Auditory descending neurons in the prothoracic network of the bush cricket *Ancistrura nigrovittata*

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ethics statement
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List of abbreviations
aN = acoustic neuropile
AN = ascending neuron
AT = acoustic trachea
CA = crista acustica
CS = calling song
dB = decibel
DN = descending neuron
DUM = dorsal unpaired median
GABA = γ-aminobutyric acid
LDN = local descending neuron
LF = low frequency
HF = high frequency
ON = omega neuron
PBS = phosphate-buffered saline
PIR = post-inhibitory rebound
SPL = sound pressure level
TG1 = prothoracic ganglion
TG2 = mesothoracic ganglion
TG3 = metathoracic ganglion
TN = T-fibre
WN = white noise
Introduction

All animals rely on sensing the outside world to survive. Due to the complexities of the natural world, perception comes in many different forms. Information as distinct as visual, auditory, electrical, and chemical can act as the basis of perception. How important a sensory modality is for any given species depends on its evolutionary past, life history, ecological niche, and anatomy, all of which are deeply interconnected.

Insects, by far the most speciose animal taxon (Stork, 2018; Stork et al., 2015), are a remarkable example of this diversity of perception. Bumblebees can sense and discriminate electrical fields and use the information for foraging decisions (Clarke et al., 2017, 2013; England and Robert, 2022). Moths can detect miniscule amounts of airborne sex pheromone (Roelofs and Arn, 1968; Roelofs and Comeau, 1968). Leafhoppers have a complex vibratory communication repertoire including choruses (Eriksson et al., 2011; Hunt and Morton, 2001; Kuhelj et al., 2016). These examples are modes of perception and communication that evade our attention, but many insects use modalities that are conspicuous to humans. Perhaps the most noticeable among these is the auditory behaviour of some insect species.

Diverse insects such as bees, flies, and mosquitos produce sounds audible to humans. Yet, arguably the most well-known examples of auditory signalling for any invertebrate group are orthopterans. Orthoptera consists of two suborders: Caelifera, containing grasshoppers and allies, and Ensifera, containing crickets, bush crickets, grigs and other related taxa (Resh and Cardé, 2003). Both groups have numerous species that can perceive sound, as well as produce it. In fact, it is the biggest animal group with acoustic communication, surpassing all sound-producing vertebrate taxa in number of species (Song, 2018).

There are contending opinions regarding the evolutionary age of Orthoptera (Chang et al., 2020; Misof et al., 2014; Nel, 2021). Nevertheless, both the order and its suborders are generally accepted to be monophyletic (e.g. Flook et al., 1999; Jost and Shaw, 2006; Song et al., 2020, 2015). The ancestral orthopteran lacked hearing organs as well as specialised structures to produce sound, which evolved independently, in some cases multiple times, within both orthopteran suborders (Desutter-Grandcolas, 2003; Gwynne, 1995; Meier and
This complex evolutionary history is evident in the diversity of hearing organs and mechanisms of sound production, especially within Caelifera (Song et al., 2020). Due to a combination of their diversity, striking acoustic communication, simplicity of rearing in the lab, and the relative ease of access to the nervous system, ensiferans have been a popular model for studying auditory behaviour for a long time (early examples: Autrum, 1940; Graber, 1875; Regen, 1913, 1914; see Bailey and Rentz, 1990; Hall and Robinson, 2021; Hedwig, 2014; Hoy et al., 1998; Huber et al., 1989 for detailed overviews). In terms of understanding neuronal mechanisms of auditory perception, some of the best-studied species including the model organism used in this study, belong to Ensifera. Therefore, I will take a detailed look into ensiferan acoustic communication, with a special focus on the peripheral structures and neuronal processes enabling auditory perception.

Their considerable diversity, besides making Orthoptera an interesting taxon to study, also lead to a significant fragmentation of research efforts. For true crickets (Gryllidae), three genera (*Acheta, Gryllus, Teleogryllus*) are subject of the majority of studies. There is much more variety in the literature on bush crickets (Tettigoniidae): the genera *Ancistrura, Copiphora, Decticus, Mecopoda, Mygalopsis, Neoconocephalus*, and *Tettigonia*, among others, have been studied intensely, though partly with different focal points. Therefore, I will indicate taxonomic affiliations and highlight systematic differences when needed. All names and classifications of taxa/species are based on Orthoptera Species File Online (Cigliano et al., 2023).

**Acoustic communication in Ensifera**

Using sound for intraspecific communication and predator avoidance is widespread within Ensifera (see Greenfield, 1997; Hall and Robinson, 2021 for a review). In most species, males “sing” and females use these signals to find the males for mating (positive phonotaxis) (Heller, 1990; Robinson, 1990). In some bush cricket taxa, e.g. Phaneropterinae, females respond to the male calling song (CS), usually with a short click (duetting) (Heller et al., 2015; Zhantiev and Korsunovskaya, 1990).
Hybridisation between sympatric species can have significant fitness costs for offspring (e.g. Muhlfeld et al., 2009; Sota and Kubota, 1998; Veen et al., 2001). Thus, singing may not only be used for localisation, but also to establish species specificity. Both male phonotaxis and female response behaviour is released based on different properties of the perceived song (Bailey and Robinson, 1971; Hill, 1974; Robinson et al., 1986; Schul et al., 1998; von Helversen and Wendler, 2000; Zhantiev and Korsunovskaya, 1990). For many crickets, dominant frequency and pulse rate are the main factors for song recognition (Hennig et al., 2016; Hill, 1974; Hill et al., 1972; Jang and Gerhardt, 2006; Oldfield, 1980; Pollack and Hoy, 1979; Thorson et al., 1982; Ulagaraj and Walker, 1973; Walker, 1957). For duetting phaneroptериne, the female needs to reply within a narrow time window to be recognised by the male (Heller and von Helversen, 1986; Zimmermann et al., 1989). External and internal factors such as age or temperature can affect the recognition criteria significantly (Atkins et al., 2008; Atkins and Stout, 1994; Pires and Hoy, 1992a; Sanborn, 2006).

As mentioned above, hearing is also used for predator avoidance, e.g. from bats (Bailey and Haythornthwaite, 1998; Zuk and Kolluru, 1998). Auditory signalling introduces two sources of predation danger: (1) from eavesdropping predators, and (2) from exposure during phonotaxis. For example, geckos find calling males using their song, but prey on the attracted females (Sakaluk and Belwood, 1984). Bats can exploit both methods to hunt ensiferans (Raghuram et al., 2015; ter Hofstede et al., 2017). They represent a significant source of pressure on the evolution of both sound production behaviour (Belwood and Morris, 1987; Faure and Hoy, 2000; Heller, 1995; Morris and Beier, 1982) and acoustic perception/response (Farris and Hoy, 2000; Libersat and Hoy, 1991; Pulver et al., 2022; Römer and Holderied, 2020).

Acoustic signals do not exist in isolation. In their natural environment, insects use multiple sensory channels to send signals and to make decisions. For example, visual cues improve phonotactictic accuracy (Bailey et al., 2003; Böhm et al., 1991; von Helversen and Wendler, 2000). In close proximity, chemical (Tregenza and Wedell, 1997) and vibratory (Morris et al., 1994) signals become increasingly relevant for mating. Especially sound and vibration are closely related sensory channels.
Acousto-vibratory communication

Sound and vibration have traditionally been investigated as separate modalities, but they intersect on multiple levels. Sound signals, especially high intensity ones such as CS, produce substrate vibrations (Broder et al., 2021; Hill et al., 2019; Kalmring et al., 1990; Stölting et al., 2002). Ensiferan ears are thought to have evolved from scolopidial organs that functioned as proprioceptors or vibration receptors (Boyan, 1993; Strauß and Stumpner, 2015). Further downstream in the nervous system, acousto-vibratory interneurons (often called VS or SV neurons) are widespread in bush crickets (Kalmring et al., 1983; Kalmring and Kühne, 1980; Kühne, 1982; Sickmann, 1997; Sickmann et al., 1997; Silver et al., 1980). On the behavioural level, added vibration enhances phonotactic acuity (Latimer and Schatral, 1983; Stiedl and Kalmring, 1989).

Despite this close relationship, there are few reports of ensiferan acousto-vibratory behaviour in the literature; most studies focus on near-field vibratory communication (Morris et al., 1994) or describe successive use of sound and vibration (Korsunovskaya and Zhantiev, 2022; Montealegre-Z and Morris, 1999; Morris, 1980; Sarria-S et al., 2016). To my knowledge, there is only a single report of simultaneous acousto-vibratory signalling in any orthopteran, from a pseudophylline bush cricket (Stumpner et al., 2013).

Singing

Though the word “singing” prompts the mental image of sound coming out of a mouth, ensiferan singing differs drastically from this image. Most species use specialised structures on the forewings in the second thorax segment. During singing, a sclerotised region of the wing (scraper or plectrum) is moved against a row of teeth (stridulatory file or pars stridens) (Morris, 1998; Rössler et al., 2006). This mechanism is called tegmino-tegmina stridulation. Closing of the wings produces the bulk of the energy contained in the signal (Montealegre-Z and Mason, 2005). For some species, the wing movements associated with singing can be more complex than a repetitive opening and closing (Walker and Dew, 1972). The sound generated by the motion of the file and scraper is amplified by specialised areas (harp and mirror) of the wings (e.g. Bailey, 1970; Bennet-Clark, 2003; Keuper et al., 1988; Nocke, 1971).
Furthermore, the properties of a species’ CS are highly dependent on environmental factors, especially temperature (Jang and Gerhardt, 2007; Martin et al., 2000; Toms, 1992; Tschuch, 1985; Walker and Cade, 2003; Walker, 1975).

Tegmino-tegminastridulation is thought to be ancestral for all ensiferans (Song et al., 2020), but there are differences on multiple levels between different species. For example, one analysis indicates that different wing veins evolved into the scraper and the file in different taxa (Desutter-Grandcolas et al., 2017). In general, there are differences between crickets and bush crickets, as well as within each taxon, in the location of the scraper and the file, and in the gross morphology of the wings (e.g. Chivers et al., 2016; Duncan et al., 2021; Jonsson et al., 2021; Koch et al., 1988; Michelsen, 1998; Montealegre-Z et al., 2006).

Hearing (peripheral)
To understand ensiferan acoustics, we need to understand the ensiferan ears. In their seminal paper, Yack and Fullard (1993) defined three criteria to accept an insect organ as an ear: (1) anatomical specialisation, (2) response to environmentally relevant sounds, and (3) behavioural output to sound perception. I have already covered the latter point in detail in the previous sections; I will focus on the anatomy of the ears and the mechanics of hearing, thus on criteria (1) and (2).

