# “Can you hear me?” Investigating the acoustic communication signals and receptor organs of bark beetles

by

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# Abstract

Many bark beetle (Coleoptera: Curculionidae: Scolytinae) species have been documented to produce acoustic signals, yet our knowledge of their acoustic ecology is limited. In this thesis, three aspects of bark beetle acoustic communication were examined: the distribution of sound production in the subfamily based on the most recent literature; the characteristics of signals and the possibility of context dependent signalling using a model species: *Ips pini*; and the acoustic reception of bark beetles through neurophysiological studies on *Dendroctonus valens*. It was found that currently there are 107 species known to stridulate using a wide diversity of mechanisms for stridulation. *Ips pini* was shown to exhibit variation in certain chirp characteristics, including the duration and amplitude modulation, between behavioural contexts. Neurophysiological recordings were conducted on several body regions, and vibratory responses were reported in the metathoracic leg and the antennae.

# Acknowledgements

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## 1.1 Introduction

### Acoustic communication in insects

Acoustic communication among insects has been documented since the writings of classic Greek philosophers (see Claridge, 2005). Many species in a wide range of insect taxa have been described to use acoustic signals to communicate. The orders Orthoptera, Hemiptera, Lepidoptera and Hymenoptera include the most well-known examples (Claridge, 2005). These insect signals have been studied extensively in the past century for their ecological functions, behavioural contexts, physical characteristics as well as their evolution. We have learned that insects rely on complex acoustic signals in various stages of their life cycle (Gogala, 2005), that they use acoustic cues for species recognition (Greenfield, 2016), communicate to show their quality during courtship (Byers et al., 2010), defend themselves against predators using sounds (Ruxton et al., 2004), and utilize acoustic signals in many other ways as well. Another important part of acoustic behaviour is acoustic reception and its neural basis, which has also been extensively studied in acoustic insects (Mason and Pollack, 2016). From these studies, one can realize that insects can detect vibrations through air, water or solid surfaces, and that they are capable of detecting the smallest changes in the temporal, spectral, and amplitude spectrum of acoustic signals (Mason and Pollack, 2016). Studies in the field of insect acoustics have shown us a vast, complex world of insect acoustic communication, which in some senses ranges far beyond what is known for any other animal groups.

Because of the enormous number of species, it is the nature of many fields in Biology that certain taxa receive less attention from scientists than others. In the field of insect acoustics, one of these groups is the order Coleoptera. Even though this insect group has the highest variety of sound producing organs, the number of publications on beetle acoustics is relatively low (Wessel, 2005). The earliest mention of sound production in beetles is from the 17$^{th}$ century (see Wessel, 2005). Beetles produce sounds through stridulation, which involves rubbing specialized body surfaces against each other. These surfaces are collectively referred to as the stridulatory organ of the insect (Wessel, 2005). Beetles are highly sclerotized insects, and since any of their sclerotized body parts can be modified into some sort of stridulatory apparatus, this could be one of the main reasons why coleopterans have the highest number of different sound producing organs. However, while morphological features of stridulatory organs have been well represented in the scientific literature, very little is known on the acoustic behaviour and acoustic reception in Coleoptera species (Wessel, 2005; Greenfield, 2016). Within acoustic coleopterans, this thesis focuses on an ecologically and economically important, but as for acoustic studies, rather neglected group: bark beetles.

## Introduction to bark beetles

The subfamily of bark and ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) consists of approximately 6000 species (Jordal and Cognato, 2012). These insects are ecologically important, as they promote carbon and nutrient recycling as part of the forest ecosystems worldwide (Vega and Hofstetter, 2014). Bark beetles generally spend most of their life cycle inside their host trees. They feed on the phloem, construct galleries under the tree bark, and also mate and lay their eggs inside the tree. The larvae

also spend the entire time of their development inside the galleries, which they expand as they feed, producing stereotypical patterns in the phloem. The adult beetles then leave their original host tree to find new hosts and mating opportunities (Vega and Hofstetter, 2014). Most bark beetle species attack dead or weakened trees, however some aggressive species are known to have periodic outbreaks, when they attack healthy trees in mass, making them the single most destructive forests pests (Vega and Hofstetter, 2014). These attacks have enormous environmental and economical impact, which is the reason why this insect group is extensively studied.

The majority of bark beetle studies focus on the chemical ecology and chemical communication of the most aggressive species, such as the infamous mountain pine beetle (*Dendroctonus ponderosae*) (Coulson, 1979; Raffa, 2001; Raffa et al., 2008). The results of this great amount of research led to many important discoveries regarding the different pheromone components and their role in chemical communication. This aided the development of new pest control technologies, such as pheromone lures that are currently being used for species specific capture of beetles for both pest control and research purposes (Lindgren, 1983). While these chemical signals are very important for long range communication, and essential for aggregation and mate finding, many bark beetle species are also known to use short range acoustic signals to communicate with conspecifics within short distances (Barr, 1969). To date however, this form of communication has not received as much attention as chemical communication, even though it has similar possible applications in species recognition (Lindeman, 2016) and pest management (Hofstetter et al., 2014). Furthermore, there is currently no knowledge on the acoustic reception of bark beetles.

The goals of this thesis are therefore to: 1: Review the literature in order to summarize our current knowledge on bark beetle acoustic communication and the distribution of sound production within the subfamily (section 1.2 of this chapter), 2: Characterize the acoustic signals of a model species and assess the possibility of context dependent signalling (Chapter 2), 3: Investigate possible acoustic receptor organs (Chapter 3).

## 1.2 Acoustic communication and sound producing organs in bark beetles

### Acoustic communication in Scolytinae

Even early descriptions of bark beetles have noted sound production in certain species (Barr, 1969), however focused researched on these sounds and sound producing organs only began in the second half of the 20$^{th}$ century. The first and only extensive review of bark beetle acoustic communication was written by Barbara Barr (Barr, 1969). This review included an extensive collection of references and a proper summary of the current knowledge on the number of sound producing species and the different types of sound producing organs. This work, combined with all the research that has been done since, illuminates a diverse and complex acoustic communication system, however, there are still many outstanding questions about the signals themselves, their functions, and the reception of acoustic signals by the beetles.

Sound production has been documented in several behavioural contexts such as courtship, same sex interactions inside the gallery, or while being handled (Barr, 1969). This has led many studies to conclude that there is context dependent acoustic signalling in a number of bark beetle species, and they often described signals such as “stress”,

"courtship" or "rivalry". Most of them however either lack proper analysis of signal characteristics (e.g. Ryker and Rudinsky, 1976; Oester et al., 1978; Ryker, 1988), or have issues regarding their sampling methods or basic definitions (Swaby and Rudinsky, 1976). More recent studies though report evidence suggesting context dependent signalling in some species (Fleming et al., 2013; Lindeman and Yack, 2015). The issue of context dependent signalling in bark beetles is further discussed in Chapter 2 of this thesis.

Currently there are 107 bark beetle species that are known or suspected to produce sounds (Table 1.1). While only a few of these species had their sounds recorded, they have all been described to possess sound producing organs. Similar to studies on chemical communication, most of the research on sound production was done on the most aggressive and most common genera, such as *Dendroctonus*, *Ips* and *Scolytus*. As such, behavioural information on acoustic communication is only available for a few specific species. The presence of sound producing organs on the other hand has been observed for many other species and so this is currently the best source of information on the taxonomic distribution of sound production in the subfamily Scolytinae.

## Sound producing organs of Scolytinae

Bark beetles, like other coleopterans, produce sounds through stridulation (Barr, 1969). The stridulatory organ consists of two parts, both of which comprise sclerotized structures on the cuticle: the pars stridens and the plectrum. The distinction between the two is not always evident. The pars stridens is often described as the more complex part, that has more sclerotized teeth as opposed to the plectrum, which often consists of a single ridge or a pair of processes, although that is not always the case. From the

literature, it appears that the general assumption is that the pars stridens has most of the vibrational properties necessary to produce the chirp's spectral and temporal characteristics, however, this has never been tested. The plectrum is defined as the excitatory part of the stridulatory organ (Barr, 1969). Almost all bark beetle species known or suspected to stridulate possess one of the following three types of stridulatory organs: Elytro-tergal, Gula-prosternal or Vertex-pronotal (Fig.1.1). These are named after the location of the pars stridens and the plectrum on the beetle's body (Barr, 1969).

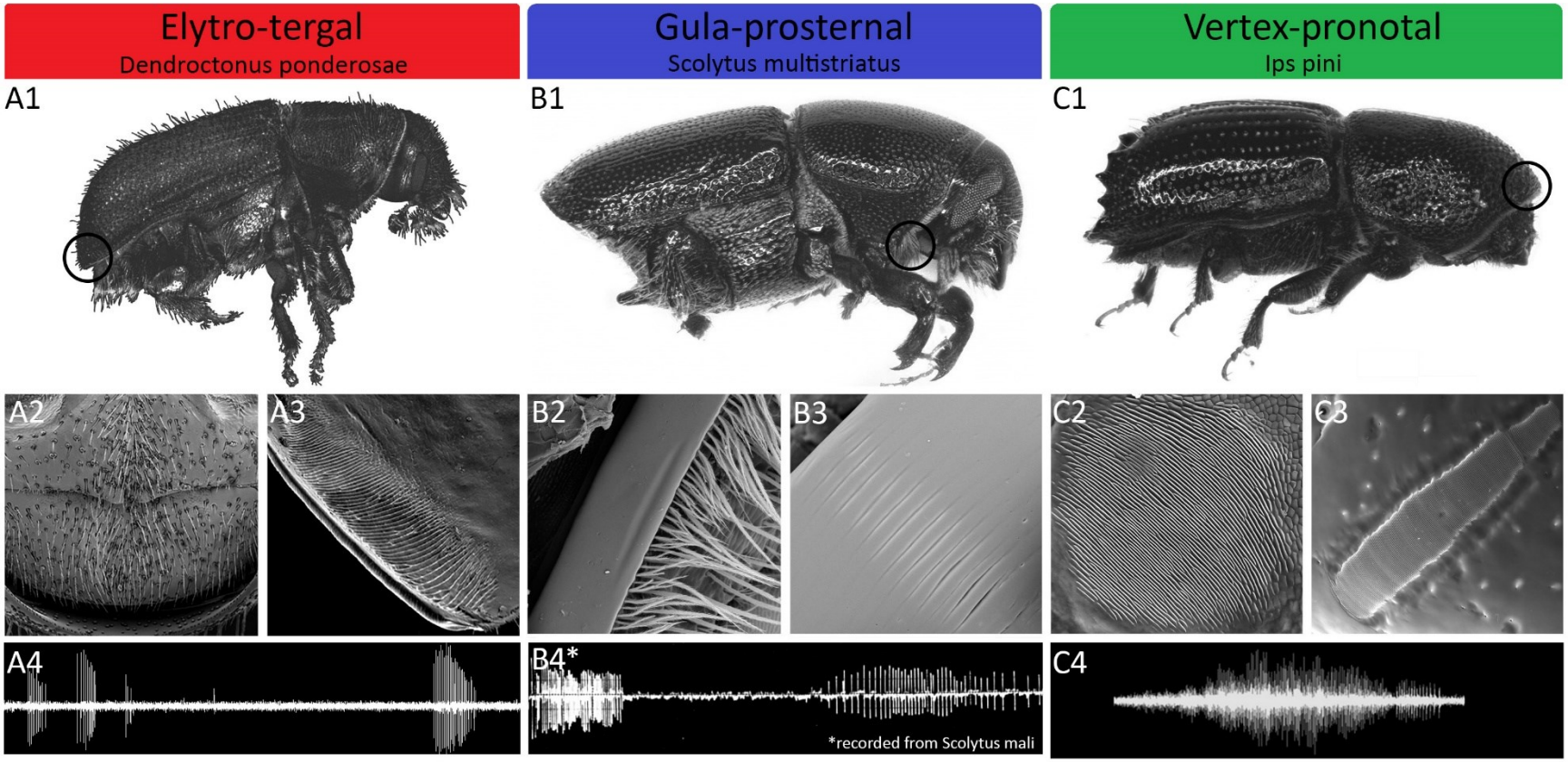


**Figure 2.1** The three most common types of stridulatory organs on three representative species: *Dendroctonus ponderosae* (A1), *Scolytus multistriatus* (B1) and *Ips pini* (C1). Each stridulatory organ consists of two parts: the plectrum (A2, B2, C2) and the pars stridens (A3, B3, C3). The approximate position of the organs is marked on each beetle with a circle. Also showing typical chirps produced by each organ type (A4, B4, C4). (Due to lack of recording from *S. multistriatus*, B4 is showing chirps of *S. mali*, which possess' the same type of stridulatory organ (Rudinsky et al., 1978)) (Credits: A1, A2, A3, A4: Fleming et al., 2013; B4: Rudinsky et al., 1978; C2, C3, C4: Sen Sivalinghem)

Elytro-tergal stridulation

The Elytro-tergal type stridulatory organ is the most common among sound producing bark beetles, as it has been described in all stridulating bark beetle genera except for *Gnathotrichus, Ips, Pseudoips and Scolytus* (Table 1.1). The pars stridens of this type is located on the underside of the elytra and consists of a file with a series of teeth. The plectrum for this organ is a modification of one of the abdominal tergites. A typical example is the stridulatory organ of *Dendroctonus* species (Fig.1.1A). In this genus, while both male and female beetles have been reported to stridulate, the male organ is always more prominent (Michael and Rudinsky, 1972; Rudinsky and Michael, 1973; Rudinsky et al., 1976). The mechanism of sound production has been described in detail in *Dendroctonus valens* (Lindeman, 2016). In these species, the plectrum (which is a pair of processes located on the 7$^{th}$ abdominal tergite) locks onto the file of the pars stridens on the underside of the left elytron at the start of the stridulating motion, and suffers deformation as the abdomen moves posteriorly, which builds up a store of elastic energy. When this energy is released, the plectrum moves along the pars stridens, exciting the teeth along the way (Lindeman, 2016). In these species, along with *Dendroctonus ponderosae*, males are known to include brief interruptions in the chirps that convey information to the receiver (Fleming et al., 2013; Lindeman and Yack, 2015). These interruptions are achieved by relocking the plectrum on the pars stridens during the stridulating motion of the abdomen (Lindeman, 2016).

Gula-prosternal stridulation

The Gula-prosternal organ is almost exclusive to *Scolytus* species, although it has also been found in some *Ips* and *Pseudoips* species as well (Table 1.1). Here the ridges of

the pars stridens are located on the ventral side of the head, while the plectrum consists of a single or double file on the anterior edge of the prosternum (Fig.1.1 B2). While the exact method of stridulation is yet to be described in these species, sound production in *Scolytus mali* (both male and female) has been associated with the upward and downward motion of the head, which results in "double chirps" (Rudinsky et al., 1978) (Fig.1 B3). Similar movement has been described in *Pseudoips concinnus*, along with a slower, anterior-posterior motion as well (Oester and Rudinsky, 1975).

Vertex-pronotal stridulation

Most *Ips* species (except for *Ips typographus* and *Ips typographus japonicus*), as well as *Cryphalus fluvus, Dryocoetes autographus, Gnathotrichus retusus* and *Orthotomicus tosaensis* possess Vertex-pronotal stridulatory organs (Table 1.1). The distinction of the plectrum and the pars stridens is not as obvious in this type as for the others, as both parts can have a high number of ridges (Barr, 1969). However, because the structure on the dorsal side of the head always has significantly more ridges than the other part on the underside of the pronotum, it is generally referred to as the pars stridens while the latter is referred to as the plectrum (Barr, 1969; Swaby and Rudinsky, 1976; Sasakawa and Yoshiyasu, 1983). The most studied species that possesses this type of organ is the pine engraver beetle, *Ips pini* (Barr, 1969; Swaby and Rudinsky, 1976) (Fig. 1.1C). The complete stridulating motion is unknown, but the nodding motion of the head of female *Ips* beetles (only females possess vertex-pronotal organs) is directly associated with sound production (Swaby and Rudinsky, 1976, see Chapter 2). Interestingly, these beetles do not produce double chirps, suggesting that only one part of the up-down motion results in sound production (Fig. 1.1C3). In Chapter 2 of this thesis, the results

show that female *Ips pini* produce chirps with different durations and amplitude envelope shapes in different behavioural contexts, which indicates that females have some level of control over the temporal and amplitude characteristics of the produced chirps (see Chapter 2).

Unknown types of sound producing organs and new discoveries

Up until the past few years, only the above three types of stridulatory organs (Elytro-tergal, Gula-prosternal, Vertex-pronotal) were reported in any sound producing bark beetle species. However, beside clear chirps, some studies have described short clicks (defined as brief sounds that consist of a low number of pulses (Broughton, 1963)), mainly in *Ips* species, from the sex that does not possess any distinct stridulatory apparatus (Oester and Rudinsky, 1975; Rudinsky, 1979). This suggests that the acoustic communication of bark beetles could be more complex than previously thought. This is also supported by recent studies that have described or suggested new types of stridulatory organs. Ivan Kerchev from the Russian Academy of Sciences has found that *Polygraphus proximus* males have a secondary stridulatory organ besides the already identified Elytro-tergal organ (Kerchev, 2015). The pars stridens of this organ is located on the outer rim of the elytra, while spines on the tibia of the hind leg act as the plectrum. Another study by the same researcher also described stridulatory movements of the hind leg during courtship, although no recordings were made (Kerchev, 2014).

The idea of multiple stridulatory organs of different types in one species has been suggested by other studies as well: in *Cryphalus fluvus* (Vertex-pronotal and Elytro-tergal in males (Sasakawa and Sasakawa, 1981)) and *Dryocoetes autographus* (Vertex-pronotal and Elytro-tergal in females (Sasakawa and Yoshiyasu, 1983)). *Orthotomicus angulatus*

males and females have different types of organs (Elytro-tergal for males, Gula-prosternal for females) (Sasakawa and Yoshiyasu, 1983). Interestingly, a 'mysterious' wing spine was found on male coffee berry borers *(Hypothenemus hampei)* (see Table 1.1), which is hypothesised to be the remnant of a stridulatory organ (Vega et al., 2015). These discoveries suggest that there are alternatives to the three commonly known stridulatory organs and that other types might be confirmed in the future.

## 1.3 Discussion

Bark beetle acoustic communication has been studied for more than a century. The first decades of this research were summarized almost 50 years ago, and that review is still a good reference for researchers wanting to gain a general idea about bark beetle acoustic ecology (Barr, 1969). This chapter aimed to build on this work by collecting what knowledge has been gathered over the past 48 years on the taxonomic distribution of stridulation as well as the currently known methods of sound production. The most important conclusion that comes from this short review is that the field of bark beetle acoustics has much room to grow. There are many species, including species within the genus *Ips*, *Dendroctonus*, *Scolytus* and other common acoustic genera that have never been examined for stridulatory organs, and most of the species that do possess organs have never been recorded or properly analyzed for different signal characteristics. More knowledge about the taxonomic distribution of sound production could aid future research looking into the phylogenetic relationships between the different sound producing bark beetle groups, and could potentially aid evolutionary studies on the origins of sound production in the subfamily. The newly found stridulatory organs, and the discovery that there can be multiple organs in the same species, and even on the same

beetle suggests that the sound producing system of these insects is much more complex than previously thought. Learning about the acoustic ecology of bark beetles has already shown potential for species recognition (Lindeman, 2016) and new pest management technologies (Hofstetter et al., 2014). The acoustic behaviour of bark beetles is therefore relevant from both a scientific, environmental and economical point of view, which is why further studies in this field are strongly encouraged.

**Table 1.1** List of species in the subfamily Scolytinae that are known or proposed to stridulate, or possess possible stridulatory organs. Citations in brackets are cited in Barr (1969) but not included in the reference list of this thesis. E-T: Elytro-tergal, V-Pn: Vertex-pronotal, G-Ps: Gula-prosternal

| Genus and species | References | Type of stridulatory organ |
|---|---|---|
| *Chaetoptelius vestitus* | Barr, 1969 (Wichmann, 1912; Russo, 1926) | E-T |
| *Cryphalus fluvus* | Sasakawa and Sasakawa, 1981 | ♂ V-Pn and E-T ♀ E-T |
| *Cryphalus laricis* | Sasakawa and Sasakawa, 1981 | ♂ E-T |
| *Cryphalus piceae* | Sasakawa and Sasakawa, 1981 | ♂E-T |
| *Dactylipalpus sp*. (no species given) | Barr, 1969 (Kleine, 1932) | E-T |
| *Dendroctonus adjunctus* | Barr, 1969 (Hopkins, 1919; S. L. Wood, 1963) | ♂ E-T |
| *Dendroctonus approximatus*[1] | Barr, 1969 (Hopkins, 1909; Lyon, 1958; S. L. Wood, 1963); Yturralde and Hofstetter, 2015 | ♂ E-T ♀ E-T |
| *Dendroctonus aztecus* | Barr, 1969 (S. L. Wood, 1963) | ♂ E-T |
| *Dendroctonus brevicomis*[2] | Barr, 1969 (Hopkins, 1909; Lyon, 1958; Tate and Bedard, 1967); Rudinsky and Michael, 1973 | ♂ E-T ♀ E-T |
| *Dendroctonus frontalis* | Barr, 1969 (Hopkins, 1909; Lyon, 1958; Osgood and Clark, 1963; S.L. Wood, 1963); Rudinsky and Michael, | ♂ E-T ♀ E-T |

[1] Previously assumed to be synonym with *D. parallelocollis* (Barr, 1969; Wood, 1982)
[2] Synonym with *D. barberi* (Wood, 1963)

| | | |
|---|---|---|
| | 1973 | |
| *Dendroctonus jeffreyi* | Barr, 1969 (Hopkins, 1909; Wichmann, 1912; S.L. Wood, 1963) | ♂ E-T |
| *Dendroctonus micans* | Barr, 1969 (Hopkins, 1909; Lyon, 1958; S.L. Wood, 1963) | ♂ E-T |
| *Dendroctonus murrayanae* | Barr, 1969 (Hopkins, 1909; Lyon, 1958) | ♂ E-T |
| *Dendroctonus obesus*[3] | Barr, 1969 (Hopkins, 1909; Lyon, 1958) | ♂ E-T |
| *Dendroctonus ponderosae*[4] | Barr, 1969 (Hopkins, 1909; Lyon, 1958; McCambridge, 1962; S.L. Wood, 1963; Cole and Weenig, 1967); Rudinsky and Michael, 1973 | ♂ E-T ♀ E-T |
| *Dendroctonus pseudotsugae* | Barr, 1969 (Hopkins, 1909; Chapman, 1955; Lyon, 1958; S.L. Wood, 1963; Jantz and Johnsey, 1964; Allen et al., 1958; McMullen and Atkins, 1962; Jantz and Johsey, 1964; Rudinsky, 1968); Rudinsky and Michael, 1973 | ♂ E-T ♀ E-T |
| *Dendroctonus punctatus* | Barr, 1969 (Hopkins, 1909; S.L. Wood, 1963) | ♂ E-T |
| *Dendroctonus rufipennis* | Rudinsky and Michael, 1973 | ♂ E-T ♀ E-T |
| *Dendroctonus simplex* | Barr, 1969 (Hopkins, 1909; S.L. Wood, 1963) | ♂ E-T |
| *Dendroctonus terebrans* | Barr, 1969 (Hopkins, 1909; S.L. Wood, 1963) | ♂ E-T |
| *Dendroctonus valens* | Barr, 1969 (Hopkins, 1909; Lyon, 1958; S.L. Wood, 1963); Rudinsky and Michael, 1973 | ♂ E-T ♀ E-T |
| *Dendrosinus bourreriae* | Barr, 1969 (Schwarz, 1920); Kirkendall et al., 2014 | ♂ E-T?[5] |
| *Dryocoetes autographus* | Sasakawa and Yoshiyasu, 1983 | ♂ V-Pn<br>♀ V-Pn and E-T |
| *Gnathotrichus materiarius* | Barr, 1969 (Schedl, 1931) | ?[6] |

