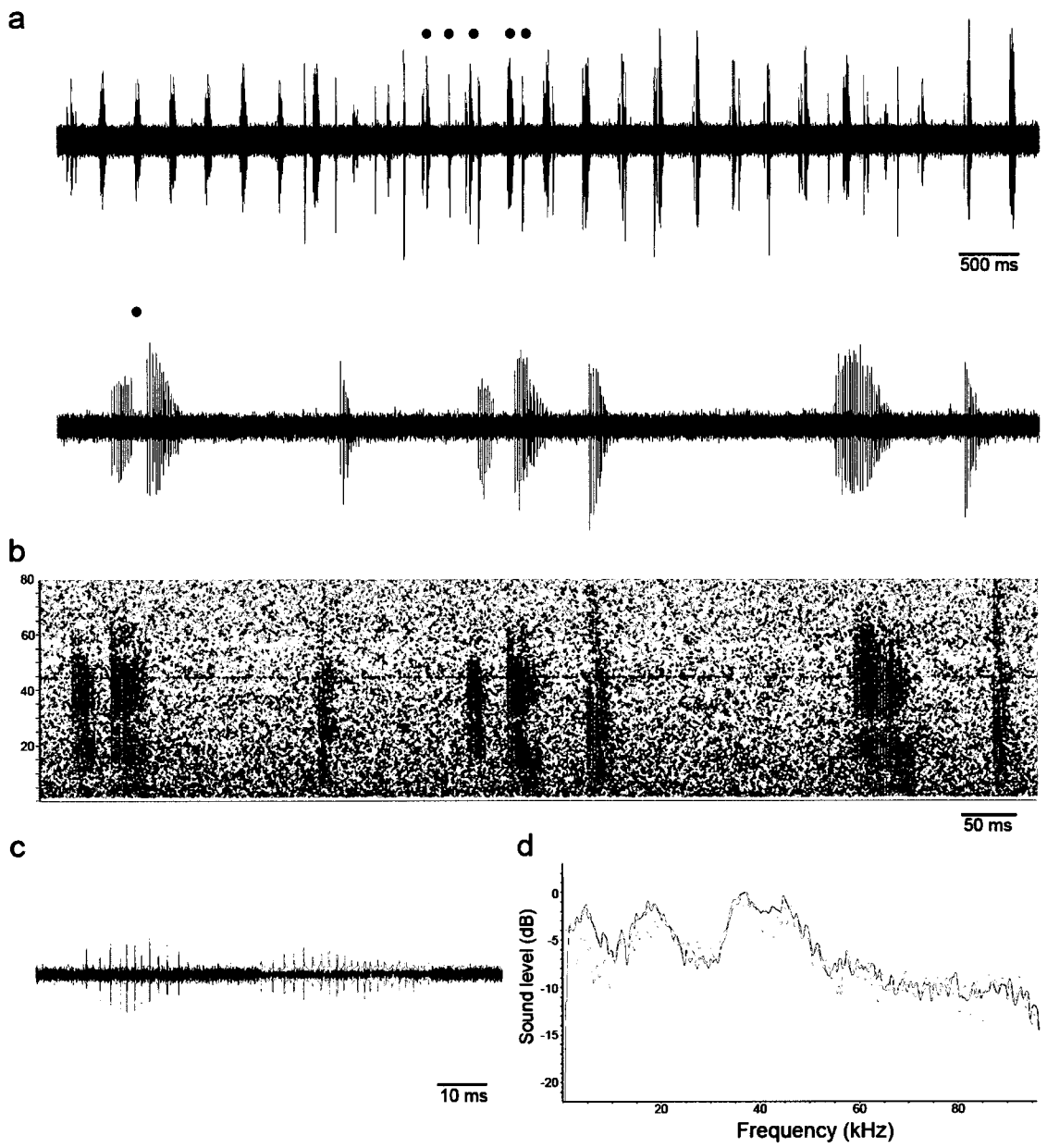

a
500 ms
b
80
60
40
20
50 ms
c
10 ms
d
0
-5
-10
-15
-20
Sound level (dB)
20
40
60
80
Frequency (kHz)

Figure 2.11 Vibratory component of male-male chirps. **a**) Oscillogram illustrating the vibrational component of the same signal presented in Figure 2.10 filtered in Raven to remove the bottom 400 Hz. **b**) Five chirps from the train in (**a**) (black circles), shown at an expanded time scale, with a corresponding spectrogram. **c**) A single chirp from (**b**) (black circle), showing the individual subunits of the complex chirp. **d**) Spectra of a single chirp component visible in part (**c**).

Figure 2.11

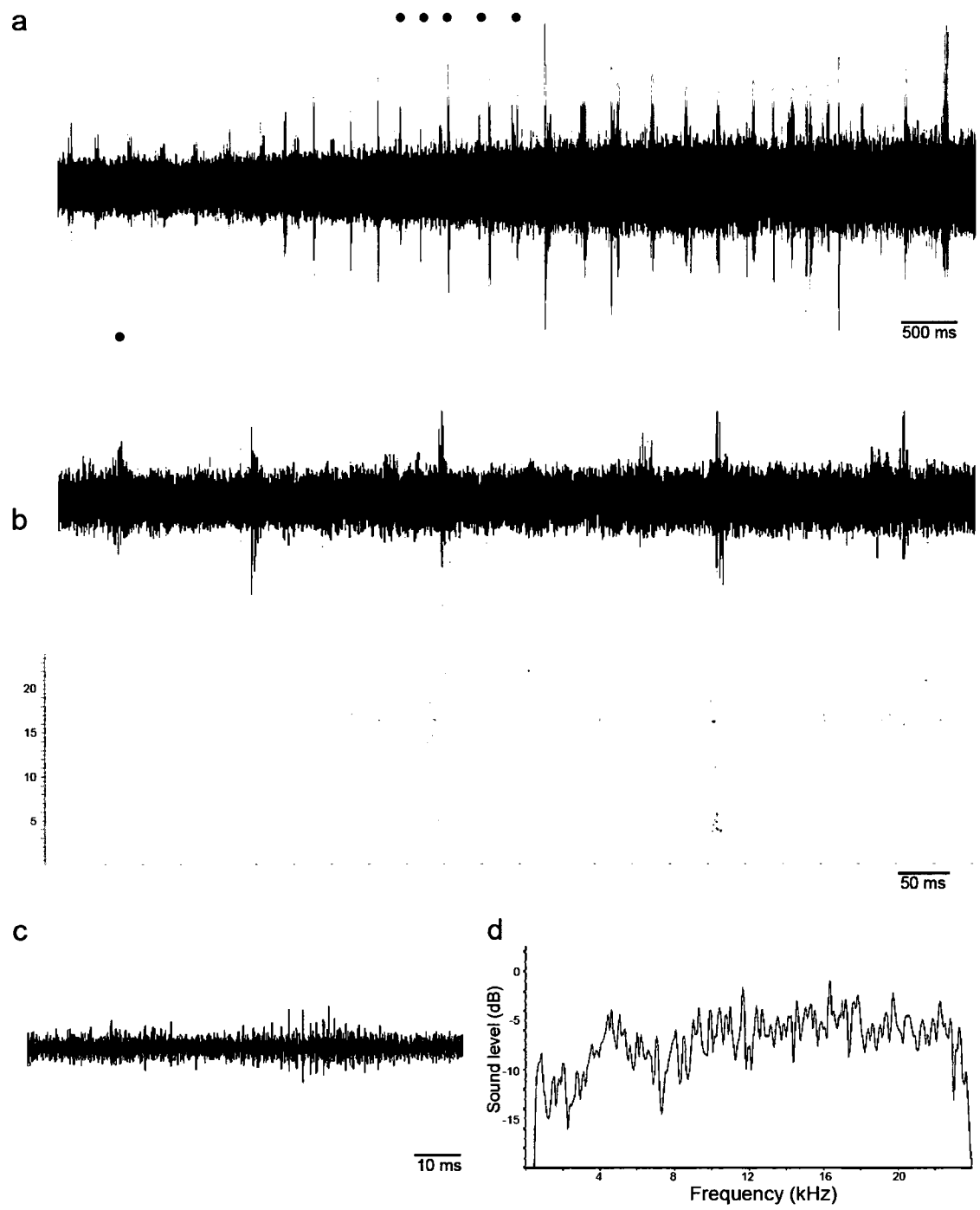
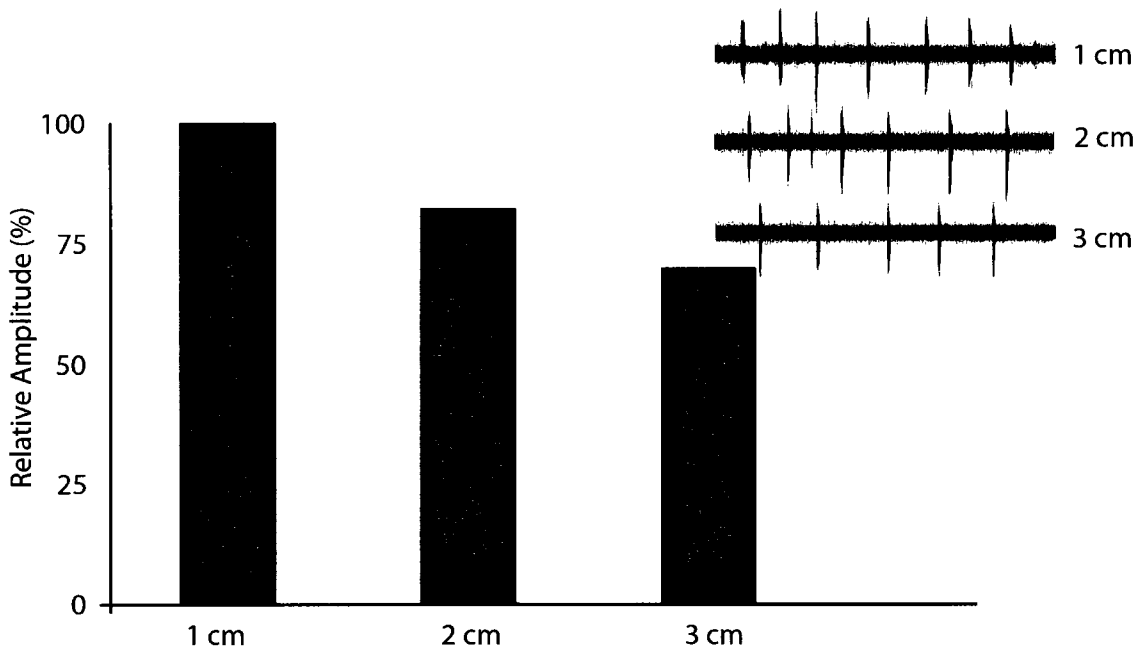

a
500 ms
b
20
15
10
5
50 ms
c
10 ms
d
Sound level (dB)
0
-5
-10
-15
4
8
12
16
20
Frequency (kHz)

Figure 2.12 Mean percent decay of amplitude of solid borne male-male signals over a range of distances (1, 2, and 3 cm from source) (n=5 animals per distance setting). Inset oscillograms show representative traces at each distance.

Figure 2.12



Comparisons between signals

Comparisons of the temporal characteristics of the airborne sounds showed statistically significant differences in all cases with two exceptions: duration between the complex signals of stress and male-male, and the number of components between complex male-female and male-male signals (data not shown). Temporally each signal type is different, conveying different information dependent on the context of the signal.

Comparisons were conducted using the spectral (frequency peak) characteristics for the both signal types (simple or complex) produced by the male beetles, in three situations: stress, male-male (territorial), and male-female. A non-paramteric Kruskall-Wallis analysis of variance was conducted to test for differences across the different signal types. Any significant difference observed between the signal types, was indicated by a superscript letter above the corresponding bar (Figures 2.13, and 2.14). This test was chosen due to the fact that the distribution of the data pertaining to frequency deviated slightly from normality. Differences between the signal types were established using a Wilcoxon post-hoc analysis. The F1 peak for the male-female simple signal differed significantly from the other two, contexts, while the stress chirp showed a significant difference in the F4 frequency peak (Figure 2.13). All simple chirps differed significantly in their F5 peak (Figure 2.13). Significant differences were observed in several frequency peaks, across components for complex chirps (Figure 2.14). Stress signals differed significantly across all peaks in component 1. A significant difference was also observed in the F1 peak of component 1 between male-female, and male-male chirps. Within component 2 a significant difference was observed in the F1 peak and the F4 peak for

male-female and stress signals respectively. Component 3 a significant difference was observed when comparing male-male to the other two signal types.

Sliding scale histograms were created to show the distributions of dominant frequencies in both the simple and complex signals in all signaling situations (Figures 2.15, 2.16). In the case of simple signals the dominant frequency distribution follows a similar pattern across the three different signal types. Simple signals all showed a major peak in the sonic range with male-male and male-female simple signals showing two more minor peaks occurring further in the ultrasonic range. It should be noted that there was a much smaller sample size available of male-female simple signals, which may have an effect on the resulting histograms. When comparing complex signals the three signal types behave in a similar fashion but each component had a different distribution of dominant frequencies. Component 1 (Figure 2.16a) showed on average the highest concentration of ultrasonic dominant frequencies across the three signal types. Component 2 (2.16b) had a more even distribution of dominant frequencies across signal types. Component 3 (2.16c) showed a higher concentration of low dominant frequencies. However component 3 also had the most erratic number of samples since not all complex chirps analyzed possessed 3 components. Observations across the signal types revealed a much more even distribution of frequencies within the male-female type signals. Male-female signals showed two major dominant peaks across all 3 components (Figure 2.16). In contrast, male-male signals showed a higher number of dominant peaks occurring in the ultrasonic range (Figure 2.16).

Statistical comparisons of the frequency domain of the substrate borne vibrations between rivalry and courtship vibrations showed no significant difference. Although

frequencies observed in the rivalry situation were on average approximately 2 kHz higher (Table 2.3).

Figure 2.13 Histogram showing distribution of mean frequency peaks for simple chirps across three chirp types, error bars indicate standard deviation. Sample sizes and descriptive statistics from table 2.2. Triads of letters indicate the results of a Kruskal-Wallis analysis of variance comparing the mean frequency peaks. A significant difference in frequency peak is indicated by the presence of different letters.

Figure 2.13

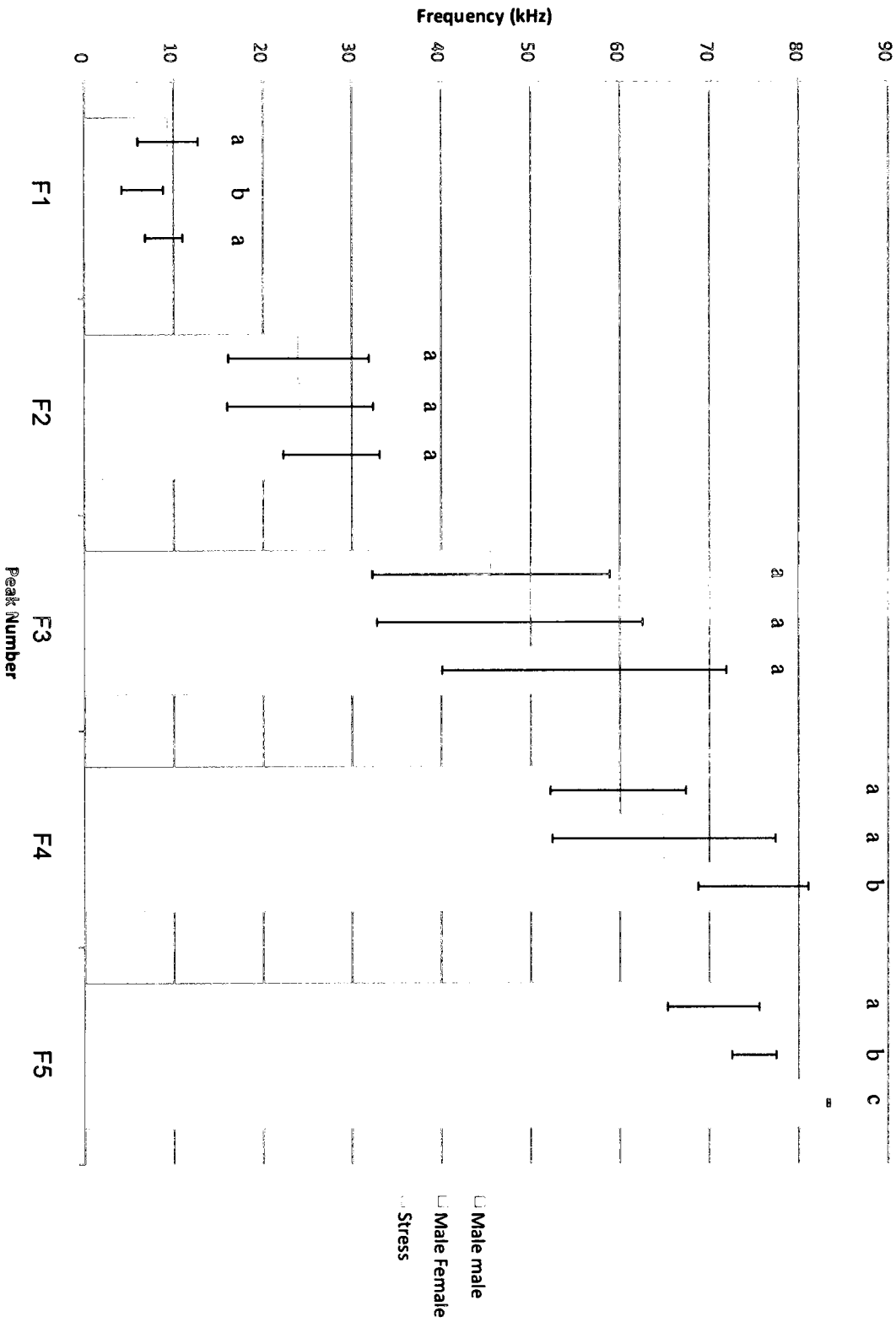

Frequency (kHz)
0
10
20
30
40
50
60
70
80
90
F1
F2
F3
F4
F5
Peak Number
a b a
a a a
a a a b a b c
Male male
Male Female
Stress

Figure 2.14 Histogram showing distribution of mean frequency peaks for complex chirps across three chirp types, error bars indicate standard deviation. Sample sizes and descriptive statistics from table 2.2. Triads of letters indicate the results of a Kruskal-Wallis analysis of variance comparing the mean frequency peaks. A significant difference in frequency peak is indicated by the presence of different letters.