Ensiferans have similar sensory organs in all three pairs of legs, named the complex tibial organ. It consists of subgenual, intermediary, and tibial organs (crickets)/crista acustica (CA; bush crickets) (Lakes and Schikorski, 1990). In the forelegs, the latter part of the organ is specialised for sound perception, with an increased number of sensory cells, modified tracheal structure, and two regions of thin cuticle called tympana (Eibl, 1978; Houtermans and Schumacher, 1974; Kalmring et al., 1994; Lin et al., 1994; Sickmann et al., 1997), similar to vertebrate eardrums. Though the ear is specialised for sound perception, even non-hearing species have a similar complex tibial organ, though they lack the specialisations in hearing species (Lakes-Harlan et al., 1991; Strauß et al., 2014; Strauß and Lakes-Harlan, 2008a, 2010).
Ensiferan ears are systems with multiple inputs. The tympana are backed by an air-filled trachea, also called the acoustic trachea (AT) (Fig. 1). CA is located on the dorsal side of this trachea and is covered by a fluid-filled channel on the opposite side (Friedrich, 1927; Schneider et al., 2017), the content of which is different from the haemolymph (Montealegre-Z. et al., 2012; Sarria-S et al., 2023). The posterior end of the AT leads to an opening on the side of the thorax, called spiracle. This represents an alternative pathway for sound to act directly on the internal side of the tympana. In crickets, AT on both sides of the body are also connected by a transverse trachea and a medial septum, which plays a role in the directionality of the hearing system (Löhe and Kleindienst, 1994). Thus, bush cricket ears have two and cricket ears have four sound inputs. This system can be defined as a pressure difference receiver with unequal inputs (see Michelsen and Larsen, 2008 for an overview of sound pressure receiving systems).

Incoming sound is passively amplified by AT (Celiker et al., 2020). The shape of the AT is the main factor defining its amplification properties (Celiker et al., 2022; Michelsen et al., 1994; Stumpner and Heller, 1992). In most bush crickets, it is shaped like an exponential horn (Heinrich et al., 1993), though other shapes with different amplification mechanics are commonly seen in different taxa (Celiker et al., 2022). Sound amplification through AT is especially prevalent for frequencies between 10 and 60 kHz (Bailey and Römer, 1991; Bailey, 1998; Heinrich et al., 1993; Hill and Oldfield, 1981; Hummel et al., 2011; Jonsson et al., 2016) and it often overlaps with the dominant frequency of the species’ CS (Bailey, 1993; Celiker et al., 2022; Woodrow and Montealegre-Z, 2023). Some bush cricket taxa (e.g. Pseudophyllinae) have many species with high ultrasound CS (>60 kHz). In these species, the power in the dominant

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**Figure 1.** Cross section of a bush cricket ear in the foreleg tibia. Crista acustica is located between air-filled trachea and a liquid-filled channel. a, anterior; AT, acoustic trachea; atym, anterior tympanum; CA, crista acustica; d, dorsal; ptym, posterior tympanum. Modified from Vavakou et al. (2021).
frequency range reaches the ear predominantly amplified through the external surface (Michelsen and Larsen, 1978), where a resonant chamber around the tympana is created by cuticular flaps (Mason et al., 1991; Pulver et al., 2022; Woodrow and Montealegre-Z, 2023).

The indirect sound input through AT is also responsible for directionality of the ears. Between 10 and 60 kHz, where the sound through AT dominates the total energy input, the diffraction around the ear is negligible as the tibia is a very small structure. However, since AT openings are on both sides of the thorax, there is significant diffraction around the body, which is much larger than the tibia (Michelsen, 1971). As a result, ensiferan ears are inherently directional, with a higher response to ipsilateral sound (Michelsen et al., 1994; Stumpner and Heller, 1992). This increased distance between the entry points of the sound into the system also leads to interaural intensity differences, which are used for phonotaxis (Pollack, 2003; Rheinlaender et al., 2006; Schöneich and Hedwig, 2010).

When a sound wave reaches the ear, it displaces the tympana and CA in a complex pattern. When tympana are immobilised, hearing is lost (Kleindienst et al., 1983; Nocke, 1975), but the complete removal of the anterior tympanum has contradictory results (Lewis, 1974; Oldfield, 1982). There is no direct correlation between the displacement of the tympana and the neuronal response to sound (Hummel et al., 2011). The “hinged flap” movement of the tympana (Bangert et al., 1998; Nowotny et al., 2010) transfers the mechanical energy to structures adjacent to CA, though the exact mechanism may differ between species (Montealegre-Z. et al., 2012; Vavakou et al., 2021). As a result, travelling waves form on CA; comparable to those in the mammalian cochlea, the travelling waves are tonotopic based on the frequency of the sound (Palghat Udayashankar et al., 2012). CA motion is not linear (Palghat Udayashankar et al., 2014) and the shear caused by the uneven movement of CA is thought to open ion channels in the sensory cells (Hummel et al., 2016). Furthermore, a mismatch between the motions of parts of CA sharpens the displacement tuning of CA (Vavakou et al., 2021).

CA is composed of a series of sensory cells (15-116 in bush crickets; Strauß, 2019) lined on a proximal-distal axis wrapped in support structures. Each sensory cell is sharply tuned to a part of the total hearing range of the ear (Fig. 2) (bush crickets: Kalmring et al., 1978; Lin et al.,
In crickets, the sensory cells in the tibial organ are organised in distinct populations with different physiological properties, but the majority is tuned to the male CS frequency (Esch et al., 1980; Imaizumi and Pollack, 1999, 2001). In bush crickets, the ear as a whole is usually broadly tuned with the lowest thresholds around 20 kHz (Hill and Oldfield, 1981; Lin et al., 1993; Schul and Patterson, 2003). There are taxa with a mismatch between the tuning of the ear and the song frequency, which can be explained through the specialised mating system of these species (Bailey and Römer, 1991; Bailey and Gwynne, 1988).

The sensory cells in the crista acustica are ordered tonotopically; cells tuned to low frequencies (LF) are on the proximal and cells tuned to high frequencies (HF) on the distal end (Stöltting and Stumpner, 1998). Tonotopy of the cells overlaps with the tonotopy of sound-induced CA motion (Vavakou et al., 2021). Though CA evolved from vibratory organs, HF sensory cells respond only to sound (e.g. Kalmring et al., 1978). Yet, recent data from *Tettigonia* claim bimodality for all cells in CA (compare Stritih-Peljhan et al., 2022; Zhantiev and Korsunovskaya, 2021, 2023 for a detailed discussion using both recent and previously published data).

Sensory cells of the CA project only into a restricted area of the prothoracic ganglion (TG1) (Rehbein, 1973). This central neuropile is called the anterior ring tract or anterior intermediate sensory neuropile, and is a part of the medial ventral...
association centre (see Pflüger et al., 1988; Römer et al., 1988; Tyrer and Gregory, 1982 for different neuropile naming conventions); it is commonly called the auditory neuropile (aN). The tonotopic arrangement of the sensory cells is reflected in their projection patterns within aN as well (Ahi et al., 1993; Oldfield, 1983, 1988; Römer, 1983; Stölting and Stumpner, 1998): LF cells project to the most anterior portion of aN and with increasing frequency, the projection areas shift towards the ventral and posterior parts of the neuropile (Fig. 2). Within aN, sensory cells form synapses with auditory interneurons (Römer et al., 1988).

Hearing (central processing)

Auditory interneurons in TG1 make up the first level of auditory processing. These can be classified into four groups: local or intraganglionic, ascending, descending, and T-fibres. Local neurons project solely within TG1. Ascending neurons (AN) have axons going to the suboesophageal ganglion and/or the brain. Descending neurons project to lower thoracic and abdominal ganglia. T-fibres have both ascending and descending axons. Auditory information from the sensory cells is processed and filtered by interneurons within TG1 and relayed to the brain by ascending neurons (bush crickets: Stumpner and Nowotny, 2014; crickets: Pollack and Hedwig, 2017; grigs: Mason and Schildberger, 1993).

Despite millions of years of evolution separating ensiferan branches, auditory interneurons in TG1 are conserved to a surprising degree across different species. Many interneurons can be observed in multiple conspecific individuals, as well as in different species. These are called “identified neurons” (see Comer and Robertson, 2001; Kandel, 1976 for discussions on the concept). Though demonstrating true homology between species requires developmental methods (e.g. Boyan, 1993), I will treat cells that are both morphologically and physiologically similar as homologous, as is done commonly for ensiferan neurons (e.g. Hennig, 1988; Molina and Stumpner, 2005; Nebeling, 2000; Schul, 1997; Stumpner, 1997).

Many auditory interneurons are first order, i.e. they receive direct input from sensory cells. They have low response latencies and their frequency tuning corresponds to a sum of the sensory cells with which they project to overlapping areas (e.g. Boyan, 1984; Römer et al.,
1988; Wohlers and Huber, 1978). They can also receive inhibitory inputs from other interneurons (Horseman and Huber, 1994; Selverston et al., 1985) as well as additional polysynaptic excitation (Faulkes and Pollack, 2001).

Certain aspects of the prothoracic auditory processing network have been thoroughly investigated. For example, a local interneuron, named omega neuron (ON), is found as a mirror pair in most ensiferans and enhances directionality by contralateral inhibition (crickets: Casaday and Hoy, 1977; Popov et al., 1978; Wohlers and Huber, 1978, 1982; bush crickets: Molina and Stumpner, 2005; Römer, 1983; Zhantiev and Korsunovskaya, 1983). Each ON receives excitatory input mainly from the soma-ipsilateral ear (Watson and Hardt, 1996) and inhibits the contralateral ON and ascending neurons (e.g. Horseman and Huber, 1994; Molina and Stumpner, 2005; Selverston et al., 1985).

Ascending neurons relay different information content to the brain. In many species, there are two identified ascending neurons, AN1 and AN2. In crickets, these neurons are associated with distinct and opposite behaviours. AN1 is tuned to LF (dominant frequency for most cricket CS) and is responsible for positive phonotaxis towards CS (Atkins et al., 1992; Lv et al., 2020; Schildberger, 1988; Schildberger and Hörner, 1988; Wohlers and Huber, 1982). In contrast, AN2, which is predominantly inhibited by LF stimuli, induces bat avoidance behaviour to ultrasound (Casaday and Hoy, 1977; Harrison et al., 1988; Moiseff and Hoy, 1983; Popov and Markovich, 1982; Rheinlaender et al., 1976; Wohlers and Huber, 1978).

Ascending neurons in bush crickets present a more complicated system. There are species with multiple identified ascending neurons (up to four, e.g. Stumpner and Molina, 2006). Only AN1 is found in many species in different subfamilies and is thought to be homologous between species and with AN1 in crickets (Conocephalinae: Römer, 1987; Phaneropterinae: Stumpner, 1997, 2002; Tettigoniinae: Hardt, 1988; Römer et al., 1988; Schul, 1997). AN1 in different bush cricket species have different spectral tuning patterns, but they can all encode the temporal structure of the conspecific CS. Another set of ascending neurons with similar spectral and/or temporal properties cannot be homologised at all due to morphology not being shown in these older findings (Kalmring et al., 1983; Oldfield and Hill, 1983;
Rheinlaender, 1975). In species with unusually LF or HF calling songs, ascending neurons tuned to these frequencies project to significantly different parts of aN (BFA-1 in *Mecopoda*: Kostarakos and Römer, 2015, 2018; unnamed ascending neuron in *Leptophyes*: Hardt, 1988). Therefore, homology of these neurons with other bush cricket AN1 depends on the approach used (see Neuhofer et al., 2008 for a discussion of evolutionary adaptation vs. conservation of coding properties).