[3] Synonym with *D. engelmanni* (Wood, 1963)
[4] Synonym with *D. monticolae* (Wood, 1963)
[5] Produces sounds, no available description of organs (Kirkendall et al., 2014)
[6] Stridulatory organ not described

| | | |
|---|---|---|
| *Gnathotrichus retusus* | Barr, 1969 | ♀ V-Pn |
| *Gnathotrichus sulcatus* | Barr, 1969 (Schedl, 1931) | ?[7] |
| *Hylastes angustatus* | Barr, 1969 (Marcu, 1931) | E-T |
| *Hylastes ater* | Barr, 1969 (Knoche, 1904; Wichmann, 1912, 1927; Munro, 1917; Marcu, 1931) | ♂ E-T |
| *Hylastes attenuatus* | Barr, 1969 (Marcu, 1931) | E-T |
| *Hylastes brunneus* | Barr, 1969 (Marcu, 1931) | E-T |
| *Hylastes cunicularius* | Barr, 1969 (Wichmann, 1912, 1927; Munro, 1917; Marcu, 1931) | ♂ E-T |
| *Hylastes macer* | Barr, 1969 | ♂ E-T |
| *Hylastes opacus* | Barr, 1969 (Marcu, 1931) | E-T |
| *Hylastes parallelus* | Sasakawa and Yoshiyasu, 1983 | ♂ E-T ♀ ?[8] |
| *Hylastes plumbeus* | Sasakawa and Yoshiyasu, 1983 | ♂ E-T ♀ E-T |
| *Hylastinus fankhauseri* | Barr, 1969 (Marcu, 1931) | E-T |
| *Hylesinus californicus*[9] | Barr, 1969; Vernoff and Rudinsky, 1980 | ♂ E-T ♀ E-T |
| *Hylesinus crenatus* | Barr, 1969 (Wichmann, 1912; Kleine, 1921; Marcu, 1930b) | E-T |
| *Hylesinus fraxini*[10] | Barr, 1969 (Knoche, 1904; Wichmann, 1912; Kleine, 1921; Marcu, 1930b); Rudinsky and Vallo, 1979 | ♂ E-T ♀ E-T?[11] |
| *Hylesinus oleiperda* | Barr, 1969 (Kleine, 1921; Marcu, 1930b); Rudinsky and Vallo, 1979 | ♂ E-T |
| *Hylesinus oregonus*[12] | Vernoff and Rudinsky, 1980 | ♂ E-T ♀ E-T |
| *Hylesinus orni*[13] | Barr, 1969 (Marcu, 1930b) | E-T |
| *Hylurgopinus rufipes* | Barr, 1969 (Kaston, 1936); Lyons, 1982 | ♂ E-T ♀ E-T |

[7] Stridulatory organ not described
[8] Never examined
[9] Synonym with *Leperisinus californicus* (Lyal and King, 1996)
[10] Synonym with *Leperisinus fraxini* (Lyal and King, 1996)
[11] Plectrum missing (Rudinsky and Vallo, 1979)
[12] Synonym with *Leperisinus oregonus* (Lyal and King, 1996)
[13] Synonym with *Leperisinus orni* (Lyal and King, 1996)

| | | |
|---|---|---|
| *Hylurgops glabratus*[14] | Barr, 1969 (Wichmann, 1912; Marcu, 1931) | E-T |
| *Hylurgops interstitialis* | Sasakawa and Yoshiyasu, 1983 | ♂ E-T ♀ E-T |
| *Hylurgops palliatus*[15] | Barr, 1969 (Knoche, 1904; Munro, 1917; Wichmann, 1912; Marcu, 1931) | E-T |
| *Hylurgops rugipennis* | Barr, 1969; Oester et al., 1978 | ♂ E-T ♀ E-T?[16] |
| *Hylurgus ligniperda* | Barr, 1969 (Knoche, 1904; Wichmann, 1912; Marcu, 1931); Sasakawa and Yoshiyasu, 1983; Menier and Carle, 1976 | ♂ E-T ♀ E-T |
| *Hypothenemus hampei* | Vega et al., 2015 | ♂ wing spike[17] |
| *Ips avulsus* | Barr, 1969 | ♀ V-Pn |
| *Ips bonanseai* | Barr, 1969 | ♀ V-Pn |
| *Ips calligraphus* | Barr, 1969 | ♀ V-Pn |
| *Ips confusus* | Barr, 1969 | ♀ V-Pn |
| *Ips cribricollis* | Barr, 1969 | ♀ V-Pn |
| *Ips grandicollis* | Barr, 1969 | ♀ V-Pn |
| *Ips latidens* | Barr, 1969 | ♀ V-Pn |
| *Ips lecontei* | Barr, 1969 | ♀ V-Pn |
| *Ips montanus* | Barr, 1969 | ♀ V-Pn |
| *Ips pini*[18] | Barr, 1969; Oester and Rudinsky, 1975 | ♂ ?[19] ♀ V-Pn |
| *Ips plastographus* | Barr, 1969 | ♀ V-Pn |
| *Ips ponderosae* | Barr, 1969 | ♀ V-Pn |
| *Ips sabinianae* | Barr, 1969 | ♀ V-Pn |
| *Ips sexdentatus* | Barr, 1969 (Wichmann, 1912, 1927; Krausse, 1917; Nunberg, 1950; Michalski, 1961) | ♀ V-Pn |
| *Ips tridens tridens* | Oester and Rudinsky, 1975 | ?[20] |

[14] Synonym with *Hylurgops decumanus* (Pfeffer, 1936)
[15] Synonym with *Hylastes palliatus* (Munro, 1917)
[16] Plectrum missing, did not stridulate (Oester et al., 1978)
[17] Possible new type of stridulatory organ (Vega et al., 2015)
[18] Synonym with *Ips oregoni* (Lanier et al., 1980)
[19] Produces clicks, organ unknown (Oester and Rudinsky, 1975)

| | | |
|---|---|---|
| *Ips typographus* | Rudinsky, 1979 | ♂?[21] ♀ G-Ps |
| *Ips typographus japonicus* | Sasakawa and Yoshiyasu, 1983 | ♀ G-Ps |
| *Ips woodi* | Barr, 1969 | ♀ V-Pn |
| *Kissophagus Chapuis sp.* | Barr, 1969 (Marcu, 1931) | E-T |
| *Orthotomicus angulatus* | Sasakawa and Yoshiyasu, 1983 | ♂ E-T ♀ G-Ps |
| *Orthotomicus tosaensis* | Sasakawa and Yoshiyasu, 1983 | ♂ V-Pn |
| *Phloeoborus Erichson sp.*[22] (no species given) | Barr, 1969 (Kleine, 1932) | E-T |
| *Phloeophthorus rhododactylus* | Barr, 1969 (Wichmann, 1912) | E-T |
| *Phloeosinus aubei*[23] | Barr, 1969 (Zocchi, 1956) | E-T |
| *Phloeosinus punctatus* | Barr, 1969 | ♂ E-T |
| *Phloeotribus caucasicus* | Barr, 1969 (Marcu, 1931) | E-T |
| *Phloeotribus scarabaeoides* | Barr, 1969 (Russo, 1938) | ♂ E-T |
| *Phloeotribus spinulosus*[24] | Barr, 1969 (Wichmann, 1912) | E-T |
| *Polygraphus grandiclava*[25] | Barr, 1969 (Wichmann, 1912) | E-T |
| *Polygraphus jezoensis* | Kerchev, 2015 | ♂ E-T |
| *Polygraphus major* | Lyal and King, 1996 | ♂ E-T |
| *Polygraphus poligraphus* | Barr, 1969 (Wichmann, 1912) | E-T |
| *Polygraphus proximus* | Sasakawa and Yoshiyasu, 1983; Kerchev, 2015 | ♂ E-T and Elytro-tibial[26] ♀ E-T |
| *Polygraphus rufipennis* | Rudinsky et al., 1978 | ♂ E-T ♀ E-T |
| *Polygraphus subopacus* | Barr, 1969 (Wichmann, 1912) | E-T |
| *Pseudips concinnus*[27] | Barr, 1969; Oester and Rudinsky, 1975 | ♂ G-Ps ♀ G-Ps |

[20] Produces clicks, organ unknown (Oester and Rudinsky, 1975)
[21] Produces clicks, organ unknown (Rudinsky, 1979)
[22] Synonym with *Phloeotrupes sp*. (Lyal and King, 1996)
[23] Synonym with *Phloeosinus bicolor* (Mendel, 1983)
[24] Synonym with *Phthorophloeus spinosus* (Mendel, 1983)
[25] Synonym with *Pseudopolygraphus grandiclava* (Swaine, 1918) and *Pseudopolygraphus cembrae* (Schedl, 1934 cited in Avtzis et al., 2008)
[26] Newly discovered type of stridulatory organ (Kerchev, 2015)
[27] Synonym with *Ips concinnus* (Cognato, 2000)

| | | |
|---|---|---|
| *Pseudips mexicanus*[28] | Barr, 1969 | ♂ G-Ps ♀ G-Ps |
| *Pseudohylesinus nebulosus* | Oester et al., 1981 | ♂ E-T |
| *Pteleobius Kraatzi* | Barr, 1969 (Kleine, 1921; Marcu, 1930b) | E-T |
| *Pteleobius vittatus* | Barr, 1969 (Kleine, 1921; Marcu, 1930b) | E-T |
| *Scolytus abietis* | Equihua-Martinez and Furniss, 2009 | ♂ G-Ps ♀ G-Ps |
| *Scolytus carpini* | Barr, 1969 (Scholz, 1905) | G-Ps |
| *Scolytus claviger*[29] | Barr, 1969 (Wichmann, 1915) | G-Ps |
| *Scolytus intricatus* | Barr, 1969 (Chapman, 1869; Gahan, 1900; Scholz, 1905; Kéler, 1922) | G-Ps |
| *Scolytus laevis* | Barr, 1969 (Scholz, 1905) | G-Ps |
| *Scolytus mali*[30] | Barr, 1969 (Chapman 1869; Gahan, 1900; Scholz, 1905); Rudinsky et al., 1978 | ♂ G-Ps ♀ G-Ps |
| *Scolytus multistriatus* | Barr, 1969 (Gahan, 1900; Scholz, 1905); Jefferies and Fairhurst, 1982 | ♂ G-Ps ♀ G-Ps |
| *Scolytus opacus* | Equihua-Martinez and Furniss, 2009 | ♂ G-Ps ♀ G-Ps |
| *Scolytus pygmaeus* | Barr, 1969 (Scholz, 1905) | G-Ps |
| *Scolytus ratzeburgi* | Barr, 1969 (Gahan, 1900; Scholz, 1905) | G-Ps |
| *Scolytus scolytus*[31] | Barr, 1969 (Chapman, 1869; Gahan, 1900; Scholz, 1905); Jefferies and Fairhurst, 1982 | ♂ G-Ps ♀ G-Ps |
| *Scolytus ventralis* | Macias-Samano et al., 1998 | ♂ ?[32] |
| *Tomicus brevipilosus* | Sasakawa and Yoshiyasu, 1983 | ♂ E-T ♀ E-T |
| *Tomicus minor*[33] | Barr, 1969 (Lindemann, 1875; Knoche, 1904; Wichmann, 1912; Kleine, 1920; Wolff, 1920; Marcu, 1931) | ♂ E-T |

[28] Synonym with *Ips mexicanus* (Cognato, 2000)
[29] Synonym with *Eccoptogaster platystylus* (Blackman, 1934)
[30] Synonym with *S. pruni* (Barr, 1969)
[31] Synonym with *S. destructor* and *S. geoffroyi* (Jefferies and Fairhurst, 1982)
[32] Stridulatory organ not described
[33] Synonym with *Blastophagus minor, Hylesinus minor, Hylurgus minor, Myelophilus minor* (Barr 1969; Lanne et al., 1987)

| | | |
|---|---|---|
| *Tomicus piniperda*[34] | Barr, 1969; Sasakawa and Yoshiyasu, 1983; | ♂ E-T ♀ E-T |
| *Xylechinus pilosus* | Barr, 1969 (Wichmann, 1912) | E-T |

[34] Synonym with *Blastophagus piniperda, Hylesinus piniperda, Hylurgus piniperda, Myelophilus piniperda* (Barr 1969; Lanne et al., 1987)

# Chapter 2. Acoustic communication in the pine engraver bark beetle: Do signals vary between behavioural contexts?

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Contribution statement

Andras Dobai wrote the manuscript, collected, interpreted and analyzed data. Sen Sivalinghem contributed the sound files, Dr. Raul Guedes contributed to statistical analyses and figures, and Dr. Jayne Yack contributed to the overall experimental design and logistics. All authors contributed to manuscript preparation.

## 2.1 Abstract

Acoustic communication is taxonomically widespread in bark beetles and proposed to play an important role in a variety of social and defensive behavioural contexts. Yet our understanding of how signals vary between contexts is currently limited. This study tests the hypothesis that acoustic signals vary between behavioural contexts in the female pine engraver beetle (*Ips pini* (Say) (Coleoptera: Curculionidae: Scolytinae)). Female *Ips pini* produce acoustic chirps using a vertex-pronotal stridulatory organ. Randomly sampled chirps generated under three contexts- distress, predation and premating- are compared for their duration, number of pulses, interpulse intervals, pulse

rate and amplitude envelope shapes. Results show that during premating events, chirps are significantly longer in duration, and tend to have a higher proportion of descending amplitude envelopes than chirps occurring during distress and predation events. Chirps produced during distress and predation conditions are indistinguishable from one another. In contrast to results from previous bark beetle studies, no support was found for categorizing chirps as 'interrupted' or 'uninterrupted' types based on temporal patterns. The functional significance of context dependent variation in chirp characteristics is discussed. Previous studies on acoustic communication in bark beetles are limited due to a general lack of objective sampling and measurement criteria for characterizing signals. Recommendations are outlined for future studies on the functions and evolution of acoustic communication in bark beetles.

## 2.2 Introduction

Acoustic communication by airborne sounds and solid-borne vibrations is widespread amongst insects (Dumortier, 1963; Greenfield, 2002; Cocroft and Rodríguez, 2005). Signals are associated with most aspects of insects' life history, including, but not limited to, attracting and choosing mates, locating food, and defense against conspecifics or predators (Alexander, 1967; Ewing, 1989; Greenfield, 2002; Yack, 2016). There is extensive research on how signal characteristics vary between and within species, and the factors leading to this variation (e.g. Alexander, 1961; Gerhardt and Huber, 2002; Greenfield, 2002; Sueur, 2005). Between species, signal variation has been studied mostly on calling songs in relation to species recognition and female choice (e.g. Gerhardt and Huber, 2002; Greenfield, 2002, 2016; Boulard, 2005; Heller, 2005; Henry, 2005; Hoikkala, 2005; Sueur, 2005; Stewart and Sandberg, 2005). Within species, studies on signal variation have focused on the calling and courtship songs that communicate information about the condition of signallers (Greenfield, 2002; Tregenza et al., 2006). Within species acoustic signals can also vary between behavioural contexts.

Many insects are reported to signal in a variety of behavioural contexts including various stages of mating, aggressive or other social interactions (Alexander, 1961; Gerhardt and Huber, 2002; Stölting et al., 2004; Guerra and Mason, 2005; Conrad et al., 2010; Balakrishnan, 2016). Empirical studies characterizing variation between signals produced in different contexts are lacking for most insects (but see examples for cicadas (Sueur and Aubin, 2004), crickets, (Zuk et al., 2008), and *Drosophila* (Hoikkala, 2005; Ritchie et al., 1998)). Understanding the signalling repertoires of a species provides important information about the communicative functions of different signal traits, and

allows us to develop hypotheses on the function and evolution of communication signals. In this study, an objective and quantitative approach is taken to sample and characterize context dependent signals of bark beetles (Scolytinae).

Bark beetles (Coleoptera: Curculionidae: Scolytinae) are ecologically and economically important insects that play a key role in forest ecosystems, promoting carbon and nutrient recycling (Vega and Hofstetter, 2014). Some aggressive species however can be devastating parasites, causing billions of dollars in damage to the global forest industry (Vega and Hofstetter, 2014). Due to their importance, extensive research has focused on their sensory ecology and life history traits (Vega and Hofstetter, 2014). Most of this research has been on chemical communication, which has led to improvements in our understanding of their biology, as well as pest control methods (Coulson, 1979; Raffa, 2001; Raffa et al., 2008). Another key form of communication for these insects is acoustic communication, although this sensory modality has received comparatively little attention.

Acoustic signalling in bark beetles is taxonomically widespread and proposed to play important roles in many aspects of life history (Barr, 1969; Lyal and King, 1996). Many species are reported to signal in more than one behavioural context, but whether chirps vary between contexts is poorly understood due to lack of quantitative analysis of signal characteristics for most species (but see Fleming et al., 2013; Lindeman and Yack, 2015; Yturralde and Hofstetter, 2015). Several studies refer to different signal types, such as “attraction”, “premating”, “rivalry” or “distress” chirps, but whether chirp characteristics differ between contexts requires verification (e.g. Barr, 1969; Ryker and Rudinsky, 1976; Oester et al., 1978; Ryker, 1988). Other studies refer to two types of

chirps: simple and interrupted in different contexts, yet again the distinction between these two lacks quantification (Michael and Rudinsky, 1972; Ryker and Rudinsky, 1976). Understanding how signals vary between contexts can be further complicated when signals are recorded under artificial conditions that may not represent natural conditions (e.g. Wilkinson et al., 1967; Swaby and Rudinsky, 1976; Yturralde and Hofstetter, 2015). This study takes an empirical approach to sampling, analyzing and comparing characteristics of chirps in different behavioural contexts using the pine engraver beetle, *Ips pini* (Say) as a model.

*Ips pini* occurs across North America, breeding beneath the bark of various pine and spruce trees (Thomas, 1961; Barr, 1969). Colonization is initiated by males by attacking damaged or weakened trees, excavating the initial nuptial chamber, and releasing pheromones to attract both females and other males to the tree (Pureswaran et al., 2000). As described for most *Ips* species, *I. pini* have a polygynous mating system (Thomas, 1961; Schmitz, 1972; Kirkendall, 1983; Reid and Roitberg, 1994), whereby harems are formed by males by attracting 2-3 females to the entrance of the nuptial chamber (Swaby and Rudinsky, 1976). As females arrive, entry to the chamber is usually blocked by the male. Copulation occurs following successful entry, after which egg galleries are excavated by the females. The galleries remain occupied by both the male and the females until the end of the egg laying process (Reid and Roitberg, 1994; Robertson, 1998). The chemical sensory ecology of *Ips* spp. has been studied in detail (Teale et al., 1991; Seybold et al., 1992; Miller et al., 1996; Robins and Reid, 1997; Robertson and Roitberg, 1998; Pureswaran et al., 2000; Sallé and Raffa, 2007). While

acoustic communication has been reported for several species, comparatively less is known about this sensory modality.

Sound production has been reported for both male and female *I. pini* (Oester and Rudinsky, 1975; Swaby and Rudinsky, 1976); however, only females have been described to possess stridulatory organs (Barr, 1969). *Ips pini* possesses a vertex-pronotal type of stridulatory organ comprising the pars stridens and the plectrum, both consisting of a series of ridges or 'teeth'. The pars stridens is located on the dorsal surface of the head, and the plectrum near the anterior end of the undersurface of the pronotum (Barr, 1969; Fig. 2.1). The pars stridens is considered to be the more complex of the two structures, and the primary resonating structure involved in sound production (Barr, 1969; Swaby and Rudinsky, 1976). The plectrum is simpler with fewer and less organized ridges (Swaby and Rudinsky, 1976). By rubbing the pars stridens against the plectrum through a nodding motion of the head, chirps are produced (Barr, 1969; Swaby and Rudinsky, 1976).

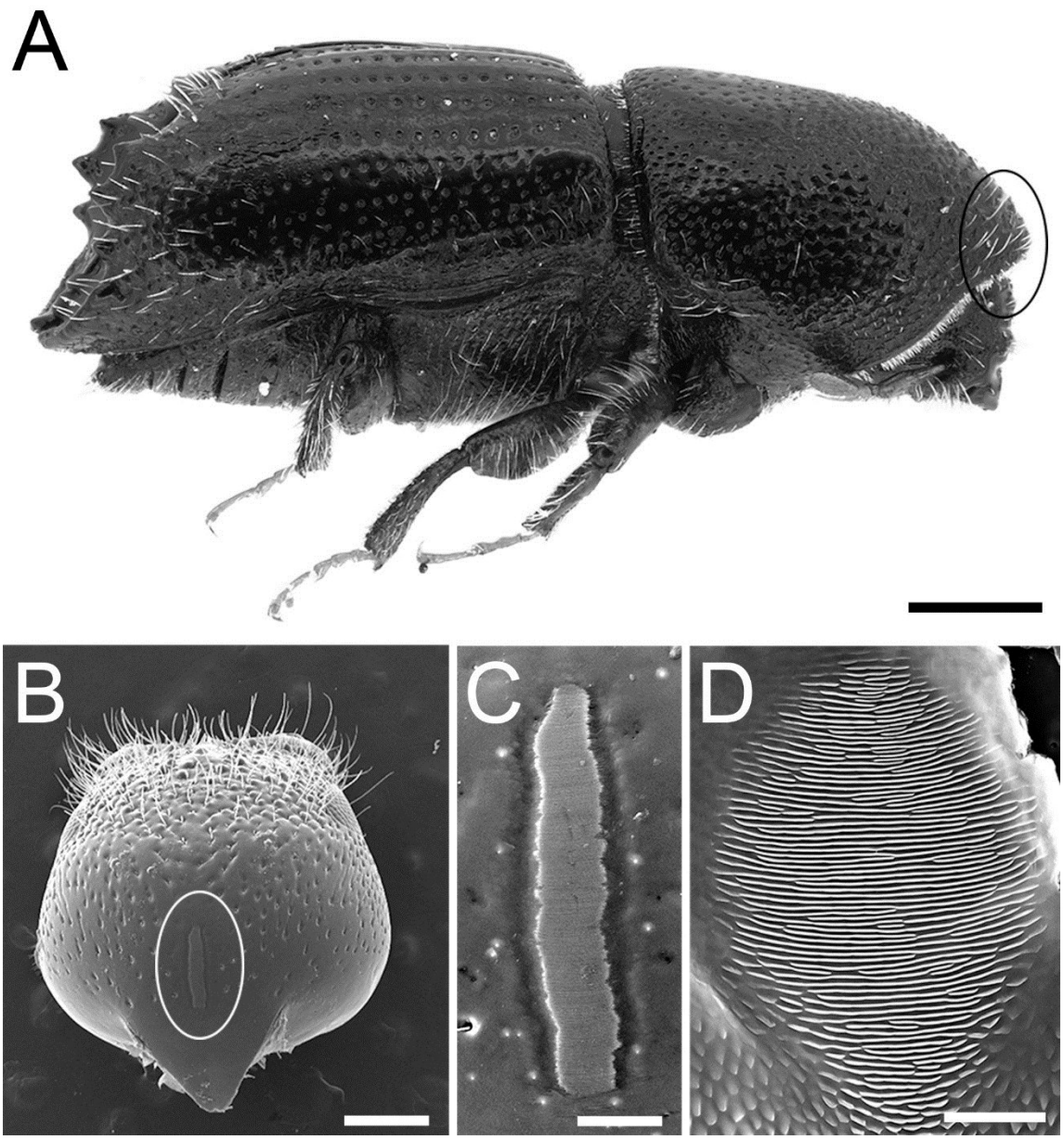


**Figure 2.1** Vertex-pronotal stridulatory organ of female *Ips pini*. A: Lateral view of the beetle showing the general region of the stridulatory organ (circled region). B: Dorsal view of the head with the rest of the body removed and the pars stridens highlighted.C: Higher magnification of the pars stridens on the vertex. D: Plectrum shown on the ventral side of the anterior pronotum. Scale bars: A: 500 µm, B: 250 µm, C, D: 50 µm

*Ips pini* females are reported to signal acoustically in different behavioural contexts: during distress (handling), attraction (female introduced to male) and 'rivalry'

(multiple females in egg gallery) (Swaby and Rudinsky, 1976). Swaby and Rudinsky (1976) report that certain temporal signal characteristics are context dependent. However, lack of objective criteria for quantifying signal characteristics, including how chirps are defined, what criteria are used to define 'interruptions', and how chirps were sampled for analysis, render the results inconclusive and precludes validation or comparison with other studies. It is also important to note that *I. pini* 'stress' chirps, like for many other bark beetle studies, were elicited under artificial conditions, and therefore characteristics may not reflect those of chirps produced under natural stressful situations, such as being attacked by a predator. Assessment of context dependent signal variation requires objective quantification of signal traits to replace the more arbitrary analysis methods used in previous studies.

The purpose of this study is to test the hypothesis that acoustic signal characteristics in *Ips pini* are context dependent. Objective methods are used to sample chirps between three contexts (distress, predation, and premating) and compare their temporal characteristics (duration, number of pulses, and average interpulse interval (IPI)). The traditional handling method is used to simulate artificial stress in distress trials, as well as predation by natural predator to assess differences between sound characteristics in artificial and natural conditions. In addition to these temporal characteristics that are commonly used to characterize acoustic signals in insects, this study also, to the best of our knowledge, is the first to quantitatively analyze the amplitude envelope shape of chirps using curve fitting of non-linear regressions. The paper also includes comparison of the findings with previous studies, and recommendations for future research on bark beetle acoustic analysis.