Figure 2.14

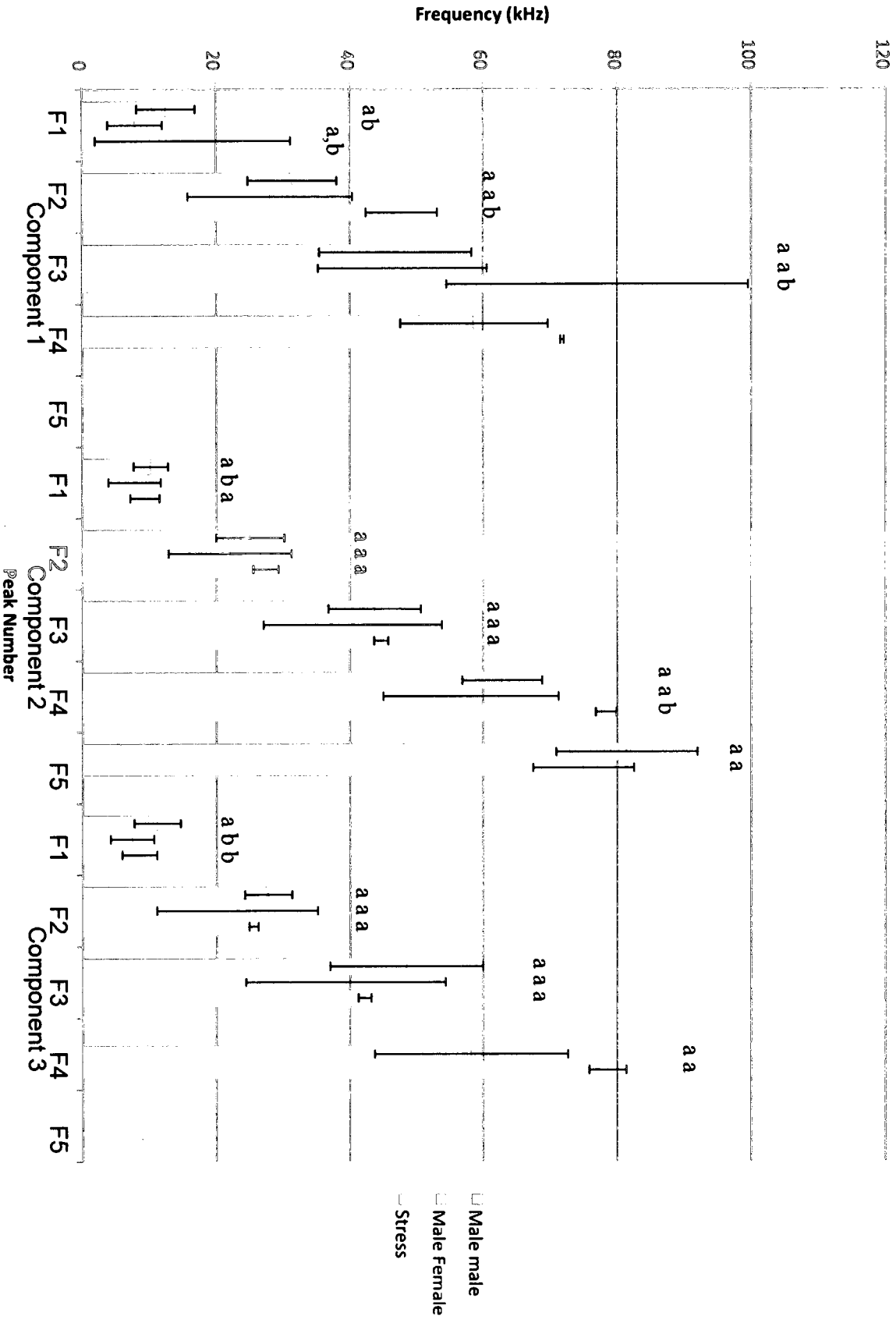

Frequency (kHz)
0
20
40
60
80
100
120
F1
F2
F3
F4
F5
Component 1
Component 2
Component 3
Peak Number
Male male
Male Female
Stress

Figure 2.15 Sliding scale histograms showing peak frequency distribution across three simple signals for each behavioral condition. Inset shows representative trace of a stress simple signal (scale = 10 ms).

Figure 2.15

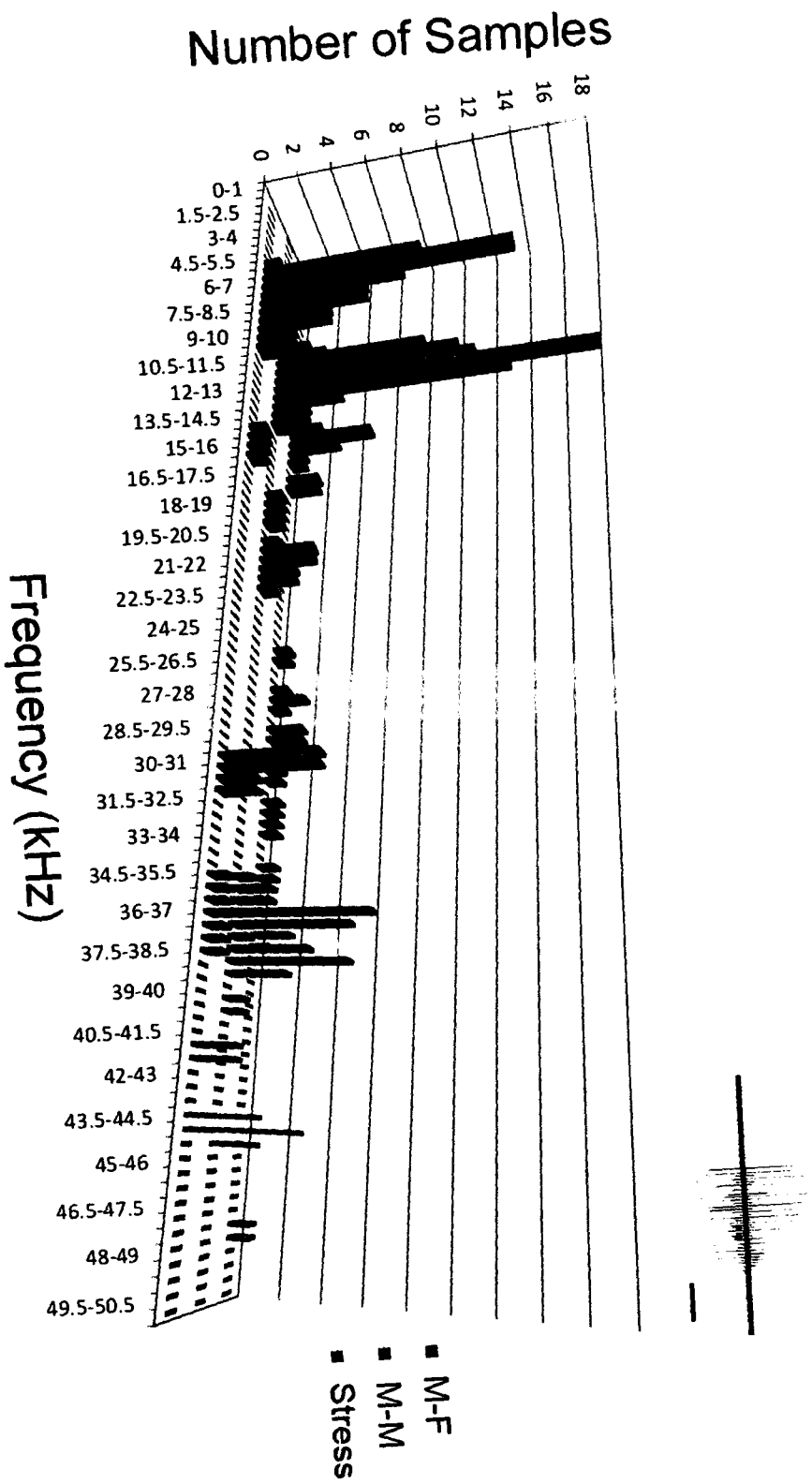
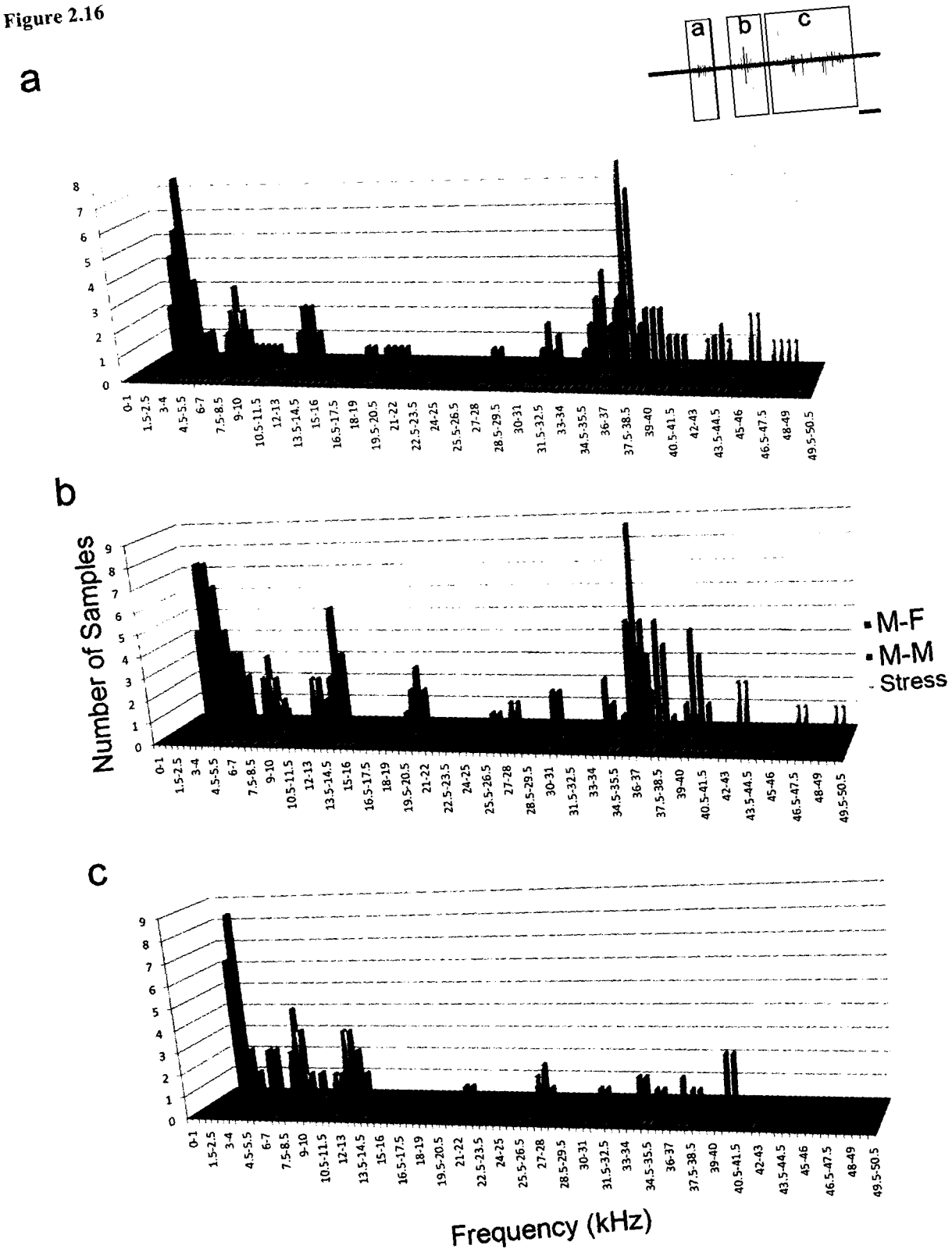

Number of Samples
0
2
4
6
8
10
12
14
16
18
Frequency (kHz)
0-1
1.5-2.5
3-4
4.5-5.5
6-7
7.5-8.5
9-10
10.5-11.5
12-13
13.5-14.5
15-16
16.5-17.5
18-19
19.5-20.5
21-22
22.5-23.5
24-25
25.5-26.5
27-28
28.5-29.5
30-31
31.5-32.5
33-34
34.5-35.5
36-37
37.5-38.5
39-40
40.5-41.5
42-43
43.5-44.5
45-46
46.5-47.5
48-49
49.5-50.5
M-F
M-M
Stress

Figure 2.16 Sliding scale histograms showing peak frequency distribution across three complex signals. **a)** Dominant frequency distribution across all signal types for component 1 of the complex signals for each behavioral condition. Inset shows representative trace of a male-female complex signal. Boxes have been placed around each of the individual components (scale = 20 ms); **b)** component 2 **c)** component 3.

Figure 2.16



2.5 Discussion

General Discussion on Sound Production

The present study was aimed at describing, in detail, the acoustic signals produced during various behavioural contexts in the bark beetle, *Dendroctonus ponderosae*. Acoustic signaling forms an integral part of the bark beetle communication system, and has been suggested to function in attraction, courtship, species identification, territoriality, and spacing (Barr, 1969; Ryker, 1988). To date, research surrounding these acoustic signals has only shown temporal characteristics, such as chirp duration, the number of components per chirp, and the number of tooth-strikes per chirp (Ryker and Rudinsky, 1976; Yandell, 1984; Ryker, 1988). In addition, despite what is known about the signals, to date, there has been no mention of the medium of signal transmission (air or solid borne). The present study demonstrates that acoustic information contained within the signals of the bark beetle *D. ponderosae* is transmitted through air employing a broadband range of sonic and previously unreported ultrasonic frequencies. This study also reports the presence of a novel substrate-borne component to the signals. This study further suggests the necessity for more investigation into the acoustic and vibratory communication in this species, and other bark beetles species.

The presence of acoustic signals, termed "stress signals", produced by male *Dendroctonous ponderosa* during periods of distress has been observed and described in previous studies (Barr, 1969; Michael and Rudinsky, 1972; Yandell 1984). Such signals have been described as being primarily simple chirps (Rudinsky and Michael, 1972; Ryker and Rudinsky, 1976), with variation noted in the duration and the number of tooth-strikes between populations (Yandell, 1984). All individuals when being handled

produced audible acoustic signals, the majority of which were simple. Two of the ten individuals, however, generated purely complex chirps when disturbed, suggesting the possibility of a multi-component signaling structure, where the latter component is only seen during specific condition such as the internal motivational level of the individual, which can be affected by various factors not accounted for in this study. Another possible reason for the presence of these complex signals is the presence of female pheromone in the environment. Although no females were present at the time of testing, exposure to females prior to testing might have been responsible for the presence of complex stress signals. Additional recordings and behavioural studies can determine if complex signals are also associated with stress, and provide insight into the function of these signals.

The fact that stress signals are evoked by 'attack' stimuli suggests that they could play a defensive role (Masters, 1980). Defense signals in insects, have been categorized into two types: 1) startle signals, and 2) warning/aposematic signals (Masters, 1980). It is unlikely that *D. ponderosae* stress signals are aposematic, as to date no deterrents, such as a chemical or bite, has been observed to follow stress signals (Wood, 1982, Lewis and Cane, 1990). It is therefore possible that these stress signals function to startle potential predators.

Two predictions were made regarding the stress signal as an anti-predator response. If the stress chirp is indeed an anti-predator response, then: 1) the onset of the signals should be at the beginning of an attack, and cease once the attack is ended, as is the case with *D. ponderosae*, seen from this study; and 2) the natural predators are capable of detecting these airborne signals, yet to be tested. The major insect predators of *D. ponderosae* includes, clerid beetles (Coleoptera: Cleridae), robber flies (Diptera:

Asilidae), minute pirate bugs (Hemiptera: Anthocoridae) and to a lesser degree, longhorn beetle larvae (Coleoptera: Cerambycidae) (Schmid, 1969; Moore, 1972; Reeve, 1997). Natural predators of *Dendroctonus* spp. also include birds; particularly woodpeckers (Picidae) have been listed as a natural predator to *D. ponderosae* (Koplin, 1972; Fayt et al., 2004). The present study shows that *D. ponderosae* stress signals possess low frequency components with peak energies falling between 7 - 10 kHz, which is well within the audible range of birds (0.5 – 10 kHz) (Dooling, 2002). It is unknown if predatory insects attacking *Dendroctonus* (e.g. Cleridae, Anthocoridae) are capable of hearing. However, one could surmise that if the pine beetles emitted stress signals while in the grasp of a predatory insect, the vibrations incidental to the stridulation might assist in startling the predator allowing the pine beetle to escape (see Lewis and Cane, 1990). In beetles of the genus *Ips* it is the females which produce acoustic signals, Lewis and Cane (1990) showed that deimatic stridulation produced by *Ips calligraphus* females disturbed predatory clerid beetles, enhancing their chances for release by the predator. Lewis and Cane (1990) suggest that although there is no aposematism present in the acoustic signals, their presence is sufficient to cause the predatory beetle to drop the *Ips* prey item, which under natural conditions would be enough to effectuate a successful escape. Further investigations into the behavioural significance of these stress signals in relation to attacks from natural predators, both clerids and woodpeckers, are needed to determine the function of 'stress' signals.

Male-female Interactions

During male-female interactions, males placed in the proximity of the borehole of a female will walk toward the hole, and begin chirping. The present study showed that

in the context of male-female signals, both complex and simple signals were present (Figure 2.6). The proportion of occurrence of each signal type (complex or simple) was temporally dependent, and was observed to change over time (Figure 2.9). I distinguished between mate attraction (complex) chirps, where the male will chirp to announce his presence prior to entering the hole, and courtship (simple) chirps, after the male has entered the female's nuptial chamber. Within the context of this study, chirps were seen to cease after 5 minutes, at which point presumably the two beetles were engaged in copulation.

Attractant signals were complex, and occurred primarily during early stages of the male-female interaction, comprising ~70% of those signals present during the first minute of the encounter down to ~22% during the 5^{th} minute of the encounter (n=5) (Figure 2.9). Once the female has accepted the male, and allows him to enter, courtship signals, simple type chirps were dominant (Figures 2.6 and 2.7) making up 77% of the observed signals during the 5^{th} minute of the trial (n=5). These results agree with previous studies on male-female encounters within *D. ponderosae*, where Ryker and Rudinsky, (1973) found that the first signals generated by the males were complex, and this was followed in the later stages of courtship by simple chirps. According to Ryker and Rudinsky (1973), simple chirps were similar to the stress chirp in structure and duration. My study shows that, although the simple courtship signals appear qualitatively to be similar to simple stress signals, the two signals differ significantly in their duration and frequency distribution at peaks F1, F4, and F5 (Figure 2.13), and are thus two distinct signals produced in different contexts.

Attractant signals have been suggested to function primarily in species recognition, as these signals vary between species, but remain consistent within a species (Yandell, 1984). Further investigation is necessary in making comparisons of peak frequencies across species in order to conclusively determine whether this signal functions in species recognition. This study found that the temporal and spectral characteristics of attractant chirps remained consistent within the test species. Females were observed to reject males in 2 trials. Observations of signaling in these two trials showed a predominance of simple signals throughout the trial. The females' rejection of these two males suggests that females decide to accept or reject males at this stage. Male-female (complex) signaling might convey information other than the species and sex, which the female can utilize to make decisions to allow or reject the male. Further studies into female mate choice based on male weight and individual signal intensity could be conducted in order to determine if parameters of the male signal convey fitness information. Information about the signal emitter can be embedded into the various parameters of signals and in the timing of the signals such as signaling rate (Endler, 1992). For example, the intensity of the signals could indicate the energetic output of the caller, which could indirectly provide information about the sender's size and weight (Endler, 1992).

Female *D. ponderosae* have also been shown to produce territorial chirps associated with courtship encounters with males as well as clicking signals during gallery formation in densely populated trees (Ryker and Rudinsky, 1976). In this study, I observed a third type of signal during male-female interaction, which I presume to be produced by the female in response to the males (Figure 2.8).

Female chirps during courtship have been suggested to function as a "territorial chirp", as it occurs in the presence of both males or intruding females, and is thought to announce her presence in the burrow (Ryker, 1988). The presumed female's response to the male was clearly observed as a simple chirp occurring during the first minute of an encounter. This signal has been called a female territorial signal by previous researchers (Ryker and Rudinsky, 1976). However its use has been suggested in both male-female and female-female encounters, possibly suggesting use in species recognition and not just territoriality.

Male-male Interactions

When two males were confined in the small arena, both males began to stridulate. Unlike in the case of male-female signaling where complex and simple chirps occurred in a chronological sequence, both complex and simple chirps were interspersed randomly throughout the male-male interactions, with an overall larger proportion of chirps being simple. Previous reports on signals generated during male-male interactions showed variability in the temporal characteristics, which was context specific (Yandell, 1984). Ryker and Rudinsky (1976) recorded from 2 males confined (not in the presence of female frass) noting only the presence of complex chirps (Ryker, 1988). This present study, however, detected simple chirps in addition to the expected complex chirps (Figures 2.10). My study was also in agreement with results from Yandell (1984), who recorded from interacting males, and noted the presence of both complex and simple chirps. This again suggests the complexity in the bark beetle communication system. Male-male signals were significantly different from male-female signals temporally, suggesting that this signal type is conveying information relevant to the context in which

it is employed. Although the presence of simple chirps within this signal type is in agreement with some of the literature it should be noted that the presence of pheromone in the air, as well as the fact that both males were intact might have confounded this result. Future studies using one silenced male in the presence of an intact male would make signal analysis much clearer.