In ensiferans as well as grasshoppers, processing in the brain is required for high-level operations such as pattern recognition or decision making (Bauer and von Helversen, 1987; Pires and Hoy, 1992b; Ronacher et al., 1986). One intensely studied aspect of auditory brain networks is the recognition of the conspecific CS in crickets. Different theories have been proposed about the circuitry and mechanism of the putative CS recognition network (see Ronacher, 2019 for a review). Early studies described multiple local brain neurons, which were mostly broadband (Boyan, 1980, 1981). Later on, systems based on neurons with band-pass filtering properties (Schildberger, 1984, 1988) and Gabor filters (Clemens and Hennig, 2013) were suggested. However, most recent investigations have made a compelling case for a CS recognition network based on coincidence detection (Hedwig, 2016; Kostarakos and Hedwig, 2012, 2015; Schöneich et al., 2015; Zhang and Hedwig, 2023). The proposed network is flexible and can be adapted temporal parameters of CS of different species (Clemens et al., 2021). In contrast, there is very limited information about local brain neurons in bush crickets and no proposed processing networks so far (Ostrowski, 2009; Ostrowski and Stumpner, 2010, 2013).

T-fibres have also been investigated, though to a lesser extent than ascending and omega neurons. TN1 is found in multiple species and characterised by large axons and rapid habituation to repeated stimulation (bush crickets: Faure and Hoy, 2000; Nebeling, 2000; Rheinlaender and Römer, 1986; Römer et al., 1988; Schul, 1997; Suga and Katsuki, 1961; Triblehorn and Schul, 2009; crickets: Atkins and Pollack, 1987a; Wohlers and Huber, 1982; grigs: Mason and Schildberger, 1993). TN1 is proposed to act as a “novelty detector”, specifically to separate bat echolocation calls from the conspecific calling song, in one conocephaline bush cricket genus (*Neoconocephalus*: Prešern et al., 2015; Schul et al., 2012; Schul and Sheridan, 2006; Triblehorn and Schul, 2013).
Unlike the previous examples, descending neurons (DN) received little attention from researchers. There are four identified DN in crickets (Atkins, 1987; Atkins and Pollack, 1987a, 1987b). Only one of these (DN1) was found in another species (Wohlers and Huber, 1982, 1985). In contrast, 15 DN in 8 genera from 5 different bush cricket subfamilies have been described (Fig. 3) (Conocephalinae: Römer, 1987; Römer et al., 1988; Mecopodinae: Kostarakos and Römer, 2015, 2018; Phaneropterinae: Korsunovskaya and Zhantiev, 1992; Stumpner and Molina, 2006; Zhantiev and Korsunovskaya, 1990; Phaneropterinae and Tettigoniinae: Hardt, 1988; Tettigoniinae: Römer, 1985; Sickmann, 1997). Some of these cells are possibly homologous, though it is difficult to establish homology, since most previous studies do not include detailed descriptions of DN; in one case, data are limited to a single staining without information on the physiological response. Nevertheless, two general morphological classes can be observed. First is a “lateral” type with little to no arborisations
within aN, but extensive branching in other areas, implying possible multimodal response properties (left on Fig. 3). These cells are mostly tuned to LF and constitute the majority of DN previously described in bush crickets. DN with predominantly aN branches, which will be called “bushy” here, are the smaller group (right on Fig. 3). DN3 is the only cell with a posterior cell body. Only five DN (DN1 and DN3 in Isophya: Korsunovskaya and Zhantiev, 1992; BFD-1 in Mecopoda: Kostarakos and Römer, 2015; TH1-DC-S4 and TH1-DC-S1 in Decticus: Sickmann, 1997) are sensitive to HF sound and all have rather broad frequency tuning.

In contrast to the neurons, for which a clear function has been proposed or shown, there are no theories regarding the role of DN within the prothoracic network or for behaviour. Furthermore, the existing data on bush cricket DN are fragmented over numerous studies with various focal points and from species with very different behaviours. To characterise this group of auditory neurons and to elucidate their role in behaviour, I used Ancistrura nigrovittata, the bush cricket species with the best-characterised prothoracic auditory network (see Cillov and Stumpner, 2022; Stumpner and Molina, 2006; Stumpner and Nowotny, 2014 for an overview of the identified interneurons).

Acousto-vibratory system in Ancistrura nigrovittata

A. nigrovittata is a brachypterous phaneropterine bush cricket found in Southeast Europe, from Serbia to Greece (Cigliano et al., 2023; Harz, 1969). It is a duetting species; females reply to the male calling song and males use this response to locate the females. The male CS consists of a group of 5-9 syllables (7 ms each, separated by 22 ms gaps; each syllable consists of three individual pulses), followed by a final trigger syllable after ~400 ms (Dobler et al., 1994a, 1994b; Heller and von Helversen, 1986), comparable to closely related species (Stumpner and Meyer, 2001).

So far, only the calling song of this species has been described; there is no information on near-field courtship behaviour and only a brief mention of vibratory signals (only as a byproduct of the female reply or for aggression; Heller and von Helversen, 1986). Behavioural experiments carried out in our lab with A. nigrovittata by our collaborator, Nataša Strith-
Peljhan, PhD from National Institute of Biology in Ljubljana, Slovenia, provide the first data on acousto-vibratory communication in this species, which I present here.

![Figure 4. Acousto-vibratory communication in A. nigrovittata. Upper trace: sound spectrogram; lower trace: recordings of vibration. (A) Recording of the calling song. Initial wing movements are soundless (black arrow); males produce previously unreported double trigger syllables (yellow arrow). (B) Female responses to CS. Wing movements can be silent (black arrow) or accompanied by short clicks (white arrows). (C) Vibratory female response to CS. Alongside the auditory click responses (white arrows), females produce high amplitude tremulations (orange arrows).](image)

Our data show multiple novel aspects of acousto-vibratory behaviour of A. nigrovittata. For example, male stridulation during CS always produces vibration as a byproduct, though the first 1-2 wing movement cycles are often not accompanied by sound (Fig. 4A), as was previously reported (Heller and von Helversen, 1986). Males often produce double trigger syllables (Fig. 4A), which has never been shown previously. Similarly to males, wing movements during female replies do not always produce sound (Fig. 4B). Furthermore, female replies are not purely auditory; females often respond with high amplitude vibratory signals, produced with abdominal movements (tremulation) (Fig. 4C). Tremulation is used more often when the male is singing on the same plant as the female, presumably perceived using the vibrational component of CS.
Observations reveal a distinct near-field communication pattern. When males and females are on the same plant, CS is often replaced with a courtship song, which has the same temporal pattern as CS without the trigger syllable (Fig. 5A). Males actively search for the females as they sing. During pauses in their movements, females respond using only tremulation signals (Fig. 5B). This is followed by the male immediately approaching the female.

In very close proximity, male courtship behaviour becomes predominantly vibratory. Males produce series of tremulation signals; these often consist of groups of 2-4 pulses but can range from single pulses to groups of ten (Fig 6A). Interestingly, these pulses are often accompanied by acoustic ticks, typically at signal onset (Fig. 6B), suggesting the animals could be communicating in the near-field using multimodal signals. As mentioned above, there are many multimodal interneurons in bush crickets, which could underlie this integrative process on the neuronal level.

Compared to many other bush cricket species, A. nigrovittata has a complex CS; as a consequence, multiple factors are relevant for song recognition, for which the temporal pattern plays a central role. Females recognise a song as conspecific and respond only if both the pattern of the syllable group as well as the gap between this group and the trigger syllable resemble the natural song (Dobler et al., 1994a). Presumably, the female reply needs to be within a narrow time window after the male trigger to be recognised, as is the case for other duetting phaneropterines (Heller and von Helversen, 1986; Robinson et al., 1986; Zimmermann et al., 1989).
Unique among Phaneropterinae (Heller et al., 2015), carrier frequency of the song is also used for recognition (Dobler et al., 1994b). Within the closely related genera Ancistrura and Barbitistes (Ullrich et al., 2009), song carrier frequency is usually correlated with body size (Stumpner, 2002). Calling song of A. nigrovittata is an outlier; peak frequency is around 15 kHz and therefore much lower than is predicted by body size. Females, however, sing at the expected frequency (peak at ~27 kHz; Dobler et al., 1994b).

One remarkable property of the duets in barbitistine bush crickets is the very short latency of the female response (Heller and von Helversen, 1986; Stumpner and Meyer, 2001). In A. nigrovittata, the female reply latency is ~30 ms, but can be as low as 18 ms (Dobler et al., 1994a; Heller and von Helversen, 1986). Most known auditory brain interneurons in crickets (Schildberger, 1984; Schöneich et al., 2015; though compare with low latencies in Atkins et al., 1988; Boyan, 1980) and bush crickets (Ostrowski, 2009; Ostrowski and Stumpner, 2010, 2013) have much higher latencies than that of the female reply. This mismatch suggests a thoracic “reflex”, which is gated by the song recognition network in the brain, could directly induce the female reply in response to the male trigger syllable, as was previously suggested (Dobler et al., 1994a; Robinson et al., 1986; see Hedwig, 2006; Poulet and Hedwig, 2005 for a similar example for low-latency phonotaxis in crickets). Neurons descending from TG1 to TG2 would be prime candidates to bridge the gap.

On the peripheral level, A. nigrovittata matches the typical bush cricket blueprint. CA consists of on average 36 primary sensory cells that are arranged tonotopically, which is also represented in their projection patterns within aN (Stumpner, 1996). Recordings from both
the tympanal nerve and from individual sensory cells show that the maximum sensitivity of
the ear is around 20 kHz, regardless of sex (Dobler et al., 1994b; Stumpner, 1996). Overall, the
intensity thresholds for neuronal activity at the ear and behavioural responses for both males
and females are remarkably similar (<10 dB, Dobler et al., 1994b).

Numerous auditory interneurons in the prothoracic network of *A. nigrovittata* have been
identified. Unlike many other bush crickets, including closely related species, AN1 is not tuned
broadly to the whole hearing range of the ear, but more sharply to the dominant frequency
of CS (Stumpner, 1997). The enhanced tuning is a result of HF inhibition (Stumpner, 1998) and
can be seen as an evolutionary adaptation to the lower frequency of *A. nigrovittata* CS, since
closely related species have broadly tuned AN1 despite inhibitory input (Stumpner, 2002).

Another ascending neuron (AN5-AG7) represents a counterpart to AN1; it is tuned sharply to
the frequency of the female response due to LF inhibition (Stumpner, 1999a, 1999b). Its cell
body is in the fifth abdominal ganglion, but its only extensive arborisations are within aN and
in the brain. AN5 could play a role in recognising the female response, since its spiking
threshold matches that of the male phonotaxis behaviour and the inhibitory input it receives
could underlie the recognition time window, which has been shown in other phaneropterine
species (e.g. Zimmermann et al., 1989).