## 2.3 Materials and methods

### Animals

*Ips pini* were collected at Herbert's Corner (Carleton University Forest, Ottawa, ON, Canada), and the Central Experimental Farm Arboretum (Ministry of Agriculture and Agri-Food Canada (AAFC), Ottawa, ON, Canada) between April and September during 2011 and 2014. Adult beetles were collected using Lindgren traps (Contech Enterprises Inc., Victoria, B.C., Canada) baited with ipsdienol and lanierone. Traps were hung on red pine (*Pinus resinosa*), white pine (*P. strobes*), and jack pine (*P. banksiana*) trees. Males were distinguished from females by the presence of an enlarged 3rd declivity spine (Wood, 1982). Separated males and females were stored in plastic containers with moist paper towels and phloem shavings and kept at 5-8˚C for a maximum of two weeks until used in one of the procedures described below. Voucher specimens were preserved in 90% ethanol and stored at Carleton University.

Checkered beetles, *Thanasimus dubius* (Coleoptera: Cleridae: *Thanasimus*), common predators of *I. pini* (Aukema and Raffa, 2004), were also collected from the above-mentioned traps. Individuals were placed in separate plastic containers covered with moist paper towels and kept at 5-8˚C as above until later use during predation trials.

### Morphology

To image the sound producing structures in females, beetles were prepared for scanning electron microscopy by separating the pronotum and head from the rest of the body using an insect pin. Specimens were mounted on aluminum stubs and double-coated with gold-palladium (Hummer VII SEM Sputtering System, Anatech Ltd., Alexandria,

VA) prior to imaging using a variable pressure scanning electron microscope (Tescan Vega II XMU, Czech Republic).

## Signalling contexts

Sounds were recorded under three different contexts- distress, predation, and premating- using an Avisoft customized condenser microphone (model CMPA-P48/CM16, Berlin, Germany) and stored as .wav files to a data recorder (model FR-2, Fostex, Boonton, NJ; sampling rate: 192 kHz). All trials were conducted in a sound-attenuated chamber (model C-14A MR, Eckel Industries of Canada, Morrisburg, Ontario, Canada) at temperatures of 20-22ºC.

### Distress

To assess any differences between signals produced during natural predation (see below) and artificial stress conditions, females were stimulated to signal following a procedure used to elicit distress signals in other *Ips* spp. (Swaby and Rudinsky, 1976) and bark beetle studies (McCambridge, 1962). This condition is referred to in the current study as distress. Each female was held by the abdomen, and sounds recorded 1 cm from an Avisoft condenser microphone (Fig. 2.2A). Distress signals were recorded for 26 females.

### Predation

Predation trials were used to represent natural stress conditions. These trials were conducted by placing an individual *I. pini* female in a glass petri dish with a checkered beetle which had been food deprived for 48 hours (Fig. 2.2B). Acoustic signals produced during attacks were recorded with an Avisoft condenser microphone placed

approximately 2cm above the beetle, and signals were stored as .wav files to a Fostex data recorder. All predation trials were conducted in a sound attenuated chamber and videotaped (Sony Handycam HDRHC5/HC7, California, U.S.A.). Predation trials were conducted on 19 females.

Premating

Premating trials were conducted on red pine log bolts (40 to 50 cm in length) sealed on each end with paraffin wax to prevent dehydration. Prior to conducting premating trials, each log was inoculated with 4-15 males. Individual males were placed in drilled holes (diameter ~0.5 cm), spaced 8-10 cm apart. A 1.5 mL micro-centrifuge tube (with the conical base cut off) was placed over the hole and sealed with reusable adhesive putty (Staples® Canada Inc.) to prevent escape. All males were given 48h to build nuptial chambers prior to introducing females. Inoculated logs were kept at room temperature (22 to 24˚C) in an insect rearing facility.

On the day of recording, an inoculated log was transported to a sound attenuated chamber, and a female was introduced near the entrance hole. An Avisoft microphone was placed 2 cm from the hole (Fig. 2.2C), and all recorded signals were stored using a Fostex data recorder. These trials were recorded for up to 6.5 minutes of the encounter, after which time signalling subsided. Premating trials were conducted on 19 females, all paired with different males.


**Figure 2.2** Experimental setup for the three behavioural contexts. A: Distress: *Ips pini* females were held between the fingers with the head facing the microphone. B: Predation: Beetles were paired with natural predator (*T. dubius*) in a petri dish. C: Premating: Females were placed near the entrance of a nuptial chamber with a male inside. Scale bars: A,B,C: 2 mm

## Sound analyses

Up to 10 chirps per female were analyzed for each behavioural context. As there is currently no generally accepted quantitative definition of a chirp for bark beetles, it was defined as "the shortest sound which appears unitary to the human observer's unaided ear" (sensu Broughton, 1963). The beginning and end points of chirps were defined using Raven Bioacoustics Research Program (Pro 1.4 Beta version, Cornell Laboratory of Ornithology, Ithaca, NY, USA) as the first and last pulse distinguishable from the background. Chirps were sampled by generating random times in excel and selecting the chirps within the sound file closest to those times.

Each chirp was analyzed for specific temporal and amplitude characteristics. Spectral characteristics were not analyzed in this study due to the different sound environments in which beetles were recorded. Temporal characteristics measured included the following: chirp duration, number of pulses, and interpulse interval (IPI) (see Fig. 2.3). Pulse rate was calculated using the chirp duration and the number of

pulses. These characteristics were measured with Avisoft Bioacoustics Sound Analysis and Synthesis Laboratory program (Avisoft-SAS Lab Pro, version 4.53, Berlin, Germany).

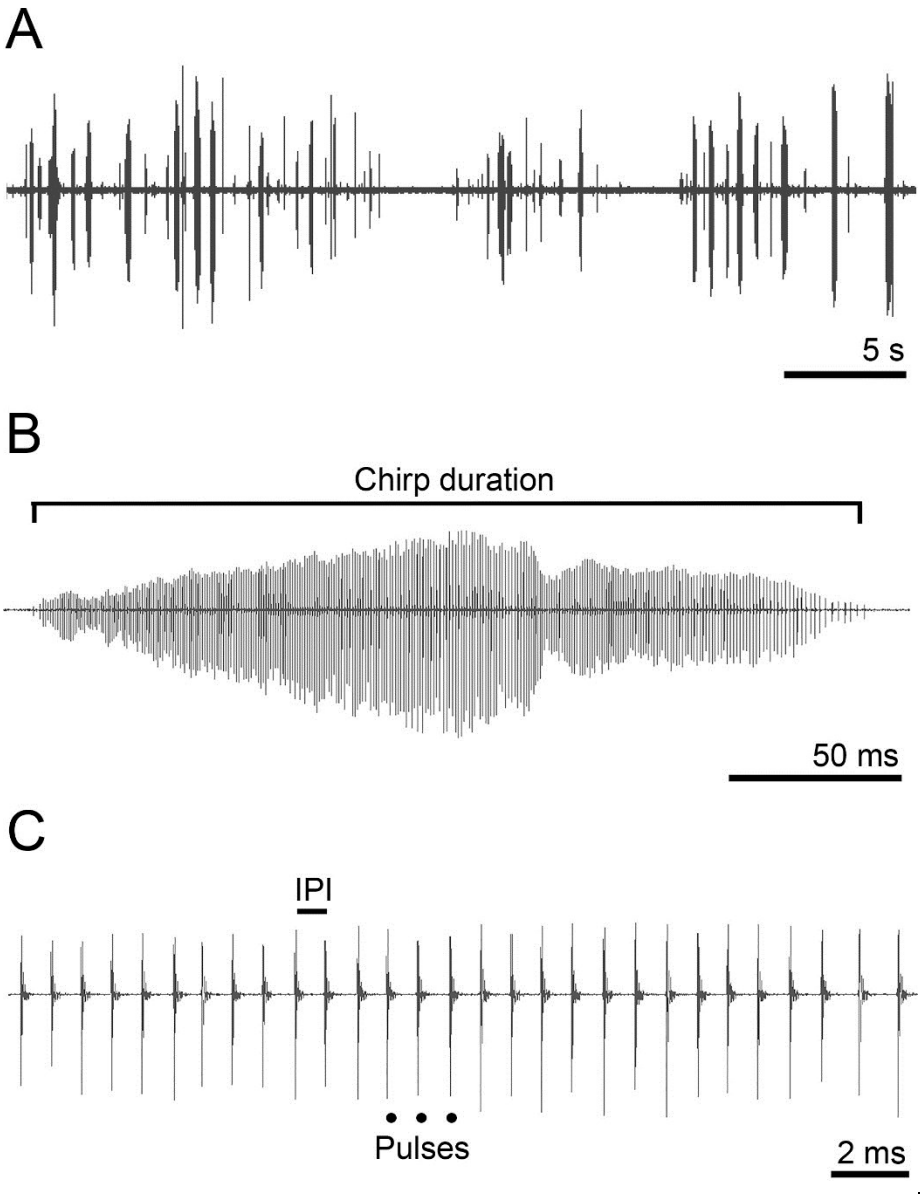


**Figure 2.3**: Temporal measurements of female *Ips pini* chirps. A: Chirp train recorded during distress (handling). B: Single chip with measurement of chirp duration shown. C Individual pulses within a chirp with measurement of interpulse interval (IPI) shown.

A quantitative method was developed to analyze the proportions of different amplitude envelope shapes of chirps. This required quantification of both the envelope shape and the different shape types. The amplitude envelope was obtained for each chirp as amplitude vs. time curves using the amplitude and time values of each pulse within the chirps. Three basic shape types were identified: “bell shaped”, for chirps that have the highest amplitude in the middle portion; “ascending”, for chirps with pulses that gradually increase in amplitude towards the end, and finally, “descending”, which is the opposite, where pulses generally decreased in amplitude (see Results). A sample of 80 chirps (separate from the sample used for the three behavioural conditions) was gathered by two observers, and identified as the best representatives of the three shape types (24 to 30 for each shape). The amplitude envelopes of these chirps were then used in Table Curve 2D software to generate model curves for the three types. Finally, these functions were fitted on the original sample of chirps for the different behavioural contexts using the same software (Table Curve 2D) to sort the amplitude envelope shape of the chirps into categories.

## Statistical Analyses

Previous studies were able to distinguish two types of chirps in *Dendroctonus* species based on regular interruptions of the same length: simple (without any interruptions) and interrupted (Lindeman and Yack, 2015). Interruptions within chirps were also claimed to hold importance for *I. pini* (Swaby and Rudinsky, 1976). In the current study, it was predicted that if there were distinct chirp types based on temporal pulse patterns (i.e. simple or interrupted), the frequency distribution of interpulse interval values collected from all chirps would be binomial: the first peak being the average IPI

and the second peak would be due to the regular interruptions of similar length in a high number of chirps. Frequency distribution of IPI values derived from chirps selected from all contexts was recorded and subjected to curve-fitting to recognize the model of frequency distribution (TableCurve 2D; Systat, San Jose, CA, USA); the frequency distribution models were selected based on parsimony, high F-values (and mean squares), and steep increases in $R^2$ with model complexity.

Assessment of differences in temporal characteristics was done by first averaging all parameters (duration, number of pulses and average IPI per chirp) for each individual. These averages were used for statistical analysis. A canonical variate analysis (CVA) was performed to determine whether significant differences are present between contexts (PROC CANDISC; SAS Institute, Cary, NC, USA). Individual ANOVAs and Fisher's LDS test ($P < 0.05$) per parameter were used to determine which parameter differs significantly (PROC GLM; SAS). Normality and homoscedasticity assumptions were checked before CVA and ANOVA and no data transformation was necessary (PROC UNIVARIATE; SAS).

The shapes of amplitude envelopes per time were subjected to regression analyses again using the curve fitting procedure of TableCurve 2D and selecting the most suitable models based on the same criteria used to recognize the models for frequency distribution of IPI. A Chi-square contingency table was run to determine if the proportion of each envelope shape was independent from the context (PROC FREQ; SAS). A Kruskall Wallis test was subsequently used to analyse differences between contexts regarding envelope shape proportions (PROC NPAR1WAY; SAS).

## 2.4 Results

### General chirp characteristics and types

Females produced chirp trains (Fig. 2.3A) in all three behavioural contexts tested. Chirps consisted of a pulse-train (Fig. 2.3B). All chirps sampled under all three contexts were first analyzed together to assess the range of chirp characteristics, and to determine if there is quantitative evidence for dividing chirps into simple and interrupted categories. In total, 588 chirps from 64 females were analyzed. There was a wide range in the temporal characteristics across all chirps (Table 2.1). To assess whether chirps could be categorized based on regular interruptions, a frequency distribution analysis of IPIs was performed to determine whether interruptions of similar duration were regularly occurring across all chirps sampled (Fig. 2.4A), or in at least one of the behavioural contexts (Fig. 2.4B-D). The analysis showed that only unimodal distributions can be fitted to the IPI data, suggesting that there are no regular interruptions of similar length within the chirps produced in the observed conditions (Fig. 2.4). One hundred percent of the chirps fit one of the three basic shapes (ascending, descending, bell shaped (Fig. 2.5)) defined by curve fitting. This indicates that these three shape categories are sufficient for quantitative analysis of amplitude envelope shapes between contexts (see below).

**Table 2.1** Temporal characteristics of *I. pini* chirps recorded in different behavioural conditions

| | Chirp Duration (ms) | | Number of pulses | | IPI (ms) | | Pulse rate (# pulses/s) | |
|---|---|---|---|---|---|---|---|---|
| | Range | Average (± SEM) | Range | Average (± SEM) | Range | Average (± SEM) | Range | Average (± SEM) |
| All | 29.2-501.3 | 137.3 ± 2.6 | 14-385 | 147.31 ± 2.78 | 0.02-135.49 | 0.94 ± 0.01 | 79.75-2703.72 | 1160.76 ± 20.24 |
| Distress | 29.2-429.4 | 122.3 ±5.4 | 14-369 | 145.76 ± 7.34 | 0.02-135.49 | 0.95 ± 0.06 | 79.75-2703.72 | 1223.99 ± 37.06 |
| Predation | 38.9-328.3 | 133.1 ±6.8 | 19-305 | 137.66 ± 6.45 | 0.17-35.70 | 0.92 ± 0.05 | 288.94-2233.51 | 1241.59 ± 30.54 |
| Premating | 33.5-501.3 | 159.7 ±8.9 | 17-385 | 152.91 ± 6.91 | 0.19-48.95 | 1.06 ± 0.06 | 146.34-2094.54 | 1007.19 ± 28.42 |

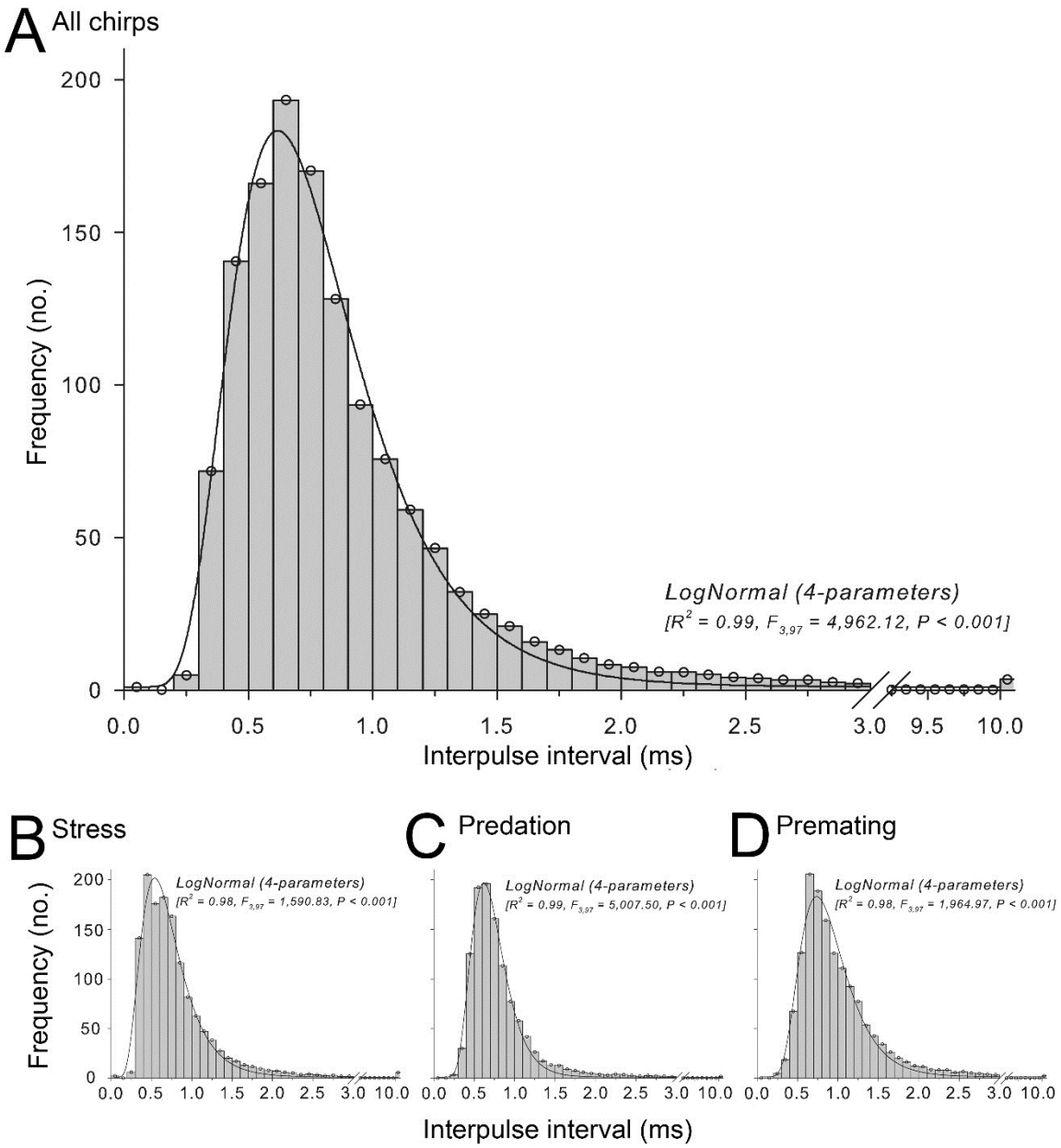


**Figure 2.4:** Frequency distribution histogram of *Ips pini* chirp IPIs showing unimodal distribution in all behavioural contexts. A: All chirps. B: Distress. C: Predation. D: Premating.

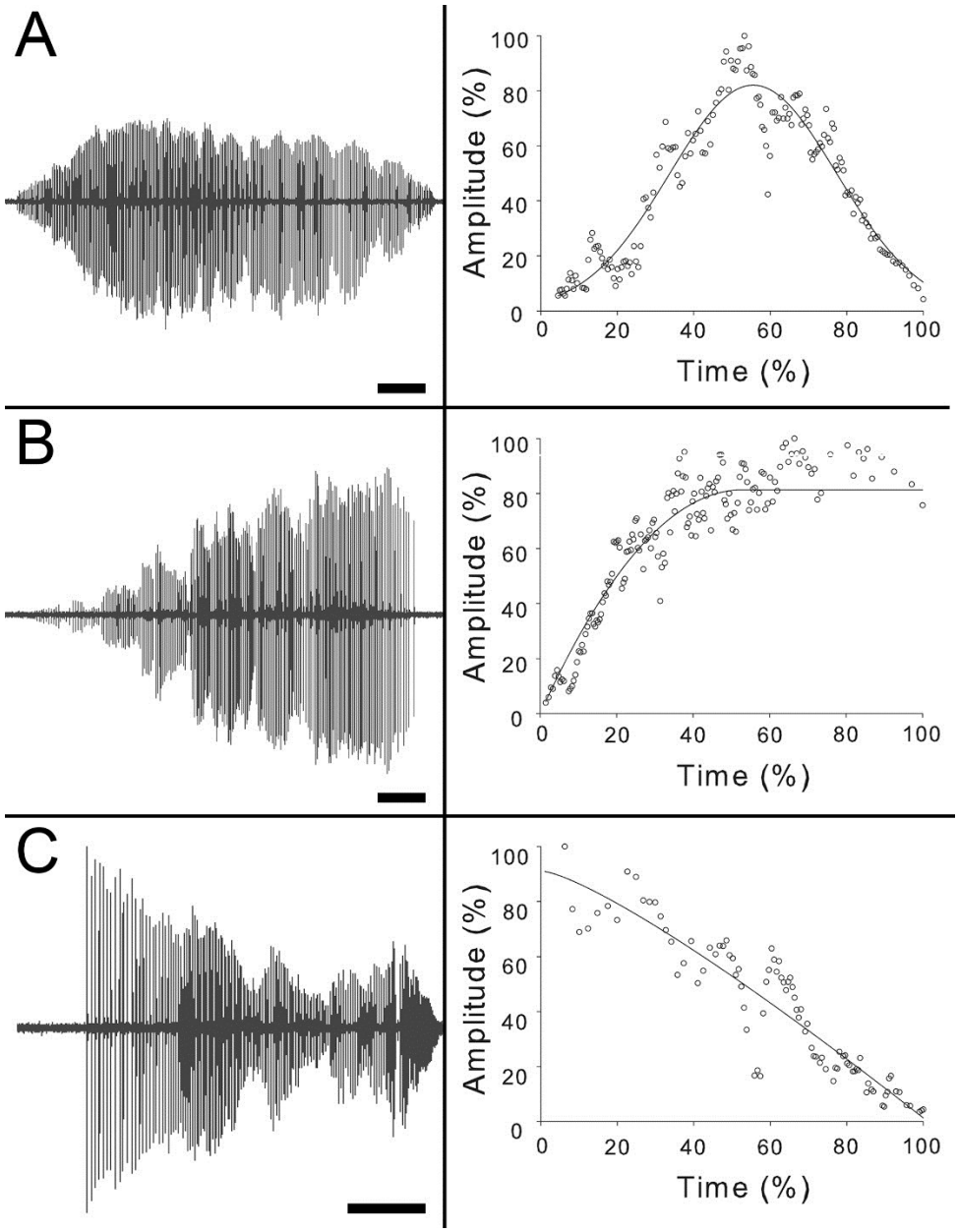


**Figure 2.5** Basic amplitude envelope shapes of female *Ips pini* chirps calculated by curve fitting on selected representative chirps, shown with examples. A: Bell shaped. B: Ascending. C: Descending. Scale bars: A,B,C: 20 ms

## Context-dependent chirp characteristics

Females produced chirps in all three behavioural contexts tested. Light pinching of the female abdomen elicited distress chirps. While being grasped by the mandibles of *T. dubius* predators, females exhibited struggling behaviour characterized by erratic leg

movements, while nodding their heads up and down, with opened mandibles; the head nodding elicited chirps. During premating trials, all females admitted into male chambers produced acoustic signals.

## Temporal characteristics

Significant differences were observed in chirp characteristics between behavioural contexts. CVA analysis showed that distress and predation were undistinguishable while being significantly different from premating (approximated $F_{6,110} = 3.56$, $p < 0.05$) (Fig. 2.6A). Individual ANOVA tests confirmed the previous analysis and showed that only duration was significantly different between premating and the other two contexts ($F_{2,59} = 8.03$, $p < 0.05$) (Fig. 2.6B). Average temporal measurements from the three contexts are summarized in Table 1.