Comparisons between signals

In a previous study on *Dendroctonus* acoustics, Yandell (1984) suggested species recognition as a possible function for the presence of acoustic signals. Yandell observed that muted males were allowed entry into the female gallery, suggesting the signal is important in the mating system. The present study suggests that the frequency spectra of the signals produced by *D. ponderosae* differ depending on the behavioural context of their production. Observations of the harmonic frequency peaks showed a difference in peak energy across the different contexts (e.g. male-male). Suggesting that perhaps information is not only encoded into the temporal character of the signal but perhaps also the spectral characteristics. Comparisons of the signals employed during the different contexts revealed differences both in the make up of the harmonic peaks (Figures 2.13 and 2.14), as well as in the distribution of dominant peak energy(Figures 2.15 and 2.16). These results suggest that *D. ponderosae* may be capable of discriminating between the dominant frequencies, gaining information both from the temporal domain as well as the frequency domain. The presence of a dominant frequency difference across signal types suggests that the signals are conveying information relevant to the context they are used in. Frequency information gathered might be pertinent to species recognition as well as

the overall fitness of the signaler (e.g. Nickle, 1976). Additionally, female beetles are exposed to a multitude of sounds within their natural environment, perhaps frequency discrimination is a way for a beetle listener to determine who is signaling. The use of frequency discrimination has been shown in bush katydids, which are able to separate conspecific signals from the ambient noise of other signaling animals in the vicinity based on frequency discrimination (e.g. Nickle, 1976). The results presented herein suggest that the frequency domain is informative to the listener, further comparisons with signals produced by other bark beetle species could be useful in determining the function of frequency within bark beetle communication. While, this study would suggest that frequency differences are deliberate actions of the beetle, it is also worth noting that some frequency deviations may have occurred due to other biotic factors present. Attenuation from the gallery can not be overlooked as a possible source of frequency deviation in this case, since the animals are producing their signals from within a burrow under bark, it is conceivable that this may have an effect on the frequencies available on the surface.

Substrate Borne Vibrations

In addition to acoustic, air-borne signals, male stridulations also produced substrate vibrations. This study demonstrates for the first time the availability of the substrate as a medium through which communication within *D. ponderosae* can occur. Vibrational signals traveling through the phloem layer were recorded during both mate attractant and rivalry contexts. The amplitude of the vibrational component of the signals observed are comparable in their amplitude/velocity to communication signals employed by other insects, which typically fall between 0.1 - 2 mm/s, depending on the substrate (Michelsen et al., 1982). There is also some indirect behavioural evidence that bark

beetles are communicating through solid substrates. In particular, there is evidence that females generate clicks when another female is boring nearby (Rudinsky and Michael, 1973). If indeed they are communicating vibrationally, then some likely receptor organs for receiving any substrate borne signals could be: the subgenual organ, or the antennae.

Unlike airborne acoustic signals, communication using vibratory signals in beetles is poorly understood. However, vibratory communication is observed across numerous taxa, and it is believed that this form of communication might be more widespread across many organisms than previously thought. Contrary to previous assumptions that vibrations are primarily used in localization, recent studies have shown that complex information can be embedded into vibratory signals, and such signals are used during various behavioural contexts including mate attraction, courtship, aggression, prey detection, predator avoidance (Hill, 2001).

In bark beetles, there is no morphological or physiological evidence to indicate reception of these various signals. There is, however, one piece of circumstantial behavioural evidence that both airborne and vibratory signals may be important for communication. Rudinsky et al. (1973) demonstrated that females responded to previously recorded male attractant chirps, which were played back through a piezoelectric ceramic disk, by releasing pheromones. It is possible that these females were picking up the airborne component of this signal or that the airborne signals could have indirectly stimulated vibration receptors by vibrating the walls of the female beetle's enclosure.

The results of this study on the characterization of the acoustic signals available to *D. ponderosae* lay the groundwork for further research to gain a better understanding of

the potential receptor mechanisms used by these insects to not only communicate with others in their environment but, perhaps with the environment itself. The capacity to detect ultrasound in a wood-boring beetle such as *Dendroctonus* could be instrumental not only in species recognition and mating, but also in finding host plants, as drought stressed trees have been shown to produce ultrasonic emissions as a result of cavitation of the xylem (Mattson and Haack, 1987; Haack et al., 1988).

Chapter 3. Anatomy and Putative Receptor Structures of *Dendroctonus ponderosae*

3.1 General Introduction on Anatomy

The function of hearing in insects has been well documented in a wide range of contexts including intraspecific communication, predator avoidance, and host-parasite interactions (eg. Roeder, 1967; Murphey and Zaretsky, 1972; Robert et al., 1996). A detailed overview of the literature on insect hearing provides numerous accounts of acoustic detection in insects. Hearing organs can occur on almost every part of the insect body, varying greatly in structure, complexity and acoustic tuning (Yack 2004). Insect sensory organs vary widely in structure from external hairs and sensillae, to internal chordotonal organs (Hoy and Robert, 1996; Yager, 1999; Virant-Doberlet and Çokl, 2004; Yack, 2004).

The purpose of this study is to 1) review external anatomy of *D. ponderosae*, and identify putative sensory organs for hearing or vibration detection, and 2) review the structure of *D. ponderosae* nervous system in search of possible sites for neurophysiological recording. Morphological observations of the external body features will be made, coupled with fine dissections of the central nervous system.

Insect hearing can be generally divided into two sub-categories, airborne receptors, and solid-borne receptors. Solid borne receptors as their name implies are sensitive to signals transmitted through the solid substrate such as soil, vegetation, water, or wood. Some examples of these receptors include the subgenual organ and campaniform sensillae (reviewed in Yack, 2004; Virant-Doberlet and Çokl, 2004).

Airborne sounds can be divided into two subcategories, far- or near-field sounds. Far field sounds are transmitted as fluctuating pressure waves, which due to their

wavelike nature, are capable of conveying a sonic signal over long distances (Greenfield, 2002). Far field receptors include the tympanal hearing organs, and the cercal hair cells (for review see Hoy and Robert, (1996); Yager, (1999); Yack, (2004)). Near field sound occurs closer to the sound source (typically within 1 wavelength). The pressure waves cause a displacement of air particles, which are capable of mechanically moving lightweight structures in their path. Near field sounds are typically of a lower frequency, and are only perceived over relatively short distances (Greenfield, 2002). Near field sound receptors include trichoid sensillae and the Johnston's organ (reviewed in Yager, 1999; Yack, 2004).

To determine the presence of an acoustic receptor organ, three lines of evidence are sought: 1) the insect must possess a morphologically distinct receptor structure; 2) the nerves associated with the structure must respond to biologically relevant sounds; and 3) the sound should mediate an adaptive behavioral response. Confirmation of any of these criteria requires a broad understanding of both the external and the internal anatomy of the examined organism.

Although receptors vary in morphology across different taxa, generally four main types of acoustic sensory organs have been described: tympanal ears, trichoid sensillae, Johnston's organ, and subgenual organs. The first three organs are used for detecting air-borne acoustic vibrations, where the final organ is primarily utilized for detecting substrate-borne vibratory signals.

Tympanal ears are typically characterized by three morphological features: 1) a localized thinning of the cuticle, typically appearing as a membrane delineated by a ring of more sclerotized cuticle called the tympanum membrane; 2) an associated enlarged

trachea or air filled sac directly adjacent to the tympanal membrane, which creates a resonating chamber; and 3) an innervation of the tympanal membrane through a chordotonal organ (Hoy and Robert, 1996). Tympanal ears respond to airborne acoustic vibrations, which strike the tympanal membrane causing it to vibrate, slightly deforming the sensory cell (Hoy and Robert, 1996; Yager, 1999; Yack, 2004). They specifically detect the pressure changes in the air caused by the movement of air particles caused by the stridulatory mechanism of the signal emitter (Ewing, 1989), and are considered far-field sound receptors.

Tympanal ears have been shown to occur in at least 8 insect orders, evolving independently across different families within the same order (Yack and Fullard, 1990; Yager and Spangler, 1995; Hoy and Robert, 1996; Yager, 1999; Yack, 2004). The use of tympanal ears for intraspecific communication by beetles has yet to be shown, as the ears discovered in Coleoptera to date have only been linked to predator avoidance (Forrest et al., 1995; Yager and Spangler, 1995).

The trichoid sensillum receptor type consists of a cuticular outgrowth or hair innervated by either single or multiple neurons. These receptor organs can be specialized to mediate a response to disturbances in air currents, such as those produced in the proximity of a near-field sound source. Trichoid sensillae have been implicated in sound reception in *Elliptorrhina chopardi,* a species of cockroach known to produce acoustic signals for intraspecific communication (Sueur and Aubin, 2006). These receptors are also employed in the context of predator avoidance by several species of lepidopteran larvae, which react to the near field sounds produced by the wing beats of approaching parasitoids (Markl and Tautz, 1975; Taylor, 2009).

Within Scolytidae trichoid sensillae have mainly been discussed in the context of chemoreception of their broad spectrum of pheromones released (Payne et al., 1973). Borden and Wood (1966) suggested that the sensory hairs present on the antennae of *Ips paraconfusus* might be used in phonoreception as well as chemoreception. This however remains a suggestion, having never been shown conclusively. More recent research has shown that these are likely chemodetectors (Payne et al., 1973). The fact that the trichoid sensillae have been shown to be chemoreceptive in other scolytids might pre-empt these from being acoustic receptors.

Another specialized structure for detecting near field sounds is the Johnston's organ. Johnston's organs are chordotonal organs located in the enlarged pedicel of an insect's antenna at the base of the flagellum (the final antennal segment) (Johnson and Triplehorn, 2004). The flagellum of the antenna sits loosely inside a special socket allowing it to vibrate at a specifically tuned resonant frequency in response to a near-field sound. Johnston's organs are quite complex, containing thousands of mechanoreceptor units, which transduce the antennal vibrations into electrical impulses, recognized by the insect's nervous system (Field and Matheson, 1998; Göpfert and Robert, 2000). Johnston's organs typically react to low frequency sounds from 150-500 Hz (Viran-Doberlet and Cokl, 2004). One example of the Johnston's organ can be found on the distal elongated flagellum of each antenna of the female culicid mosquito *Toxorhynchites brevipalpis*, which are capable of detecting sounds produced by the male as he modulates his wing beat frequency (Göpfert and Robert, 2000).

In Coleoptera, the Johnston's organ can be found in the whirligig beetle, *Gyrinidae* where it is used for both visual as well as vibrational cues on the water's

surface during intra- and inter- specific interactions (Bendele, 1986). Johnston's organ has also been observed in other members of the family Curculionoidea (Hix et al., 2003). However it has never been described in the family Scolytidae.

Substrate-borne vibrations in insects are typically received through one of two vibrational receptor types, either campaniform sensillae, or leg scolopidial organs also referred to as the subgenual organ (Yager, 1999; Virant-Doberlet and Cokl, 2004; Yack, 2004). Campaniform sensillae have been described as small, innervated dome-like structures, sensitive to cuticular deformation (Gullan and Cranston, 2005). Often located on the membranous intersegmental joints of legs. They generally function as proprioreceptors, monitoring the forces generated by the muscles in the insect leg, but can also act as vibrational receptors. They are typically most sensitive to low frequency vibrations below 100 Hz (Virant-Doberlet and Cokl, 2004), possibly due to their non-specific use as mechanoreceptors.

Subgenual organs are more complex scolopidial organs, located in the tibiae of the thoracic legs, just beneath the joint. Insects possessing this type of organ typically show some enlargement or swelling of the tibia at the receptor site, as exemplified by the honeybee *Apis mellifera* (Storm and Kilpinen, 1997). In grasshoppers each subgenual organ is characterized by a fan-shaped flap of tissue innervated by 1-400 sensory cells (Moran et al., 1975; Virant-Doberlet and Cokl, 2004). The subgenual organ is a very broadband organ in terms of its frequency tuning- capable of detecting very low amplitude substrate displacements at frequencies from 700 Hz, all the way up to ~5 kHz (Devetak, 1998; Yack, 2004). It has been suggested that the primary function of the subgenual organ is the registering of substrate borne vibrations.

To date there has been no official description of a subgenual organ present in Coleoptera (Virant-Doberlet and Cokl, 2004). However putative chordotonal organs have been suggested to occur in the fore-coxae of *Callosobruchus maculatus,* a chrysomelid beetle in the family *Bruchidae*, which is a pest of grains (Ramaswamy and Monroe, 1997). Other reports exist suggesting the use of vibratory cues within other families of beetles, such as Tenebrionidae, which have been suggested to use a substrate tapping behaviour in intraspecific communication (Slobodchikoff and Spangler, 1979). These reports however, remain speculative and have not conclusively proven the presence of a subgenual organ in any species of coleopteran.

Anatomy of D. ponderosae

A complete review of the general external morphology and nomenclature of the genus Scolytidae is presented in Hopkins' Practical information on the Scolytid beetles of North America (1909). This document stands as the foremost important document with respect to the external anatomy of *D. ponderosae*. Snodgrass (1935), and Johnson and Triplehorn (2004), have also been used as guides of insect anatomy, establishing the accepted principles and nomenclature.

The central nervous system in insects is composed of a double chain of ganglia joined by longitudinal connectives. This network of ganglia can be divided into four major parts: the brain, the subesophageal ganglion, the thoracic ganglia, and the abdominal ganglia (Johnson and Triplehorn, 2004). The brain and thoracic ganglia are further subdivided. The brain comprises: the proto-, deutero-, and trito-, cerebrum, while

the thoracic ganglion is comprised of the pro-, meso-, and meta-thoracic ganglia. The thoracic ganglia are followed by a series of abdominal ganglia (Calder, 1989).

Coleoptera are the most diverse order in terms of gangliar fusion ranging from complete fusion of all thoracic and abdominal ganglia, to 3 and 8 completely separate thoracic and abdominal ganglia respectively (Calder, 1989). Anatomical descriptions of the nervous system within Coleoptera exist for several different families- which range in complexity- most showing only the ventral nerve cord and some of the more major nerve roots : Dytiscidae (Holste, 1910), Scarabaeidae (Edmonds, 1974), Curculionidae (Calder,1989), Carabidae (Heath and Evans, 1990), Chrysomelidae (Mann and Crowson, 1983), and Cincindellidae (Yager and Spangler, 1995) to name just a few. To date the finest description of the beetle nervous system has been that of *Dytiscus marginalis* L. (Coleoptera: Dytiscidae) as described by Holste (1910).

Holste (1910) presented a detailed analysis of the entire nervous system of *D. marginalis*. Despite its being published almost a century ago, this text has served as the central reference for any work discussing beetle nervous system anatomy. Apart from Holste (1910), most texts simply describe the ventral nerve cord and do not discuss the major nerve roots or lateral branches.

To date, no fine descriptions have been made elaborating on the gross morphology of Scolytidae. Calder (1989), however, presented the basic structure of the ventral nerve cord in Curculionoidea. Among these, he also examined the Australian scolytid beetle, *Hylurgus ligniperda*. His study revealed that the meta- and meso- thoracic ganglia as well as the abdominal ganglia were all fused. Dissections in Calder (1989) were very basic and no nerve roots or branches were shown.

The objectives of this study are the following: 1) to identify putative locations for sensory organs externally; 2) to provide a dissection guide to access the central nervous system in *D. ponderosae*; and 3) to describe the main features of the gross anatomy of the nervous system. The findings presented in this study will help pave the way for future experiments, neurophysiological recordings, and histological descriptions of putative receptor organs.

3.2 Methods

Animals

Adult *D. ponderosae* were reared from naturally infested bolts of ponderosa pine (*Pinus ponderosa*) obtained from Doug Linton, (Pacific Forestry Division, NRCan) collected from a region just south of Merrit, BC, Canada (49°52'31.51"N, 120°53'34.09"W). Bolts were stored in sealed 15 gal. Sterlite containers at 3-5°C in a walk-in type fridge, and brought to room temperature (20°C) as beetles were required for experiments. As the animals emerged from their galleries they were stored individually in Eppendorf tubes with a small portion of bark beetle medium (Insect Production Services, Sault Ste. Marie) to provide food and moisture. Once experiments were completed adult beetles were fixed and stored for later use. The bolts of ponderosa pine which previously housed the beetles were autoclaved and destroyed.

External and General Anatomy of *Dendroctonus ponderosae*

The nomenclature, as applied to the anatomical structures (both internal and external) of *D. ponderosae*, were derived from several sources. Chief among these with

respect to external anatomy was a treatise on the genus *Dendroctonus* by A.D. Hopkins (1909). This text was supplemented with more modern accounts of general insect anatomy such as Johnson and Triplehorn (2004). With respect to the nervous structures, the primary source for nomenclature was the description provided by Holste (1910) detailing the nerves of *Dytiscus marginalis*.

Following is a list of abbreviations used in the figures and text (nomenclature adapted from the 1910 Holste text):

External morphology

abd = abdomen
ant = antenna
c = club
el = elytron *(a)*
f = funniculus
h = head
JO = johnston's organ
ml = membranous lobes
p = pedicel
ps = *pars stridens*
s1-s8 = spiracles *1 – 8*
sc = scutelum
th = thorax
tm = tympanal membrane
tr = trachea
sc = scape

Ganglia

agc = abdominal ganglion complex
gfr = frontal ganglion
gs = supraoesophageal ganglion
gi = suboesophageal ganglion
gtr = corpora cardiaca allata
T1 = Prothoracic ganglion
T2 = Mesothoracic ganglion
T3 = Metathoracic ganglion

Nerves

cm = connective or commissural nerve
cms = circumoesophageal commissural nerve
na = antennal nerve
nal = alary nerve
nca I = prothoracic anterior coxal nerve
nca II = mesothoracic anterior coxal nerve
nci I = inferior coxal nerve
nci II = mesothoracic inferior coxal nerve
ncm II = second commissural nerve
ncp I = prothoracic posterior coxal nerve
ncp II = mesothoracic posterior coxal nerve
ncp III = alocoxal posterior nerve
nel = elytral nerve
nfr = frontal nerve
ng = stomatogastric nerve
nis I = prothoracic ischadic nerve
nmd = mandibular nerve
nis II = mesothoracic ischadic nerve
nis III = metathoracic ischadic nerve
no = optic nerve
nst I = sternal prothoracic nerve
nst II = mesothoracic sternal nerve
nst III = metathoracic sternal nerve
nrts = primary jugular nerve

Light Micrographs of External Anatomy of *Dendroctonus ponderosae*

External anatomical features were characterized using both C&C (Chauthani and Callahan, 1966) fixed, and freshly sacrificed material. All observations were made through an Olympus SZX12 (Olympus Corporation, Tokyo, Japan) light microscope. Micrographs were obtained by taking pictures with the coupled Zeiss AxioCam MRC5 Digital camera (Carl Zeiss Micro Imaging GmbH, Göttingen, Germany) processed through AxioVision software (Rel 4.7.2). Drawings of anatomical features were made using a Wild Heerbrug Leitz Drawing Tube (Leica 10446993, Heerbrug, Germany).

Nervous System Dissection of *Dendroctonus ponderosae*

Initial Preparation

Dissections were carried out on adult beetles fixed in C&C fixative solution. Gender was confirmed by gently moving the elytra to the side to view the 7th abdominal tergite (Lyon, 1958; Barr, 1969). Fixed adults were kept in solution in a refrigerator at 5° C, and were brought out as needed. Dissections were conducted on both males and females in order to determine the extent of variation, if any existed, between the sexes. All dissections were carried out in Petri dishes lined with Sylgard 184 (Dow Corning, Midland, Michigan). Specific dissection methods were written up as follows.