As evidenced by AN1 and AN5, inhibitory effects play a central role in shaping the tuning of
various prothoracic interneurons. So far, two sources of inhibition have been discovered. First,
as in many other ensiferan species, ON is a central component of contralateral inhibition
(Molina and Stumpner, 2005). Curiously, this effect decreases significantly after treatment
with picrotoxin, which blocks γ-aminobutyric acid (GABA) receptors, even though ON likely
has histamine (Skiebe et al., 1990) or serotonin (Hörner et al., 1995) as a neurotransmitter.
The second inhibitory influence in the prothoracic network is a cluster of GABAergic dorsal
unpaired median (DUM) neurons. DUM neurons are a group of local neurons that were
extensively studied in grasshoppers (Bräunig and Pflüger, 2001; Thompson and Siegler, 1993).
Although only a small subset of the cluster is GABAergic in grasshoppers, *A. nigrovittata* has a
more significant GABAergic population that constitutes a filter bank, since different neurons
have different spectral and temporal response properties (Lefebvre et al., 2018; Stumpner et
Additionally, DUM neurons receive inhibitory input themselves as well (Lefebvre et al., 2018; Stumpner et al., 2020). The auditory DUM cluster is possibly an evolutionary adaptation to the extended hearing range of bush crickets, since a similar cluster is not found in crickets (Cillov, 2020).

Three other ascending neurons, two T-fibres, and one local neuron have also been described in A. nigrovittata. One of the ascending neurons (AN3) and the local neuron (SN2) are broadly tuned and possibly act as reference neurons (Cillov and Stumpner, 2022; Stumpner and Molina, 2006). AN4 is similarly broadly tuned but has a higher response threshold and is the only ascending neuron with a posterior cell body. AN2, like AN1, is tuned to the dominant frequency of CS, but is much less sensitive (Stumpner and Molina, 2006). TN1 is a highly directional phasic neuron that responds well to ultrasound. Consequently, it was suggested to play a role in bat detection in different bush cricket species (Faure and Hoy, 2000; Libersat and Hoy, 1991; Schul et al., 2000). Finally, TN3 is a broadband neuron that generates action potentials only at high stimulus intensities and is therefore thought to play a role in local processing (Stumpner and Molina, 2006).

There is surprisingly little data regarding the final major group of auditory neurons, descending neurons. Only a LF descending neuron (DN5) has been briefly described in a previous publication (Stumpner and Molina, 2006). Further two (DN2a and DN4) have only been shown as a single staining (Stumpner and Nowotny, 2014). Preliminary results reveal at least six DN in the same animal and multiple different morphological as well as physiological cell types (Andreas Stumpner, unpublished results). These data indicate an unexpected aspect of the diversity of prothoracic auditory processing that is so far unexplored. Combined with previous behavioural data that strongly suggest a thoracic network as the trigger for the female reply in low latency phaneropterine duets, descending neurons are an interesting study subject with possible implications on the behavioural level. Therefore, the aims of this study were the extensive morphological and physiological characterisation of the prothoracic DN population in A. nigrovittata using intracellular recording, staining, and immunohistochemistry, as well as exploring the possible role of DN in triggering the low latency female response, especially with a focus on projections in TG2 and comparisons between neuronal and behavioural thresholds.
Material & methods

All details regarding chemicals, solutions, equipment, and further methods can be found in Appendix A, in the order in which they appear in the main text.

Animals

*Ancistrura nigrovittata* (Brunner von Wattenwyl, 1878) (Orthoptera: Ensifera: Tettigoniidae: Phaneropterinae) bush crickets were caught near Methoni, Central Macedonia, Greece and reared in captivity. Animals were kept in semi-transparent cages (40 x 30 x 25 cm from hatching until the subadult stage, 55 x 45 x 50 cm in subadult and adult stage; H x W x D, respectively) with mesh openings in a 12h:12h light/dark cycle at 25°C and 50% air humidity. Additional heat was supplied by 40W incandescent lightbulbs. Leaf-bearing twigs of raspberry (*Rubus idaeus*), bird cherry (*Prunus padus*), blackberry (*Rubus* sp.), dog rose (*Rosa canina*), thicket dog rose (*Rosa corymbifera*), sloe (*Prunus spinosa*), common hazel (*Corylus avellana*) and dogwood (*Cornus* sp.), as well as nutrient supplement (Nekton) and water were provided *ad libitum*. Containers with a 1:1 mix of fine sand and vermiculite were placed into the cages as egg-laying medium. The containers were regularly removed and the medium sieved to separate the eggs, which were placed in petri dishes containing the same medium. After being stored at RT for a few weeks, the eggs were incubated in a fridge at 4°C between 6 months and 3 years. When moved back to RT, the eggs started hatching within 1-3 weeks, though the viability of the eggs decreased drastically if the eggs were kept in the cold for >1 year (personal observation; Andreas Stumpner, personal communication). Both wild-caught and lab-reared (F1-F8) animals were used for experiments.

Acousto-vibratory behaviour recordings by Nataša Stritih-Peljhan, PhD

Pairs of male-female animals were placed on plants (*Rubus* sp.) in an arena (t = 24 °C ±1) illuminated with red light. Females were acoustically isolated preceding the experiments. Pieces of reflective foil were placed on different areas of the plant; these were used as recording sites for vibration. Sound and vibration were measured simultaneously with a condenser microphone and laser vibrometer, respectively; both feeds were digitised and
recorded using a laptop PC running Raven Pro 1.6. Female signals were converted to lower frequencies using a bat detector, as their frequency exceeded the range of the microphone; therefore, they appear as LF signals in the recordings. The arena was also recorded using a camcorder. All following figures from these experiments were made with Raven Lite 2.0.5.

Dissection and intracellular recording
Animals were anaesthetised with CO$_2$ and fixed ventral side up to a holder using melted wax; mid- and hindlegs were immobilised by fixing to the holder in a neutral standing position, forelegs were fixed to a copper wire with putty. A tungsten wire heated with a DC power supply was used to form a tub around the median prothorax with a 1:1 mix of wax and rosin. TG1 was exposed by carefully removing the overlying cuticle using fine scissors. TG1 was superfused with saline solution (modified from Fielden, 1960). The ganglion sheath was permeabilised with solid collagenase for 25 s. TG1 was stabilised from above and below using steel rings. The holder was placed in an anechoic chamber lined with sound absorbent foam. Forelegs were separated from the copper wire and fixed to a plastic fork, which was connected to a body speaker, in a similar position.

Micropipettes were pulled from thick-walled borosilicate capillaries (1.0/0.58 mm) using a horizontal puller. One pipette was filled with 1 M KCl and inserted into aN to monitor neuronal activity during acoustic stimulation with 16 kHz stimuli. Hearing threshold was measured as the lowest intensity with a field potential. Further pipettes were filled with Lucifer Yellow, neurobiotin, Alexa 555, Alexa 633, CF568, or CF633 and the shafts were backfilled with 1 M LiCl (Lucifer Yellow), 1 M KAc (neurobiotin), or 1 M KCl (all other dyes). Pipettes were inserted into a holder with an Ag/AgCl wire; the holder was moved using a mechanical micromanipulator. Movement of the pipette was monitored visually through a stereomicroscope and was illuminated by a light source located outside of the anechoic chamber. Another Ag/AgCl wire was inserted into the abdomen to be used as a reference electrode. Signals were amplified using an intracellular bridge amplifier, converted to digital with a 20 kHz sampling rate, and stored on a desktop computer running Spike2.
Cells were impaled in the cell body or nearby neurites, which are found in an anterolateral cluster. A standard high amplitude stimulus programme consisting of 5 kHz, 16 kHz and white noise (WN) signals was used while searching for neurons. Neurons were characterised using stimulus programmes described in detail below and stained ionophoretically for up to 10 min using 0.5-2 nA depolarising (neurobiotin) or hyperpolarising (all other dyes) current. When possible, multiple cells from the same hemiganglion were recorded from and stained with different dyes in one animal. Recordings were monitored acoustically with headphones and visually on a digital oscilloscope throughout the experiment. Afterwards, TG1, TG2, and TG3 were excised in an intact chain when possible. Tissues were fixed with paraformaldehyde for 30-60 min and stored in phosphate-buffered saline (PBS).

Stimulation
Two dynamic broadband speakers were situated on both sides of the animals in the setup (distance 37 cm, angle 85°). These were calibrated annually (±2 dB) using two condenser microphones connected to an amplifier. The body speaker used for vibration stimuli was calibrated using the laser vibrometer in the acousto-vibratory setup described above. Multiple vibration calibrations were run using different angles between the laser vibrometer and the body speaker. All stimuli were generated using a custom-made computer (Lang et al., 1993) and amplified with custom-made amplifiers. The following stimulus programmes were used for the experiments.

**50/70/90 dB SPL test**
50 ms pulses (1.5 ms ramps) with 50, 70, or 90 dB SPL amplitude and increasing carrier frequency (3-50 kHz for 50 and 70 dB SPL, 3-38 kHz for 90 dB SPL; one pulse/frequency) 335 ms apart were presented to initially test the whole frequency range. These programmes were presented from both sides consecutively.

**R-L-16/28**
100 ms pulses (2 ms ramps) with 16 or 28 kHz carrier frequency and increasing amplitude (30-90 dB SPL in 10 dB SPL steps; five pulses/amplitude) 350 ms apart (385 ms when changing sides) were presented alternatingly from either side to test the directional response. 50/70
test and R-L-16/28 programmes were used to determine the more sensitive side for each cell based on the response strength, which was used to present later stimuli programmes for that cell.

**50/70 dB SPL scan**

50 ms pulses (1.5 ms ramps) with 50 or 70 dB SPL amplitude and pseudorandom carrier frequency (3-50 kHz; five pulses/frequency) 300 ms apart (335 ms when changing frequency) were presented to characterise frequency tuning.

**XX-10**

50 ms pulses (1.5 ms ramps) with a given (XX; range 2-46 kHz) carrier frequency and increasing amplitude (30-90 dB SPL in 10 dB SPL steps; five pulses/amplitude) 300 ms apart (335 ms when changing amplitude) were presented to measure thresholds and characterise saturation responses at different frequencies. For 2 and 3 kHz programmes, the tested amplitudes were 50-100 dB SPL.

**WN-300**

300 ms WN pulses (2 ms ramps) with increasing amplitude (30-84 dB SPL in 9 dB SPL steps; five pulses/amplitude) 1300 ms apart (1335 ms when changing amplitude) were presented to record responses to longer stimuli and measure the change in phasic-tonic responses in relation to amplitude.

**vibscan**

100 ms pulses with increasing amplitude (five pulses/amplitude) 600 ms apart grouped in two or three (200-500 Hz, or 50-200-500 Hz) carrier frequency steps that are 1100 ms apart were presented from the body speaker to test vibration sensitivity. Since the computer generating the stimuli was made for acoustics, stimulus amplitudes for this programme were set in “dB” values and measured afterwards (Appendix B).