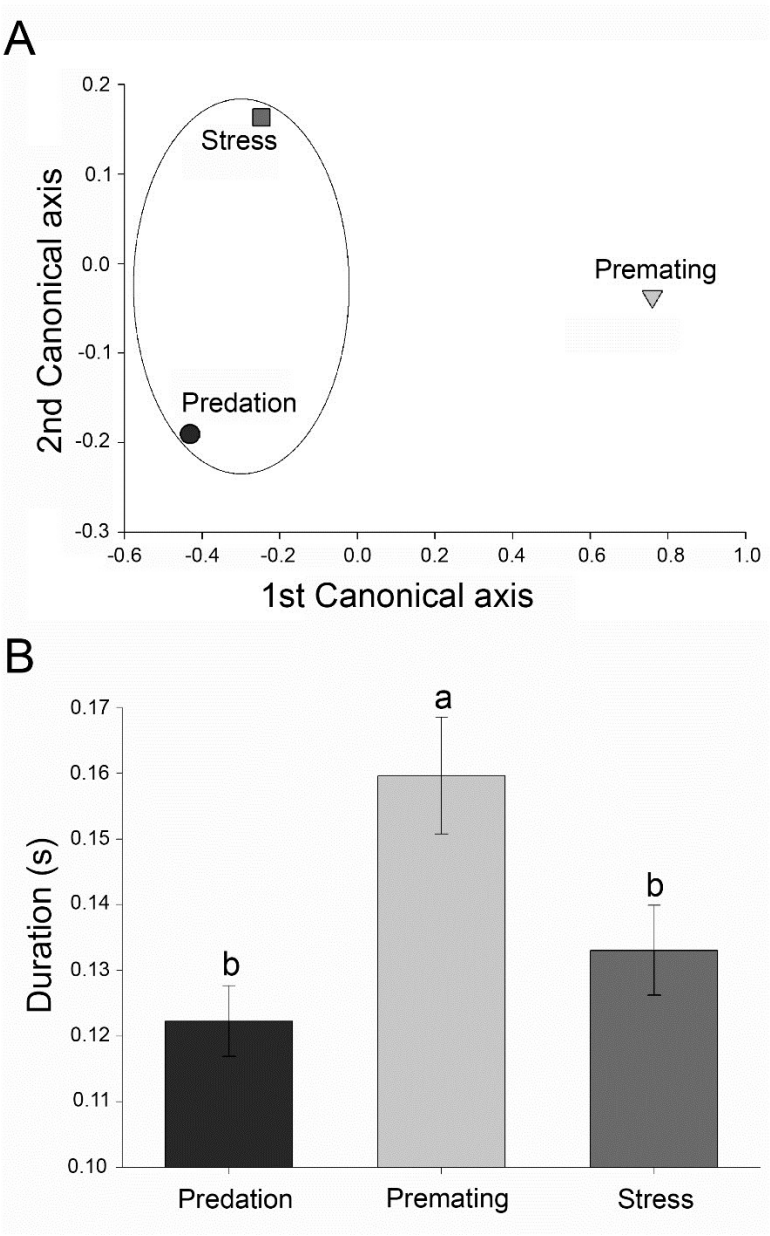


**Figure 2.6** Statistical results showing significant differences between the temporal chirp characteristics from the three behavioural contexts. A: CVA diagram graph showing premating chirps being significantly different from other chirps while distress and predation being undistinguishable; the symbols are centroid of treatments represent class mean canonical variates and the large ellipsis grouping then indicate lack of significant difference (approximated F-test at $p < 0.05$), based on the Mahalanobis distance ($D^2$) between class means (i.e., behavioural contexts). B: Bar graph of the average chirp duration (± SEM) in the three behavioural contexts; different lower case letters at the top of each bar indicate significant differences by Fisher's LSD test ($p < 0.05$). Duration was significantly longer during premating conditions.

## Amplitude envelope

The chi-square contingency table showed that frequency of each envelope shape differed between the three contexts ($\chi^2 = 33.70$; df = 4; $p < 0.05$). The Kruskall Wallis test showed that the "descending" shape was significantly more frequent in the premating context (Fig. 2.7).

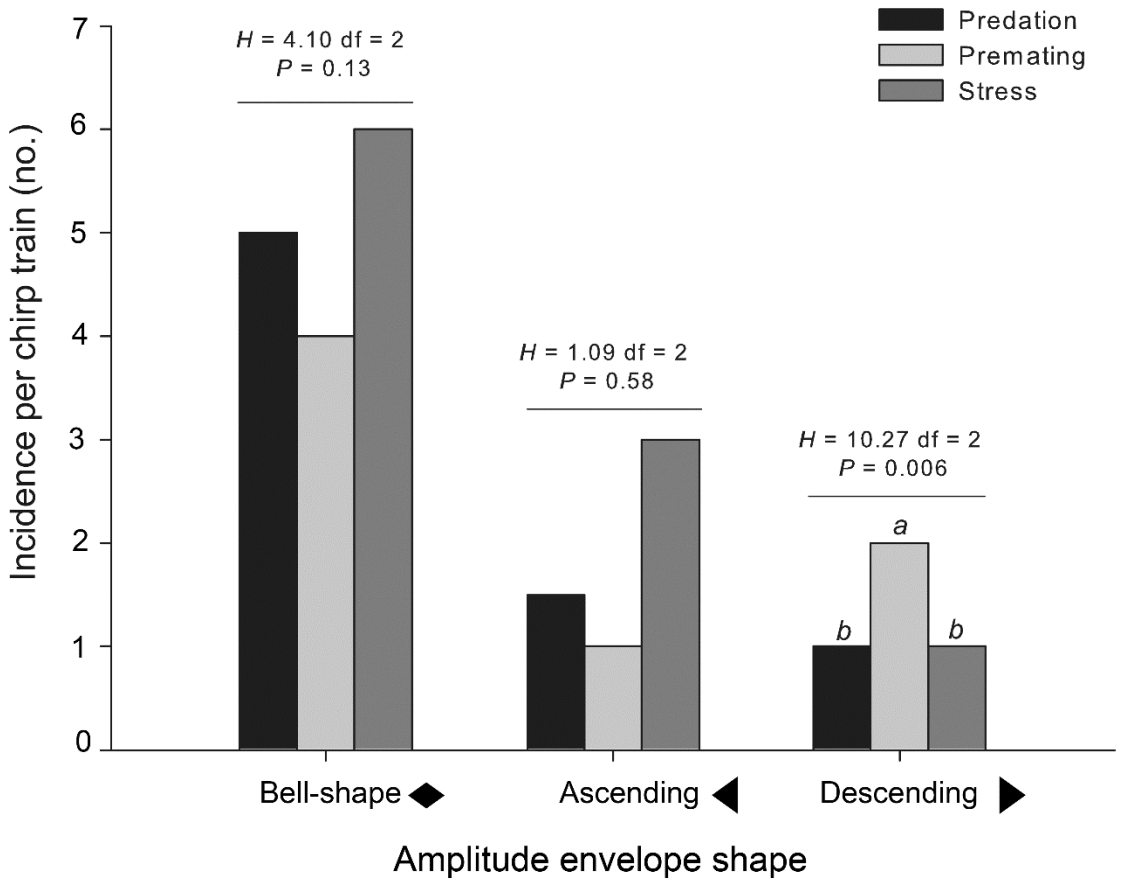


**Figure 2.7** Bar graph showing the occurrence of the three basic amplitude envelope shape types in the three behavioural contexts. Bell shaped chirps were the most common in all contexts. Relative occurrence of descending chirps was significantly higher during premating than in the other two contexts.

## 2.5 Discussion

This study shows that acoustic chirps in female pine engraver beetles are consistently produced in the three contexts tested- distress, predation, and premating. The results show significant differences between the temporal and amplitude envelope characteristics of chirps in different contexts, suggesting that context dependent signalling is present in this species. The functional significance of variation in chirp characteristics between contexts, and recommendations for future comparisons of signal characteristics in bark beetles, are discussed.

### Do *Ips pini* produce different chirp types?

Bark beetle species have been proposed to exhibit two chirp types based on regular interruptions: simple (or uninterrupted) and interrupted (e.g. *Dendroctonus* spp. (Michael and Rudinsky, 1972), *Ips* spp. (Oester and Rudinsky, 1978)). However, currently there is little quantitative evidence that such types exist. In *I. pini* the distribution of IPIs is unimodal for all chirps whether analyzed together, or for the three different contexts. Therefore, in disagreement with previous *Ips* spp. studies (Oester and Rudinsky, 1978), the results of this study do not support the hypothesis that *I. pini* produces categorically different chirp types, as has been confirmed for at least one *Dendroctonus* species (Lindeman and Yack, 2015). *Ips pini* has a completely different sound producing mechanism from *Dendroctonus* species (Barr, 1969). Males of stridulating *Dendroctonus* species possess an elytro-tergal type mechanism, where the pars stridens is located on underside of the elytra, and the plectrum is a single pair of ridges on the 7th abdominal tergite (Lyon, 1958). This conceivably allows for finer motor control of the plectrum resulting in a more precise mechanical manipulation of

interruptions (Lindeman, 2016). Such fine motor control necessary for inserting regularly spaced interruptions may not be feasible for *I. pini*. While different chirp types cannot at present be ruled out entirely for *I. pini*, as these may exist in other behavioural contexts not tested in this study, at present this research does not support previous categorization of chirps into distinct types.

## Distress signalling under natural and artificial conditions

Most bark beetle species, including *I. pini*, are reported to generate 'distress', 'stress' or 'disturbance' signals (Barr, 1969; Ryker and Rudinsky, 1976). Signals are elicited by holding the insect between fingers and squeezing slightly (Swaby and Rudinsky, 1976). This method, however, is not representative of any natural stressful conditions, such as predation, and it is possible that the sound characteristics do not represent natural conditions. Unfortunately, there is only one study that tested acoustic behaviour during predator attack, and it did not include analysis of emitted signals (Lewis and Cane, 1990). In the current study, natural stress conditions were created by pairing the beetles with one of their natural predators, *T. dubius,* and the recordings from these trials were compared with those emitted during handling. No significant differences were found between chirps recorded under natural and artificial stress conditions; therefore, it is concluded that in *I. pini*, handling is an accurate representation of stress, and can be considered a valid means of evoking distress signals for future studies.

## Premating chirps

Chirps produced during premating are significantly longer than chirps during distress or predation. However, the number of pulses and mean IPIs are not significantly

different between contexts. This might be the result of the small increase in the number of pulses combined with a slightly lower rate, or it could suggest that during premating interactions, females increase chirp duration by introducing random interruptions within a chirp. During premating interactions, while acoustically signalling, females also engage in jostling behaviour by repeatedly pushing against the male elytral declivity with their frons, and occasionally biting and scraping male elytral spines (Schmitz, 1972). It is possible that these physical interactions can introduce random interruptions, causing longer chirp durations. However, the exact mechanism of generating longer duration premating chirps is not clear and requires further investigations on signalling mechanisms. Regardless of how they are produced, longer chirps can play an important role during mate choice in insects and this may also be the case for bark beetles.

Longer signals are easier to detect (see Pohl et al., 2013), and can provide information about signaller quality. Signal characteristics that provide honest information about signaler phenotypic and/or genetic quality are usually under directional selection, and show much variation between individuals (Johnstone, 1995; Pomiankowski and Møller, 1995). Longer signals are often more costly than shorter ones (Prestwich, 1994) and have been shown to be more attractive to the opposite sex in other acoustic animals, such as field crickets (Hedrick, 1986), lesser wax moths (Jang and Greenfield, 1996), tree frogs (Gerhardt et al., 2000), and spiders (Parri et al., 2002). It is hypothesised that in bark beetles, premating signals provide information about the signaler's physical attributes (Byers et al., 2010; Lindeman and Yack, 2015), and higher effort in signalling may relate to better physical condition, as shown for other acoustic insects (Bertram et al., 2006). It is important to note that there is currently no information on bark beetles'

capability to detect airborne sounds and that it is possible that these signals are perceived as substrate borne vibrations. While this would mean that many of the characteristics of the sounds would be transformed or lost during the transfer between the substances, differences in chirp duration and the amplitude envelope are known to be translated into the vibration component of bark beetle sounds (Fleming et al., 2013; Lindeman, 2016), meaning that the observed significant differences would most probably be translated as well.

This study shows that female *Ips pini* signals have a broad range of chirp durations, and may be highly variable between individuals. However, future studies should examine variations in signal characteristics between females, and whether these traits can provide information about quality and/or species. In contrast, distress signals, which presumably function for momentarily deterring predators, are generally shorter in duration and more intense (Masters, 1980). The effectiveness of these signals is usually short-lived; predators can become habituated to longer signalling. Therefore, producing long distress signals can be counterproductive. The effectiveness of distress signals in bark beetles remains unknown, and untested empirically (but see Lewis and Cane, 1990).

## Amplitude envelope shape

The amplitude envelope shape of insect sounds can be as important as the temporal attributes (Ronacher, 2016). In grasshoppers, species specific amplitude modulation is directly related to the sound producing mechanism of the given species, and is important for the listener (von Helversen and von Helversen, 1998). In the grasshopper, *Chorthippus biguttulus*, changing the amplitude shape decreased the attractiveness of that signal - signals with a descending shape were very attractive;

whereas signals with an inverted shape (ascending) were rejected (Schmidt et al., 2008). Despite the importance of amplitude envelope shape, quantitative methods for the analysis of the amplitude-time envelope shape have not been used to assess insect acoustic signals.

To analyze relative amplitude characteristics of *I. pini* chirps, a curve fitting based method was developed. By quantifying the amplitude envelope using the software to match with pre-defined curves, we minimize subjectivity. The results show that "bell shaped" is dominant for all contexts, while "descending" is significantly more frequent during premating conditions. The change in the envelope shape might be the result of a different signalling motion during premating. For example, during premating signalling, females might only stridulate using part of the pars stridens. This suggests that females may have some control over the envelope shape, which possibly requires effort. In turn, these descending chirps could be preferred by the male. It would be important to know whether these insects are capable of detecting changes in the envelope shape. However, to this date, there is no information on the sound detecting organ of bark beetles. Furthermore, studies on the morphology and kinetics of the sound producing mechanism might show why "bell shaped" seems to be the dominant envelope shape for these species. These findings suggest that the amplitude envelope can possibly be part of the context dependent signal characteristics of *I. pini* chirps, and it is recommended that this chirp characteristic be considered in future acoustic analyses.

Bark beetles produce acoustic signals in several different behavioural contexts but little is understood about how these signals vary, owing partly to a lack of objective and quantitative methods used to characterize acoustic signals in this group. This study takes

a first step in applying quantitative analytic methods to sample and analyze signal characteristics in a bark beetle, *Ips pini*. The results show that premating chirps are of longer duration than defensive chirps, supporting the hypothesis that signal characteristics vary between conditions. It is recommended that future comparative studies on bark beetle acoustics follow similar methods to facilitate meaningful comparisons of acoustic communication signals within and between populations of this economically and ecologically important insect group. Finally, it is recommended that more research focuses on context dependent signal variation in other insect groups, as this knowledge will provide insights into the selection pressures on insect communication signals in general.

## 2.6 Acknowledgements

We thank C. Wood and the experimental farm and arboretum, and Carleton University for allowing us to hang collecting traps. This research was funded by the Natural Sciences and Engineering Research Council of Canada (JEY), the Canadian Foundation for Innovation (JEY), the Ontario Innovation Trust (JEY), the Ontario Ministry of Economic Development and Innovation (JEY), and the National Council of Scientific and Technological Development (CNPq, Brazilian Ministry of Science and Technology (RNCG).

# Chapter 3. Investigating the acoustic receptor organs of bark beetles

## 3.1 Introduction

Acoustic sensory organs are widespread among insects (Mason and Pollack, 2016). They can have a variety of different functions including conspecific communication (Greenfield, 2016) and predator detection (Pollack, 2016). The term "acoustic signal" has a broader definition in insects than in most other animals, as signals can be transmitted through air, water or solid substrates, and can include both pressure and displacement waves (Yack, 2004; Windmill and Jackson, 2016). Pressure waves propagating through air (also known as far field sound) are detected by insects by various tympanal ears (Yack, 2004; Windmill and Jackson, 2016). These organs come in many varieties and can be found in almost anywhere along the insect's body (Hoy and Robert, 1996), yet they all share the same basic structural characteristics. A thin cuticle membrane, called the tympanum covers an air chamber usually near an enlarged spiracle. The sensory apparatus, called the chordotonal organ, is associated with the membrane (Yack, 2004; Windmill and Jackson, 2016). The complexity of each of these components can vary greatly depending on the function of the ear. Some insect groups are capable of detecting the displacement component of sound, known as near field sound, as these signals are only detectable in close range (Yack, 2004; Windmill and Jackson, 2016). Near field signals are often detected in these insects by the Johnston's organ, located on the antenna of the insect (Yack, 2004; Windmill and Jackson, 2016). The Johnston's organ is a large chordotonal organ that is attached to loosely fitted flagella on the pedicel, which propagate the displacement of air particles to the sensory cells (Yack, 2004).

Besides airborne sound, an estimated 195,000 insect species use substrate-borne vibration as form of communication (Cocroft and Rodríguez, 2005). While any seta or similar structure can be used to detect solid-borne vibration, the primary vibration receptor organ found in almost all insect orders (except currently for Diptera and Coleoptera) is the subgenual organ (Yack, 2016). This consists of a chordotonal organ located in the proximal tibial area of one of the 3 pairs of legs of the insect. The vibration of the surface is propagated through the cuticle and the hemolymph to the sensory cells (Yack, 2016). While there has been extensive research on the acoustic reception of some insect orders, such as Orthoptera and Hemiptera, other groups have received much less attention. One of these is the order Coleoptera, which is the focus this chapter.

While many coleopteran species have been documented to communicate acoustically (Wessel, 2006; Greenfield, 2016), much less is known about acoustic reception. It is one of the two insect orders currently believed to lack a subgenual organ for vibration detection (besides Diptera (Yack, 2016)). Johnston's organ has only been described in the family Gyrinidae (Henrikson and Stenson, 1992), and tympanal ears are only found in certain tiger beetles (Cicindellidae; Spangler, 1988) and scarab beetles (Scarabaeidae; Forrest et al., 1997) (Fig. 3.1). The tympanal hearing organ found in multiple tiger beetle species is located on the first abdominal tergum, and it is most sensitive when the elytra are open and the beetles are flying. These ears are reported to detect ultrasound, which induces an evasive landing manoeuvre in flying tiger beetles, suggesting that the function of the ear is to detect bat echolocation (Spangler, 1988). Studying the auditory afferent revealed an exclusive sensitivity to ultrasound with the ear being tuned at 30 kHz (Yager and Spangler, 1995). Four scarab genera (*Cyclocephala*,

*Dyscinetus*, *Euetheola* and *Oxygrylius*) have also been found to possess tympanal organs as part of the cervical membrane behind the head (Forrest et al., 1997). These ears were also found to be tuned to ultrasound at around 40 kHz, and the neural response to sound was found to be aligned with startle behaviour (Forrest et al., 1997). These findings sum up the current knowledge of coleopteran acoustic reception. As such, for most acoustic beetle groups there is currently no information on hearing, which includes the subject group of this thesis: bark beetles.

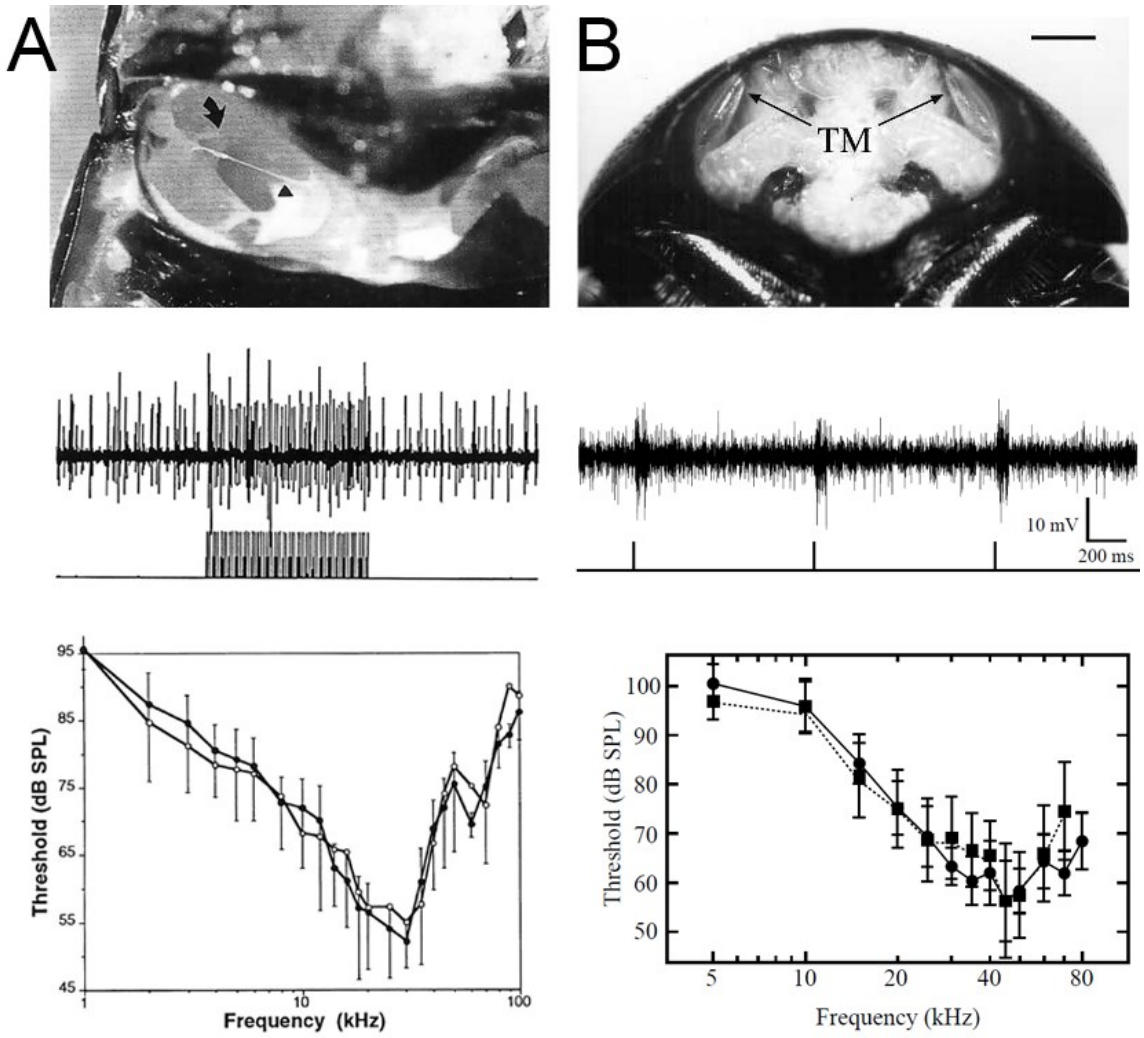


**Figure 3.1** Tympanal ears in Coleoptera. A: tiger beetle (*Cicindela marutha*) B: scarab beetle (*Euetheola humilis*). Top: Images of the tympanal ears (tympanum removed on A, head removed on B). Arrow (A): auditory nerve. Scalebar (B): 1mm. Middle: neural response to stimulus (A: auditory afferent at 30 kHz B: neck connective at 40 kHz) Bottom: tuning curves. TM: tympanal membrane. Images taken from Yager and Spangler (1995) for A; Forrest et al. (1997) for B.

While many species of the subfamily Scolytinae have been described to produce sounds, or to possess stridulatory organs (see Chapter 1), the form of sound reception is currently unknown. Because of this, one cannot assume that sound reception is happening in the acoustic far field. All occurrences of acoustic communication between conspecifics so far has been observed while the beetles were in close proximity either at the entrance

of the nuptial chamber or inside the galleries (e.g. Barr, 1969; Swaby and Rudinsky, 1976; Fleming et al., 2013; Lindeman and Yack, 2015), which leaves the possibility open that signals are received through the acoustic near field or as substrate-borne vibrations. There are a few reasons however why far field acoustic reception is possible in these beetles: the complexity of acoustic signals produced by bark beetles (see Chapter 2; Fleming et al., 2013; Lindeman and Yack, 2015) which is unprecedented in near field communicating insects, the lack of subgenual organs in Coleoptera that is believed to be the most common vibration receptor organ of insects (Yack, 2016), and the assumable low vibratory quality of the phloem which would need to be the substrate for solid vibrational communication (Sivalinghem 2011; Fleming et al. 2013). Bark beetles were also shown to react to playback of conspecific sounds from a distance (Rudinsky et al., 1973). Yet, as there is currently no direct evidence against any forms of acoustic reception, all possibilities should be examined. Anatomical studies by previous students in the Yack lab have identified a thin membrane on the metathorax of *Ips pini*, *Dendroctonus ponderosae* and *D. valens* and described it as a possible tympanal organ (Fig. 3.2B). It has also been found that there is a nerve originating from the metathoracic ganglion that innervates the area of the membrane, possibly including acoustic afferent nerve fibers (Gall-Duncan, 2015). Tympanal membranes have been described in at least 17 different positions so far in insects (Strauß and Lakes-Harlan, 2013), which leaves the possibility open for another, currently undiscovered tympanal membrane somewhere along the beetle's body. Other possible areas of acoustic reception are the legs (in case of substrate-borne vibrations) (Fig. 3.2C), and the antennae (for near field reception) (Fig. 3.2D).