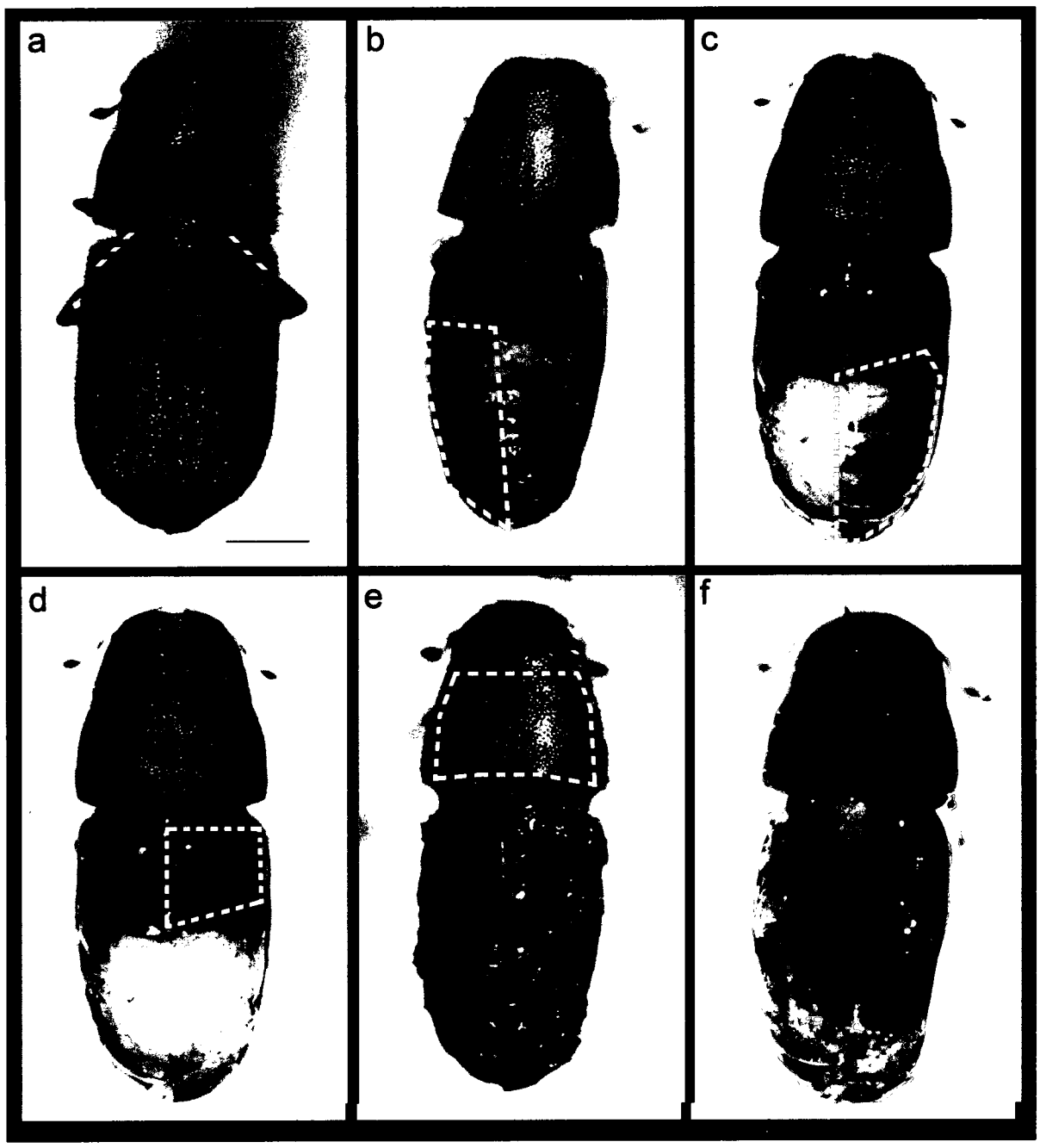
Dorsal Preparation

Dissections of the ventral nerve cord were carried out on whole C&C fixed or freshly sacrificed specimens. Since many nerve roots project dorsally it was thought that by dissecting from two different orientations more information could be gained by minimizing damage to the delicate nerve branches. Fixation was carried out by injecting the abdomen of living animals with C&C fixative, using a 29.5 gauge needle (B-D Allergy Syringe, Beckton Dickinson Consumer Health Care, Franklin Lakes, NJ) on a 1 cc syringe. The fixed animal was stored in an Eppendorf tube with C&C fixative until it was used, at a minimum 24 hours between time of fixation and use of the tissue.

Using a dissecting microscope, under low magnification, the general external anatomy of the insect was examined (Figure 3.1). The beetle was secured to the Sylgard by placing a minutin pin directly through the back of the head, perpendicular to the substrate. By pinning the beetle in this manner any damage to the abdominal region was avoided. It should be noted that this method may have compromised the frontal lobes of the brain region (proto cerebrum).

Figure 3.1 Dorsal dissection of female *D. ponderosae*. **a**) Dorsal aspect of a female beetle, elytra and metathoracic wings are removed by making cuts to the attachment points located beneath the hatched lines. **b**) & **c**) A series of shallow cuts are made to remove the abdominal tergites, by making one cut up the midline of the animal the tergites can be peeled back exposing the digestive and reproductive structures. Cuts are then made along the lateral margins of the tergites and they are removed. **d**) With the abdomen of the animal fully exposed cuts can now be made through the thoracic plate. **e**) Once the thoracic plate is removed the pronotum can then be cut allowing access to the brain region of the insect. (scale bar **a** - **f** = 1 mm)

Figure 3.1



The outer forewings (elytra) were carefully teased apart and clipped off at the base with a pair of fine pointed Dumont medical biology forceps (Fine Science Tools Inc., North Vancouver, BC) and a pair of coarse student Vannas spring scissors (Fine Science Tools Inc.) (Figure 3.1 a). The next step was to remove the metathoracic wings by gently extending them and clipping the anterior notal wing process at the mesoscutal lateral edge (Figure 3.1 b). With both sets of wings removed the animals four body sections were made visible: head, pronotum, thorax, and abdomen (Figure 3.1 b).

Fine pointed spring scissors (Fine Science Tools Inc.) were used to make 2 longitudinal incisions, one along the pleuron on the lateral margin of the insect body and one directly down the middle of the abdomen (Figure 3.1 b). Both incisions spanned the entire length of the abdomen. A cross cut was then made along the anterior margin of the fist abdominal tergite where it meets the scutellum allowing 2 triangular sections to be peeled away (Figure 3.1 c). This exposed the animal's internal structures such as, the digestive tract, the sex organs, and the excretory system (Figure 3.1 c and d).

Next, two longitudinal cuts were made along the pleural sutures of the metascutum, 2 transverse cuts along the top and bottom margins, and one longitudinal cut down the scutellar groove. This allowed the release of the thoracic tergum from the rest of the integument. In order to detach the scutellum, several shallow cuts were made directly beneath the cuticle to release the flight muscles attached to it (Figure 3.1 d).

Directly beneath the scutellar plate the flight muscles were now visible, these were removed using careful shallow cuts and gentle pulling (Figure 3.1 d). Any digestive organs, malpighian tubules, and digestive tract were carefully removed leaving a large gap with only muscles and the ventral nerve cord (Figure 3.1 e). Staining of nervous

structures was achieved using Janus Green B vital stain (Yack, 1993), which increased the contrast of nervous tissue against the rest of the animal (Figures 3.1 e and f). Next the head capsule was split using an improvised tool consisting of a small fragment of a double-edged razor blade (Tiger Brand) held in an Xacto blade holder (X-Acto Knife #1, X-Acto Brand). The head capsule was then gently pried apart exposing muscles and the optic lobes as well as the supraesophageal ganglion. The preparation was kept moist by pipetting drops of distilled water directly adjacent to the animal's body, allowing the water to permeate the animal without disturbing the delicate structure of the nervous system.

Ventral Preparation

The C&C fixed or freshly sacrificed animal was pinned ventral side up on a sylgard lined petri dish. The animal was dissected ventrally in order to examine those nerve roots and branches, which project from the ventral side of the ventral nerve cord. The animal was secured to the sylgard by placing a minutian pin directly through the mouth emerging from the back of the head, while a second minutian pin was placed through the abdomen close to the apex of the elytra (Figure 3.2 a).

Once secured in place, using coarse dissection scissors, all 6 legs were removed to facilitate the ventral dissection. Two shallow sagittal cuts, one through the anterior edge of the sternum, and the second along the caudal edge of the sternum were made (Figure 3.2 b).

This was followed by two longitudinal cuts through the pleuron along the lateral suture of the thorax, allowing for free motion of the sternal plate. Next the scissors were carefully inserted just beneath the sternal plate. Very shallow cuts were made to sever the

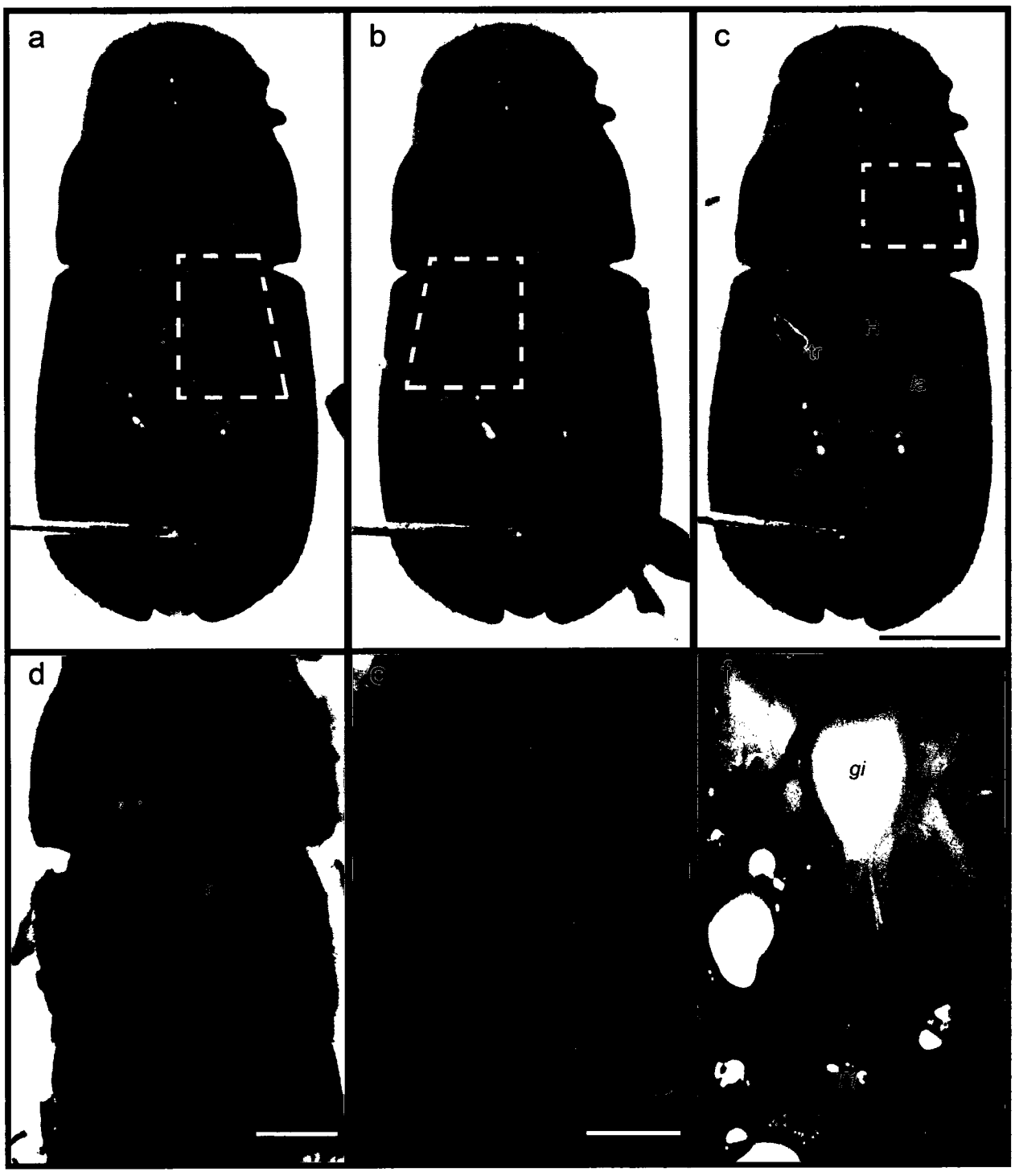
underlying muscle from its attachment points on the inner surface of the sternal plate. Cuts were also made around the sockets for each of the 6 legs. The sternal plate was then removed and discarded (Figure 3.2 c).

The overlying thoracic muscles were removed exposing the fused meso- and meta-thoracic ganglia (Figures 3.2 d and e). As the individual parts of the central nervous system were exposed a vital stain (Janus Green B) was applied in order to increase the relative contrast between the nervous system and the surrounding tissues (Figure 3.2 d). Once the meso- and meta- thoracic ganglia were fully exposed, three additional shallow cuts were made, one between the anterior pair of coxae on the prothorax, and two on either side of the prothorax. This exposed the prothoracic ganglion.

To expose the subesophageal ganglion, a cut was made along the medial suture visible on the ventral side of the head directly caudal to the mouth. Cuticle and muscles were removed avoiding any trauma to the ventral nerve cord. The head capsule was then split exposing the optic lobes and the brain.

Figure 3.2 Ventral dissection of *D. ponderosae* male. **a**) The adult male *D. ponderosae* is pinned ventral side up with two pins, one through the oral cavity and the other through the abdomen to secure it in place. The dissection is then facilitated by the removal of all of the thoracic legs (scale bar **a**, **b**, and **c** = 1000 μm). **b**) First a longitudinal cut is made along the medial suture of the sternal sternal plate. **c**) A second group of shallow cuts is then made in order to remove the sternal plate, revealing several trachea (tr), ventral longitudinal muscles (H), and the insertion point of the (*lA)* tergosternal muscles. **d**) Once the overlying muscles are removed the meso- meta- thoracic (T2, T3) and abdominal gangliar complex (agc) are the first to become visible, in this image the metathoracic coxa are also visible (scale bar **d**, **e**, and **f** = 500 μm). **e**) A lower magnification image, with the abdominal sternites removed to expose abdominal nerve connectives. **f**) Image of the suboesophageal ganglion (gi) and prothoracic ganglion (T1), gi appears white as it has not yet been exposed to Janus Green B.

Figure 3.2



3.3 Results

External Anatomy

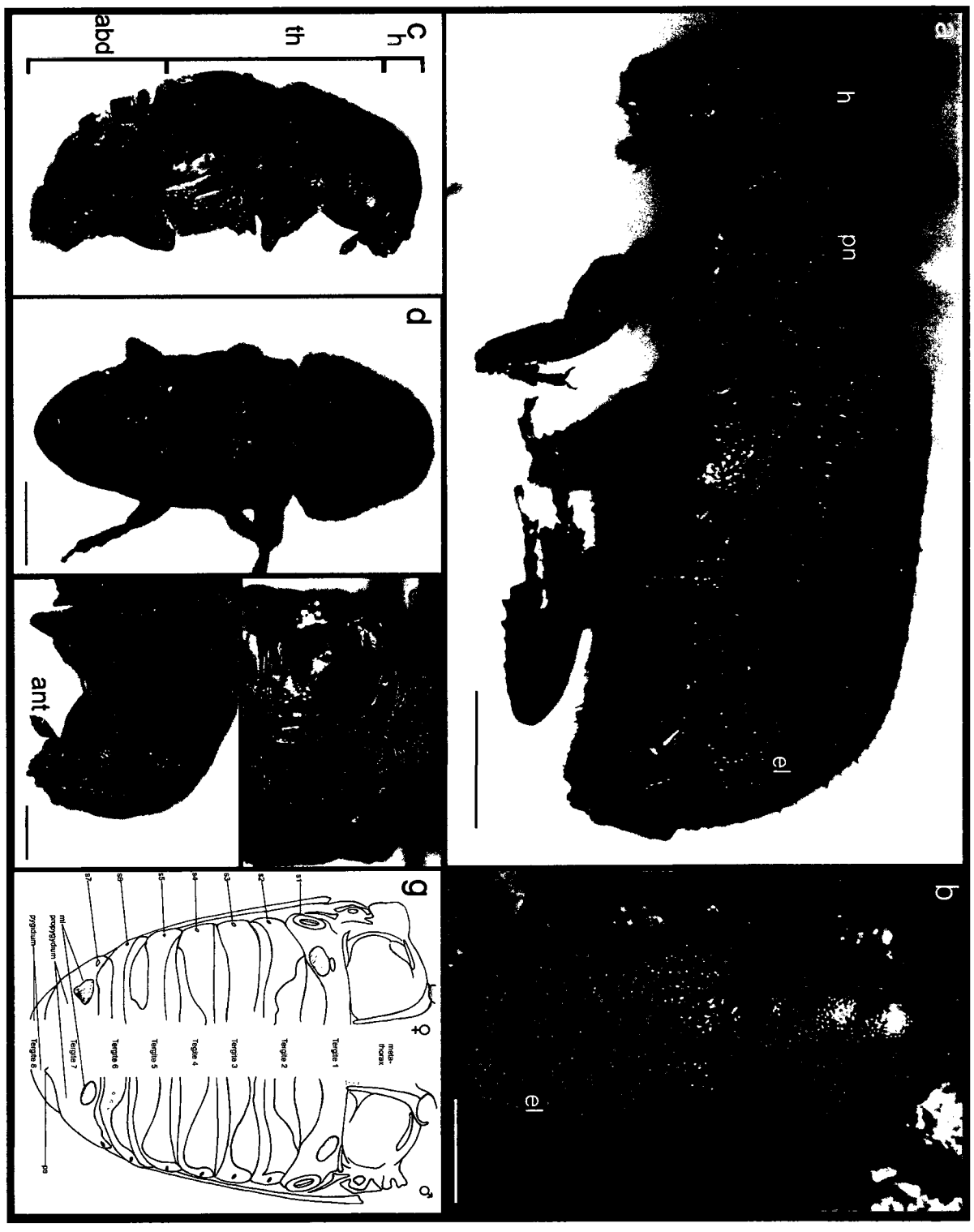
General external anatomical features were only examined to afford the reader a degree of familiarity with the general body plan of the beetle (Figure 3.3), as there has already been a treatise on the external anatomical features of *D. ponderosae* by Hopkins (1909). More detail was given only when a putative structure warranted further investigation with respect to its possibility as a sensory organ. Unless otherwise noted all descriptions presented herein will refer to both sexes.

The body of the beetle is divided into three segments: the head, thorax and abdomen (Figure 3.3 a-d). Short chitinous setae cover all of the external surfaces of the animal (Figure 3.3).

The head of the mountain pine beetle is small and round possessing a pair of relatively large hardened mandibles enabling the animal to bore through its woody substrate. The antennae sit on either side of the head directly anterior the lower part of the compound eye (Figures 3.3 f and 3.5). *Dendroctonus* antennae are club type, elbowed, and covered in short setae, which have been implicated in chemoreception (Hopkins, 1909; Lyal and King, 1995).

Figure 3.3 General external anatomy of *D. ponderosae.* **a)** Lateral aspect of a male specimen. Indicated on the figure are head (*h*), pronotum (*pn*) and elytra (*el*). Also visible on this specimen are the dense setae, which cover the animal's body (scale bar = 1mm). **b)** Dorsal view of a male beetle in a natural resting position on the bark of *Pinus ponderosae* (scale bar = 1 mm). **c) & d)** Lateral and dorsal views (respectively) of a female beetle with elytra removed. This figure shows the 3 divisions of the body head (*h*), thorax (*th*), and abdomen (*abd*). This specimen's legs were removed to facilitate positioning and for later dissection. (scale bar c & d = 1mm) **e)** Close up view of the dorsal thoracic scutellum (*sc*). (scale bars e and f = 500 μm) **f)** Close-up view of the head of a male specimen clearly illustrating the antenna (*ant*), and antennal insertion point. **g)** Drawing of the abdominal tergites of both sexes of *D. ponderosae*, female and male. This figure illustrates the individual differences observed between 2 individuals and presents a better representation of the sexually dimorphic character of the 7th abdominal tergite. The caudal margin of the 7th abdominal segment shows the sexual dimorphism where the *pars stridens* (*ps*) is present only in the male. In both specimens several membranous lobes (*ml*) are present on the 1st and 7th abdominal tergites. Spiracles (s1-s7) are visible along the outer edge of both sexes.