The following stimulus programmes use artificial CS or duets with the same basic pattern. Each programme changes a different temporal or spectral property of CS or duet. CS is a group of 7 ms pulses separated by 22 ms gaps followed by a 7 ms trigger syllable after a 350 ms gap. Duet has the same pattern as CS with an added 3 ms click after 35 ms.
zpau
500 ms WN adaptation pulse and WN CS (4-23 syllables to keep the total duration of CS fixed) with pseudorandom gaps between the syllables (2-61 ms; five repetitions/gap duration) and 70 dB SPL amplitude without trigger syllable were presented.

zpul
500 ms WN adaptation pulse and WN CS (5-9 syllables to keep the total duration of CS fixed) with pseudorandom syllable duration (2-25 ms; five repetitions/syllable duration) and 70 dB SPL amplitude without trigger syllable were presented. zpau and zpul were used to characterise temporal characteristics.

antwfreq
500 ms WN adaptation pulse and 16 kHz CS with 60 dB SPL amplitude, followed by female reply with 60 dB SPL amplitude and pseudorandom carrier frequency (12-42 kHz; five repetitions/frequency) were presented.

antwlat
500 ms WN adaptation pulse and 16 kHz CS with 70 dB SPL amplitude, followed by 28 kHz female reply with 60 dB SPL amplitude after a pseudorandom gap (5-85 ms; five repetitions/gap duration) were presented. anwfreq and antwlat were used to characterise responses to artificial duets with temporal and spectral features that deviate from the natural range of the conspecific CS.

antwtrig
500 ms WN adaptation pulse and 16 kHz CS with 70 dB SPL amplitude with a pseudorandom gap between the syllable group and trigger syllable (100-500 ms; five repetitions/gap duration) were presented to record possible responses independent of the trigger syllable, when this was outside of the natural range of the conspecific CS.

antwprim
500 ms WN adaptation pulse, followed by 16 kHz CS with 70 dB SPL amplitude repeated 30 times, followed by 16 kHz CS with 70 dB SPL amplitude without trigger syllable repeated 10 times were presented to “prime” the song recognition network with the conspecific CS and to record possible responses in the absence of a trigger syllable after the last 10 CS.
Data analysis

Intracellular recordings were initially analysed with custom-made Spike2 scripts (written by Heribert Gras and Andreas Stumpner). A spike threshold was manually set on a low-pass filtered (2 ms) copy of the signal to measure spike counts and latencies. For non-spiking cells or ambiguous recordings, spike counts were not evaluated. To measure graded potentials, the signal was low-pass filtered (3.5 ms); negative components were removed by subtracting half of the sum of a rectified and an unrectified copy of the signal; action potentials were removed with a high-pass filter (0.2 Hz, Butterworth first order); if the signal exceeded the baseline (100 ms preceding the stimulus) ± three standard deviations, the area between the signal and the baseline was measured as the graded potential. The quantified responses were further analysed and visualised using matplotlib (Hunter, 2007) in Python programming language (van Rossum and Drake, 2009). Data from the same animal were represented with mean ± standard deviation (SD); when data from different animals were pooled, standard error of the mean was used for the error bars (SEM).

Backfills

Backfills were performed to visualise whole clusters of cells. Animals of either sex were anaesthetised with CO₂; head, legs and tip of the abdomen were cut off and the organs were removed posteriorly. Subsequently, the animal was cut dorsally along the body axis and pinned dorsal side up. TG1-TG2 or TG2-TG3 connective (anteriorly), or nerve 3 of TG2 (proximally) were backfilled overnight with neurobiotin. Afterwards, TG2 was excised, fixed with paraformaldehyde and stored in PBS. Silvia Gubert and Andreas Stumpner kindly performed these procedures and Andreas Stumpner kindly imaged the ganglia.

In some animals used for intracellular recordings, TG1-TG2 connective was backfilled anteriorly overnight with neurobiotin following the experiment. Afterwards, TG1, TG2, and TG3 were excised in an intact chain when possible, fixed with paraformaldehyde, and stored in PBS.
Histology

Immunostaining against choline acetyltransferase, an enzyme involved in acetylcholine synthesis, was performed to reveal if DN cluster was cholinergic (protocol modified from Stumpner et al., 2020). This cluster has not been stained in any histological study against other neurotransmitters so far (Hörner, 1999; Hörner et al., 1996, 1995; Spörhase-Eichmann et al., 1992; Stumpner et al., 2020). TG1 was dissected using the same method as for the backfills, fixed with paraformaldehyde for 1 h, washed with PBS (pH = 7.4; 2x10 min), embedded in 5% agarose, and sectioned with a vibratome (80-150 μm). Sections were incubated in PBST (pH = 7.4) for three to five days. Subsequently, unspecific binding was blocked by treatment with blocking buffer for 3 h. Sections were incubated overnight in primary antibody (1:400; mouse anti-choline acetyltransferase or rabbit anti-choline acetyltransferase), washed with PBST (pH = 7.4; 4x20 min), and incubated overnight in secondary antibody (1:500; donkey anti-mouse or donkey anti-rabbit). Finally, sections were washed with PBST (pH = 7.4; 4x 20 min) and mounted on a slide with a DABCO solution.

Some tissues that were previously stained in intracellular recordings were embedded in low viscosity agar and sectioned parasagittally. Tissues stored in PBS were dehydrated with increasing concentrations of ethanol (50, 70, 90, 96, 100%; 10 min each). Subsequently, they were treated with propylene oxide (2x15 min) and a 1:3 mix of agar:propylene oxide (1 h), then incubated in a 1:1 mix of agar:propylene oxide overnight. Afterwards, they were treated with a 3:1 mix of agar:propylene oxide (1 h), embedded in the final medium, and cured at 70°C for 48 h. Embedded tissues were sectioned (10 μm) with a microtome and mounted on a slide. Silvia Gubert kindly performed these procedures.

Microscopy

Tissues previously stained with neurobiotin in intracellular recordings were processed preceding microscopy. They were dehydrated with increasing concentrations of ethanol (50, 70, 90, 96, 100%; 10 min each), treated with xylene (2 min), and rehydrated (same steps in dehydration in reverse order). They were washed with PBS (2x10 min), incubated in 2 mg/ml collagenase-dispase at 37 °C, washed with cold PBS to stop the enzymatic reaction (2x10 min),
washed with PBST (3x10 min), then incubated overnight in 1:400 Cy3 streptavidin conjugate at 4°C. Finally, they were washed with PBST (3x15 min) and PBS (3x15 min).

Preceding imaging, all wholemount ganglia were dehydrated with increasing concentrations of ethanol (50, 70, 90, 96, 100%; 10 min each), cleared with methyl salicylate, mounted on a slide with methyl salicylate, and imaged with a confocal microscope. Confocal image stacks were used to create maximum or depth-colour-coded projections to convert cell morphology to 2D with LAS X and FIJI (Schindelin et al., 2012).

Figures
Stainings were used as maximum or depth-colour-coded projections; some were retraced and scanned to better highlight morphological details. Confocal projections and scans were visually optimised in Adobe Photoshop. Data traces were imported from Spike2 into Adobe Illustrator for reformatting. Figures were assembled in Illustrator.
Results

Descending axon backfills

Backfills of the axons descending from TG1 show highly variable numbers of stained cells. In general, more cell bodies are distinguishable than neurites and there are less stained cells in TG2-TG3 backfills, since not all axons descend further than TG2. Up to ca. 50 cell bodies are found in an anteroventral cluster on either side of the midline in TG1 (Fig. 7A). In some ganglia, two distinct soma populations are visible: a smaller and more anteromedian group (up to 15) and a bigger posterolateral group, though this distinction is not consistent (Fig. 7B). In contrast, not more than 15 neurites can be seen, all of which are anterior to aN (Fig. 7C). Any possible neurites that project directly through aN cannot be identified well, as the signal-to-noise ratio suffers from the overall strong staining within aN. Only in a few cases are the connections between the neurites and cell bodies visible (Fig. 7D). Based on the backfills, there could be up to 50 DN in the same anterior cluster, though the real number is likely lower in light of the low number of previous descriptions. Furthermore, backfills do not provide any information on physiological properties, so the number of neurons involved in auditory processing is possibly much lower.

Intracellular recordings

A total of 220 cells in 116 animals were recorded from and stained to varying degrees. 140 cells were not stained to an extent that allowed morphological characterisation; in 83 of these the cell body was stained so that the neuron could be assigned to the DN cluster. In 11 of the remaining cells, stainings could not be unambiguously matched to recordings due to multiple unintentionally stained cells; in ganglia with two unequally stained cells, the better staining was accepted as unambiguous if the difference was substantial. 19 cells were stained enough to identify them as DN, but the fine details of the morphology could not be resolved. The remaining 50 cells were used to establish identifiable cell types. The general morphological classes (lateral vs. bushy) from the literature, which have been summarised in the Introduction, could also be reproduced in this study. Eight different subtypes were characterised, though some groups have significant heterogeneity.
Figure 7. DN stained through backfills of descending axons. (A) Overview of a prothoracic ganglion with extensive staining, typical for backfills. DN cell body cluster (white circle) is visible in most stainings, but neurons crossing the midline are less distinguishable (blue rectangle, inset in C). (B) Two distinct soma populations are visible in some ganglia. The more anteromedian population (white circle) is always smaller than the posterolateral (orange ellipse). (C) Neurites are less visible than cell bodies and predominantly to the anterior side of aN (some distinct neurites marked with white arrows). Background staining hinders identification within aN (blue ellipse). (D) The connections between the cell bodies and neurites are visible in very few cases (white arrows). (B), (C), and (D) are depth-colour-coded (cold = ventral; warm = dorsal).

Local descending neuron (LDN) (n=17)

The neuron with the highest number of complete recordings and detailed stainings is LDN: a local neuron that has a cell body within the anterolateral DN cluster and has a very similar gross morphology to other DN but has no functional descending axon. Though LDN often has a thin neurite that projects posteriorly, this rudimentary axon is not intersegmental (Fig. 8A). LDN has intense arborisations on both sides of the midline (Fig. 8B) and throughout the whole aN, which predominantly delimits its dendrites (Fig. 8C). Though its lateral branches have some interindividual variability, the overall projection pattern is very consistent (Fig. 8D).
Figure 8. Morphology of LDN. (A) Wholemount drawing of LDN. (B) Depth-colour-coded projection stained with neurobiotin (cold = ventral; warm = dorsal). (C) Sagittal section through aN with stained LDN (a, anterior; v, ventral). Projections are extensive throughout aN. (D) Interindividual variability in LDN. Though the lateral branches vary, the central projections are always centred around aN. Dashed line indicates midline. Modified from Cillov and Stumpner (2022).