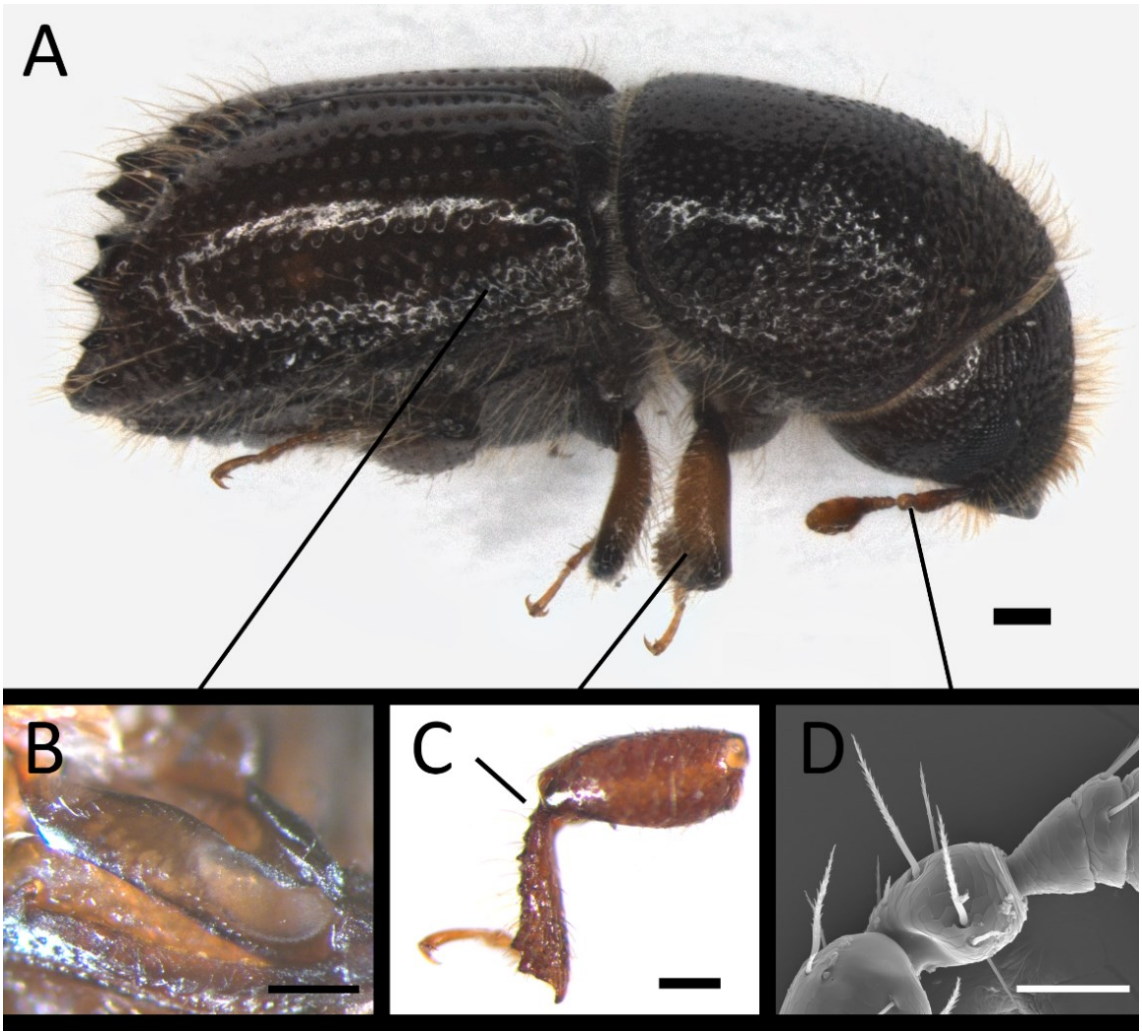


**Figure 3.2** Possible hearing or vibration sensory organs of bark beetles. A: Female *I. pini* B: Putative tympanal ear on the metathorax (elytron removed) C: Prothoracic leg with possible location of vibration receptor organ D: Pedicel of *I. pini* antenna. Scalebars: A, B, C: 200µm D: 50µm. Credits: B,C: Sen Sivalinghem D: Kyle Lemay

One of the most important methods for analyzing acoustic reception is electrophysiology (Mason and Faure, 2004). By characterizing the neural response to the acoustic signals, one can identify what part of the signal is actually received by the insect and what characteristics hold importance. This method is also very important for narrowing down possible anatomical locations for receptors when there is no observable

morphological evidence. As a result, some forms of extra- and intracellular electrophysiological experiments have been included in all projects that characterized newly discovered hearing organs, including the ones on the two tympanal ears found in Coleoptera (Yager and Spangler, 1995; Forrest et al., 1997). For the final chapter of my thesis I used extracellular electrophysiology with hopes to narrow down the possible location of acoustic reception in bark beetles as well as to identify the form of vibration these beetles are specialized to receive. The goals of this chapter were the following:

I. **Development of protocols for neurophysiological measurements on bark beetles**. The current literature on bark beetle neurophysiology is limited to electro-antennogram recordings which are not applicable to acoustic studies (e.g.: Dickens and Payne, 1977; Ranger et al., 2014). As such, methods for preparing the insects, exposing the areas of interest, making successful recordings, and delivering various forms of acoustic stimuli all needed to be developed, based on protocols for other insect species. Since there is currently no reason to believe that different acoustic bark beetle species detect sounds differently, I decided to use *D. valens* for these experiments instead of *I. pini* that was used in Chapter 2. *Dendroctonus valens* is one of the largest known bark beetle species, it is available through trapping, it is known to have a relatively complex acoustic ecology, and there is information available on the species' acoustic signal characteristics (Lindeman and Yack, 2015; Lindeman, 2016), making it ideal for these highly exploratory experiments (Fig. 3.3).

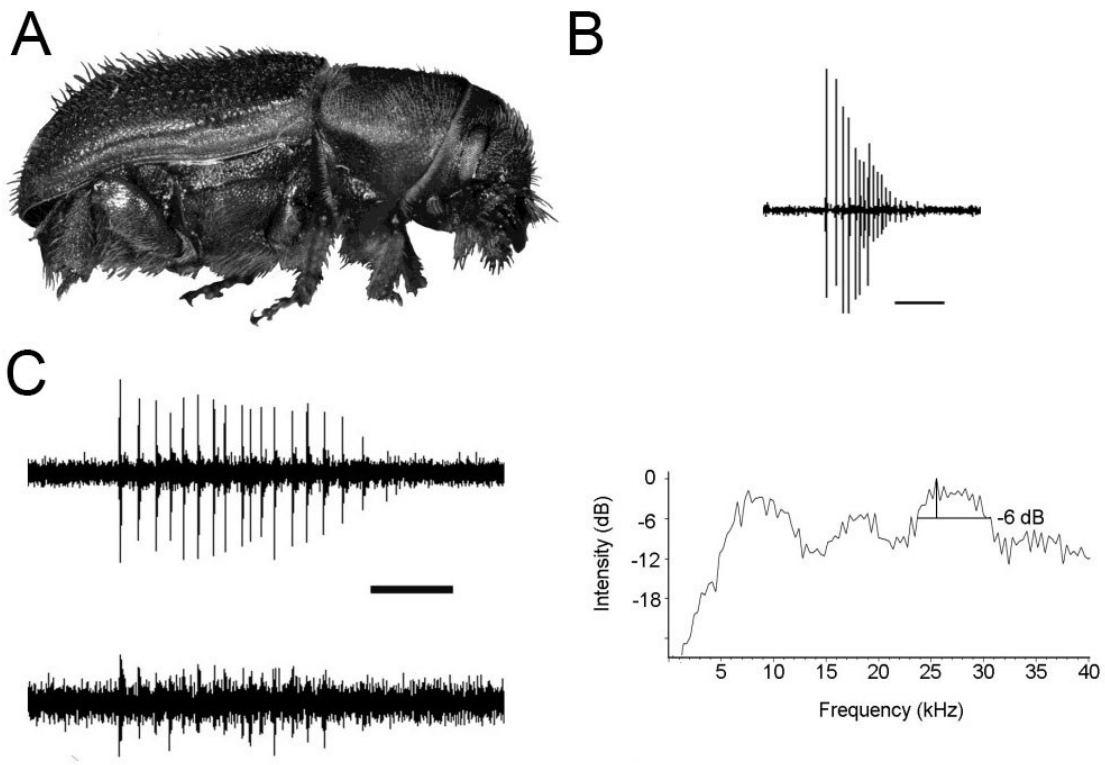


**Figure 3.3** Acoustics of *D. valens* A: Male *D. valens*. B: Example of a chirp (top) and power spectrum (bottom) of *D. valens* chirps showing peaks at 7 kHz and 27 kHz. C: Airborne (top) and substrate-borne (recorded on the phloem) vibration (bottom) components of a *D. valens* chirp. Scalebars: B: 10 ms C: 20 ms Images taken from Lindeman (2016).

II. **Identifying regions of acoustic reception in *D. valens.*** Three main areas were examined for neural responses: 1) The thoracic nervous system, with a focus on the metathoracic nerve previously identified to innervate the putative tympanal membrane on the thorax, as well as other available nerves and the connectives; 2) All three pairs of legs; 3) The antennae. Stimuli included all three forms of vibrations (far field, near field, substrate-borne vibration) in various frequencies (see Methods for details).

## 3.2 Methods

### Insects

Live *D. valens* specimens were collected using Lindgen funnel traps with *D. valens* lure (Synergy Semiochemicals Corp., Burnaby, BC, Canada) that were placed on various pine (*Pinus spp.*) trees in the Arboretum of the Central Experimental Farm (Ottawa, ON, 45.391021, -75.70489). Beetles were stored in plastic vials with red pine (*Pinus resinosa*) phloem shavings and moist paper between 5-10°C at Carleton University. Some of these beetles were used to inoculate red pine logs following the protocol described by Lindeman (2016). Beetles emerging from these logs were also used for the experiments. Voucher specimens were kept in 70% alcohol and stored at Carleton University.

### Immobilizing and dissecting specimens

In order to obtain successful electrophysiological recordings, the *D. valens* specimens needed to be restrained of any movement that could possibly interfere with the electrodes. At the same time, it was also important to not compromise any possible acoustic receptors. For these reasons, the specimens were placed on 2 mm diameter wooden rods and were fixed in position using a minimum amount of warm wax (Veet$^{tm}$ Stripless Warm Wax, Reckitt Benckiser Group plc, Slough, United Kingdom). The position of the insect on the rod and the steps that followed the immobilization depended on the type of the experiment (Fig. 3.4).

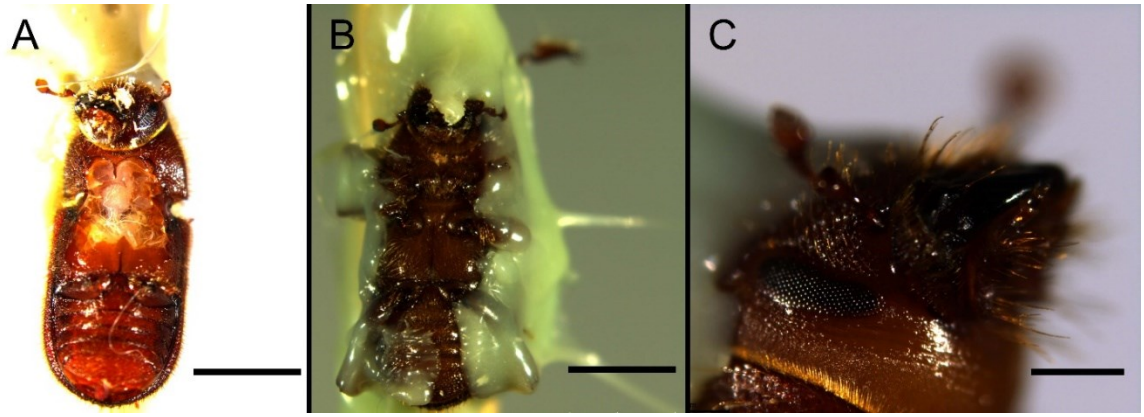


**Figure 3.4** Images of beetles prepared for neurophysiological studies. A: Exposed nervous system experiments. B: Legs. C: Antennal recordings. Legs and part of the ventral cuticle removed in A. Scalebars: A, B: 2mm C:500µm.

Exposed nervous system experiments

Due to the exploratory nature of the experiments, the goal of these trials was to expose the nervous system in the ventral thoracic area and look for responses from the previously discovered metathoracic nerve, the main connectives, and any other possible parts of the body. This required minimal usage of wax as well as the minimization of invasiveness during dissection (see Appendix 2 for the detailed dissection protocol). Once they were successfully immobilized, the beetles were transferred to the Leica M205C stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany) for dissection (Fig. 3.4 A). Pictures and videos of the dissection process were recorded by the Leica DMC4500 microscope camera operated through the Leica LAS4.3 Software (Leica Microsystems GmbH).

Identification of nerves, connectives and ganglia were based on the neural maps of *D. ponderosae* made by Terrence Gall-Duncan (2015) (Fig. 3.5). If the desired structures were successfully exposed, the opened area of the cuticle was covered by a

drop of Lepidoptera saline (Paul, 1974) to prevent tissue death, and the insect was transferred to the neurophysiology rig.

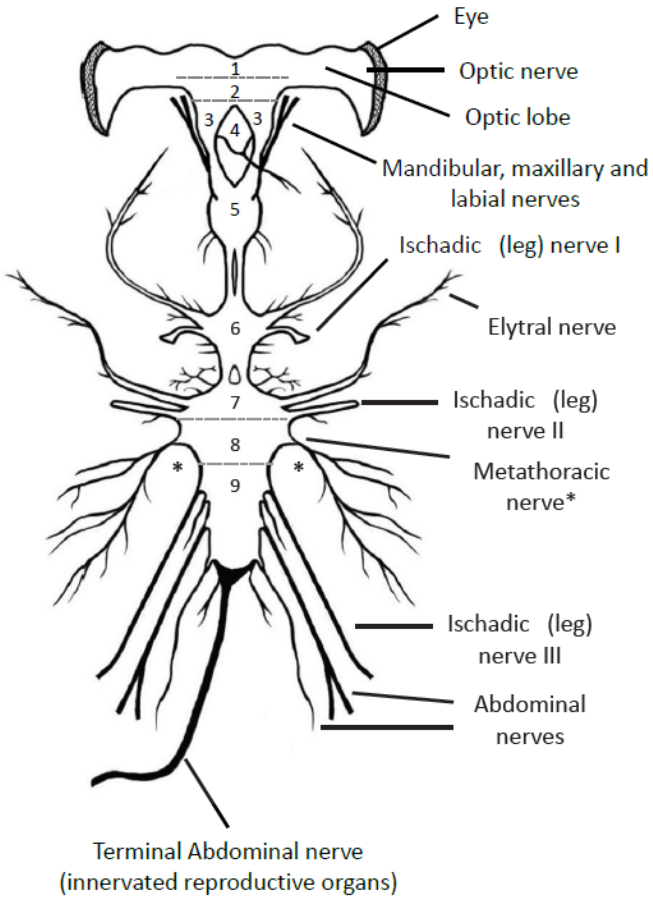


**Figure 3.5** Neural map of *D. ponderosae* which was used as a reference for the extracellular recordings. The * identifies the metathoracic nerve that was found to innervate the putative tympanal membrane (Gall-Duncan, 2015). Figure taken from Gall-Duncan (2015).

Vibration experiments on the legs

Various ways of immobilization and dissection were tested to optimize recording from the leg. Successful recordings however were made only when beetles were placed in the wax on their dorsal side, with the distal portion of the leg covered in wax (Fig. 3.4 B).

While this position of the legs is not natural for the insects, this allowed for more options for electrode placement. Dissection was limited to making a small hole on the cuticle of the femur or the coxa in a small number of experiments in order to insert the electrodes. However, most trials were done without any dissection.

### Near field experiments on the antenna

The immobilization for antennal experiments was done the same way as for the exposed nervous system experiments (see above). Special care was needed to immobilize the head while leaving the antenna free from wax. Dissection was limited to removal of the legs in order to prevent the insect from touching the electrodes. Because of the use of tungsten needle electrodes (see below), further dissection was unnecessary.

## Electrophysiology

### Thoracic nerves and connectives

Stainless-steel hook electrodes were made for exposed nervous system recordings. Electrodes were electrically etched from 0.01" stainless steel rods (AM Systems, Sequim, WA, USA) in 4% hydrochloric acid solution (method modified from Grundfest et al., 1950). The rods were repeatedly immersed into the HCl solution while a 1Hz AC current was delivered with a function generator (SG 1274 Heath Company, USA) through the rod and a stainless-steel wire immersed in the same solution. Once a desired taper was achieved, the rods were soldered to 0.032" male electrode pins (AM Systems) and were insulated with generic shrink wrap. A hooked end was formed for recording electrodes using forceps. During experiments, the reference electrode was inserted into the abdominal area of the beetle, and the recording electrode was used to lift the desired nerve above the saline level during recordings. The nerves were immersed back into the

saline after each trial in order to keep them from dehydration. The following nerves were tested with this method (see Fig. 3.5 for reference): metathoracic nerve, pro-mesothoracic connective, subesophageal-prothoracic connective (neck connective), ischadic nerve I and III, elytral nerve, first abdominal nerve. Stimuli for these trials included all three types of acoustic stimuli (far field sound, near field sound, substrate-borne vibration). All trials included stimuli at multiple frequencies (see below) that were later separated for analysis. Each frequency trial in each recording included at least 7 stimulus bursts.

For field potential recordings from certain thoracic areas (subesophageal-prothoracic connective, pro-mesothoracic connective, putative tympanal organ area) tungsten needle electrodes were made from tungsten rods (0.004”-0.008”, AM Systems) using the same electro-etching technique as above, but with the use of 10% potassium-hydroxide solution. For one experiment, premade, coated tungsten electrodes were used (1MΩ, 3”, World Precision Instruments, Hitchin, UK).

Leg recordings

For field potential recordings from the pro-, meso- and metathoracic legs, the same tungsten needle electrodes were made as for the thoracic nerve experiments (see above). Both electrodes were inserted into the coxa or the femur, either by penetrating the cuticle with the electrodes, or by making a small hole on the desired structure’s cuticle with scissors. Vibration stimuli between 100 and 1,000 Hz were presented to each leg (see below).

Antennal recordings

For antennal recordings, tungsten needle electrodes were made the same way as for the leg recordings (see above). Both electrodes were inserted into the base of the

antennae, with care not to penetrate deeper than necessary into the head capsule (just deep enough to get a neural signal). Near field acoustic stimuli was presented between 100 and 20,000 Hz (see below).

Differential signals were amplified using a G.R.A.S.S. P55 pre-amplifier, and were digitized and recorded along with the stimulus data using a TDT RP 2.1 processor (Tucker-Davis Technologies, Gainesville, FL), as well as a Fostex FR-2 data recorder (Fostex Corp, Los Angeles, CA, USA).

## Signal delivery

Pure tone sounds were designed in OpenEx software and were generated by a TDT MX6 processor (Tucker-Davis Technologies). Early experiments included sine wave stimuli with square envelope, which was later modified to signals with ramped envelopes (see Fig. 3.8B and C in Results as examples). Acoustic stimulus bursts (both near field and far field) were 30 ms in duration, and bursts were delivered at every 1 or 2 seconds respectively during each trial. Low frequency sounds (300 to 3000 Hz) were amplified using TDT SA1 amplifier (Tucker-Davis Technologies) and were delivered using a generic woofer. High frequency sounds (4000 to 25,000 Hz) were delivered using a generic tweeter. Due to the exploratory nature of these studies, stimulus amplitude values were not measured. Speakers were placed 30 cm from the specimen during far field and low frequency near field experiments, and 3 cm during high frequency near field experiments (while there are currently no known insect species that detect high frequency near field signals, the rationale behind these trials was to playback the dominant frequency range of *D. valens* signals (see Fig. 3.3B)).

Vibration stimuli were designed the same way as airborne sounds (see above), but with 100 ms bursts every second for the first round of experiments, and 30 ms bursts every 1 or 2 seconds for the rest. Signals were amplified by a B&K Type 2718 power amplifier, and were delivered using a B&K Type 4810 mini-shaker (Bruel and Kjaer, Denmark). A wooden rod (4 mm diameter) with a narrowed wax coated tip (1 mm diameter) was attached to the shaker; the tip was the part that was directly in contact with the beetles.

## Data analysis

Experiments were monitored live using a TDS2002C oscilloscope (Tektronix, Inc., Beaverton, OR, USA) and an AM systems audio-monitor (AM Systems). The recordings were viewed and analyzed using custom made MatLab (MathWorks Inc., Natick, MA, USA) scripts that were written in collaboration with Conrado Denadai. Recordings made by the Tucker Davis equipment did not have high enough sampling rate to record the full shape of stimulus bursts at higher frequencies (see for example Fig. 3.6D), however those were verifiable on the wave files recorded by the Fostex data recorder. Stimulus artefacts were identified on the neural channel of the recordings from their shape, number of peaks, and/or duration being seemingly identical to the stimulus, if their amplitude appeared to change linearly with the stimulus amplitude, or if their frequency was close to identical to the stimulus when listening to the recording. After initial observation, recordings were deemed successful if action potentials with amplitudes at least twice the background noise were observable, and if the number of identifiable artefacts were low or were easy to remove by deleting part of the recording.

In order to minimize both subjectivity and false positive results, two independent analyses were run for every successful recording, referred to as T-test 1 and T-test 2. For both analyses, stimulus beginning and end times were identified using the stimulus information recorded by Open Ex and by using the "findpeaks" MatLab function on the stimulus channel of the recordings. Different parameters were measured on the neural channel for the two tests. For T-test 1, the average amplitude before and during stimulus was compared for each stimulus burst in order to see if there is a change in activity that might not be detectable above the background noise amplitude. The neural channel's amplitude values for the duration of each stimulus burst were averaged individually for each sample. A similar set of average amplitude values were gathered from 30 or 100 ms (equal to stimulus burst duration) before the beginning of each stimulus, with a time window identical to the stimulus burst duration. The two sets of samples were then tested for significant differences using a paired T-test. For T-test 2, the number of spikes above the background noise were counted for 100 ms-s after the stimulus start time, and were compared to the number of spikes during the same time window in-between stimulus bursts. The neural spikes above a certain threshold (identified for each recording based on the observable noise or stimulus artefact amplitude) were identified using the "findpeaks" MatLab function. The number of neural spikes within 100 ms after the beginning of each stimulus burst was then compared to the number of neural spikes within the same time window exactly in between stimulus bursts using a two sample T-test. T-test 1 and T-test 2 were run for each stimulus frequency within each successful recording. The two tests were designed to compensate for each other's faults: T-test 1 tested for changes in the baseline activity during stimulus bursts, but was prone to false positive results when

stimulus artefacts were present in the neural recording. T-test 2 was more subjective due to the arbitrary way of choosing the threshold amplitude, but was not as sensitive to stimulus artefacts. As such, a response was only confirmed if both tests returned positive results consistently at a certain frequency or in a certain frequency range.

Visualization of the average activity during, before and after the stimulus was done by compiling the sampled neural traces with a time window 3 times the duration of the stimulus burst (see figures in Results).

## 3.3 Results

### Thoracic nerves

Fourteen beetles were successfully dissected and tested for neural response, producing 41 useful recordings from the various nerves and connectives tested. No response was observed in response to the stimulus during any of the recording sessions. Results of T-test 1 and T-test 2 analyses are summarized below. For a complete list of all T-test 1 and T-test 2 results, see Appendix 3.

#### Metathoracic nerve

Eight successful recordings were done on the metathoracic nerve of 6 beetles (Fig. 3.6). Far field sound stimulus frequency varied between 300 Hz and 15,000 Hz. All T-test 1 results showed no significant difference in the neural activity before and during the stimulus. All except one T-test 2 results ($p<0.05$ for 1 out of 3 7,000 Hz trials) showed no significant differences between the number of spikes during and in-between stimulus bursts.