Figure 3.3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Putative receptor structures in *Dendroctonus ponderosae*

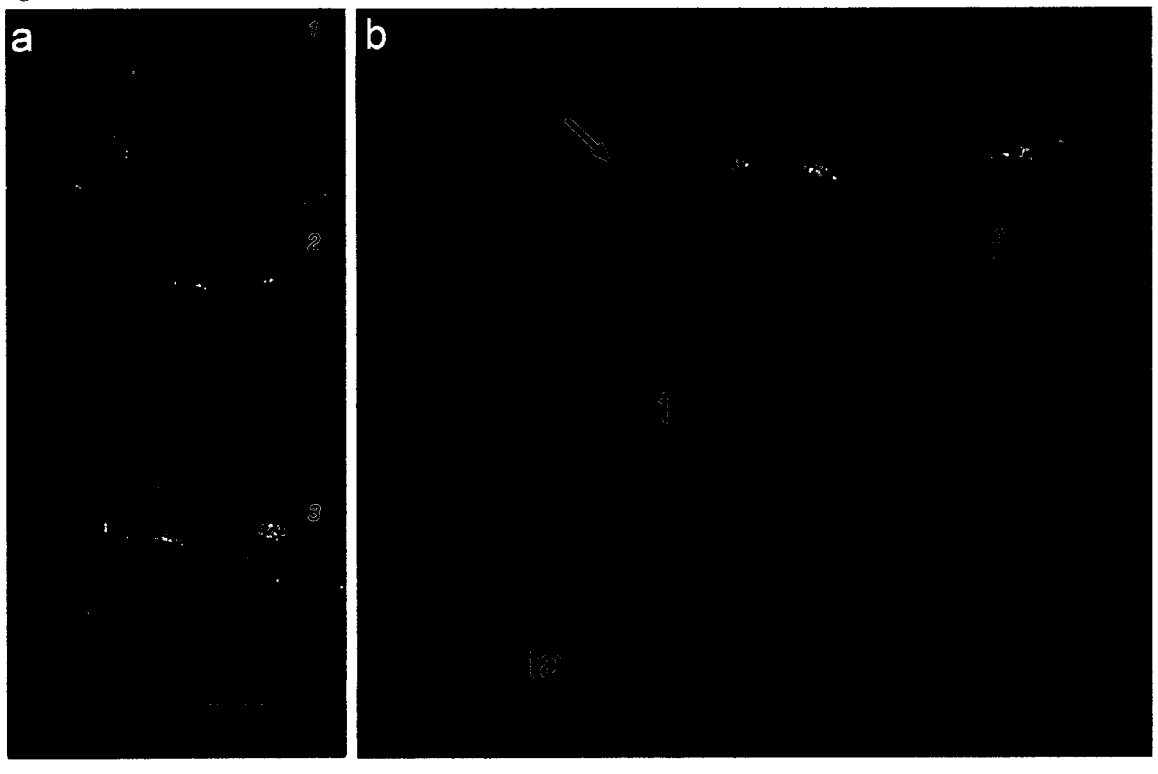
Putative structures were observed along the integument of the animal. The results of investigations on both external and internal features of putative receptor structures are presented. Structures explored included the possibility of a subgenual organ, trichoid sensillae, a Johnston's organ, and a tympanal organ.

The possible subgenual organ

The tibiae of *D. ponderosae* are thin and flattened with a granulated surface, covered in dense setae (Figure 3.4). Along the posterior margin is a ridge of conical tubercles giving the edge a serrated appearance. The presence of scolopidial organs present in the legs of *D. ponderosae* was not directly examined. External morphological examinations of the legs of *D. ponderosae*, showed no distinct enlargement of the tibia as would be expected in animals possessing a subgenual organ (Vilhelmsen et al., 2008).

Figure 3.4 Detailed anatomy of the leg of *D. ponderosae,* **a)** Image of 3 legs taken from the right side of a male specimen, *1* prothoracic, *2* mesothoracic, and *3* meta thoracic legs (scale bar = 1000 μm). Legs from both sexes were observed to be identical. **b)** Close-up image of mesothoracic leg. The arrow indicates the relevant location for a possible subgenual organ; *ta,* tarsi; *t,* tibia; *f,* femur (scale bar = 200 μm)

Figure 3.4

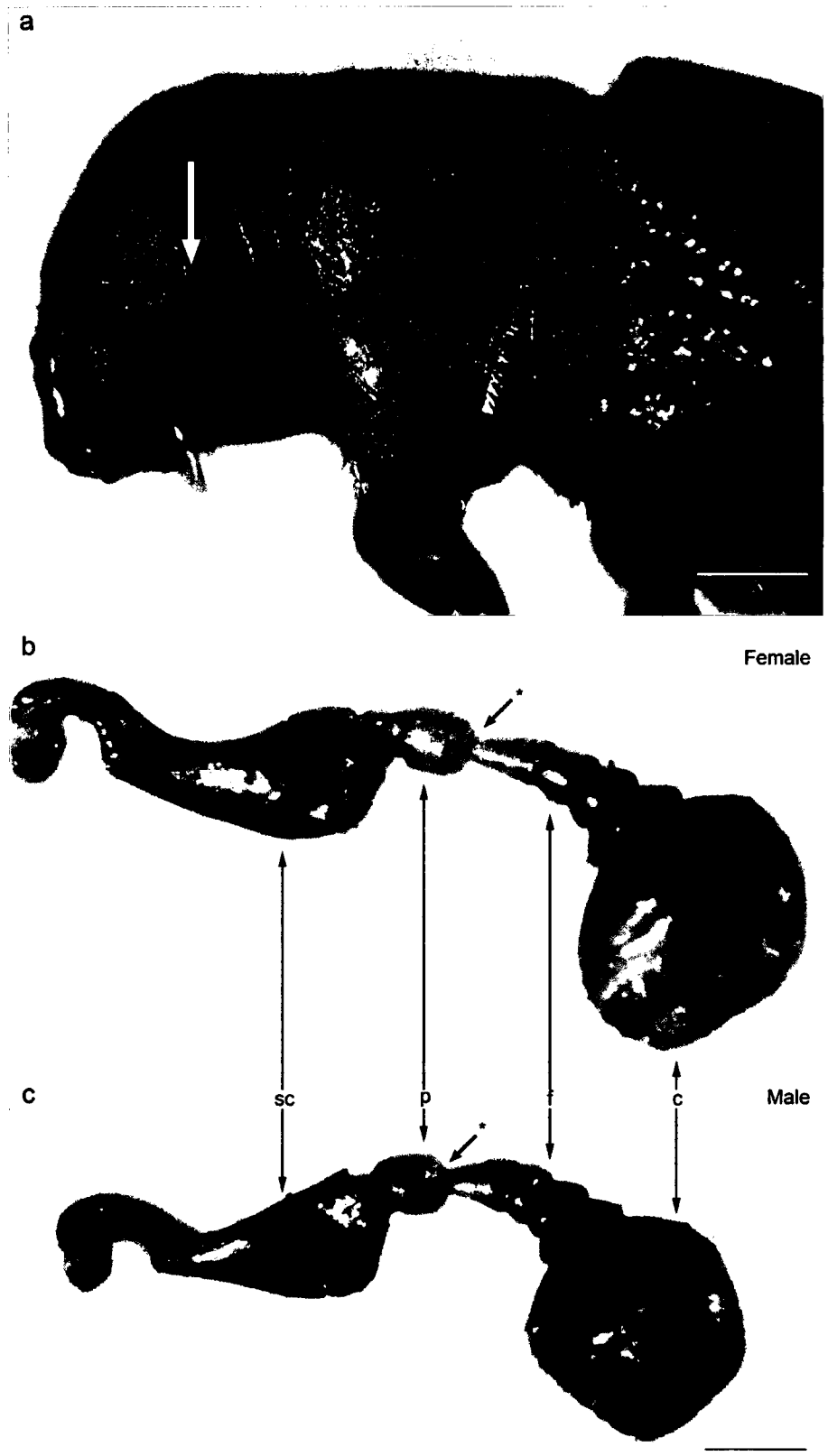


The possible Johnston's organ

Dendroctonus ponderosae possess clubbed-elbowed antennae, divided into 3 parts. Sexual differences exist only in terms of the size of the antennae, which are proportional to the size of the adult beetle (Figure 3.5). The most distal portion to the body is the club of the antennae. Four lateral sutures divide the club into sections, each with its own linear array of setae. Following the club is the funniculus, which is made up of 5 segments: the first 3 are relatively equal in size, while segments 4 and 5 are at least double the length (Figure 3.5). The final segment of the funniculus is the pedicel (Hopkins, 1909) (Figure 3.6). All segments of the funniculus are lightly setose, except for the pedicel, which is completely smooth. The pedicel is roughly double the diameter of the previous segments and sits directly above to the scape. The scape connects the antenna to the head of the animal, inserting into a shallow depression or scrobe directly anterior the margin of the eye (Figure 3.5). Should there be a Johnston's organ present, it would occur at the juncture between the funniculus and the pedicel (Figure 3.5). External observations of the structures present on the antennae of *D. ponderosae* revealed no structures consistent with a Johnston's organ as described in other species. However internal anatomical explorations within the antennae are necessary to conclusively exclude the presence of a Johnston's organ in this species.

Figure 3.5 Putative Johnston's organ on a *D. ponderosae* female head. **a**) Magnified image of the head of *D. ponderosae*, the arrow indicates the base of the funniculus, a possible location for the putative Johnston's organ. (scale bar: 500 μm). **b & c**) A close up image of the antenna of a female and a male *D. ponderosae* (respectively), *sc*- scape, *p* – pedicel, *f* – funniculus, *c* – club, asterisk (*) indicates possible location for Johnston's organ. (scale bar = 100 μm).

Figure3.5

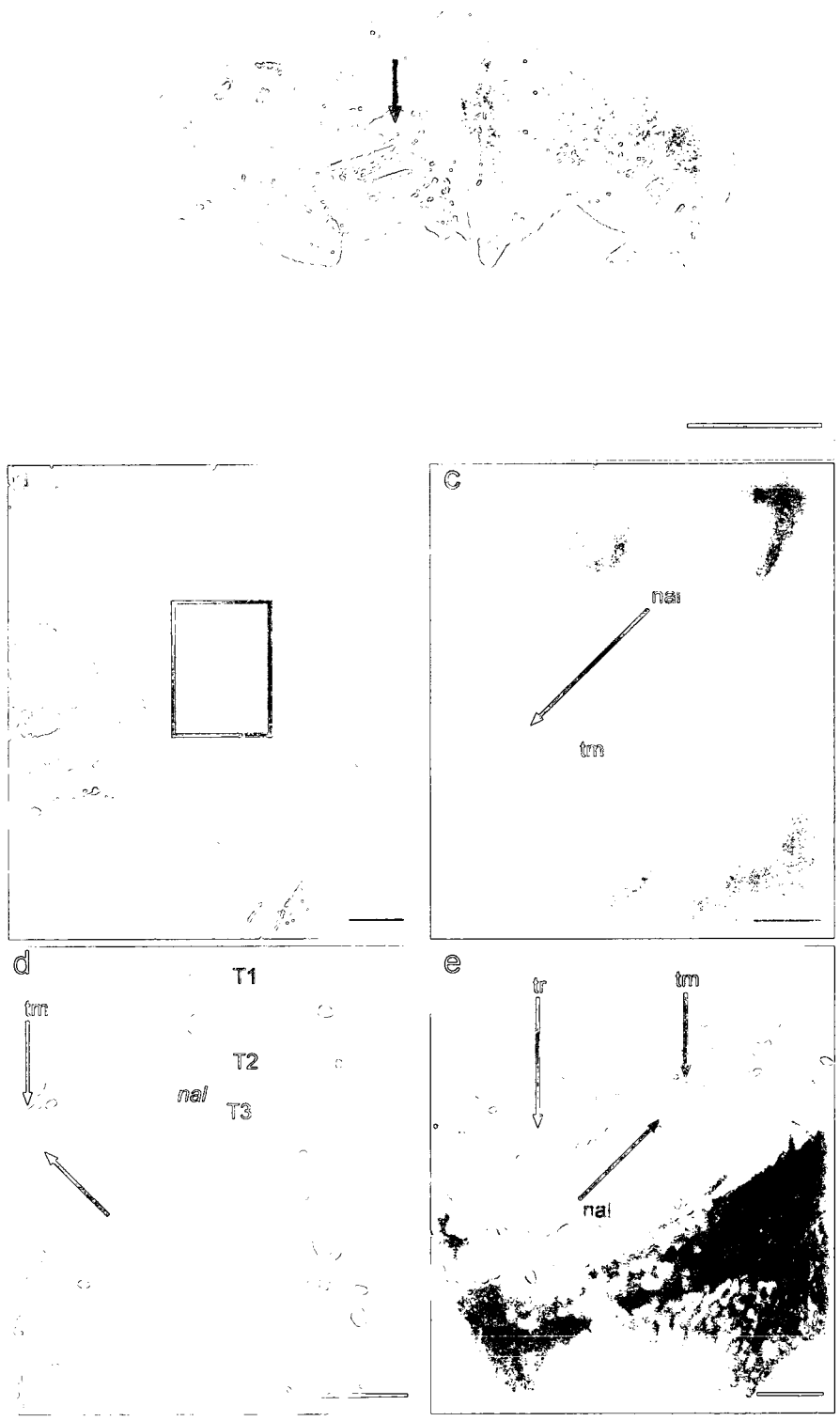


Putative tympanal structures

Examinations were carried out to explore the possibility of a tympanal acoustic receptor. Preliminary observations were made in regions of the body known to support tympanal structures in other coleopterans. These sites revealed no specialized structures or innervation as observed in Scarabaeidae and Cincindellidae. Observations of the rest of the body of *Dendroctonus* revealed several sites that appeared promising due to the presence of membranes or differently sclerotized cuticle, as seen in Figure 3.6. However, most were not innervated or had no associated air sac. The most promising of the observed sites was not visible in the normal resting state of the animal when the elytra were closed. However, removal of elytra revealed a distinctive thinning of the cuticle along the lateral margin of the caudal edge of the thorax, directly adjacent to the wing attachment site, and anterior to the first abdominal segment (Figures 3.6 a and b). This thinning was delineated by margins of thicker cuticle that covered a large air-filled sac directly behind the transparent membrane (Figures 3.6 b and c). In the area adjacent to the air sac there can be seen several small trachea. Several trachea were visible throughout the area, the putative membrane is also very closely associated with the enlarged first abdominal spiracle (S1) (Figure 3.6). Injection of Janus green B behind the membrane allowed the visualization of what was later confirmed to be a projection from the alary nerve, which attached on the underside of the membrane. Internal explorations confirmed a nerve insertion point on the inner surface of the putative tympanal air sac (Figures 3.6 d and e).

Figure 3.6 Putative thoracic receptor structures on *D. ponderosae*. **a)** Light micrograph of entire body of *D. ponderosae*. Arrow indicates the location of the putative tympanal structure on the meta-thorax (scale bar = 1000 μm). **b)** Close-up of a small membranous air-sac and membrane found on the proximal lateral margin of the dorsal surface of the meta-thorax. (scale bar = 100 μm) **c)** Close up of the external anatomy of the putative receptor structure, a thinning of the cuticle delineated by harder chitin is visible. At the center of the membrane (tm) a possible attachment point for the alary nerve projection (nal) can be observed. (scale bar = 200 μm) **d)** By conducting a dorsal dissection of the animal it can be seen that the alary nerve (nal) projects from the meta-thoracic ganglion (T2), extending towards the wing base with one branch extending to the putative receptor site. The arrows indicate the location of the putative structure, and the alary nerve branch (scale bar = 100 μm). **e)** Lateral view of the attachment site showing the inner surface of the membrane with small nerve extending out from between the muscles up to the base of the membrane (scale bar = 200 μm).

Figure 3.6



Nervous System Anatomy

The central nervous system in *D. ponderosae* consists of 6 distinct sections; the brain, the subesophageal ganglion (*gi*), the prothoracic ganglion (*T1*), the meso thoracic ganglion (*T2*), the metathoracic ganglion (*T3*), and the abdominal gangliar complex (*agc*). The adult nervous system in *D. ponderosae* is essentially the same between the two sexes, with the only discernible difference being related to the size difference of the individual animals. As a result from this point forward they will be used interchangeably to provide a description of the nervous system for the organism as a whole.

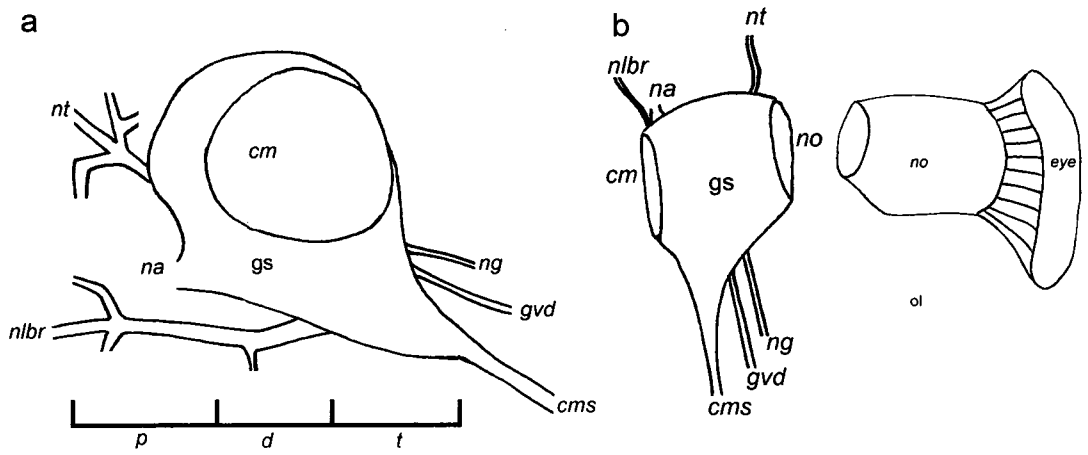
Brain and subesophageal ganglion

The brain (Figure 3.7 a) can be divided into 3 distinct regions, the proto-, deutero- and trito- cerebrum. The optic lobes begin at the eye as a fan shaped arrangement of nerves associated directly with the chitinous compound eye (Figure 3.7 b). Distally they connect to the proto-cerebral area of the supraesophageal ganglion directly via the optic nerve (*no*). No major nerve roots were observed to extend from the optic lobes. Projecting from the deuterocerebrum is the antennal nerve (*na*). This region of the brain collects the information gathered by the antennae. The tritocerebrum is the third segment of the brain and is the seat of all the major buccal nerves. From here we can see the labral nerve (*nlbr*). Projections extend dorsally from the tritocerebrum to the corpora cardiaca allata (*gtr*) via the commissural nerve (*gvd*), and also links the ventral nerve cord to the stomatogastric nerve (*ng*), which connects the brain to the stomatogastric nervous system (a secondary nervous system responsible for the innervation of the digestive and

circulatory systems). The supraesophageal ganglion connects to the subesophageal ganglion via the (*cms*), the circumesophageal commissural nerve (Figure 3.7 c).

Figure 3.7 Drawings of the brain of *D. ponderosae*. **a**) The supraesophageal ganglion (*gs*) is divided into 3 regions, the proto-cerebrum (*p*), the deutero-cerebrum (*d*) and the trito-cerebrum (*t*). **b**) Dorsal aspect of the *gs* and the optic lobe (*ol*). The gs projects laterally via the optic nerve (*no*) to the *ol* where many small projections emerge, innervating the compound eye.**c**) Schematic diagram of the lateral aspect of the gs as it connects to the subesophageal ganglion (*gi*) via the circumesophageal commissural nerve (*cms*). Several major nerves project from the supraesophageal ganglion, rostrally the (*nt*), the antennal nerve (*na*) and the labral nerve (*nlbr*). Caudal projections include the stomatogastric nerve (*ng*) which connects the ventral nerve cord to the stomodeal nervous system and the (*gvd*) which connects to the corpora cardiaca allata (*gtr*). Projections from the subesophageal ganglion (*gi*) include the mandibular nerve (*nmd*) and the (*ncm l*), the *gi* projects caudally to the thoracic ganglia via a pair of commissural nerves (*cm*).