Figure 9. Example responses of LDN. (A) LDN typically responds with phasic-tonic graded potentials without spikes (70 dB SPL; 500 ms; WN). (B) LDN response to an artificial duet (60 dB SPL; white stimuli: 16 kHz, black stimulus: 28 kHz). Modified from Cillov and Stumpner (2022).
LDN is a non-spiking neuron (Fig. 9A). It is not sensitive to vibratory stimuli. Its auditory responses are phasic-tonic and without clear inhibitory input: a sharp phasic rise in potential is followed by a fall to the tonic level, which persists up to 500 ms. There is often a phasic fall in response shortly after onset, but its extent varies significantly between cells (Fig. 9A). LDN can reproduce the conspecific CS, as well as the female reply, in its response (Fig. 9B).

![physiological response properties of LDN](image)

**Figure 10.** Physiological response properties of LDN. (A) Frequency responses of 17 LDN to a 50 dB SPL scan. Individual peaks vary, but the population as a whole is centred around 20 kHz. (B) Average response threshold of LDN (mean ± SEM, n=15-17). Similar to the response peaks, the lowest response threshold is at 20 kHz. Response of LDN (mean ± SEM, n=17) to artificial CS with varying gap (C) and syllable (D) durations. The natural parameters of the song are marked in blue.

In contrast to its morphological consistency, LDN has higher variation in its neuronal responses. Though all LDN are broadly tuned to high audio/low ultrasound frequencies, the exact tuning peak varies considerably between cells (Fig. 10A). Its lowest response threshold is around 20 kHz, closely resembling the tuning of the ear (Fig. 10B). When tested with artificial CS, its average response decreases strongly with increasing gaps between syllables.
(Fig. 10C) and decreasing syllable duration (Fig. 10D). Compared to other local neurons in *A. nigrovittata*, LDN has the lowest directionality in its responses, clearly less than primary sensory cells (Fig. 11).

A neuron morphologically resembling the previously described DN2 was stained and characterised. Its dendrites are predominantly along the primary neurite and outside aN and project very laterally (Fig. 12A). These lateral neurites are characteristically thickened, which are visible even in many weak stainings (Fig. 12A). DN2 has a posterior crossing neurite; though in rare cases, a neurite can project to the same contralateral area by directly descending on the soma-ipsilateral side (Fig. 12B). The branches on the soma-ipsilateral side usually have a beaded appearance, whereas the opposite side has mostly smooth branches, which are commonly associated with output and input synapses, respectively (e.g. Peters et al., 1986; Stumpner, 1997, 1999a). DN2 has little or no visible branches within aN. Cells stained in different animals follow the same morphological pattern: a major projection area bordering aN on the soma-contralateral side, thickened branches on the posterior end of this area and a posterior neurite that crosses the midline back into the soma-ipsilateral hemiganglion (Fig. 12C).
Figure 12. Morphology of DN2. (A) Wholemount drawing of DN2. Thickened lateral branches are characteristic for DN2 (black arrow). (B) Maximum projection of two stained DN2 (green: Lucifer Yellow; pink: neurobiotin) in opposite hemiganglia. Cell #1 has a posterior crossing neurite (white arrow) projecting to a soma-ipsilateral area (white rectangle), whereas cell #2 reaches the mirror image of the same area (magenta rectangle) with a posteriorly crossing and an ipsilaterally projecting neurite (magenta arrows). (C) Maximum projections of DN2 stained in four different animals (green: Lucifer Yellow; pink: neurobiotin). The major neurites as well as the projection areas are consistent between cells. In all cases, soma-ipsilateral branches have a more beaded structure.

Unlike LDN, DN2 produces spike trains in response to stimulation, though its auditory responses are limited to high intensities (Fig. 13A). In nearly all recordings, it is much more responsive to vibratory stimuli than to sound (Fig. 13B), presumably receiving direct excitatory input from primary sensory cells in the forelegs, which project to lateral areas of TG1 (Ebendt et al., 1994; Lakes-Harlan and Strauß, 2014; Nishino and Field, 2003; Stölting and Stumpner, 1998; Strauß and Lakes-Harlan, 2010). Some cells also receive clear inhibitory input during auditory stimulation (Fig. 13C). When present, the auditory responses are concentrated to <10 kHz though spontaneous activity can cause significant variation at HF (Fig. 13D). Responses of DN2 to difference duet parameters could not be characterised due to its low auditory sensitivity.
Figure 13. Sensory responses of DN2. (A) Example response of DN2 to a 2 kHz stimulus programme increasing in intensity. Responses are only visible at 80 dB SPL and above. (B) Example response of DN2 to a vibration programme increasing in intensity within each frequency. Note the constant strong response. (C) Example response of DN2 to a 90 dB SPL stimulus programme increasing in frequency. Note the clear inhibitory input (asterisks) (D) Frequency responses of 12 DN2 to a 90 dB SPL test. Responses are concentrated at LF, but some cells do not respond to sound at all, whereas others produce spontaneous spikes at all frequencies.

DN5 (Lateral - along) (n=3)
Another DN with a gross lateral morphology was named DN5. Although it resembles DN2, it has consistent morphological differences as well as a distinct response pattern. DN5 branches predominantly along its primary neurite like DN2, but it has significantly more projections within aN; especially within the most anterior part of aN, which is the projection area of LF sensory cells (Fig. 14A). The secondary posterior crossing neurite is not present, though individual fine projections are visible on the soma-ipsilateral side (Fig. 14B), albeit to a much smaller extent compared to DN2.
Figure 14. Morphology of DN5. (A) Wholemount drawing of DN5. (B) Depth-colour-coded projection of DN5 stained with neurobiotin (cold = ventral; warm = dorsal). Despite the lack of a secondary neurite crossing back to the soma-ipsilateral side, some branches project there directly (dashed ellipse).

Figure 15. Auditory responses of DN5. (A) Example response of DN5 to a 50 dB SPL stimulus programme increasing in frequency. (B) Example response of a DN5 to a series of equal acoustic stimuli (90 dB SPL, 50 ms, 8 kHz). Note the inhibitory potentials (asterisks) mixed with excitatory input. (C) Frequency responses of 3 DN5 to a 70 dB SPL scan. DN5 responds to LF sound and is inhibited by frequencies >20 kHz. Dashed line indicates sections of the curve below the shown y axis. (D) Average response threshold of DN5 (mean ± SEM, n=2-3).

DN5 responds to acoustic stimulation with a tonic spike train combined with graded excitatory potential. As predicted by its projection area within aN, the acoustic responses of DN5 are
restricted to LF sound (<10 kHz at 50 dB SPL) (Fig. 15A). It receives clear inhibitory input under HF stimulation and even at LF at sufficiently high intensities, though this inhibition is mixed with and masked by excitation at LF (Fig. 15B). The frequency tuning is consistent across different cells, with the response strength steadily decreasing with increasing stimulus frequency and inhibitory input >20 kHz (Fig. 15C). This linear inverse correlation is not reflected in response thresholds; DN5 is most sensitive at 6 kHz and responds to sounds above 50 dB SPL, with the thresholds quickly increasing towards both ends of the spectrum (Fig. 15D). Above 10 kHz, either data could not be pooled or thresholds were >90 dB SPL. As DN5 is strongly inhibited at HF, its responses to artificial duets could not be tested.

DN6 (Lateral - ipsi) (n=1)
A DN with an unusual morphology was recorded and faintly stained a single time. Unlike most previously described DN, DN6 arborises predominantly on the soma-ipsilateral side, but one of its neurites crosses the midline through aN and forms a minor projection area on the soma-contralateral side (Fig. 14A). DN6 responds exclusively to high intensity LF sound but is very sensitive to vibration (Fig. 14B). Due to the low acoustic sensitivity and the general lack of data, response thresholds and tuning for song parameters could not be characterised.

Figure 16. Morphology and sensory responses of DN6. (A) Wholemount drawing of DN6. Minor contralateral branches were stained too faintly to be retraced (dashed ellipse). Example response of DN6 to a 90 dB SPL stimulus programme increasing in frequency (B) and a series of equal vibratory stimuli at lowest intensity (500 Hz) (C).
DN4 (Bushy) (n=12)

DN4, previously only shown as a single staining in *A. nigrovittata* (Stumpner and Nowotny, 2014), is morphologically similar to TH1-DC-S1/S4 described in *Decticus* and to LDN. Similarly, it branches extensively throughout aN (Fig. 15A). Unlike LDN, it does not branch ubiquitously; the most anterior section of aN is not part of the projection area (Fig. 15B). Due to the dense projection pattern, interindividual morphological variation is difficult to observe (Fig. 15C). All stained DN4 share the same general structure with a single primary neurite crossing the midline through aN and arborisations concentrated in this region, but the exact latitude of the neurite in the anterior-posterior axis differs between cells.

![Figure 17](image.png)

**Figure 17.** Morphology and projection areas of DN4. (A) Wholemount drawing of DN4. (B) Sagittal sections on different levels through aN (dashed line) in the same animal with stained DN4 (a, anterior; v, ventral). Projections are extensive throughout aN except for the anterior section (magenta). Maximum (C) and depth-colour-coded (D) projections of DN4. Cells in different animals have little variation; only the trajectory of the main neurite differs visibly. (C: green: Lucifer Yellow; pink: neurobiotin; D: cold = ventral; warm = dorsal)

DN4 exhibits high interindividual variability in its responses. Most cells have a phasic auditory response that can be followed by tonic depolarisation without spikes (Fig. 18A). Some cells respond with sustained tonic spiking, the frequency of which decreases over time (Fig. 18B).
One cell has suppressed activity due to strong inhibitory input (Fig. 18C), which is especially relevant at high stimulus intensities (Fig. 18D).

Although the basic response pattern varies significantly between cells, frequency tuning of DN4 is relatively consistent. Similarly to LDN, nearly all DN4 have their tuning peak between 10-20 kHz and most cells are tuned to 16-20 kHz (Fig. 19A). Interestingly, frequency tuning is sharpened greatly when spike data are used instead of graded potentials (Fig. 19B). In congruence with the tuning of response strength, the lowest response threshold is between 10-24 kHz, with 20 kHz being the most sensitive frequency (Fig. 19C).
Figure 19. Frequency tuning of DN4. (A) Frequency responses of 12 DN4 at 50 and 70 dB SPL. Individual peaks vary, but the population as a whole is centred around 16-20 kHz. Dashed line indicates sections of the curve below the shown y axis. (B) Comparison between frequency tuning curves based on spike numbers and graded potentials at 70 dB SPL. In most cases, spike numbers are more sharply tuned than graded potentials. (C) Average response threshold of DN4 (mean ± SEM, n=4-11). Similar to the response peaks, the lowest response threshold is at 20 kHz.
In response to artificial duets, DN4 again shows very high variance between different recordings. Some cells do have flat tuning for syllable duration, whereas others have clear optima or can be linearly correlated (Fig. 20A). Similarly, tuning for gap duration is highly variable, though optimum type tuning is the most common form (Fig. 20B). When data from all cells are pooled, DN4 is flatly tuned to syllable duration and to 10 ms gap duration (Fig. 20C).