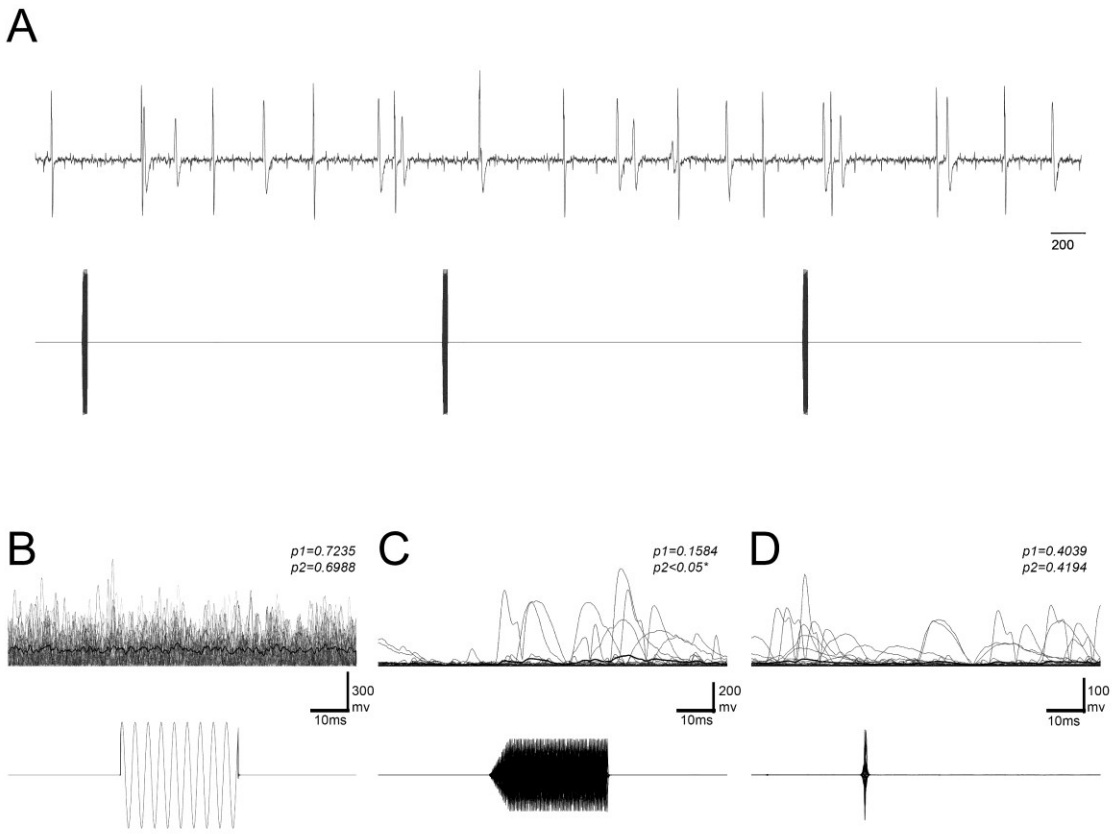


**Figure 3.6** Oscillograms of electrophysiological recordings from the metathoracic nerve of *D. valens* during far field sound stimulus. A: 6 seconds of a recording showing the neural activity (top) and 3 7,000Hz stimulus bursts (bottom). B,C,D: Compiled neural activity before, during and after 35 300 Hz (B), 22 7,000 Hz (C) and 31 1,5000 Hz (D) stimuli (full shape of stimulus bursts in D are not visible due to low sampling rate). Thick black line shows averaged neural activity of compiled recording samples. p1: p value of T-test 1 p2: p-value of T-test

Pro-mesothoracic connective

Nine successful recordings were performed on the connective of 7 beetles (Fig. 3.7). Stimulus frequency varied between 300 Hz and 25,000 Hz. All T-test 1 results showed no significant difference in the neural activity before and during the stimulus. All except one T-test 2 results ($p<0.05$ at 7,000 Hz for 1 out of 4 trials) showed no significant differences between the number of spikes during and in-between stimulus bursts. One

additional field potential recording was made from the area of the prothoracic ganglion and the connective (legs were removed). This trial had a positive T-test 1 result at 10000Hz ($p<0.05$), however, T-test 2 results were negative for all frequencies.

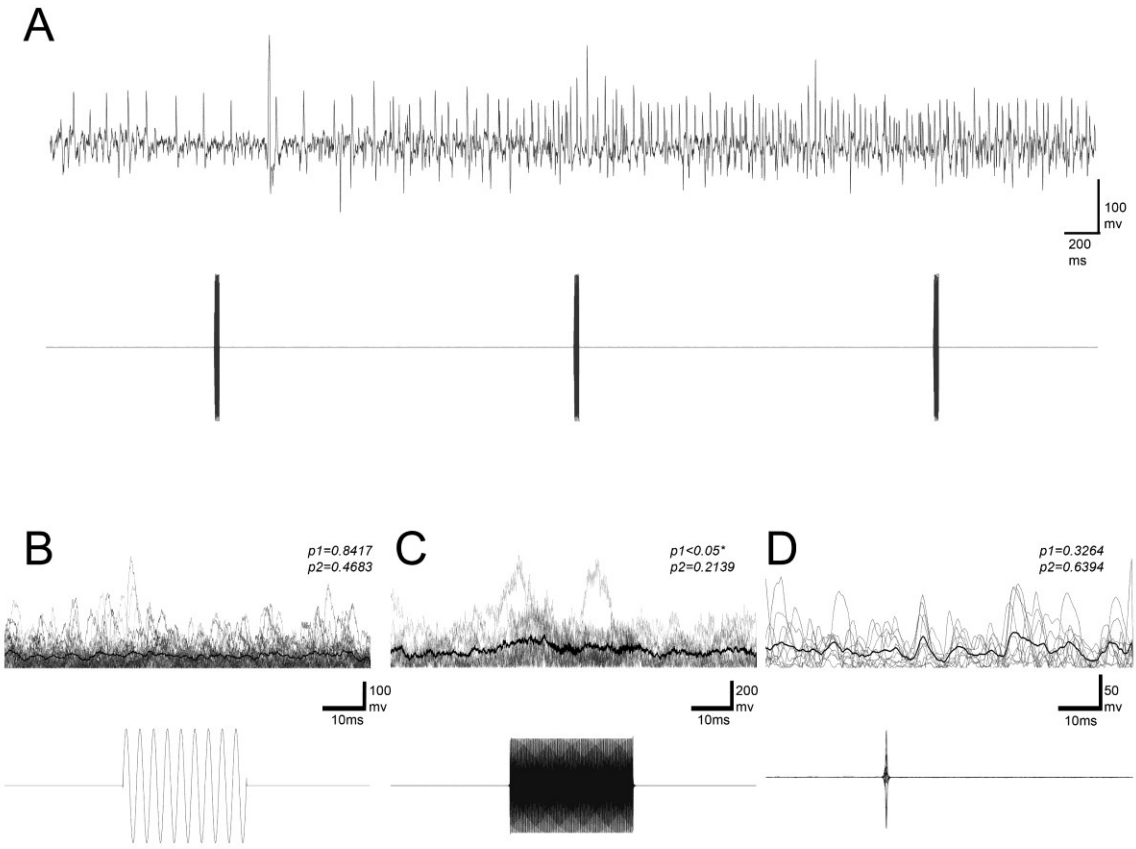


**Figure 3.7** Oscillograms of electrophysiological recordings from the pro-mesothoracic connective of *D. valens* during far field sound stimulus. A: 6 seconds of a recording showing the neural activity (top) and 3 7,000 Hz stimulus bursts (bottom). B,C,D: Compiled neural activity before, during and after 30 300 Hz (B), 12 7,000 Hz (C) and 10 25,000 Hz (D) stimuli (full shape of stimulus bursts in D are not visible due to low sampling rate). Thick black line shows averaged neural activity of compiled recording samples. p1: p value of T-test 1 p2: p-value of T-test 2

Neck connective

Exposing the neck connective proved to be one of the most difficult dissection tasks (see Appendix 2), which resulted in a low number of successful recordings. Only 3 successful recordings were done using hook electrodes, however, field potential recordings were also made from the neck area of 4 beetles using tungsten needle electrodes inserted in the segment directly posterior to the head. This added up to a total 7 recordings from the proximity of the neck connectives (Fig. 3.8). Stimuli included far field sound between 2000 and 15,000 Hz, vibration stimuli between 100 and 1, 000 Hz, and near field sound at 4,000 and 10, 000Hz. T-test 1 results for 4 frequencies showed significant difference in the neural activity before and during the stimulus at 100 Hz ($p<0.001$ for 1 out of 1 trial), 300 Hz ($p<0.001$ for 1 out of 2 trials) 500 Hz ($p<0.001$ for 1 out of 2 trials) vibration and 7,000 Hz ($p<0.05$ for 2 out of 5 trials) far field sound stimulus. It is important to note that low frequency vibrations almost always resulted in stimulus artefacts on the neural recordings that compromised T-test 1 results. All T-test 2 results showed no significant differences between the number of spikes during and in-between stimulus bursts.

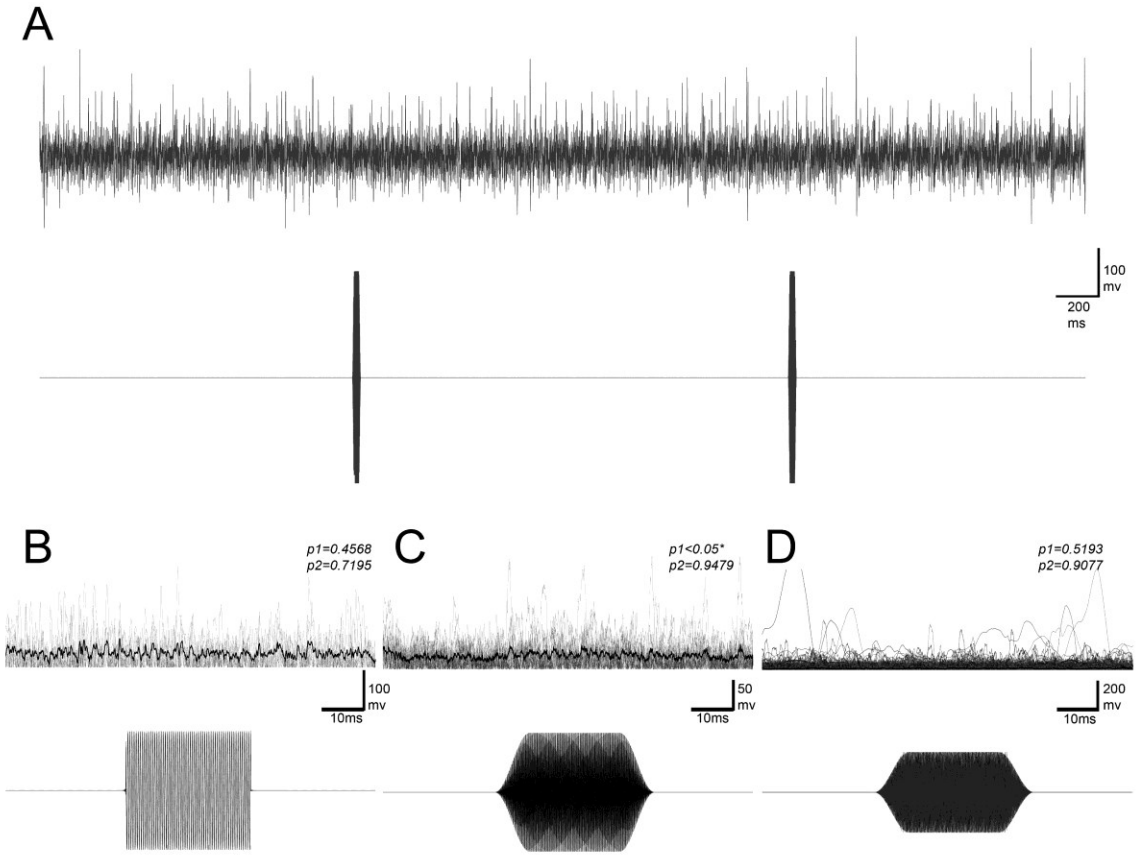


**Figure 3.8** Oscillograms of electrophysiological recordings from the neck connective of *D. valens* during far field sound stimulus. A: 4 seconds of a recording showing the neural activity (top) and 2 10,000 Hz stimulus bursts (bottom). B,C,D: Compiled neural activity before, during and after 9 2,000 Hz (B), 15 7,000 Hz (C) and 42 15,000 Hz (D) stimuli. Thick black line shows averaged neural activity of compiled recording samples. p1: p value of T-test 1 p2: p-value of T-test 2

Area of the putative tympanal membrane

After no response was observed during the recording sessions on the metathoracic nerve and the connectives, 2 additional recordings were made using coated and uncoated tungsten needle electrodes inserted into the close proximity of the putative tympanal membrane on the metathorax. Far field stimuli were presented at 7000, 17000 and 20000Hz. Both T-test 1 and T-test 2 results were negative for all frequencies.

Other nerves in the thoracic area

None of the other nerves tested had positive T-test 1 or T-test 2 results in any frequencies. These results are summarized in Appendix 3.

## Legs

Vibration stimuli during field potential recordings from the legs caused clearly identifiable stimulus artefacts even at low amplitudes, especially at lower frequencies (see Fig. 3.9B for example), which often compromised T-test 1 results. No response was observed in any frequencies on the audio-monitor or the oscilloscope during recording sessions. The T-test 1 and T-test 2 results for each pair of legs are summarized below. For a complete list of all T-test 1 and T-test 2 results, see Appendix 3.

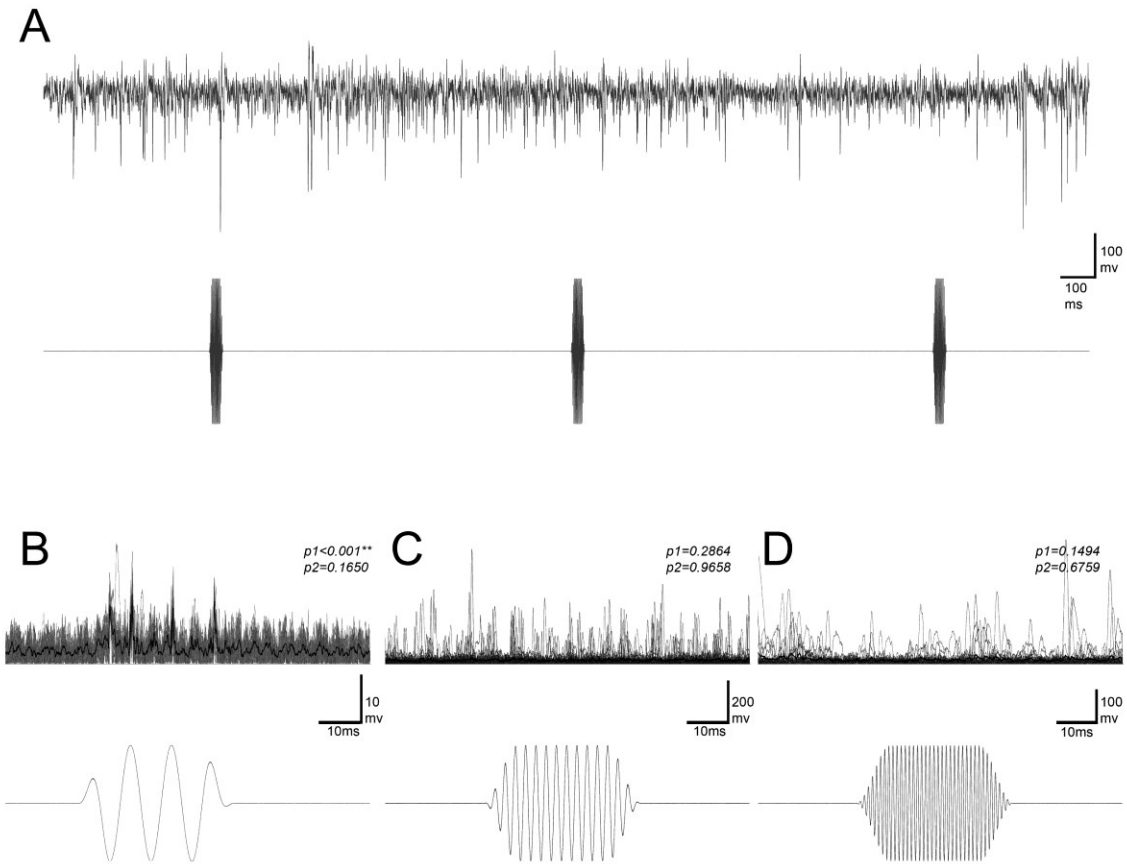


**Figure 3.9** Oscillograms of electrophysiological recordings from the prothoracic leg of *D. valens* during vibration stimulus. A: 4 seconds of a recording showing the neural activity (top) and 3 400 Hz stimulus bursts (bottom). B,C,D: Compiled neural activity before, during and after 26 100 Hz (B), 40 400 Hz (C) and 24 1,000 Hz (D) stimuli. Thick black line shows averaged neural activity of compiled recording samples. p1: p value of T-test 1 p2: p-value of T-test 2

Prothoracic legs

Nine successful recordings were made from 6 different beetles (Fig. 3.9). 12 out of 22 trials resulted in highly significant ($p<0.001$) T-test 1 results but only 1 out of 22 T-test 2 trials were positive ($p<0.001$ for 1 out of 3 200 Hz trials).

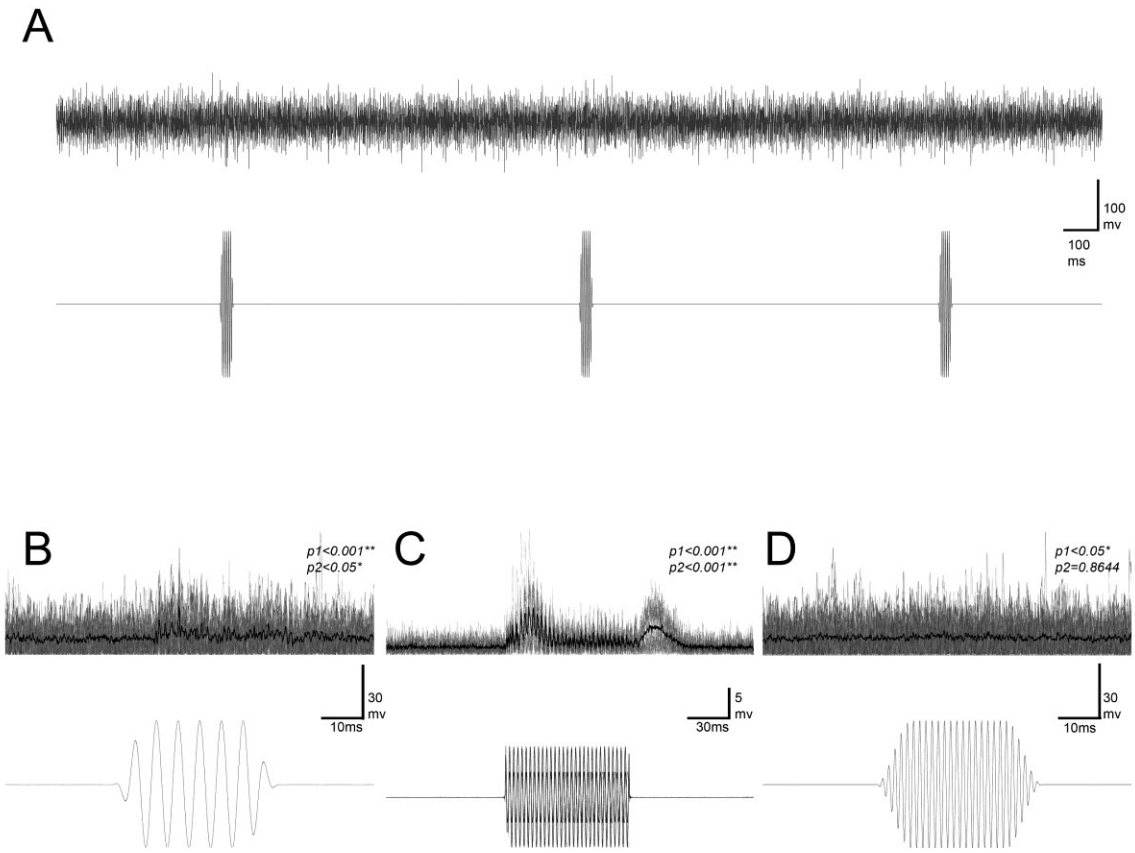


**Figure 3.10** Oscillograms of electrophysiological recordings from the mesothoracic leg of *D. valens* during vibration stimulus. A: 4 seconds of a recording showing the neural activity (top) and 3 200 Hz stimulus bursts (bottom). B,C,D: Compiled neural activity before, during and after 26 200 Hz (B), 18 300 Hz (C) and 39 700 Hz (D) stimuli. Thick black line shows averaged neural activity of compiled recording samples. p1: p value of T-test 1 p2: p-value of T-test 2

Mesothoracic legs

Five successful recordings were made from 3 different beetles (Fig. 3.10). Three out of 14 trials resulted in highly significant ($p<0.001$), while 3 others resulted in significant ($p<0.05$) T-test 1 results. Two out of 14 T-test 2 trials were positive ($p<0.001$ for 1 out of 1 300 Hz trial and $p<0.05$ for 1 out of 1 200 Hz trial). Double positive results were observed at 200Hz and 300Hz.

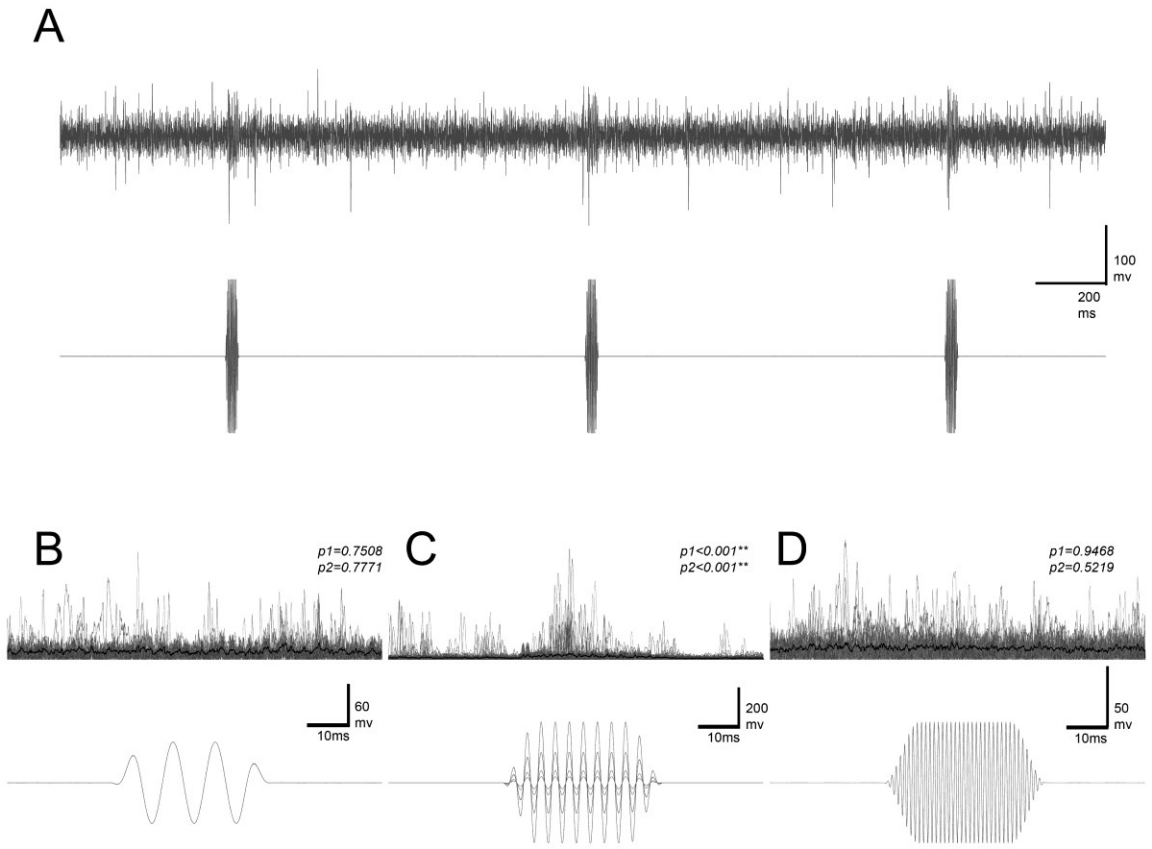


**Figure 3.11** Oscillograms of electrophysiological recordings from the metathoracic leg of *D. valens* during vibration stimulus. A: 4 seconds of a recording showing the neural activity (top) and 3 300 Hz stimulus bursts (bottom). B,C,D: Compiled neural activity before, during and after 23 100 Hz (B), 86 300 Hz (C) and 27 1,000 Hz (D) stimuli. Thick black line shows averaged neural activity of compiled recording samples. p1: p value of T-test 1 p2: p-value of T-test 2

Metathoracic legs

Five successful recordings were made from 5 beetles (Fig. 3.11). Five out of 18 trials resulted in highly significant ($p<0.001$), while 1 resulted in significant ($p<0.05$) T-test 1 results. Three out of 18 T-test 2 trials were positive ($p<0.001$ for 3 out of 3 300 Hz trials). This was the only time when multiple independent trials from different beetles resulted in all positive, highly significant results at a certain frequency.