Figure 3.7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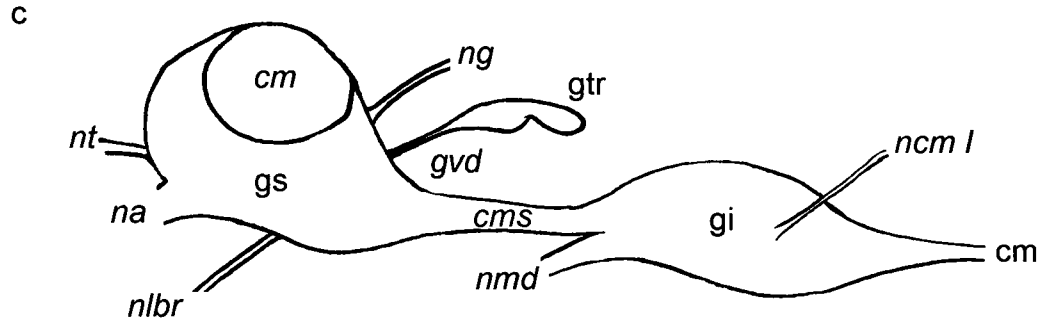
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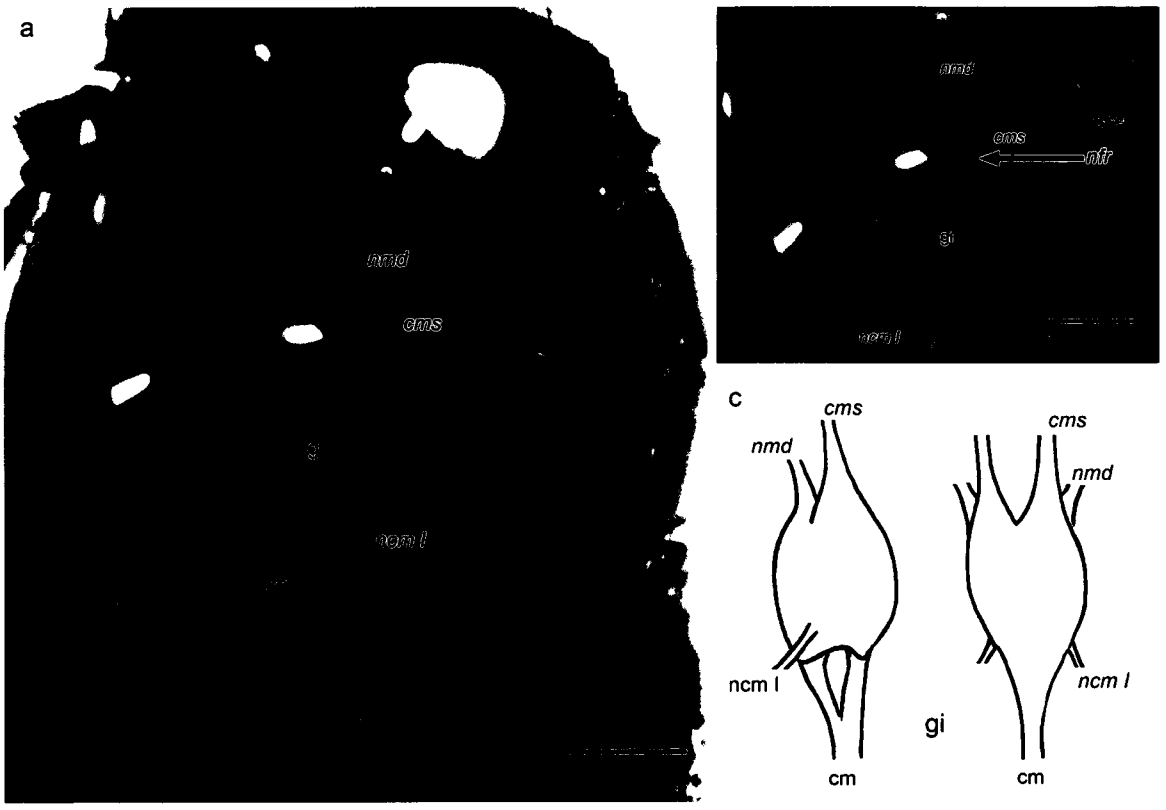
The subesophageal ganglion sits posterior to the supraesophageal ganglion just below the esophagus as its name implies (Figure 3.8). From this ganglion several connections were observed innervating the mouthparts and neck muscles. Projections to the mandibles (*nmd*), and the jugular nerve (*ncm I*) can be observed. There is also a set of very fine commissural nerves (*nfr*) that project frontally from the subesophageal ganglion, terminating at the frontal ganglion (*gfr*). The frontal ganglion was not shown as it is so small I was not able to properly visualize it. Caudally, the subesophageal ganglion connects to the ventral nerve cord via a pair of commissural nerves (*cm*), also referred to as the connective nerves.

Figure 3.8 Light micrographs and drawings of the subesophageal ganglion. **a**) A light micrograph showing the ventral surface of the subesophageal ganglion and its nerve projections. The commissural nerve (*cm*) connects the gi to the prothoracic ganglion (*T1*). **b**) Close up of the subesophageal ganglion showing the projection of the frontal nerve (*nfr*). **c**) Schematic drawings of the lateral and dorsal aspects of the subesophageal ganglion (scale bars a, b = 200 μm).

Figure 3.8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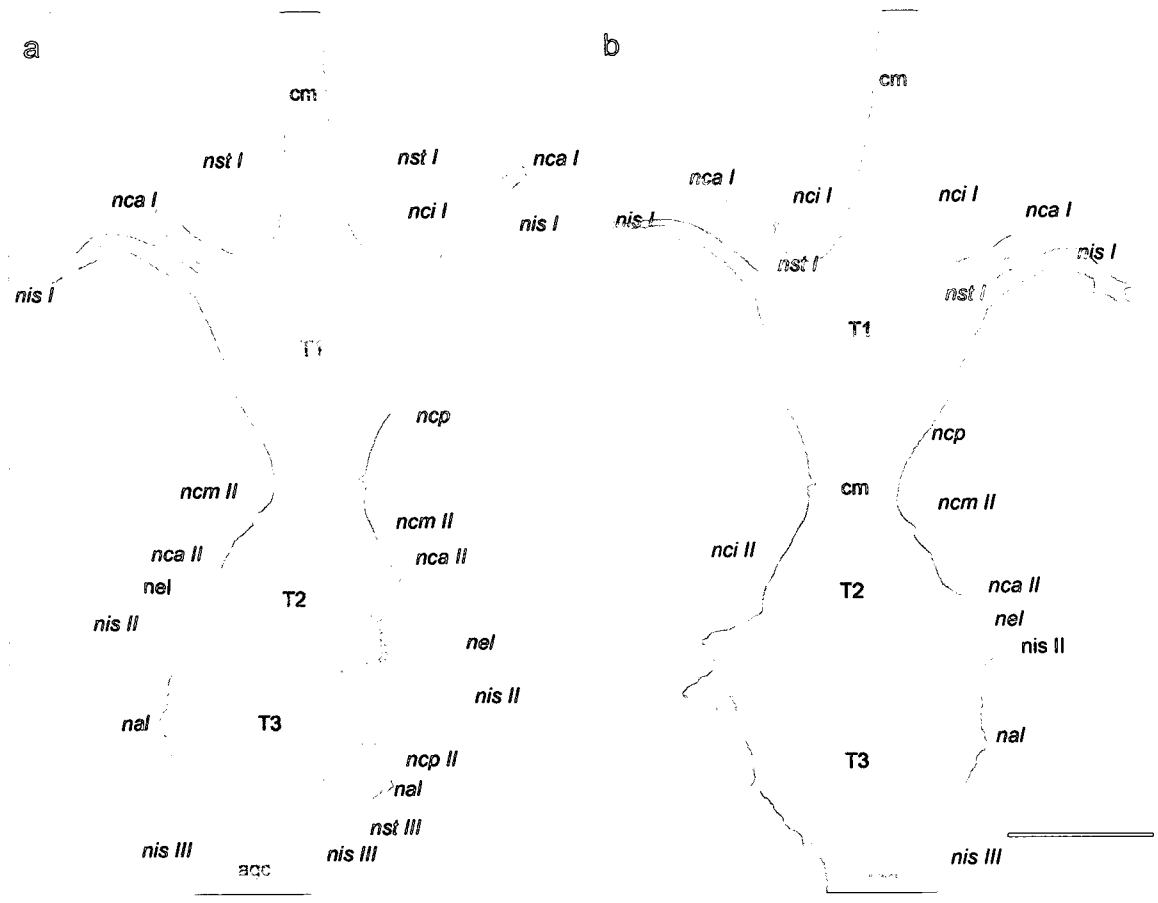
Ventral Nerve Cord – Thoracic and abdominal gangliar complex

The thoracic ganglia can be divided into 3 sections; the pro- meso- and meta-thoracic ganglia, followed typically by a set of abdominal ganglia (Figure, 3.9). In the case of *D. ponderosae* the thoracic ganglia are divided into only 2 sections: a distinct prothoracic ganglion (*T1*), followed by a larger ganglion comprised of the fused meso- and meta- thoracic ganglia (*T2-T3*) as was previously described for the genus in (Calder, 1989). Also attached to these ganglia is the abdominal gangliar complex (*agc*), a fused set of all the individual abdominal ganglia (typically one per abdominal segment). This gives the *D. ponderosae* nervous system the appearance of having only two thoracic ganglia and no abdominal ganglia. Nerve projections from the thoracic ganglia possess all the innervations to the primary centers for locomotion, such as the legs, wings and elytra. Projections from the abdominal gangliar complex serve to innervate the abdominal muscles as well as the reproductive organs and anus of the animal (Holste, 1910).

Two major nerve branches extend laterally from each side of the pro-thoracic ganglion, the prothoracic anterior coxal nerve (*nca I*), and the prothoracic ischadic nerve (*nis I*), at the distal end of the prothoracic ganglion is the prothoracic posterior coxal nerve (*ncp I*). The prothoracic ganglion also bears several smaller projections. Dorsally the prothoracic inferior coxal nerve (*nci I*), and ventrally the prothoracic sternal nerve (*nst I*). Both nerves project from the anterior end of the prothoracic ganglion. Following the thoracic commissural nerve (*cm*) are the fused meta- meso- and abdominal gangliar complex.

Figure 3.9 Light micrographs of the thoracic ganglia in *D. ponderosae*. **a**) Dorsal aspect of the thoracic ganglia, showing the pro-, meso-, and meta-thoracic ganglia (T1-T3) respectively. **b)** Ventral aspect of the same thoracic ganglion from part a) showing the nerve roots which project from the ventral surface. All visible nerve projections are described in detail in the text (scale bar = 200 μm).

Figure 3.9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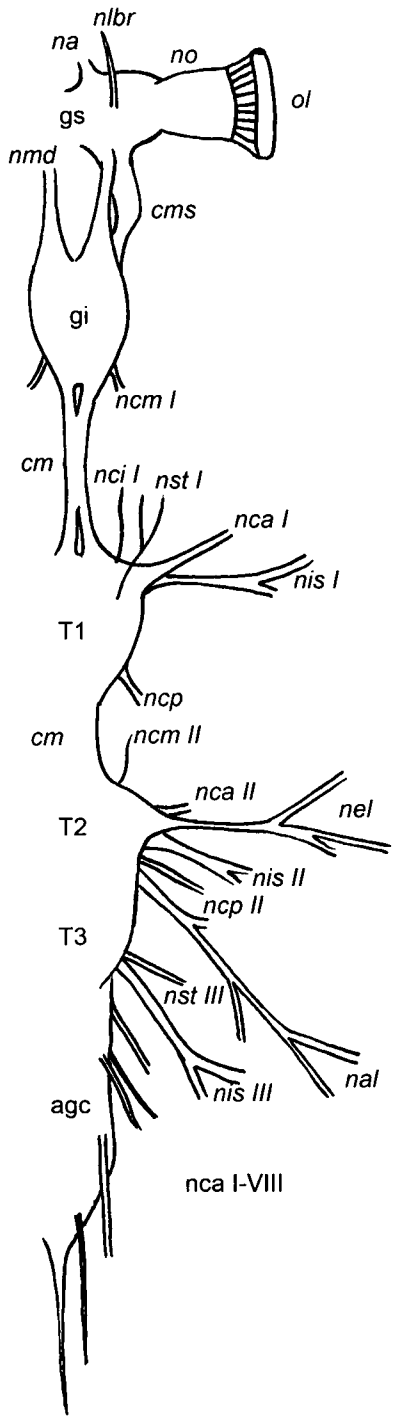
The separation of the meso-, and meta- thoracic ganglia is distinguishable by their bilobate nature, a separation punctuated at the center by a small depression. The meso- and meta- thoracic ganglia bear the nerves associated with the wing movements. These are apparent as two major lateral projections the elytral nerve (*nel*) and the alary nerve (*nal*) respectively. The same pairs of leg nerves as were seen on the prothoracic ganglion are also present on the more distal thoracic ganglia the ischadic nerve (*nis II, nis III*), the anterior coxal nerve (*nca II, nca III*), the inferior coxal nerve (*nci II, nci III*), the posterior coxal nerve (*ncp II, ncp III*), and the respective sternal nerves (*nst II, nst III*). At the anterior end of the meso-thoracic ganglion is the secondary commissural nerve (*ncm II*) (Figures 3.9, 3.10).

The abdominal gangliar complex has several projections extending from it, which weren't specifically characterized. These projections innervate the abdominal segments thereby controlling the movement of the abdomen (Holste, 1910). These nerves can therefore be suggested to control the neural aspect of sound production causing the *pars stridens* to rub against the plectrum.

Figure 3.10 Schematic drawing of the entire ventral nerve cord in *D. ponderosae* detailing the major nerve roots. Nomenclature for nerve abbreviations can in anatomy methods section.

Figure 3.10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3.4 Discussion

The purpose of this study was to identify sites capable of supporting a possible receptor organ within *D. ponderosae.* Considering the number of beetles that produce sounds it is surprising that to date ears have only been discovered in two families, Cincindellidae, and Scarabaeidae (Spangler, 1988; Forrest et al., 1995). The discovery of an acoustic receptor structure in *Dendroctonus* would therefore be a novel discovery of hearing. External morphological examinations coupled with internal dissections of *D. ponderosae* revealed one major site capable of supporting a tympanal structure (Figure 2.10). This study found no sites resembling those present on other families where acoustic reception has already been observed. This would suggest that should an ear exist in *Dendroctonus* it has either evolved independently or is not a tympanal ear at all.

Near-field Receptors

Johnston's organ, trichoid sensillae

Typically when a Johnston's organ is present the pedicel appears more enlarged at the base of the funniculus, indicating the presence of increased innervation. As can be seen in figure 3.8 the pedicel present on *D. ponderosae* morphologically speaking could possess a functional Johnston's organ. It is morphologically comparable to other curculionoid species, which possess a Johnston's organ (Hix et al. 2003). Johnston's organs are however limited in their frequency range, capable of detecting frequencies up to 500 Hz (Virant-Doberlet and Cokl. 2004). Since the signals produced by *D. ponderosae* are so broadband and possess high frequency energy, it is unlikely that a

Johnston's organ is the hearing mechanism employed. While there is a slight enlargement of the pedicel of the antennae, it seems unlikely that this would be capable of housing a Johnston's organ.

Vibration Receptors

The subgenual organs

To date hearing in Coleoptera has been limited to the realm of airborne acoustic emissions. Some evidence exists that certain species of beetles are capable of exploiting substrate borne vibrations to detect conspecifics. Gyrinidae has been shown to employ waves across the waters surface as a medium of communication (Bendele, 1986). This would suggest that it is possible that beetles are capable of detecting substrate borne vibrations. Studies in signal transmission have shown wood structures to be efficient transmitters of low frequency vibrations (Virant-Doberlet and Cokl, 2004).The presence of a subgenual organ, implicated to function as vibration detector, has not been described in Coleoptera.

Previous study (chapter 2) suggested the possibility of a vibrational component during intraspecific signals produced by the *D. ponderosae* males. However, an external examination of the morphology of the tibia of *D. ponderosae*, in this study, revealed no morphological adaptations associated with a subgenual organ. The presence of scolopidial organs in the legs of *D. ponderosae*, however, was not directly tested in this study, and it is possible, given the signaling environment of these beetles that a subgenual organ is present. The evidence from this study does not rule out the capacity of *D. ponderosae* to employ vibration detection via femoral chordotonal organs. Further investigations could examine the biological relevance of these substrate vibrations

through behavioural studies, and further investigate the neural reception to vibratory signals.

Far-field Receptors

Tympanal organs

A tympanal ear requires, 1) the presence of a differentiated area of cuticle capable of supporting a tympanic membrane, 2) an associated air sac to be used as a resonating chamber, and 3) the system must be innervated in order to transduce the mechanical energy into a nervous impulse. Inspection of the external morphology of *D. ponderosae* revealed many membranous regions consistent with criterion 1. Examinations of these membranes were limited to those membranes possessing air sacs or airways in close proximity fulfilling criterion 2. The results from this study show the presence of a site fulfilling all 3 of the previously described morphological criteria necessary for a tympanal hearing organ. However, in order to conclusively define the presence of an ear at this site, and to demonstrate that its function is detecting acoustic signals produced by conspecifics, further neurophysiogical studies are required.

To establish that communication is occurring, further studies are required to demonstrate the existence of acoustic or vibrations receptor organs, through behavioural and neural responses to acoustic stimuli. While many techniques exist for recording electrophysiological impulses in insects, the size of *D. ponderosae* might prove to be a limiting factor. Although the nerves are large enough to support a hook electrode for recording, dissection of the nervous system needs to be confined to a very small area in order to not be fatally traumatic to the insect. One way of recording such impulses in small insects is based on the idea that if a behavior is elicited then there must be a

muscular reaction, thereby allowing the experimenter to implant an electrode directly into a large group of muscles, and record the motor action potentials of the muscular contraction. Yack et al. (2007) used recordings from muscles as a rough measure of hearing in Hedylidae, a family of tympanate nocturnal butterflies. This served to establish the presence of hearing. However, concrete evidence of hearing was established by using hook electrodes (steel or tungsten) to hook directly on to the peripheral sensory nerve directly associated with the purported hearing structure (Yack et al. 2007). Further study is required to prove that *D. ponderosae* is indeed capable of hearing, and acoustic signals are biologically relevant.

Chapter 4. Playbacks

4.1 Introduction

Dendroctonus ponderosae possess a complex repertoire of acoustic signals which they employ in a range of communicative situations. These acoustic signals have been hypothesized to be an integral part of the courtship interaction by acting as cues for species recognition (Rudinsky and Michael, 1974). The previous chapter showed anatomical examinations of *D. ponderosae* and revealed several putative structures capable of functioning as an acoustic receptor organ. As can be seen in the criteria for an ear set out by Yack and Fullard (1993), the last major component remaining to be defined is the presence of an acoustically mediated behavioral and/or neural response. The main objective of this chapter was to provide evidence supporting the hypothesis that *D. ponderosae* is capable of hearing the airborne acoustic signals produced by conspecifics. This objective will be achieved by examining any acoustically mediated behavioral response in *D. ponderosae* when exposed to a manipulated conspecific signal. Attention was focused on the courtship chirps of *D. ponderosae* males, in particular the complex and simple signals produced when a male encounters a female. Male female interaction signals were chosen as they form part of a more predictable set of outcomes, and, were the most reliably reproduced signal type. Male-female signals are essential for intraspecific interactions and have been suggested to be able to elicit an acoustic response from both sexes (Ryker, 1988).