**Figure 20.** (A) Syllable and (B) gap duration tuning of four individual DN4. Flat, optimum type and linear tuning curves are all seen in DN4 population. (C) Response of DN4 (mean ± SEM, n=9-10) to artificial CS with varying gap and syllable durations. The natural parameters of the song are marked in blue. DN4 responds most strongly to syllables separated by 10 ms gaps but is not affected by syllable duration.
High interindividual variance in DN4 implies there could be multiple cells in this pool with different physiological responses but similar morphology, which makes it difficult to separate the dataset into multiple discernible cells. Yet, there are no clear correlations between frequency and temporal tunings and basic response patterns. Additionally, there are no multiple stainings where more than one cell could morphologically be classified as DN4. Therefore, I have decided to pool data from cells that were all classified as DN4 despite their high variability. The problem of classification will be reviewed in detail in the Discussion.

**DN7 (Bushy - hanging) (n=2)**

DN7 is morphologically highly similar to DN4 and LDN, except for a more posterior primary neurite and projection area within aN (Fig. 21A). There are typically several prominent branches of the primary neurite, from which most of the finer projections radiate. Though three stainings exhibit this morphological subtype, one cell has radically different responses (strong inhibition during acoustic stimulation) than the other two (Fig. 21B). Since stainings and recordings can be mismatched in some cases, I consider this incongruent dataset erroneous and only classify the acoustically responsive neurons as DN7.

As can be predicted from its more posterior projection area, DN7 is tuned to slightly higher frequencies than other bushy subtypes, with a maximum response at 24 kHz (Fig. 21B). This tuning seems to be sharpened through inhibition at lower and higher frequencies, as is also seen in other auditory interneurons in this species (Stumpner, 2002). Acoustic responses of DN7 are generally phasic, which is at least partly a result of inhibitory input with a higher latency than that of excitation, especially at higher stimulus intensities (Fig. 21D). Consequentially, DN7 does not reproduce the conspecific calling song. Lastly, DN7 does not respond to vibration.
**Figure 21.** Morphology and auditory responses of DN7. (A) Wholemount drawing of DN7. Its posterior projection area is a unique characteristic of DN7 (dashed line: approximate location of aN). Note the thicker branches that connect the fine projections with the primary neurite (magenta arrows). (B) Frequency responses of two DN7 and an outlier cell (dashed curve) at 50 dB SPL. (C) Example response to a 50 dB SPL stimulus programme increasing in frequency. (D) Example response of the same cell to stimuli at 70 dB SPL. Note the delayed inhibitory input that limits the neuronal response to a short initial time window (asterisks).

**DN8 (Bushy - split) (n=2)**

DN8 has a morphological characteristic that separates it from other bushy type DN: whereas all bushy DN arborise over the ganglion midline, DN8 has an area free of any branches directly on the midline and an area of more intense arborisations in its immediate vicinity (Fig. 22A). It arborises through most of aN except for the most posterior section (Fig. 22B), which acts as a clear morphological characteristic that separates it from other bushy subtypes.

DN8 is very similar to DN7 in its acoustic responses. It is tuned to 24 kHz, which is sharpened through inhibition at lower frequencies (Fig. 22C). Additionally, DN8 responds phasically to sound, which is also potentially caused by delayed inhibitory input (Fig. 22D). Like other bushy DN subtypes, DN8 also does not respond to vibration.
**Figure 22.** Structure of DN8. **(A)** Wholemount drawing of DN8. **(B)** Adjacent sagittal sections through aN (dashed line) in the same animal with stained DN8 (a, anterior; v, ventral). Projections are extensive throughout aN except for the posterior section (magenta). **(C)** Frequency responses of DN8 at 50 and 70 dB SPL. **(D)** Example response to a 70 dB SPL stimulus programme increasing in frequency. Note the delayed inhibitory input (asterisks).

**DN9 (Bushy - lateral) (n=1)**

Although recorded and stained only once, DN9 is one of the most interesting cells described in this study. Like other bushy type DN, it arborises extensively within aN, but also has significant lateral branches like many of the lateral type DN (Fig. 23A). This combination is reflected in its response patterns. As well as being sensitive to vibration, it also responds well to sound (Fig. 23B). This is especially striking when compared to other lateral type DN, which are typically insensitive to sound or are inhibited by it (Fig. 23C).

Like the previous two subtypes, DN9 also has receives delayed inhibitory input (Fig. 24A). However, this is only visible as a brief break in the spike train and does not lead to phasic responses as in DN7 and DN8. Highlighting this difference even further, DN9 is broadly tuned on the frequency spectrum, especially at higher stimulus intensities (Fig. 24B) unlike DN7 and DN8, which have sharper frequency tuning due to inhibitory input (Figs. 21B and 22C). This inhibitory influence also accumulates with repeated stimulation; hence, DN9 does not reproduce the conspecific song in its activity but shows a post-stimulus hyperpolarisation
instead, which lasts for multiple hundred ms (Fig. 25A), which can also be seen in other DN (Fig. 25B).

Figure 23. Morphology and response modalities of DN9. (A) Wholemount drawing of DN9 in TG1, TG2, and TG3. (B) Example responses of DN9 to vibration and sound. (C) Comparison of DN9 to multiple lateral type DN. Note the difference in the used stimulus strength: DN9 responds to 50 dB SPL stimuli significantly more strongly than lateral DN respond to 70 dB SPL stimuli.
Figure 24. Sensory responses of DN9. (A) Example response to 300 ms 39 dB SPL stimuli. Note the inhibitory input causing a dip in the auditory response (asterisks). (B) Frequency tuning of DN9 at 70 dB SPL. (C) Averaged response of DN9 to a 16 kHz 70 dB SPL artificial conspecific song without trigger syllable (described as *antwprim* in Material & Methods; mean ± SD, n=10). Note the long hyperpolarisation persisting after the end of the stimulus.

Figure 25. Long-lasting inhibition triggered by acoustic stimuli in DN. Averaged response of (A) DN9 and (B) a DN of unknown type to a 16 kHz 70 dB SPL artificial conspecific song without trigger syllable (described as *antwprim* in Material & Methods; mean ± SD, n=10). Note the long hyperpolarisation persisting after the end of the stimulus in both recordings.
Discussion

In this study, I reported multiple new descending neurons with unique morphological and physiological characteristics that have not been described previously, as well as cells that could be homologs to previously reported ones. Overall, the current data reproduce the general morphological classification of orthopteran auditory TG1 DN into “lateral” and “bushy” types. However, these groups were revealed to have hidden complexity, both in terms of neuronal diversity and interindividual variability.

A major part of the generated experimental data could not be included in the analysis, since these were recordings that could not be matched to a clearly resolved morphology. If, using other methods such as clustering, these data could also be included in the process of classification, the subtypes I propose here could be significantly refined and other possibly hidden subtypes could be uncovered. Nevertheless, classifying a diverse group of neurons with only meagre background information from previous literature was the most challenging part of data analysis and several decisions and processes need to be argued in detail.

Classification of DN

The current classification of the data can be seen partly as preliminary, as some “identified” cells (e.g. DN4) exhibit significant variation in their responses. Though high interindividual variability is not unusual for insect neurons (e.g. Cillov and Stumpner, 2022; Stölting and Stumpner, 1998; Wohlers and Huber, 1982), the concept of an “identified cell” requires a degree of consistency that can be seen as a counterargument against the current classification, especially regarding DN4. This could also imply that relatively similar cells could be within the natural variation spectrum of a single identifiable neuron, which were nevertheless separated here (e.g. DN4/DN7/DN8). If one such cell was to be stained and recorded from in different animals, it would create the impression of multiple distinct, identifiable cells, though in reality it would be variations of one neuron.

Some neurons described here, such as LDN and DN2, are very consistent in their frequency tuning, basic response patterns, and projection areas, and can reasonably be considered real
identified neurons. On the other hand, DN4, DN7, and DN8 have morphological and physiological overlap to various degrees, which hinders simple separation. Despite the similarity in gross morphology, however, DN4 is consistently different from DN7 and DN8 with tuning to lower frequencies and lack of strong inhibitory input (except for one cell shown in Fig. 18D). Furthermore, DN8 has a characteristic anterior projection pattern that sets it apart from other bushy subtypes. Yet, the gold standard for showing the existence of multiple different cells is still multiple stained neurons in the same animal (see Lefebvre et al., 2018 for a detailed discussion of neuron classification).

In the current study, 27 experiments had multiple stained cells on the same hemiganglion, albeit in many cases with vastly different structural resolutions. In one case, DN2 was found with another lateral type DN, the structure of which could not be resolved any further. In three animals, DN4 could be identified in combination with DN7 or DN8. Therefore, at least two bushy and two lateral type DN must be located in TG1 of A. nigrovittata, which is in congruence with previous data from multiple other bush cricket species (Decticus: Sickmann, 1997; Leptophyes and Tettigonia: Hardt, 1988; Mecopoda: Kostarakos and Römer, 2015, 2018), as well as with preliminary data from A. nigrovittata (Andreas Stumpner, unpublished results). Therefore, the cells described here are likely to be separate identifiable neurons and not part of the natural variation of fewer neurons.

In reverse, we have to consider that a cell described here could exist in more than one copy, e.g. as twins. In this study, none of the described cells were stained multiple times in the same hemiganglion, at least not to the extent that both could be visualised. In the only case where two cells recorded in the same hemiganglion were conspicuously similar, it turned out to be the same LDN recorded twice in a row. This could be confirmed since the same cell was stained with two different dyes during different recordings. Yet, cells that have multiple structurally identical, but physiologically diverse copies are known from grasshoppers (Marquart, 1985; Stumpner, 1989). This would be unlikely for LDN and DN2 due to the high number of recordings for each and the lack of any double copies. However, I cannot rule out the existence of multiple copies for the other DN described here.
Comparison of DN in _A. nigrovittata_ to other orthopteran DN

As mentioned in the Introduction, proving homology between cells in different species requires developmental evidence. For orthopterans, however, the amount of neurophysiological data far outweighs that of developmental data. Therefore, structural and physiological similarities have often been used to argue for homology, especially for cells that have been described in many species (e.g. ON1, see Cillov and Stumpner, 2022 for a review). I will use the same method to compare DN described here to ones in the literature.

DN2 has the most numerous and closest similarities to previously described neurons. DN2 in another phaneropterine bush cricket, LFD-1 in mocopodine bush crickets; DN1 in crickets, mole crickets and haglids; and many DN with similar morphology but especially TH1-DC/B1 and TH1-DB2 in cave crickets have a remarkably similar structure to that of DN2 (Phaneropterinae: Korsunovskaya and Zhantiev, 1992; Mecopodinae: Kostarakos and Römer, 2015; crickets: Atkins and Pollack, 1987a; Wohlers and Huber, 1982; mole crickets: Mason et al., 1998; haglids: Mason and Schildberger, 1993; cave crickets: Stritih, 2006). These cells are all characterised by only partial projections in aN, major arborisation in more posterolateral areas and a secondary neurite that crosses the midline back into the soma-ipsilateral hemiganglion. Though based on limited data for most of these studies, they also share the physiological characteristics of DN2, with tonic sensory responses, tuning to low frequencies, and sensitivity to other modalities such as wind or vibration.