## Antennae

A total 7 beetles were tested and 9 successful recordings were made. Low frequency stimulus caused distinguishable stimulus artefacts on the majority of recordings, possibly making most of the T-test 1 results for low frequencies invalid. Low (1,000 Hz and below) and high frequency (4,000 Hz and above) results are therefore presented separately. For a complete list of all T-test 1 and T-test 2 results, see Appendix 3.

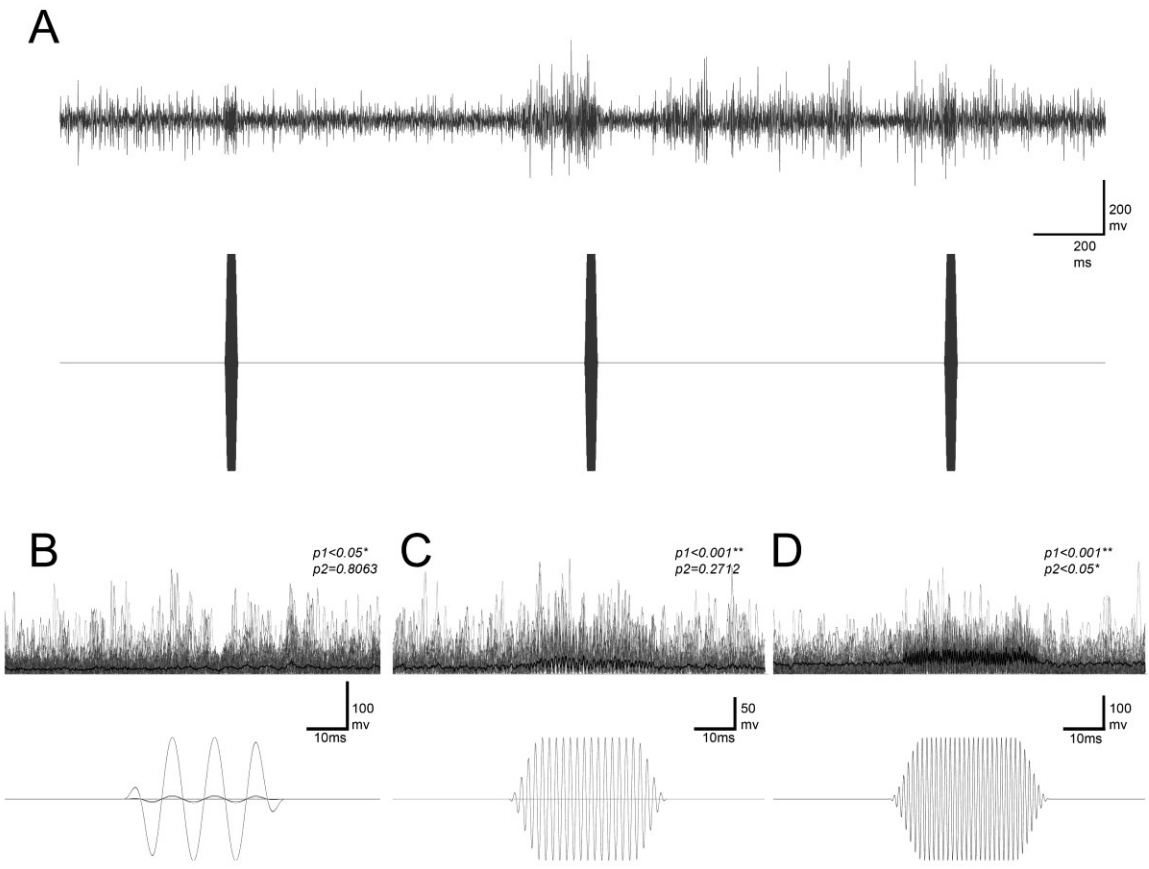


**Figure 3.12** Oscillograms of electrophysiological recordings from the antenna of *D. valens* during low frequency near field sound stimulus. A: 4 seconds of a recording showing the neural activity (top) and 3 900 Hz stimulus bursts (bottom). B,C,D: Compiled neural activity before, during and after 57 100 Hz (B), 34 600 Hz (C) and 39 900 Hz (D) stimuli. Thick black line shows averaged neural activity of compiled recording samples. p1: p value of T-test 1 p2: p-value of T-test 2

Low frequency

Three recordings from 2 beetles were made while stimulating with low frequency near field sound (Fig. 3.12). Nineteen out of 26 T-test 1 trials were positive (14 highly significant ($p<0.001$) and 5 significant ($p<0.05$)). Four out 26 T-test 2 trials were positive ($p<0.05$ for 1 out of 2 800 Hz trials, 2 out of 3 900 Hz trials, and 1 out of 3 1,000 Hz trials). Repeated double positive results were observed at 900 Hz.

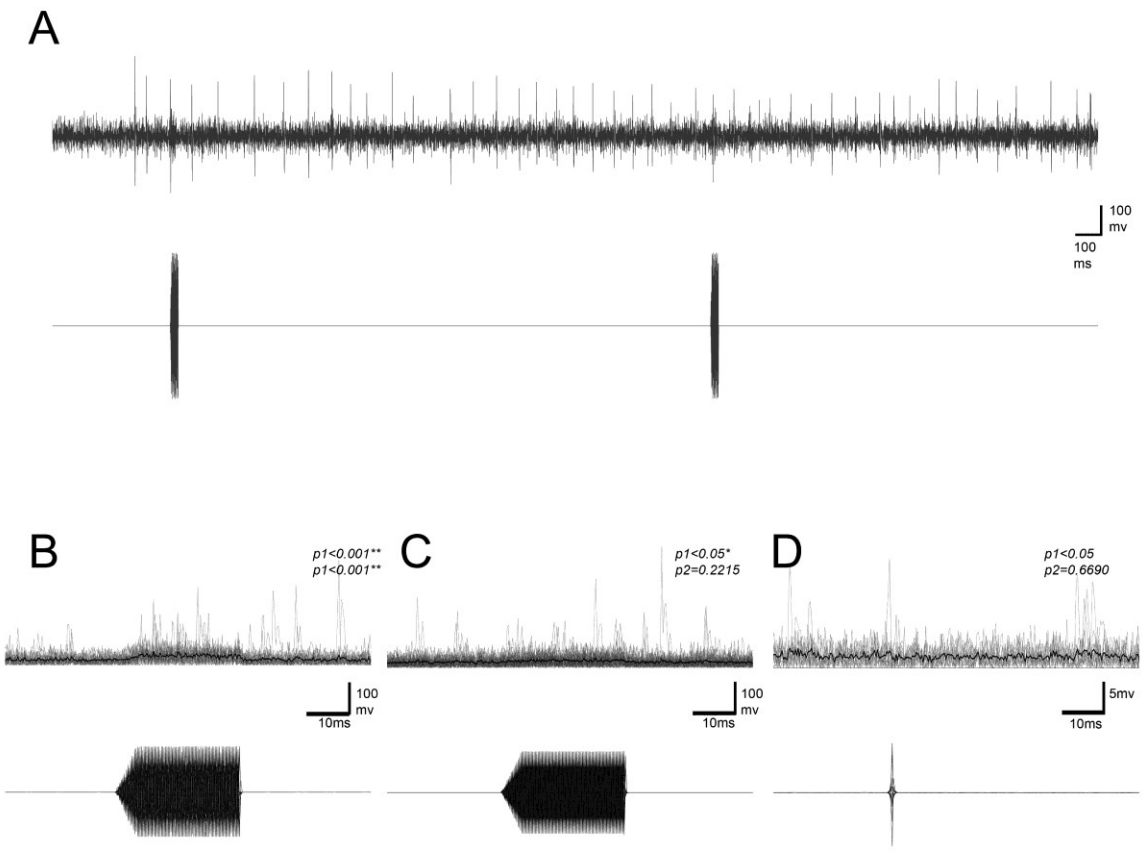


**Figure 3.13** Oscillograms of electrophysiological recordings from the antenna of *D. valens* during high frequency near field sound stimulus. A: 5 seconds of a recording showing the neural activity (top) and 2 4,000 Hz stimulus bursts (bottom). B,C,D: Compiled neural activity before, during and after 26 4,000 Hz (B), 43 7,000 Hz (C) and 13 20,000 Hz (D) stimuli. Thick black line shows averaged neural activity of compiled recording samples. p1: p value of T-test 1 p2: p-value of T-test 2

High frequency

Six recordings were made from the antennae of 5 beetles (Fig. 3.13). Three out of 20 trials showed highly significant ($p<0.001$) and 2 others showed significant ($p<0.05$) T-test 1 results. Only 1 trial resulted in positive T-test 2 results ($p<0.05$ for 1 out of 3 4000Hz trials). Double positive result was observed for 1 out of 3 4,000 Hz trials.

## 3.4 Discussion

Acoustic reception of bark beetles was assessed for the first time by using extracellular neurophysiology to test for sensory responses in the thoracic nerves and connectives, the legs and the antennae to various forms of acoustic stimuli. The recordings were analyzed using two independent analyses, T-test 1 and T-test 2. Based on the results of these analyses, the following conclusions were made on *D. valens'* ability to detect different forms acoustic vibrations:

### Far field sound reception

Prior to the experiments, far field sound was believed to be the most probable form of vibration the beetles could detect, based on the acoustic characteristics of the bark beetle sounds and because of the putative tympanal membrane that was found in 3 acoustic bark beetle species (Fig. 3.2B) (Fleming, 2010; Sivalinghem, 2011). While this membrane did not appear to have ideal displacement characteristics for a tympanal membrane (see Appendix 1 on laser vibrometry experiments on *I. pini*), the first set of experiments was focused on the metathoracic nerve that was found to innervate the membrane area, and the connectives that were afferent to it (Fig. 3.5). While a number of trials did have positive results for either T-test 1 or T-test 2 at 7,000 Hz (which is one of

the peak frequencies of *D. valens* chirps (Fig. 3.3), no consistent response was observed or measured on any frequencies either on the metathoracic nerve, or the connectives. Additional field potential recordings from the membrane area were also negative. While these results suggest that putative tympanum is not a tympanal organ, this possibility cannot be fully ruled out at this point. Future histological studies might reveal the presence of chordotonal organs associated with the membrane, which would validate further studies, including single unit recordings from the metathoracic nerve and ganglion. Far field receptors in insects are not always easy to locate and are often not morphologically obvious. For example, the tympanum of scarab beetles was found hidden behind the head (Forrest et al., 1997), and the ears of lacewings and mantids are also difficult to observe (Miller, 1970; Yager and Hoy, 1987). It is possible that bark beetles have similar 'cryptic' ears in a less obvious region of the body.

## Near field sound reception

While there are no documented cases of coleopterans detecting signals in the acoustic near field, Johnston's organs (typical near field receptors in insects) are known to be present in the order (Henrikson and Stenson, 1992), and the close proximity of bark beetles during acoustic communication leaves the possibility open that it is the displacement, rather than the pressure component of the chirps that the beetles detect. Recordings directly from the base of the antennae showed no consistent response to near field stimuli in the dominant frequency range of *D. valens*, however, double positive results were seen between 800 and 4,000 Hz including repeated double positive responses at 900 Hz. These results suggest that while communication through near field sounds is unlikely for these beetles, other forms of near field signals might be detected through the

antennae. Further studies are recommended on the effects of low frequency near field stimuli on the neural activity, as well on the behaviour of these beetles.

## Substrate-borne vibration reception

Detection of substrate-borne vibrations is universal among insects and many species are known to use vibrations to communicate (Yack, 2016). It was shown that most of the important temporal characteristics of bark beetle sounds are translated into the vibration component measured on the phloem surface during stridulation (Fleming et al., 2013; Lindeman, 2016), making it possible for these beetles to communicate through the substrate or through direct contact. For this reason, all 3 pairs of legs were tested for vibration reception. The analysis showed a consistent neural response in the metathoracic leg at 300 Hz, with highly significant double positive test results in all trials. This was the only consistent and confident positive result in all the neurophysiology experiments. Other double positive results were gathered from the middle leg as well at 200 and 300 Hz; these trials, however, were not repeated. These results suggest that there is a vibration receptor in at least one pair of legs in *D. valens* tuned around 300 Hz. However, while there is no documented information on the frequencies of *D. valens* chirps' vibration component, based on studies in *D. ponderosae* (9-70 kHz for airborne sounds, 4-18 kHz for substrate-borne vibrations; Fleming et al., 2013) it is hypothesised that the dominant frequency would be higher than 300 Hz. Further studies are therefore needed to clarify the function of this putative receptor. Anatomical and histological studies on the metathoracic leg should be performed to narrow down the location of vibration receptors and their afferent nerve. Single cell and intracellular recordings then would allow for the

construction of a tuning curve and would enable further characterization of the sensory response.

The neurophysiological studies described in this chapter did not answer the question of how bark beetles detect the chirps of conspecifics. However, they were able to cast some doubt on previous assumptions on far field sound reception and a putative ear. The results also provide a glimpse of what seems to be a much more complex acoustic sensory system than previously believed, possibly involving both near field and substrate-borne vibration receptors, suggesting that bark beetles might be capable of detecting all three kinds of acoustic vibrations. Furthermore, through the repeated trials, multiple protocols were developed for bark beetle electrophysiological experiments, that can be applied in future investigations.

# Chapter 4 General discussion

## 4.1 Introduction

Bark beetles have been referred to as "the greatest threat to North American forests" (Hopkins, 1909) for the past century, and as such, their ecology and chemical communication has been extensively studied (Coulson, 1979; Raffa, 2001; Raffa et al., 2008, Vega and Hofstetter, 2014). Through these studies, our knowledge on this subfamily has grown to great extent, and by applying this knowledge, efficient ways have been developed to control their populations, and fight against the so called "bark beetle epidemic" of the 21st century (Kayes and Tinker, 2012). Yet, even though sound producing behaviour has been described since the earliest modern descriptions of multiple important species (Barr, 1969), this form of communication did not get as much attention. Because of this, there are important questions on bark beetle acoustic ecology still unanswered to this day: 1) How taxonomically widespread is acoustic communication among bark beetles? 2) What are the characteristics of bark beetle sounds and how complex is their acoustic repertoire? 3) How do bark beetles detect acoustic signals from conspecifics and the environment? Finding the answers to these questions is important from both a scientific, and environmental perspective. For example, it could be possible in the future to use species specific acoustic pest control systems to control the population growth of dangerous bark beetle species, some of which are already under development (Hofstetter et al., 2014; Lindeman, 2016). In this thesis, the aforementioned questions on bark beetle acoustic communication were assessed using the most current literature, the most widely used acoustic and electrophysiological techniques, as well as modern statistical methods and novel quantitative analyses.

## 4.2 Sound production in bark beetles

While acoustic communication did not get as much attention as the chemical communication, a considerable amount of literature has been published on acoustic signalling in the past century. However, no general review on bark beetle acoustic communication has been written since 1969 (Barr, 1969). The goal of the first chapter of this thesis was therefore to look at the literature published since the latest review in order to summarize the current general knowledge on bark beetle acoustic ecology, focusing on the taxonomic distribution of sound production. It was found that currently there are 107 bark beetle species known to possess sound producing organs. The findings of the papers reviewed also suggest that the previous assumption that all acoustic bark beetles possess one of the three main types of stridulatory organs (elytro-tergal, gula-prosternal and vertex-pronotal) is false, as some species seem to have multiple different types of organs on the same individual (Sasakawa and Yoshiyasu, 1983), and other species were found to have previously unknown types of sound producing organs (Kerchev, 2015). What is also noticeable in the literature is that many bark beetle species have never been examined for stridulatory organs, and that only a handful of species that possess sound producing mechanisms were actually recorded producing sounds. This again shows the enormous gap in our current knowledge on acoustic communication in the subfamily Scolytinae. Future studies need to work on filling this gap by examining more species for stridulatory organs, and by recording and analyzing the chirps of more acoustic bark beetles in order gain information on the acoustic characteristics and possible functions of acoustic signals. This would be especially important for species that possess multiple different stridulatory organs, as the acoustic ecology of these species could be much more complex than

previously believed possible for bark beetles. It would also be important to investigate acoustic communication in the larvae, as currently all information on Scolytinae acoustics is from the adult stage. Once we have a more complete picture on the distribution of sound production, other studies could begin to focus on the evolution of stridulation in Scolytinae, and answer questions about the origins of this form of communication in the subfamily as well as the number of times it has evolved in the different tribes and genera.

## 4.3 Signal characteristics and context dependent signalling

Many acoustic insects, including bark beetles have been described to produce sounds in different behavioural contexts (Alexander, 1961; Gerhardt and Huber, 2002; Stölting et al., 2004; Guerra and Mason, 2005; Conrad et al., 2010; Balakrishnan, 2016). Whether these signals are different in their temporal, spectral or amplitude characteristics however, is often not tested in an objective, quantitative way. In order to confirm context dependent signalling in a certain species, it is important to identify these differences between the signals and not rely solely on the behavioural information. A number of assumptions have been made by previous papers on bark beetle signals being context dependent, often without proper analysis (Michael and Rudinsky, 1972) or with questionable methods (Swaby and Rudinsky, 1976). In the second chapter of this thesis, context dependent signalling was examined in *Ips pini*. Using modern statistical methods and a newly developed quantitative analysis of amplitude envelope shapes, this project was able to confirm context dependent signalling in *I. pini* by showing that pre-mating chirps are significantly longer in duration, and more often have descending envelope shapes than distress or predation chirps. On the other hand, it showed that previous distinction of interrupted and non-interrupted (simple) chirps (Swaby and Rudinsky,

1976) is not quantitatively justified. It was able to show that previous assumptions need quantitatively assessed in order to verify or contradict them. The results also suggest that there are differences in the complexity of communication systems between the different acoustic bark beetle species, as previous studies have shown that interrupted chirps are indeed distinguishable from simple chirps in two *Dendroctonus* species, and that their ratio within chirp trains is context dependent (Fleming et al., 2013; Lindeman and Yack, 2015). Future studies could determine the reasons behind these differences between species, and whether it is just a direct result of the differences between the sound producing mechanisms, or if the two represent distinct evolutionary stages. This study was intended to be a model for future acoustic analysis in other acoustic bark beetle species and acoustic insects in general.

## 4.5 Acoustic reception

Insects are capable of detecting acoustic vibrations in various forms, using several different kinds of receptor organs ranging from simple setae to complex tympanal ears (Mason and Pollack, 2016). Not all acoustic insect groups have received the same amount of attention from researchers when it comes to hearing. In the order Coleoptera for example, only two groups have been described to possess typmpanal ears (Spangler, 1988; Forrest et al., 1997), and only one genus has been described to use Johnston's organs to detect vibrations (Henrikson and Stenson, 1992). This is also one of the two insect orders (beside Diptera ) that is yet to be described to possess subgenual organs (Yack, 2016). Within Coleoptera, bark beetles are one of those acoustic groups where there is no information on acoustic reception. This leaves open the possibility that these beetles, while their communication is detectable in the far field, could be detecting

acoustic signals as either substrate-borne vibrations or near field sounds. In the third and final chapter of this thesis, a series of extracellular electrophysiological experiments were carried out on *Dendroctonus valens*, in order to assess bark beetles' capability to detect different forms of vibrations, as well as to explore possible locations of acoustic reception. Two independent quantitative analysis methods, T-test 1 and T-test 2 were developed in order to minimize both subjectivity and false positive results.

Three main areas were examined. The first series of experiments focused on the thoracic nerves and connectives. Special attention was given to a metathoracic nerve that was previously identified as a possible acoustic afferent (Gall-Duncan, 2015). The analysis failed to show any consistent response to far field sound stimuli on any of the tested frequencies. However, the occasional, partially positive results around the peak frequency of *D. valens* sounds make the results inconclusive, and the possibility is still open for far field reception. Further anatomical and histological studies are needed to narrow down the number of possible locations for receptors in order to use more sensitive analyses, such as single unit or intracellular measurements that could better detect more subtle responses. The second series of experiments tested for responses to substrate-borne vibration stimuli in the three pairs of legs of *D. valens*. These tests were able to show a consistent neural response in the metathoracic leg at 300 Hz. As this was the most promising result out of all the experiments, further studies on this possible vibration receptor in the metathoracic leg should be highly encouraged. Recordings from the ischadic nerve, as well as well histological studies on the metathoracic leg should be among the first steps regarding future research. Lastly, the antennae of the beetles were tested for both low and high frequency near field stimulus. While these beetles have

never been shown to rely on near field stimuli, it appears that there might be a sensory response in the antenna at around 900 Hz, although the results are not conclusive. The possible function of this putative Johnston's organ is currently unknown. Further experiments are needed, as well as perfection of the experimental protocols in order to reduce stimulus artefacts on neural recordings.

While the predictions on far field acoustic reception were neither confirmed, nor were fully denied by these experiments, other interesting aspects of bark beetle acoustic reception seemed to have started to surface as a result of this highly exploratory study. It is possible that the acoustic reception system of Scolytinae species are far more complex and unique than previously thought, which is yet another reason why further research in this field is so necessary.

## 4.6 Conclusions

The ultimate goal of this thesis was to address some important questions regarding bark beetle acoustics. Through the three chapters, the three main problems: the distribution of sound production, the characteristics of sounds, and acoustic reception were discussed, with hopes that it will start a conversation and inspire future research. It is important to notice that all three studies introduced in this thesis were able to contradict previous assumptions on bark beetle acoustics, some of which have been around for decades. It appears that the subfamily Scolytinae show a much greater variety in both sound producing organs, signal repertoire, and acoustic receptor systems than previously thought possible. All of this shows that one should be very carful assuming anything regarding these beetles, and instead, they are encouraged to truly "look under the bark".

# Appendix 1: Laser vibrometry measurements of the *I. pini* metathoracic structure

## A1.1 Introduction

Tympanal membranes have been described in various insect species, with varying complexity and ability to distinguish between frequencies (Yack, 2004). For example, the tympanal ear of the migratory locust is able to function as both the organ of sound reception and frequency discrimination (Windmill et al., 2005), while nocturnal moths are unable to recognize different frequencies (Windmill et al., 2007). Coming to such conclusions regarding the tympanal membrane characteristics of these insects was made possible partially by the use of microscanning laser Doppler vibrometry (Windmill et al., 2005; Sueur et al., 2006; Lucas et al., 2009). With this technique, the vibration velocity of the resonating membrane is being measured coherently on the whole surface of the membrane, as opposed to other types of laser vibormetry where only one specific point is measured. This allows us to see how the different regions of the membrane reacting to sound waves. In the observed species, it is generally seen that at certain sound frequencies, the tympanal membrane propagates the vibration to one or a few specific points, giving maximal displacement responses at the locations where the chordotonal organs attach to the membrane (Windmill et al., 2005). With this knowledge, we are also able to test putative tympanal membranes to assess whether they react accordingly to relevant frequencies.

## A1.2 Methods

To assess whether the methathoracic membrane of bark beetles can vibrate in response to biologically relevant sound stimuli, I measured the displacement response to

a range of sound frequencies using a microscanning laser Doppler vibrometer, located in the University of Toronto Scarborough Campus, with the assistance of postdoctoral research fellow Natasha Mhatre.