Playback experiments are used to artificially elicit a response in an organism, when exposed to a synthetic or reproduced stimulus. This way an experimenter can control for a larger number of variables while manipulating elements of the animal's

natural communication. For example, by using artificial or pre-recorded male calls when exploring aspects of male-female communication one can eliminate certain problems inherent to using living male beetles: 1) non-cooperation of the male, and 2) the possible incompatibility between two individuals (Hedrick, 1986).

Another benefit to playback experiments is the ability it affords the experimenter to manipulate the signal characteristics, allowing signals to essentially be "made-to-order," removing any flaws or imperfections introduced into the natural signal by environmental interference (i.e. ambient noise) (Hedrick, 1986). Once the signal is prepared and presented, an experimenter can observe any behavioral response to that cue. Not only does this give vital information as to what cues an animal may be attending to and what information is conveyed in the signal, but also, it is evidence that the animal is in fact capable of receiving the presented stimulus.

To date no study has focused specifically on playback experiments with relation to bark beetle communication. However, at least one study has been conducted suggesting a bilateral stream of communication in *D. ponderosae*. Ryker (1988) showed that individual females could be stimulated to produce pheromones when exposed to played back male attractant (complex) chirps through a piezoelectric ceramic disk. The purpose of Ryker's experiment was to collect pheromones from confined females, which he accomplished successfully. This may have been the first piece of evidence showing that bark beetles are capable of perceiving relevant acoustic signals. There were shortcomings to the Ryker experiment, in that it did not accurately limit the acoustic stimulus to the airborne dimension. The Ryker experiment was conducted inside a glass apparatus with several females confined within glass vials. This setup could have caused

a number of confounding problems including: the production of an undesired vibratory component to the signal, which may have altered the females' behaviours. Despite this encouraging evidence, no experiments have been conducted to date focusing specifically on the transmission of acoustic information in *D. ponderosae*. This chapter will examine the presence of acoustic communication in *D. ponderosae*, by exploiting two major communication systems in *Dendroctonus*: the communication of territoriality, and intersexual communication in a more natural setting.

Male-male Signals

Territorial communication in *D. ponderosae* was previously only linked to pheromone production (Alcock, 1982). However, as it has been shown, *Dendroctonus* beetles are very acoustically active in territorial disputes, employing rivalry stridulations in male-male encounters (Rudinsky and Michael, 1974; Yandell, 1984; Ryker, 1988). Intruder males may take part in acoustic battles with a resident, most often resulting in the intruder leaving the situation and avoiding the conflict. However, if enough information is given on the relative strength of the rival, the intruder may choose to engage in a physical dispute (Alcock, 1982). Playback of male-male type signals to a male beetle could therefore be capable of eliciting an acoustic behavioral response.

Male-female Signals

Territoriality is not the only stimulus associated with an acoustic interaction. Both males and females can also be stimulated into acoustic interactions in the presence of the opposite sex (Ryker, 1988). It has been shown that for a female to produce the 'masking

pheromone' (discussed in Chapter 1) the male does not have to enter the female's burrow, rather only the chirp of male proximal to the burrow entrance is necessary to trigger the release of pheromones (Rudinsky, 1968; Rudinsky and Michael, 1973). It has also been observed that females tend to reject males that have been silenced (Ryker and Rudinsky, 1976; Yandell, 1984). Both of these facts demonstrate the importance of acoustic communication in *D. ponderosae*, and support the hypothesis that acoustic communication plays a central role in the exchange of information within this species.

Male and female *D. ponderosae* possess a rich repertoire of signals (chemical or acoustic) that can be employed under different conditions, to communicate information regarding their physical status and/or willingness to engage in an interaction. Like males, females have been shown to not only be capable of communicating chemically but also through the use of acoustic signals. Within the females' repertoire, two types of sonic signals are employed during their interactions with conspecifics. The first is a single click, which occurs intermittently while the female is in her burrow. This signal has been assumed to serve either a territorial function or gallery spacing function within the wood, typically occuring when the female beetle is alone and undisturbed (Rudinsky and Michael, 1973; Ryker, 1988). Single click, territorial signals have been shown to cause intruder females seeking substrate for a burrow to turn away from areas with already established clicking females (Ryker, 1988). The second signal in the female repertoire, is a multi-pulse chirp. This signal is assumed to not only play a role in territoriality but also in courtship (Ryker, 1988). Typically this signal is produced when another beetle, intruder or mate, begins an interaction with a female (Ryker and Rudinsky, 1976;

Yandell, 1984). In *Dendroctonus valens*, this female stridulation has been correlated with the presence of a chirping male as well as a signal of agreement to mate (Ryker, 1988).

With respect to courtship two signal types are produced, a complex and a simple signal. When the female (or her frass) is first encountered, the male will begin stridulating, first with complex signals and changing to simple signals within the first 5 minutes of the encounter. (For a more detailed description of male sounds see Chapters 1 and 2). Since acoustic signals play such a central role in the *Dendroctonus* behavioral repertoire, I reasoned that the acoustic interaction between males and females could also be exploited in a playback situation.

4.2 Methods

Animals

Refer to methods in the previous chapter.

Signal Preparation

Airborne acoustic signals were collected using a B&K ¼" microphone Type 4939 (Naerum, Denmark) with the protective grid off, amplified through a B&K Nexus conditioning amplifier type 2690 and recorded onto a Fostex FR-2 field memory recorder. Files were saved as '.wav' type files, using 24 bit quantization at a sampling rate of 192 kHz. Recorded signals were analyzed using Raven Bioacoustics Research Program 1.3 (Cornell Lab of Ornithology, Ithaca, NY). Signals used for playback experiments were filtered using a high-pass filter at 800 Hz to remove any underlying noise (see Chapter 2 on Signal Characterization). The signal types presented to the

animals included male-male simple, male-male complex, male-female simple, and male-female complex (Figure 4.1). Presentations were carried out in 20 second intervals, a 10 second bout of stimulus signal followed by 10 seconds of silence repeated 12 times per trial. Simple signal bouts were created by selecting 3 consecutive chirps, which were repeated for 10 seconds. Complex signal bouts were created by selecting 4 consecutive complex chirps, which were repeated in succession until a 10 second stimulus was created. The signals were selected from trials conducted in chapter 2. Twenty-four bit quantization was incompatible with Avisoft. As a result the quantization of the signal had to be reduced from 24-bit wave, to 16-bit wave. Quantization was reduced using the 'save as' function in Raven Bioacoustics program, and selecting the 16-bit wave option. This had no effect on the spectral or temporal characteristics of the signal.

Signal Playback

Signals were presented to the subject animals using Avisoft Recorder USGH software, through an Avisoft Ultrasoundgate Player 116 Amplifier connected with Speakon Cabling to a Scanspeak Ultrasound Speaker (1-120 kHz) (Figure 4.2). All playbacks and recordings were carried out in an Eckel Industries sound isolating chamber (Eckel Industries, Model C-14A MR, Morrisburg, Ontario), at Carleton University. Signal intensities were calibrated by comparing peak-to-peak voltages produced by a series of pure tones of known intensity on a Tektronix model TDS 2002 oscilloscope, to the voltages obtained from signal intensities gathered in chapter 1. This yielded a voltage, which was converted to dB and calibrated to the Avisoft USG Player. Amplitude of the signals was calibrated at approximately 50-60 dB, at 4cm from the speaker horn. Reliable signal parity was confirmed by matching the voltage of the played back signal with the

original on oscilloscope, and later by comparison of spectrograms in Raven (Figures 4.3 a and b). Data pertaining to the behavioral responses were collected using the parameters indicated on figure 4.3.

The speaker was positioned 4 cm above the stage on a tripod. The amplifier gain was placed at the 2nd line of the variable gain knob (arbitrary gradations) which corresponded with the necessary intensity as shown by the signal calibration in chapter 2. Video recordings were made using a Sony HDR-HC7 high definition miniDV cassette recorder (Sony Corporation, Tokyo, Japan) with a Sony 4x Wide Conversion lens adapter. The video camera was positioned in one of two ways, depending on the scenario being tested. It was either suspended directly above the transparent stage using a JOBY gorilla pod flexible SLR tripod (Joby Inc. San Francisco, CA, USA) wrapped around the main post of a retort stand, or placed directly beneath the transparent stage on the same tripod. In both cases the camera was held directly perpendicular to the interaction. A Sony electret condenser microphone (ECM-MS907, Sony Corp., Tokyo, Japan) was hooked directly into the camcorder in order to link the sounds to the video, allowing for the analysis of response latency. Since these signals were not to be used for acoustic/spectral analysis, a Sony microphone was chosen over the flatter more high frequency B&K microphone due its the higher sensitivity.

In all conditions a baseline of behaviours was established by recording for one minute prior to the presentation of any stimulus signal. This also allowed the animal time to acclimate to its surroundings. Observations made during this 'pre-trial' period included all general activity, as well as the presence of any acoustic signaling. After the pre-trial period, a stimulus signal was presented to the animal while observing any behavioral

response. Stimuli were presented in a series of different conditions, relevant to the known acoustic behaviours.

Figure 4.1 Oscillograms of the signals used in the playback experiments. **a**) male-female complex chirps, **b**) male-female simple chirps **c**) male-male complex chirps, **d**) male-male simple chirps.

Figure 4.1

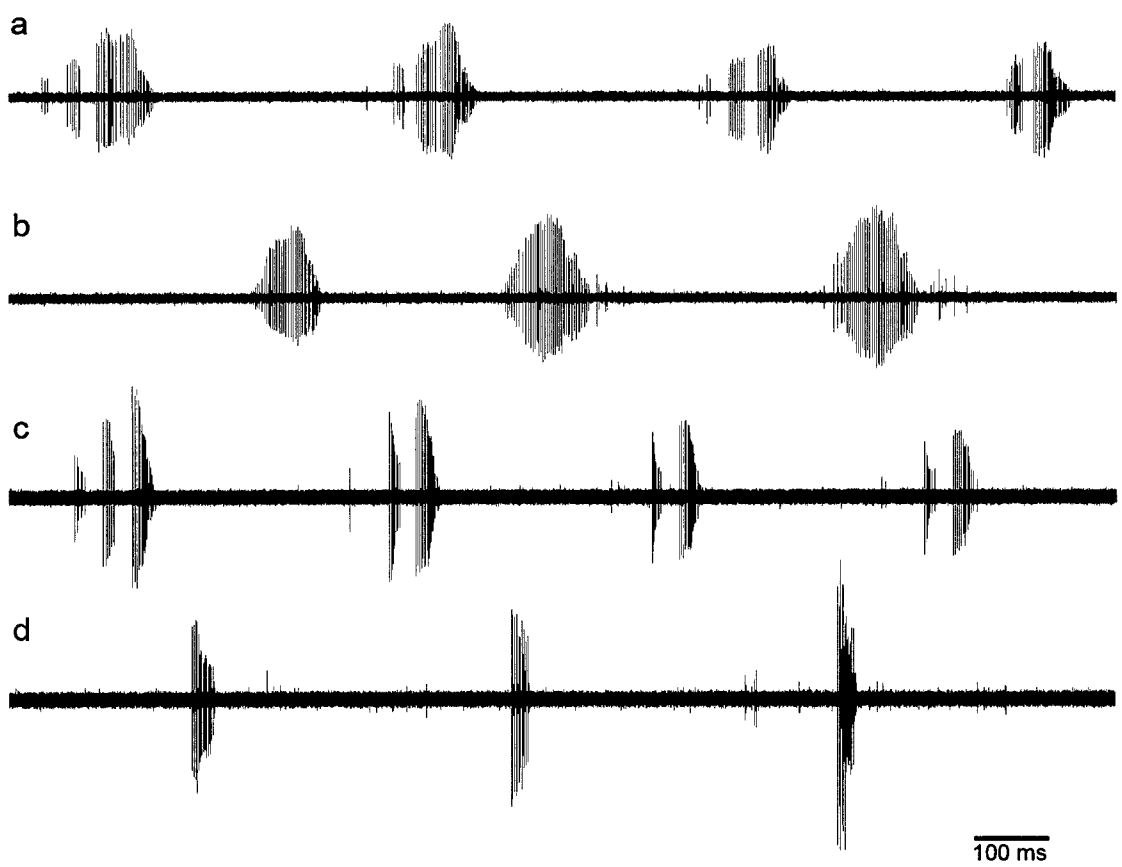


Figure 4.2 Experimental setup for playbacks. **a**) Experimental setup for airborne acoustic playback (petri dish setup), previously recorded signals are presented to the test animal in a prepared arena through an ultrasound speaker. Responses are monitored using a high definition video camera, with a wide conversion lens, perpendicular to the interaction, while audio is monitored through an external microphone. **b**) The petri dish testing arena used in male-male tests, the calibrated division are 1 cm apart. A male beetle is presented in the arena for scale. **c**) The avisoft Scanspeak ultrasound speaker used in the behavioral playbacks.

Figure 4.2

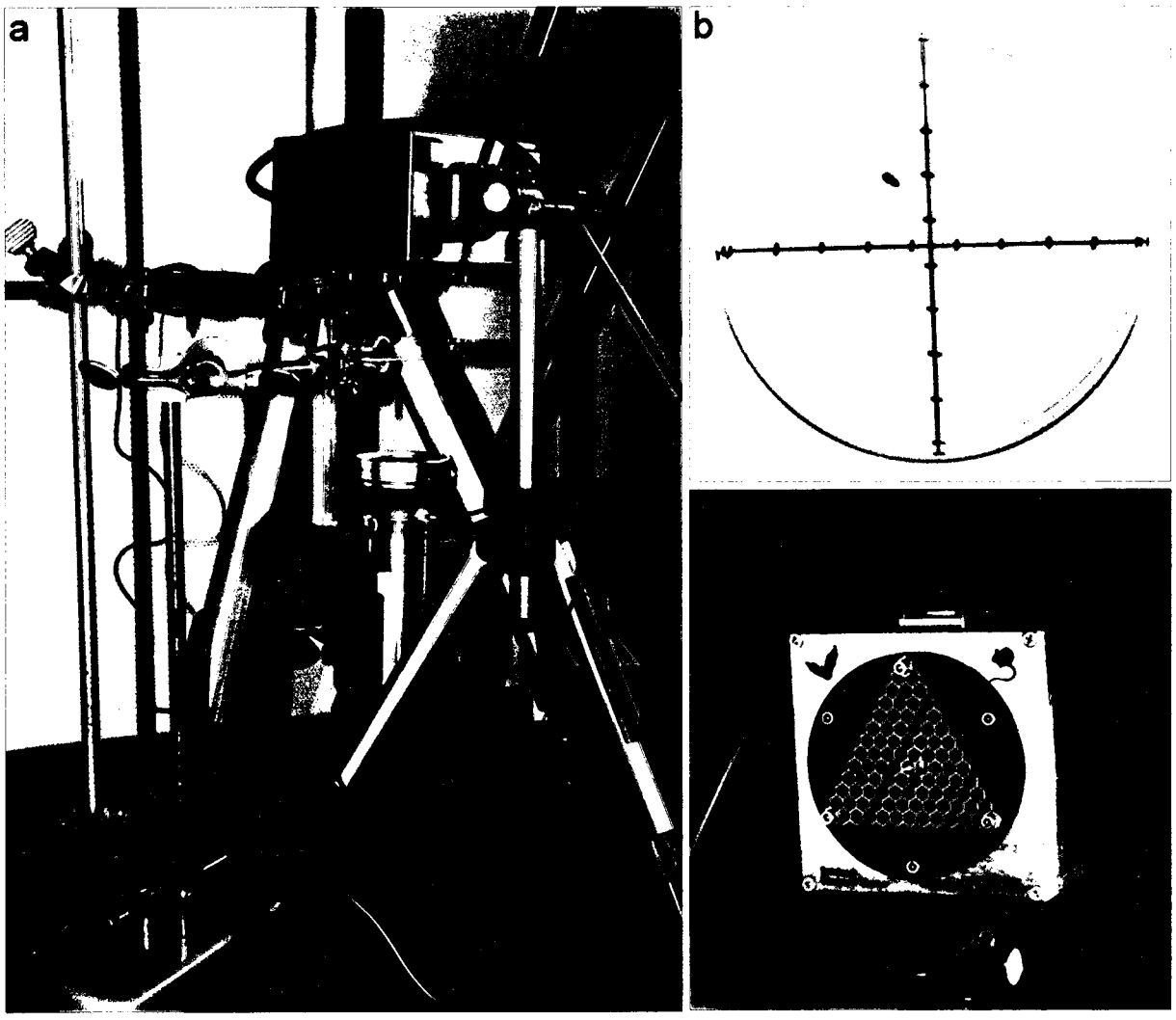
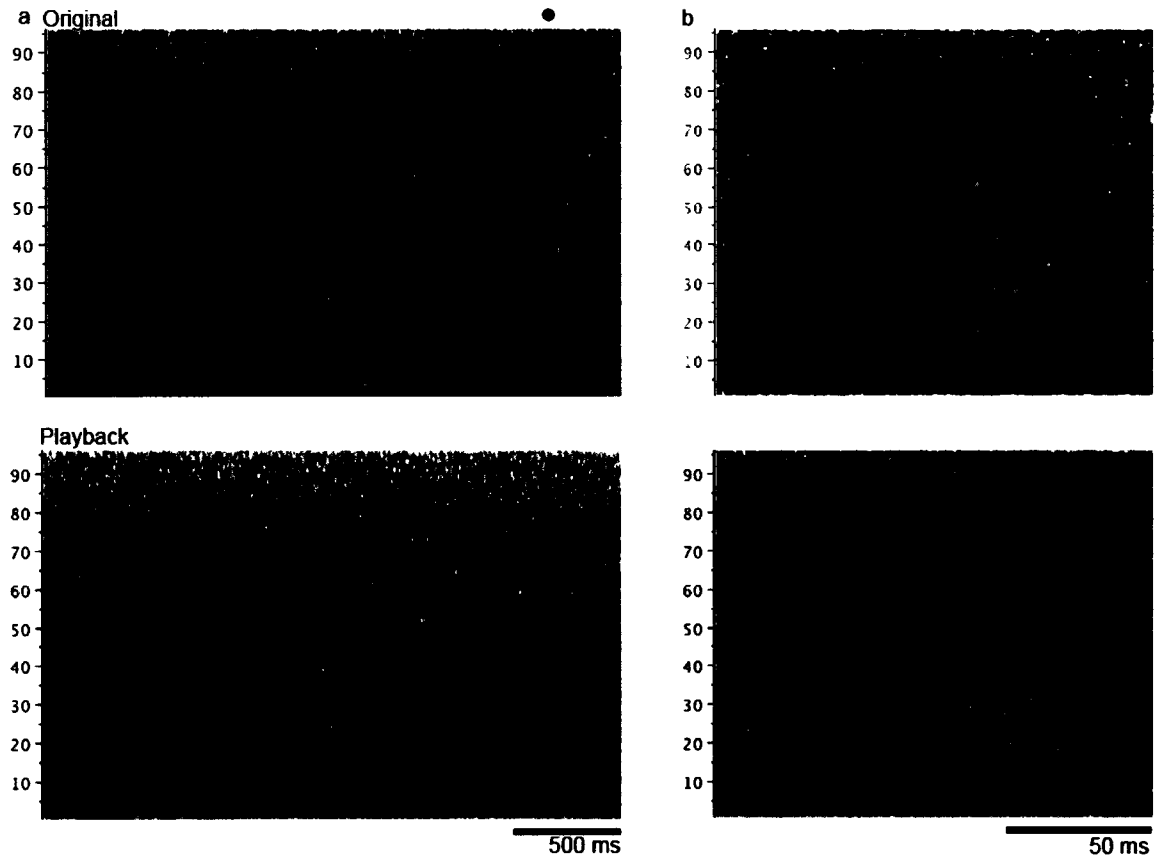


Figure 4.3 Comparison of played back versus original signals of a male-female complex interaction. A very reliable parity of frequencies can be seen between both types of signals. **a**) Spectrograms of two trains of male-female complex chirps. Dot indicates chirp magnified in **b**. **b**) Time expansion of one chirp from trains presented in **a**). Despite a small amount of introduced noise visible as the darker band occupying the frequencies below 50 kHz, it can be seen that there is a very reliable parity between the original and played back signals.

Figure 4.3



Male Setup

Twelve bouts of signals were presented to each individual per trial. Signals were presented to test animals in a small arena constructed from a plastic petri dish, with calibrated distance markers suspended with a clamp on a retort stand (Figure 4.2). A thin piece of fiberglass mesh was draped over the top of the petri dish to secure the beetle within the testing area. The method presented in Ryker (1988), used mesh topped vials, successfully eliciting a pheromone response. The use of mesh between the speaker and the animal had no observable effect on the signal properties.

This condition was designed to exploit the inherent territorial nature of *Dendroctonus,* which have been shown to actively and aggressively employ rivalry stridulations in encounters between two males (Rudinsky and Michael, 1974). A lone male was placed in the center of the arena, marked by the intersecting gridlines (Figure 4.2). The male was allowed to acclimate for a 1-minute pre-trial period, following which either a male-male simple or a male-male complex stimulus signal was presented. A positive response was qualified if any acoustic behaviour or any sudden deviation from baseline activity was observed when exposed to the sonic stimulus. No difference in baseline activity when the animal was exposed to the sonic stimulus was considered a negative response. Since the speaker was placed directly above the animal in these trials, phonotaxis was not directly assessed (i.e. positive vs. negative). However, observations were made to correlate the signal with any phonokinetic behaviour.

Responses were quantified by observing the video coupled with the acoustic trace, noting any changes in behavior after delivery of the test stimulus. Once the trial was

finished a stress response was induced as outlined in Chapter 2, to confirm the capacity of the animal to produce sound and to confirm the sex of the animal.

Female Setup

During the signal characterization trials, males that did not stridulate appropriately were denied access to the borehole or were evicted from the burrow shortly after gaining entry (personal observation). Based on this behavior the next setup involved the playback of male-female courtship signals to female beetles inside established burrows.

Females were given a small disc of ponderosa pine bark (~5.08 cm diameter, 0.5 cm thick) with a small, predrilled borehole at the center. The females were allowed to dig and establish a sham burrow entrance in the bark for 2 hours prior to testing. All females tested accepted the makeshift burrow evidenced by the presence of frass build up at the burrow entrance. It should also be noted that at no point did any of the females burrow completely through but rather remained within the bark disc. Once a female had acclimated to the bark disk, they were exposed to male sounds of both the male-female simple and male-female complex varieties. Audio and video equipment was set-up as in the male-male playback experiments. Observations of behaviour were not undertaken with female subjects, since they were within a burrow. Female responses were therefore quantified purely based on acoustic behaviour.

4.3 Results

Male-male Interactions

Two sets of trials were performed, one in which the individuals were exposed to simple male-male signals (n=3), and one where the individuals were exposed to complex male-male signals (n=3). In each case one trial showed no acoustic response despite the fact that the beetle was confirmed to be capable of producing sonic signals by inducing the stress chirp after the trial was complete. Males produced sound defensively in 57.1% of the trials as they were being placed in the arena at the beginning of the trial. However, all defensive stridulation ceased during the pre-trial period and males were completely silent prior to any stimulus being applied. The relationship between playback stimulus and acoustic response is shown in Figure 4.5.

More stridulation occurred during the male-male simple signal interactions than during the complex signal interactions with averages of 27.00 ± 24.02 (n=3) and 14.67 ± 13.58 (n=3), chirps respectively (Table 4.1). Such a small sample size excludes any measure of statistical significance. The number of chirps per trial varied greatly ranging from 2-46 chirps per trial. Phonokinesis was not assessed as the males showed continuous motion throughout the trials.

Figure 4.4 Oscillograms of stimulus and acoustic response during male-male playback. **a**) Ten seconds of a played back male-male simple signal in black followed by the "silent" 10 seconds exhibiting the male acoustic response, the male response can be seen in the series of blue chirps following the stimulus. **b**) Ten seconds of a male-played back male complex signal in black followed by the "silent" 10 seconds once again showing a train of male response stridulations.

Figure 4.4

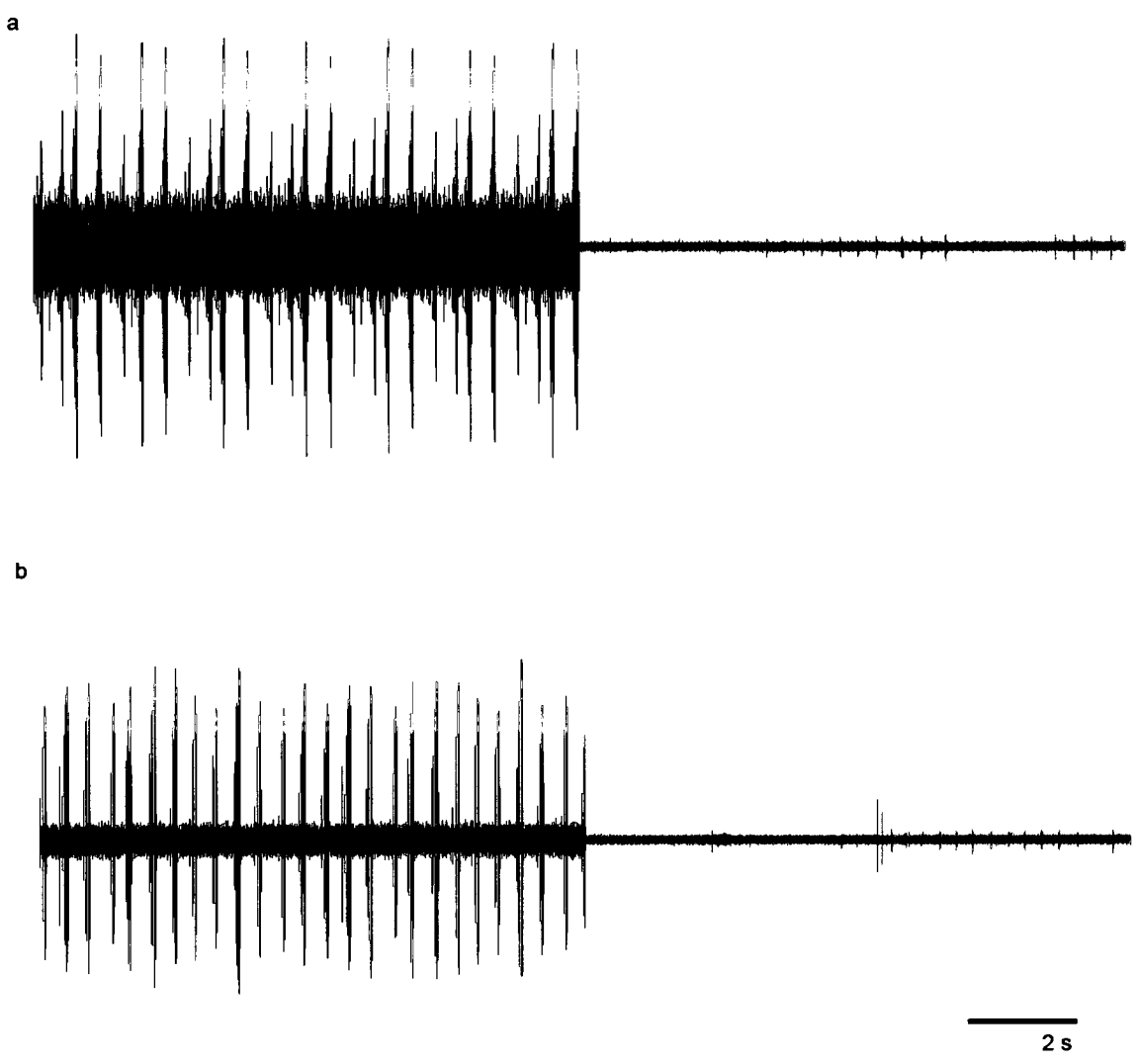


Table 4.1 Summary of results of playback study in *D. ponderosae*.

Pre-trial Period

Response Type	Stimulus Type (n=3) Male-male Simple	Complex	(n=5) Male-female Simple	Complex
Single Click	N/A	N/A	10.00 ± 6.48	11.60 ± 6.19
Multi Pulse Chirp	6.67 ± 5.86	0.50 ± 0.58	0	0

Stimulus period

Response Type	Stimulus Type (n=3) Male-male Simple	Complex	(n=5) Male-female Simple	Complex
Single Click	N/A	N/A	50.00 ± 29.19	19.20 ± 18.51
Multi Pulse Chirp	27.00 ± 24.02	14.67 ± 13.58	10.00 ± 7.71	3.8 ± 3.96

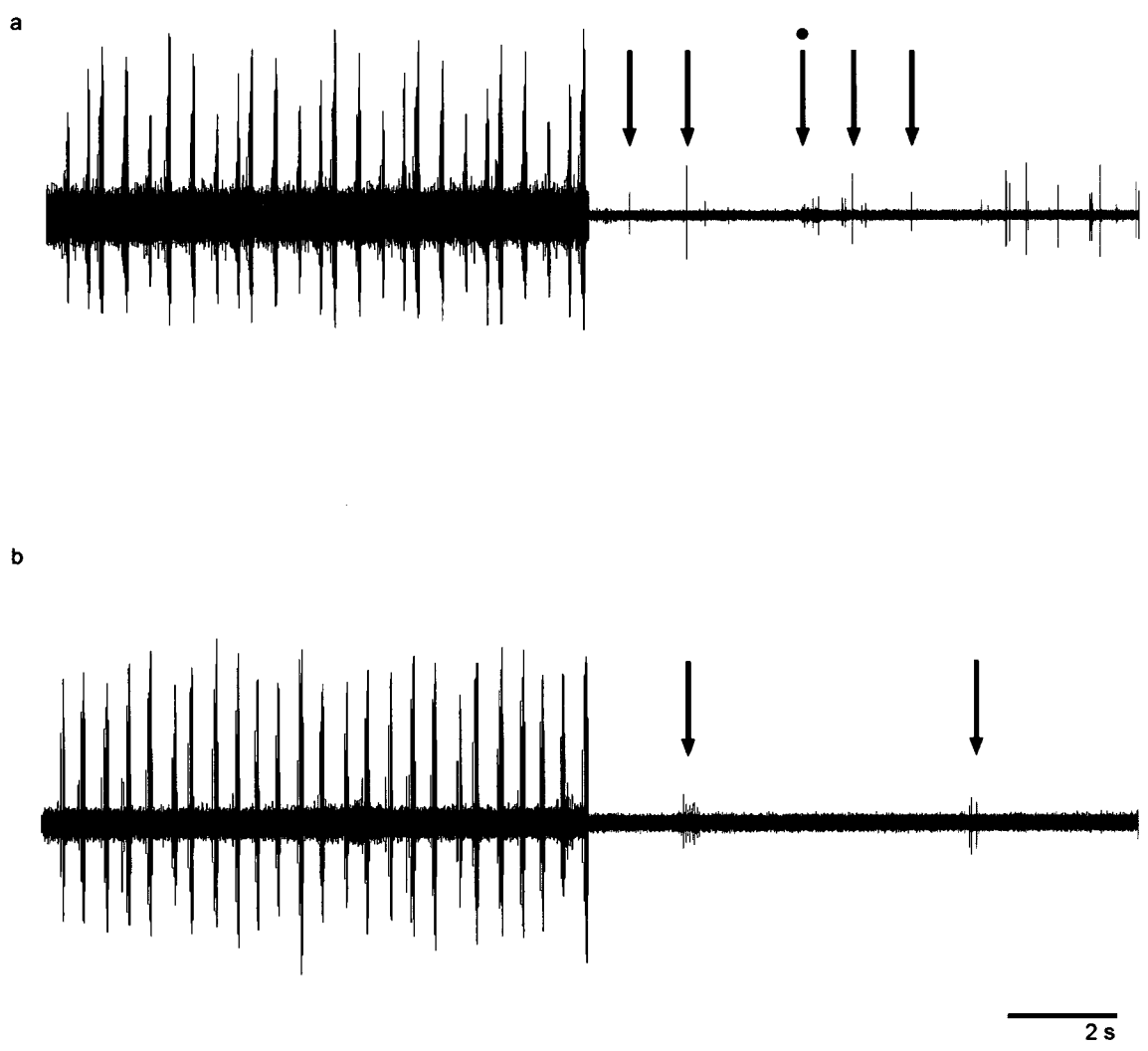
Male-female Interactions

Two sets of trials were performed, one where individuals were exposed to simple male-female signals, and the other exposed to complex male-female signals. When exposed to both signal types female beetles produced single pulse clicks throughout the pre-trial control period as well as throughout the stimulus period. There was a higher number of clicks during the stimulus period of the trial as compared to the pre-trial period. Analysis of the pre-trial period showed an average of 10.00 ± 6.48, and 11 ± 6.19 for simple and complex stimuli respectively. In contrast, the average number of single impulse clicks increased during the stimulus period with an average of 50.00 ± 29.19, and 19.20 ± 18.5,1during the simple and complex stimuli respectively. No multi-pulse chirps were observed during the pre-trial period (Table 4.1).

The relationship between playback stimulus and acoustic response is shown in Figure 4.5. Eighty percent (80%) of the females tested in each trial set showed the presence of a multi pulse female chirp (Figure 4.6). More multi-pulse chirps occurred during the male-female simple signal interactions than during the complex signal interactions with averages of 10.00 ± 7.71 and 3.81 ± 3.96, chirps respectively (Table 4.1). Female multi-pulse responses typically occurred after the 2^{nd} or 3^{rd} bout of played back signals. Single pulse clicking was extinguished after the second bout of stimulus signals. The difference in incidence of multi-pulse chirps between the 2 stimulus types was shown to be not statistically significant based on an unpaired t-test ($t(8) = 0.7142$, $p=0.4594$).

Figure 4.5 Oscillograms of stimulus and acoustic response during male-female playback. **a)** Ten seconds of male-female played back simple signal in black followed by the silent 10 seconds exhibiting the female acoustic response, predominant within this interaction is the female single pulse click as indicated by the arrows. Arrow-dot indicates a multi pulse chirp among the single clicks. **b)** Ten seconds of a played back male-female complex signal in black followed by the silent 10 seconds. Two female multi pulse chirps are visible indicated by the arrows.

Figure 4.5



4.4 Discussion

Male beetles were exposed to both simple and complex type male-male signals. The original sought after measure of acoustic reception was to be phonokinesis. However observations of locomotor behavior associated with sound reception proved to be an impossible task as the beetles never settled down, maintaining constant movement throughout the trial. When compared to the control pre-trial period exposure to male-male simple signals yielded a 405% increase in signaling behaviour, while complex signals were substantially more effective. The complete cessation of defensive signaling prior to the commencement of the trial, in addition to a lack of any signaling present, suggest that any stridulations occurred in response to the acoustic stimulus.

Female beetles were exposed to both simple and complex type male-female signals. All trials involving females, exposed to male-female relevant signals yielded an acoustic response. Single pulse clicking occurred ubiquitously throughout all trials, during both the pre-trial and stimulus periods, with an average 330% increase in frequency of clicking. Eight of the 10 trials conducted showed a second acoustic signal type. A simple multipulse chirp, the same chirp type as was observed during signal characterization (see Chapter 2: Signal characterization). These simple chirps were not observed to occur during the pre-trial recording period. Although a larger 'n' would be required to conclusively establish the presence of an acoustic response, the results presented in this study suggest that these chirps occur in response to a male generated acoustic stimulus.

The experiments conducted in this chapter were very preliminary. However the results presented herein suggest the capacity of acoustic reception in *D. ponderosae*. Although the sample sizes were small, a presumed sonic response to a sonic stimulus was observed, in both sexes. This experiment will lay the groundwork for future playback studies, and provide a vital step in the discovery of an acoustic receptor organ in *D. ponderosae*.

Chapter 5. General Discussion

5.1 General Discussion

Despite the ubiquity of sound production in the order Coleoptera, hearing in beetles remains a subject in its infancy. Functional hearing organs have only been described in two families to date: Cincindellidae (Spangler, 1988; Yager and Spangler, 1995), and Scarabaeidae (Forrest et al., 1997). The ears found in these two families are ultrasound detectors, which have been postulated to function in bat avoidance. Despite their similar function they represent an example of convergent evolution, arising on two different body parts, in two taxonomically distinct groups of Coleoptera. This study focused on the potential of hearing in a third group, Scolytidae, or more specifically, *Dendroctonus ponderosae,* the Mountain Pine beetle, a highly acoustic, and, owing to its destructive nature, economically important pest species.

Previous research into the acoustic behaviour of *D. ponderosae* had focused on sound production, while no attention was given to sound reception. Evidence presented by Rudinsky (1973) suggested the capacity for sound reception by inducing pheromone release in females of the species through the use of an acoustic stimulus. This suggested that the female beetles were capable of detecting an acoustic stimulus, but the study did not explore the underlying mechanism of sound reception. To date, no anatomical explorations have been made addressing the potential of a hearing organ in this species. Through characterization of its acoustic repertoire, its anatomy and its acoustic response to a sonic stimulus, the objective of this thesis was to find and characterize a putative acoustic receptor structure.

Characterization of the sounds produced by *D. ponderosae* provided insight into the nature of a potential hearing organ. This study demonstrated that *D. ponderosae* generate complex broadband signals. While the *Dendroctonus* signals themselves were not novel, this study demonstrated for the first time the existence of ultrasound within the *Dendroctonus* repertoire of signals. This study also demonstrated the capacity for *D. ponderosae* to generate lower frequency vibrations which can be transmitted through the substrate.

If indeed these insects are receiving vibrational signals then the most likely organ for receiving these stimuli would be the subgenual organ. Traditionally the presence of a subgenual organ is exemplified by an enlargement of the tibiae of the animal. Examinations of the external anatomy of *D. ponderosae* however, revealed no sites consistent with the adaptations necessary for a vibration detector. Although the use of vibratory cues has been suggested in some coleopterans, to date there has been no evidence of subgenual organ present in this order. While the evidence presented does not explicitly rule out the possibility of vibrational communication in *D. ponderosae*, it does suggest a higher likelihood that it is the airborne acoustic communication.

Due to the intensity and spectral characteristics of the signals produced by *D. ponderosae* both near field (Johnston's organ) and far field (tympanal ears) air borne receptors were explored. Anatomical explorations revealed that a Johnston's organ was a possible candidate, however comparisons between the accepted frequency limitations of the Johnston's organ with the signal repertoire available to *D. ponderosae* suggested a disparity. Since the signals produced by *D. ponderosae* possess so much high frequency

energy it is unlikely that a Johnston's organ is the hearing mechanism employed by these insects, suggesting a more high frequency far-field receptor.

A tympanal ear requires the presence of three morphological characters, an area of differentiated cuticle, a resonating chamber, and the necessary innervation. Examinations of the external and internal anatomy of *D. ponderosae* revealed sites, which fulfilled these three anatomical criteria. However in order to demonstrate that a site is in fact capable of detecting airborne acoustic sound, further study is required in the form of electrophysiology and/or playback studies.

Playback studies are a good way of testing the presence of acoustic reception. By presenting the animal with a biologically relevant acoustic stimulus, observations can be made regarding any reaction from the test animal. For the playback experiments conducted in this study, both male and female *D. ponderosae* were presented with biologically relevant signals recorded during chapter 2. Observations made during the playback experiments showed the existence of an acoustic response to acoustic stimuli in both sexes. While the results presented herein were highly preliminary, they do offer valuable evidence suggesting the presence of an acoustic receptor organ. This study provides a foundation upon which further research into hearing in the genus *Dendroctonus* can be built.

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