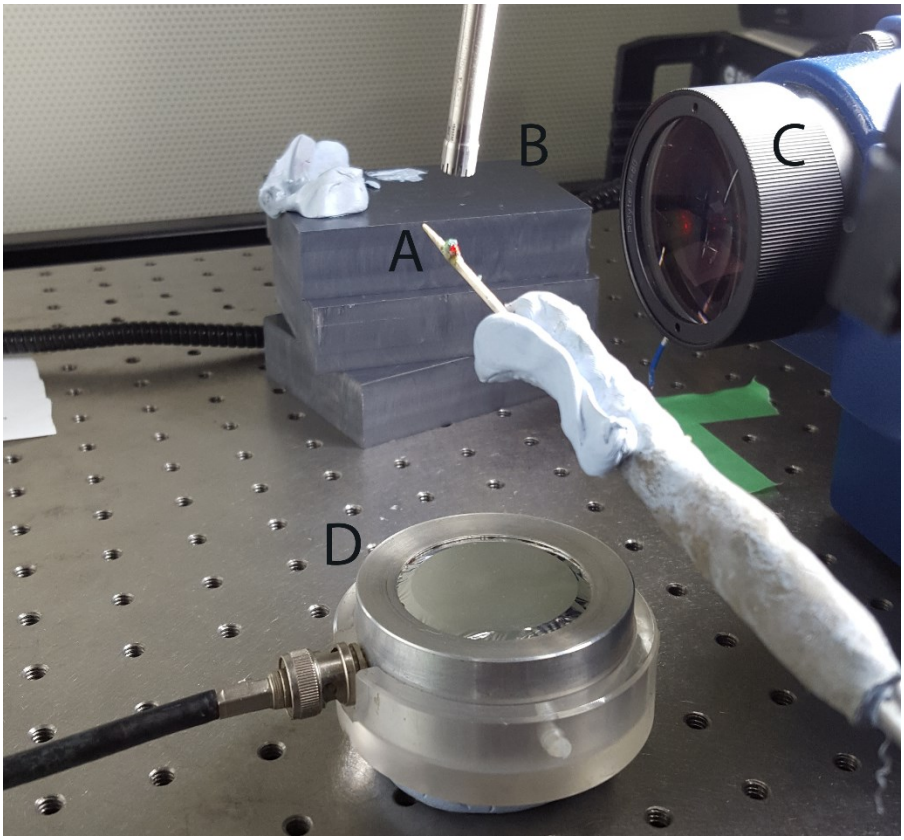


**Figure A1.1** Experimental setup for laser vibrometry measurements. A: *Ips pini* specimen fixed on toothpick; B: Microphone; C: Laser vibrometer closeup lens; D: Membrane speaker

Experiments were conducted on 9 specimens of *I. pini* (5 males and 4 females) using a Polytec PSV-400 laser vibrometer equipped with a PSV-410 close-up lens. The specimens were mounted on wooden toothpicks and fixed along the ventral surface using a mixture of beeswax and rosin. The dorsal surface of the abdomen and both elytra were left uncovered. To expose the putative ear, the elytra and the wings were removed prior to measurements, except for one experiment where the elytral response was measured

before removal. The mounted beetles were fixed on a micromanipulator using Blu-tack, and were positioned so that the putative tympanic membrane surface was in focus and perpendicular to the laser beam (Fig. A1.1A). The experimental setup also included a Bruel and Kjaer 4939 ¼ inch microphone connected to a Bruel and Kjaer 2231 SPL meter AC output, placed above the beetle for feedback analysis (Fig.A1.1B), and a speaker under the specimen facing both the beetle and the microphone (Fig.A1.1D). Separate measurements were made for lower (2.5-20 kHz) and higher (20-80 kHz) frequencies. For lower frequencies, a generic tweeter and a Realistic SA-10 solid state amplifier were used, while for higher frequencies we used a custom built ultrasonic amplifier and membrane speaker. After the laser was focused and calibrated properly, we selected the scanning surface and set up the frequency parameters and corrections for the speaker. The sound volume was set to 40 mPa at all frequencies. We used pure tones for all frequencies. We measured both the sound pressure level of the acoustic stimulus (known as a periodic chirp) and the velocity of the membrane moving when driven by this sound. From this data, the transfer function of the membrane (i.e. displacement per unit sound pressure) at each frequency was then calculated.

## A1.3 Results and Discussion:

The displacement of the putative ears was between 0.254 pm/Pa and 2.34 nm/Pa, with a general higher response between 2.5 and 7 kHz, and a highly inconsistent response in other frequencies. There was no observable difference in the displacement response between the putative ear and other cuticle surfaces. In 8 out of 9 experiments, there was no observable differentiation in the displacement between the different parts of the putative ear, as it can be observed in specialized tympanal membranes of other species

(Windmill et al., 2005). While the beetles were unable to move their abdomen, rapid deflation-inflation movements of the abdomen were observed during all experiments.

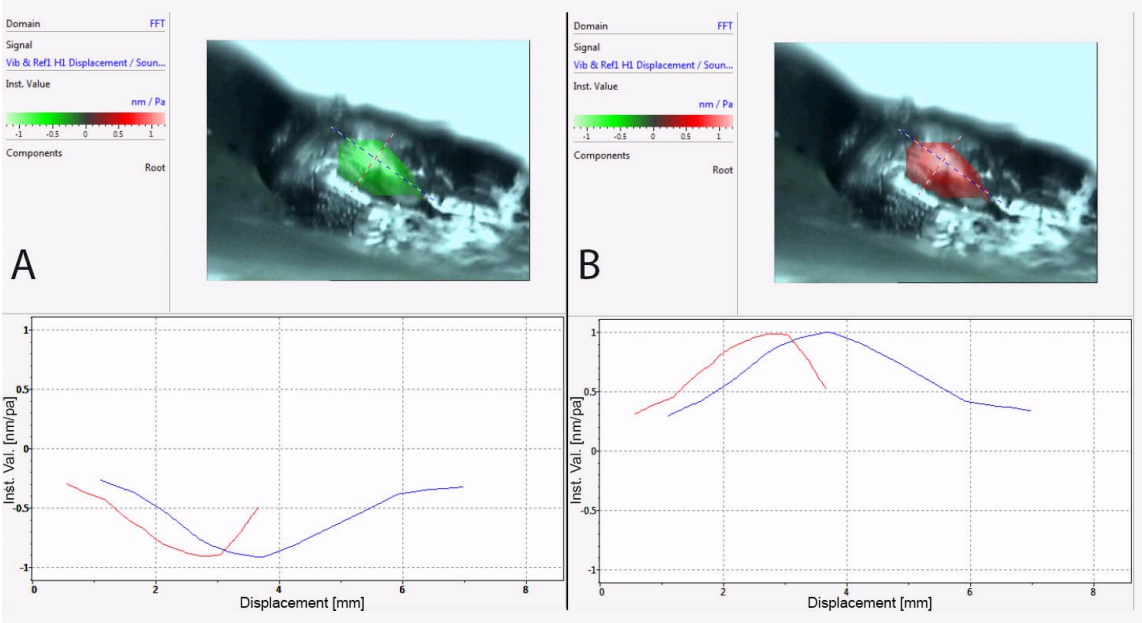


**Figure A1.2** Displacement of the putative tympanal membrane of a male *I.pini* specimen. Screenshots (A, B) were taken at two stages of the movement. Red and blue lines represent the two axis of the movement.

Although there was a general high response to frequencies between 2.5 and 7 kHz, this range is under the frequency range of *I. pini* stridulation (approximately 7-70 kHz, with peak frequencies between 11 and 27 kHz (Sivalinghem, 2011). No consistent high displacement response was registered from the putative ear to any of the relevant frequencies. The displacement did not exceed a few hundred picometers at any of the relevant frequencies, and the membrane movement did not show tympanal membrane characteristics except for the last experiment. Due to the inconsistency of the data, the results are not conclusive, and other experiments are required to assess whether the membrane can function as an ear. However, the observed rapid change of abdomen shape

during the experiments could have had an effect on the membrane characteristics of the putative ear. Other insects, like certain hawkmoth species are known to possess hearing organs that need to be inflated in order to operate (e.g. Göpfert et al., 2002). If the tympanal ear of bark beetles operates in the same manner, this could explain the observed inconsistency in the data.

# Appendix 2: Detailed dissection protocol for exposed nervous system recordings

Immobilized beetles were dissected under Leica M205C stereomicroscope (Leica Microsystems). The dissection started with the removal of all legs using watchmaker forceps. This was necessary because the frontal and mesothoracic leg sockets covered the thoracic ganglia on the ventral side, and the metathoracic legs would have been able to touch the electrodes during exposed nervous system recordings. A small area of the ventral cuticle was removed using scissors and forceps (Fig. A2.1B). Minimization of this area was necessary to preserve as many peripheral nerves as possible. The ideal size of the area to access the meso-metathoracic structures appeared to be equal to the area outlined by the pro- and mesothoracic leg sockets (Fig. A2.1B last frame). Swiping an insect pin along the inner surface of the cuticle helped detach the underlying structures from the cuticle while keeping the tissues intact (Fig. A2.1B second frame). Remaining pieces of muscle and cuticle floating in the hemolymph covering the nervous system were removed carefully using forceps and scissors (Fig. A2.1B third frame). Accessing the prothoracic nerves and the neck connective was only possible when only the prothoracic cuticle (including part of the prosternum between the leg sockets) was dissected, keeping the meso-metathoracic ganglion attached to the intact mesothoracic cuticle. During the development of the dissection technique, Janus green (Yack, 1993) was used to aid identification of nerves and ganglia. This step was removed from the protocol once the electrophysiology experiments began, and nerves were identified without any aid or with the help of a stainless-steel hook electrode (Fig. A2.1A). Dissections were recorded using a Leica DMC4500 microscope camera (Leica

Microsystems), and the videos were stored on a PC as well as external hard drives at Carleton University.

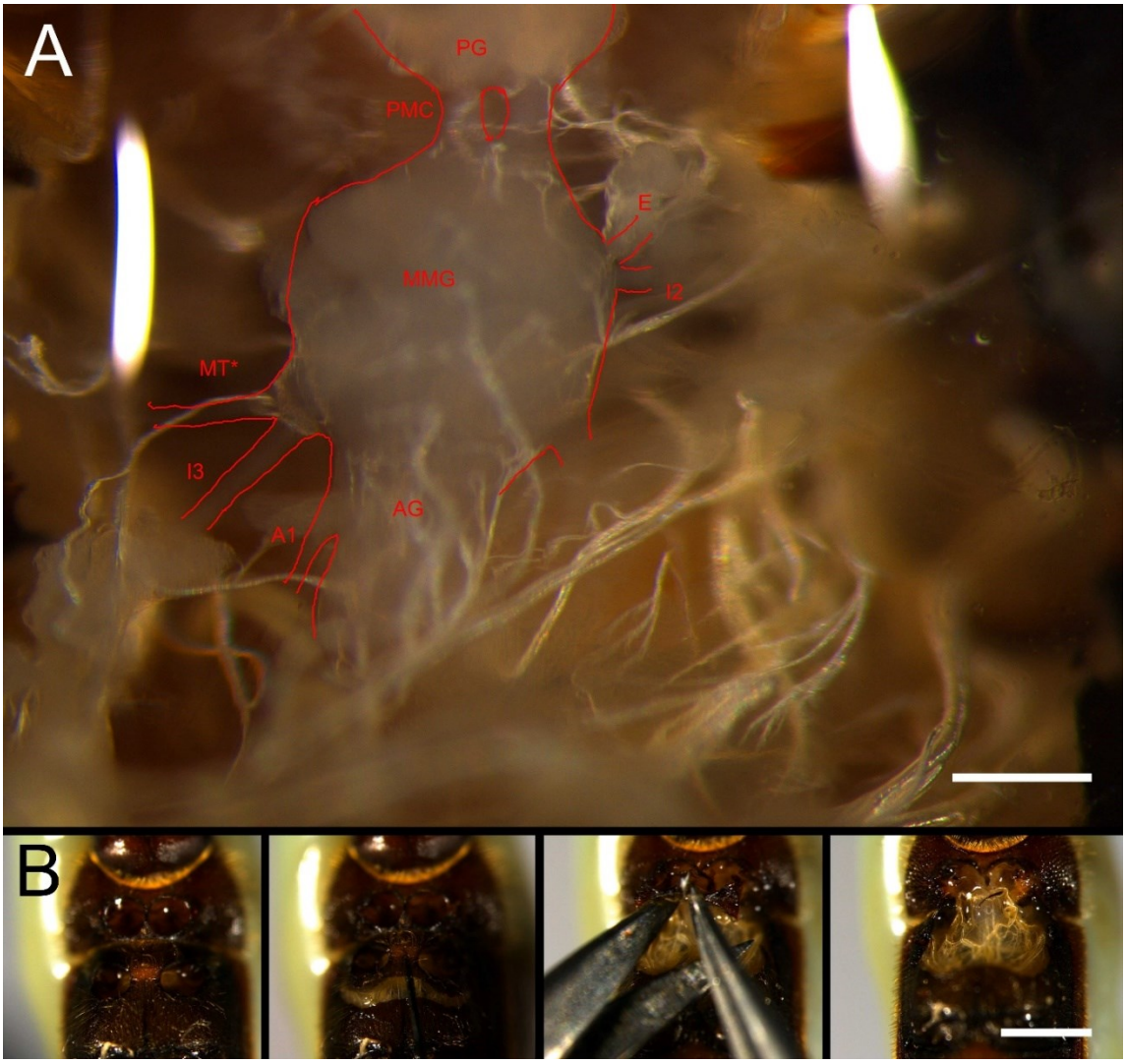


**Figure A2.1** Preparation of *D. valens* specimens for nervous system recordings. A: Ventral view of a dissected beetle with thoracic CNS structures and peripheral nerves outlined (some nerves were severed during dissection). Asterisk indicates the nerve innervating the putative tympanal organ PG: prothoracic ganglion PMC: pro-mesothoracic connective MMC: fused meso-metathoracic ganglia AG: fused abdominal ganglia E: elytral nerve I2: second ischadic nerve MT metathoracic nerve I3: third ischadic nerve A1 first abdominal nerve. B: Four frames from a dissection video showing the main dissection steps and equipment (legs were removed before the first frame). Scalebars: A: 200µm B: 1mm

# Appendix 3 Summary T-test 1 and T-test 2 results for all trials

**Table A3.1** T-test 1 and T-test 2 results from all trials. p1, p2: p-values for T-test 1 and T-test 2. v: indicates vibration trial while recording from the neck area. n: indicates near field trial while recording from the neck area

**Metathoracic nerve**

| Frequency (Hz) | # of bursts | p1 | p2 |
|---|---|---|---|
| 300 | 36 | 0.7235 | 0.6988 |
| 3000 | 12 | 0.7205 | 0.6524 |
| 5000 | 18 | 0.7193 | 0.557 |
| 7000 | 20 | 0.3245 | 0.8726 |
| **7000** | 22 | 0.1584 | **<0.05** |
| 7000 | 22 | 0.1457 | 0.9853 |
| 15000 | 43 | 0.4039 | 0.3344 |
| 15000 | 31 | 0.0923 | 0.789 |
| 15000 | 30 | 0.559 | 0.2192 |

**Pro-meso connective**

| Frequency (Hz) | # of bursts | p1 | p2 |
|---|---|---|---|
| 300 | 30 | 0.8417 | 0.4683 |
| 500 | 42 | 0.1786 | 0.9853 |
| 3000 | 24 | 0.2461 | 0.6517 |
| 5000 | 27 | 0.9452 | 0.5509 |
| 7000 | 22 | 0.2544 | 0.1726 |
| **7000** | 12 | **<0.05** | 0.2139 |
| 7000 | 29 | 0.1457 | 0.7941 |
| 7000 | 24 | 0.2597 | 0.4496 |
| 15000 | 32 | 0.0656 | 0.8809 |
| 15000 | 21 | 0.9239 | 0.8952 |
| 15000 | 13 | 0.1634 | 0.8747 |
| 15000 | 15 | 0.5151 | 0.4194 |
| 25000 | 10 | 0.3264 | 0.6394 |

**Neck connective area**

| Frequency (Hz) | # of bursts | p1 | p2 |
|---|---|---|---|
| 2000 | 9 | 0.4568 | 0.7195 |
| 4000 | 7 | 0.5075 | 0.2741 |
| 5000 | 14 | 0.6232 | 0.6576 |
| 5000 | 17 | 0.6116 | 0.8329 |
| 6000 | 16 | 0.1421 | 0.6112 |
| **7000** | 48 | **<0.05** | 0.4848 |
| 7000 | 13 | 0.5265 | 0.7101 |
| **7000** | 15 | **<0.05** | 0.9479 |
| 7000 | 9 | 0.4726 | 0.4794 |
| 7000 | 97 | 0.5664 | 0.9741 |
| 10000 | 11 | 0.879 | 0.0626 |
| 10000 | 14 | 0.4794 | 0.8122 |
| 15000 | 23 | 0.5193 | 0.9077 |
| **100v** | 54 | **<0.001** | 0.3048 |
| 300v | 99 | 0.7817 | 0.7022 |
| **500v** | 139 | **<0.001** | 0.8195 |
| 500v | 12 | 0.7452 | 0.5638 |
| **600v** | 13 | **<0.05** | 0.1086 |
| 700v | 70 | 0.4292 | 0.6661 |
| 700v | 15 | 0.7906 | 0.5334 |
| 1000v | 21 | 0.2078 | 0.1071 |
| 4000n | 12 | 0.492 | 0.6952 |
| 10000n | 57 | 0.2005 | 0.6104 |

**Putative tympanal organ area**

| Frequency (Hz) | # of bursts | p1 | p2 |
|---|---|---|---|
| 7000 | 25 | 0.5879 | 0.2485 |
| 7000 | 68 | 0.1503 | 0.442 |
| 17000 | 36 | 0.4731 | 0.8451 |
| 20000 | 11 | 0.9047 | 0.5589 |

**Prothoracic leg socket**

| Frequency (Hz) | # of bursts | p1 | p2 |
|---|---|---|---|
| 3000 | 9 | 0.2782 | 0.4649 |
| 5000 | 10 | 0.7875 | 0.4491 |
| 7000 | 14 | 0.6563 | 0.7013 |
| 7000 | 49 | 0.8031 | 0.4519 |
| **10000** | 27 | **<0.05** | 0.8077 |

**Mesothoracic leg sockets**

| Frequency (Hz) | # of bursts | p1 | p2 |
|---|---|---|---|
| 7000 | 22 | 0.9786 | 0.449 |

**Hind leg socket**

| Frequency (Hz) | # of bursts | p1 | p2 |
|---|---|---|---|
| 7000 | 28 | 0.7444 | 0.1998 |

**Abdominal nerve 1**

| Frequency (Hz) | # of bursts | p1 | p2 |
|---|---|---|---|
| 3000 | 45 | 0.9431 | 0.5156 |
| 7000 | 32 | 0.8268 | 0.8717 |
| 7000 | 7 | 0.7919 | 0.35 |
| 15000 | 8 | 0.3282 | 0.635 |
| 15000 | 12 | 0.6273 | 0.1737 |

**Elytral nerve**

| Frequency (Hz) | # of bursts | p1 | p2 |
|---|---|---|---|
| 3000 | 45 | 0.9102 | 0.4614 |
| 7000 | 15 | 0.5805 | 0.9983 |
| 15000 | 40 | 0.9616 | 0.3955 |
| 15000 | 7 | 0.2517 | 0.763 |

**Ischadic nerve 1**

| Frequency (Hz) | # of bursts | p1 | p2 |
|---|---|---|---|
| 7000 | 77 | 0.0708 | 0.7277 |
| 15000 | 13 | 0.6204 | 0.6818 |

**Ischadic nerve 3**

| Frequency (Hz) | # of bursts | p1 | p2 |
|---|---|---|---|
| 7000 | 21 | 0.4066 | 0.9379 |

**Prothoracic leg**

| Frequency (Hz) | # of bursts | p1 | p2 |
|---|---|---|---|
| **100** | 26 | **<0.001** | 0.165 |
| **150** | 61 | **<0.001** | 0.7316 |
| **200** | 32 | **<0.001** | 0.1534 |
| **200** | 43 | **<0.001** | <0.001 |
| **200** | 79 | **<0.001** | 0.0989 |
| **250** | 11 | **<0.001** | 0.7539 |
| 300 | 51 | 0.8305 | 0.5843 |
| 350 | 73 | 0.4777 | 0.9404 |
| **350** | 59 | **<0.05** | 0.423 |
| 400 | 40 | 0.2864 | 0.9658 |
| **500** | 98 | **<0.001** | 0.4676 |
| 500 | 35 | 0.102 | 0.2579 |
| 500 | 48 | 0.0478 | 0.5752 |
| 600 | 10 | 0.1269 | 0.3624 |
| 600 | 33 | 0.1901 | 0.9388 |
| 600 | 39 | 0.1774 | 0.5589 |
| 700 | 17 | 0.6211 | 0.2266 |
| 700 | 20 | 0.268 | 0.8087 |
| 800 | 47 | 0.1494 | 0.6759 |
| 800 | 41 | 0.9029 | 0.8127 |
| **900** | 36 | **<0.001** | 0.5887 |
| 1000 | 24 | 0.1124 | 0.5762 |

**Mesothoracic leg**

| Frequency (Hz) | # of bursts | p1 | p2 |
|---|---|---|---|
| **200** | 26 | **<0.001** | **<0.05** |
| **300** | 18 | **<0.001** | **<0.001** |
| **350** | 52 | **<0.05** | 0.3837 |
| **400** | 38 | **<0.05** | 0.8859 |
| 500 | 31 | 0.1329 | 0.4477 |
| **500** | 21 | **<0.001** | 0.1303 |
| **500** | 56 | **<0.05** | 0.8392 |
| 600 | 31 | 0.9622 | 0.6231 |
| **600** | 42 | **<0.05** | 0.9966 |
| 700 | 56 | 0.956 | 0.2467 |
| 700 | 64 | 0.0394 | 0.1679 |
| **700** | 39 | **<0.05** | 0.8644 |
| 800 | 20 | 0.9529 | 0.8644 |
| 900 | 32 | 0.6617 | 0.533 |

**Metathoracic leg**

| Frequency (Hz) | # of bursts | p1 | p2 |
|---|---|---|---|
| 100 | 12 | 0.7893 | 0.9207 |
| 100 | 23 | 0.7508 | 0.7771 |
| **150** | 9 | **<0.001** | 0.7891 |
| **200** | 21 | **<0.001** | 0.4027 |
| **300** | 58 | **<0.001** | **<0.001** |
| **300** | 86 | **<0.001** | **<0.001** |
| **300** | 28 | **<0.001** | **<0.001** |
| **400** | 25 | **<0.05** | 0.9088 |
| 500 | 82 | 0.7004 | 0.3595 |
| 500 | 155 | 0.4058 | 0.3415 |
| 500 | 30 | 0.5406 | 0.2545 |
| 700 | 50 | 0.0947 | 0.9599 |
| 700 | 19 | 0.1355 | 0.2545 |
| 800 | 15 | 0.9983 | 0.2954 |
| 900 | 20 | 0.4616 | 0.7298 |
| 1000 | 64 | 0.5306 | 0.5012 |
| 1000 | 7 | 0.0832 | 0.9199 |
| 1000 | 27 | 0.9468 | 0.5219 |

**Antennae (low frequency)**

| Frequency (Hz) | # of bursts | p1 | p2 |
|---|---|---|---|
| 100 | 7 | 0.4728 | 0.2204 |
| 150 | 29 | 0.0995 | 0.4735 |
| **200** | 42 | **<0.05** | 0.179 |
| **200** | 24 | **<0.05** | 0.7909 |
| 200 | 31 | 0.628 | 0.9876 |
| 250 | 26 | 0.2027 | 0.6668 |
| **300** | 17 | **<0.05** | 0.4667 |
| 300 | 48 | 0.7489 | 0.5257 |
| **400** | 26 | **<0.001** | 0.4915 |
| **500** | 31 | **<0.001** | 0.8866 |
| **500** | 29 | **<0.001** | 0.0891 |
| 500 | 34 | 0.0548 | 0.0651 |
| **600** | 16 | **<0.001** | 0.1509 |
| **600** | 30 | **<0.001** | 0.0552 |
| **600** | 45 | **<0.001** | 0.2712 |
| **700** | 33 | **<0.001** | 0.0539 |
| **800** | 30 | **<0.001** | 0.0802 |
| **800** | 30 | **<0.001** | 0.0758 |
| **900** | 39 | **<0.001** | **<0.001** |
| **900** | 25 | **<0.05** | **<0.05** |
| **900** | 26 | **<0.001** | 0.9245 |
| **1000** | 35 | **<0.001** | **<0.05** |
| **1000** | 25 | **<0.001** | 0.669 |
| **1000** | 38 | **<0.001** | 0.4361 |

**Antennae (high frequency)**

| Frequency (Hz) | # of bursts | p1 | p2 |
|---|---|---|---|
| **4000** | 33 | **<0.001** | 0.313 |
| **4000** | 26 | **<0.001** | **<0.001** |
| **4000** | 20 | **<0.05** | 0.6156 |
| **5000** | 16 | **<0.05** | 0.8266 |
| **5000** | 13 | **<0.05** | 0.9386 |
| 5000 | 28 | 0.0598 | 0.6209 |
| 7000 | 43 | 0.8541 | 0.2494 |
| 7000 | 11 | 0.9151 | 0.6379 |
| **7000** | 43 | **<0.001** | 0.2215 |
| 7000 | 81 | 0.8322 | 0.6914 |
| 7000 | 68 | 0.3458 | 0.8378 |
| 8000 | 10 | 0.22 | 0.3967 |
| **8000** | 14 | **<0.05** | 0.8742 |
| 10000 | 25 | 0.569 | 0.6909 |
| 10000 | 34 | 0.3195 | 0.3552 |
| 10000 | 13 | 0.0793 | 0.7489 |
| **10000** | 32 | **<0.05** | 0.192 |
| 10000 | 55 | 0.845 | 0.5757 |
| 10000 | 33 | 0.7402 | 0.7481 |
| 20000 | 10 | 0.4704 | 0.2332 |
| **20000** | 13 | **<0.05** | 0.7597 |

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