Despite the prevalence of data on this neuron in diverse taxa, there are only few proposed functions, which is complicated by the significantly different life histories, behaviours and anatomies of different species. In _Mecopoda elongata_, LFD-1 and LFD-2 act as signal detectors under masking (Kostarakos and Römer, 2015, 2018). This species has sympatric populations with different calling songs: a continuous trill with stronger HF components and short chirps with a lower dominant frequency. LFD-1 and LFD-2 respond to the LF chirps even when they are masked by a much stronger trill. A very similar mechanism is also present in TN1 in _Neoconocephalus_ (Prešern et al., 2015; Schul et al., 2012; Schul and Sheridan, 2006; Triblehorn and Schul, 2013), which detects bat echolocation calls under masking by calling males. However, functional similarities between LFD-1/LFD-2 in _Mecopoda_ and DN2 in _Ancistrura_ are
difficult to suggest due to the differences in the calling songs of these species; *Ancistrura* calling song has a much higher dominant frequency that lies above the response range of DN2. Furthermore, the native environment of *Ancistrura* does not have especially strong masking from conspecifics like that of *Mecopoda* or from high background noise as is the case in other habitats, e.g. rainforests (Ellinger and Hödl, 2003; Lang et al., 2005).

DN4 also shows similarities to previously described bush cricket neurons, though comparisons are much more difficult here due to the lack of full characterisations in the literature. DN reported in *Leptophyes*, which is closely related to *Ancistrura*, and *Tettigonia* only consist of a single staining without any recordings. BFD-1 in *Mecopoda* is reported with much more associated data, though the anatomical structure varies significantly between publications (compare Kostarakos and Römer, 2015, and 2018). Finally, TH1-DC-S1 and TH1-DC-S4 in *Decticus* are also remarkably similar to DN4 in terms of morphology (Sickmann, 1997). These cells are all characterised by extensive branching in aN and a broadband tuning to frequencies between 10-40 kHz. Except for the LF-tuned component in BFD-1, frequency tuning in these cells is quite similar despite the differences between the frequency distributions of the species’ calling songs (Heller, 1988; Heller and von Helversen, 1986; Kostarakos and Römer, 2015).

Finally, DN5 is morphologically similar to a descending neuron in *Mygalopsis marki* (Römer, 1987; Römer et al., 1988). Both neurons arborise mainly along the primary neurite and respond only weakly to sound. Yet, the cell in *M. marki* could also be seen as a mix between DN5 and DN2, since it has major posterolateral branches as well.

Overall, the majority of previously described DN in orthopterans could be found in *A. nigrovittata*, as well as some with highly novel response patterns and anatomical structures, e.g. DN9. Phaneropterine bush crickets are thought to have a bigger and more diverse set of auditory interneurons due to their complex song structures compared to bush cricket taxa with more repetitive songs such as *Tettigonia* (Korsunovskaya and Zhantiev, 1992; Stumpner and Nowotny, 2014). Therefore, the set of DN in *Ancistrura* does not set the baseline for how many DN are expected in any given bush cricket species. Nevertheless, both general types (“lateral” and “bushy”) are represented even in distantly related species (compare *Decticus* in
Sickmann, 1997 with Ancistrura here; see Song et al., 2015, 2020 for the evolutionary relationships between bush cricket taxa), meaning both groups should be present in all hearing bush cricket taxa. However, since neuronal architecture and, especially, function can be highly preserved over significant evolutionary time (Neuhofer et al., 2008; Stritih, 2006) or be adapted based on changing selection pressures (Stumpner, 1999a; Stumpner and von Helversen, 2001), it is difficult to predict if DN in other species would closely resemble those in Ancistrura.

Possible functions of descending neurons
Besides characterising the prothoracic DN population in Ancistrura, the ultimate objective of this study was to find the “missing link” connecting the conspecific CS and the low-latency female reply. Since the temporal pattern of the species’ duet rules out an information loop through the brain, I was hoping to find a DN that shows signs of gating or facilitation in the order of hundreds of ms in response to the natural CS, thus providing evidence for the suggestion that a purely local network is responsible for triggering the female reply.

One possible gating mechanism for song recognition would be through post-inhibitory rebound (PIR). PIR is known to play a role in sensory systems in general (e.g. Ai et al., 2018; Large and Crawford, 2002; Matsuura and Kanou, 1998). Most importantly, coincidence detection based on PIR in a small neuronal network is strongly suggested to underlie song recognition in crickets (Hedwig, 2016; Kostarakos and Hedwig, 2012, 2015; Schöneich et al., 2015; Zhang and Hedwig, 2022). This network is flexible and can be easily tuned over evolutionary time to detect different temporal patterns (Clemens et al., 2021). Long-lasting inhibitory patterns are also known from local brain neurons in Ancistrura as well (Ostrowski and Stumpner, 2013). DN9, as well as another DN of unresolved morphology, exhibit similar inhibitory effects when an artificial calling song is played, lasting ~200 ms. These cells sometimes start firing spontaneously 200-400 ms after stimulus offset, indicating PIR activity. Unfortunately, the temporal variation of such PIR is extremely high and cannot be reproduced reliably.
Other phaneropterine species, such as *Leptophyes punctatissima*, have female responses that are described as reflex-like behaviours (Zimmermann et al., 1989; Andreas Stumpner, personal communication). Yet, even in crickets, which rapidly orientate towards attractive as well as nonattractive stimuli (Poulet and Hedwig, 2005), behavioural output is highly dependent on internal state of the animal (Hedwig, 2000; Hommaru et al., 2020). In contrast, mating behaviour in *A. nigrovittata* is an extensive and highly selective process with multiple approaches and retreats (Nataša Stritih-Peljhan and Andreas Stumpner, personal communication; personal observation). Therefore, it is highly likely that the generation of the female reply is highly dependent on internal processes and can be easily disrupted in an experimental setting. Since the methods I employ are extremely invasive and include fixing the animal upside down, a position that by itself triggers postural reflexes in most insects to reorient themselves (Faisal and Matheson, 2001; Jusufi et al., 2011; Zeng et al., 2017), and making incisions in the cuticle, the overall context and internal state or motivation of the animal can reasonably be assumed not to facilitate mating behaviour. This constitutes an inherent problem of such invasive methods and can only be bypassed by switching to noninvasive methods and making inferences from behaviour or by artificially triggering desired behaviour, as is commonly done for singing in crickets (Schöneich and Hedwig, 2012).

How could we infer otherwise if DN could be involved in triggering the female reply? Any neuron involved in that process would invariably have to excite the muscles controlling the wings, which are involved in stridulation. Most singing ensiferans, including *A. nigrovittata*, produce sounds by closing the wings during stridulation (Bennet-Clark, 2003; Elliott and Koch, 1985; Heller and von Helversen, 1986; Koch et al., 1988; Montealegre-Z and Mason, 2005; Suga, 1966). This is powered by forewing elevator muscles, which are innervated by motoneurons that project from TG2 to the muscles through nerves 3 and 4 (crickets: Wang and Robertson, 1989; grasshoppers: Bentley, 1970). These motoneurons have extensive dorsolateral projections in TG2, representing a target area for any neurons involved in the process. None of the DN I describe here had dorsal branches in TG2; on the contrary, these were restricted to the ventral association centre in the far ventral side of the ganglion.

Wing motoneurons cannot be directly activated by DN due to the differences between projection areas. Polysynaptic connections, on the other hand, are not ruled out; but are they
possible? As mentioned in the Introduction, the average latency between the trigger syllable of the calling song and the female reply is ~30 ms. I assume here the shortest route between perception of the trigger syllable and generation of the female reply, i.e. connection between sensory and motor systems in TG2. First of all, the majority of recorded DN have depolarisation latencies <14 ms, indicating they receive direct input from sensory cells. The complete relay from sensory cells to activity of first-order auditory interneurons in the bush cricket brain takes ~19 ms (Ostrowski and Stumpner, 2010). The TG1-TG2 distance is much shorter than the TG1-brain distance, but I use this value for a conservative estimate. I add 2 ms for each synapse between DN and the forewing elevator motoneurons (Hennig, 1988; Vogel and Ronacher, 2007), in this case at least two. Finally, the time between action potentials in the motoneuron and onset of muscle contraction is ~5 ms (Josephson, 1973). Since orthopterans have direct, neurogenically controlled wing muscles, a single action potential is sufficient to start muscle contraction (Burrows, 1996a). This calculation allows up to two interneurons in TG2 between DN and the motoneurons generating the female reply. Therefore, a polysynaptic thoracic network might still be responsible for bridging the gap between male and female signals.

Moving away from intraspecific communication, DN could be involved in integrating audiovibratory signals from TG1 with mechanosensory or proprioceptive information from lower thoracic segments. Insects quickly adapt their behaviour on the basis of internal and external sensory input using local neural networks (e.g. see Burrows, 1996b; Ritzmann and Büschges, 2007 for reviews in grasshoppers and stick insects, respectively). Such phenomena affect even highly stereotyped behavioural patterns like positive phonotaxis (Poulet and Hedwig, 2005). A. nigrovittata is a flightless species, so the bat avoidance response common among flying orthopterans (Hoy, 1989; Hoy et al., 1989) is not expected to be a part of this repertoire. Especially for motor decisions, information from different organs dispersed throughout thoracic ganglia need to be integrated. Like DN, many mechanosensory cells from the legs (Strauß, 2017; Strauß et al., 2014; Strauß and Lakes-Harlan, 2008a, 2008b, 2010) as well as proprioceptors (Burrows, 1996c; Collin, 1985; Field and Matheson, 1998; Murrain and Ritzmann, 1988; Nishino and Field, 2003) project into ventral areas of thoracic ganglia. Since the prothoracic legs are the only source of sensitive acoustic information, input from DN could contribute significantly to any behavioural decisions. Therefore, it is possible that DN are
either connected directly with these sensory cells or project to common targets in TG2 and possibly TG3.

Multimodal communication in *A. nigrovittata*

Our collaborator, Nataša Stritih-Peljhan, provided the first data on near-field communication and behaviour in *A. nigrovittata*. As with many other insects, Ancistrura also utilises vibration in the near field, which presents a more private channel that cannot be as easily eavesdropped by predators as sound (Belwood and Morris, 1987; Morris et al., 1994; Scala et al., 2024; Stritih-Peljhan and Virant-Doberlet, 2021; ter Hofstede et al., 2017). Most reports of acousto-vibratory behaviour in orthopteran species focus on intra- or interspecific aggression (Benavides-Lopez et al., 2020; Heller and von Helversen, 1986; Morris, 1971), asynchronous use of sound and vibration (Korsunovskaya and Zhantiev, 2022; Montealegre-Z and Morris, 1999; Römer et al., 2010; Sarria-S et al., 2016), or changes in phonotaxis with multimodal stimulation (Kalmring et al., 1983; Kalmring and Kühne, 1980; Kühne, 1982; Latimer and Schatral, 1983). In bush crickets, there is only one report of simultaneous multimodal signalling: *Nesonotus reticulatus*, a pseudophylline species, uses both channels seemingly independently, leading to only occasionally multimodal signals (Stumpner et al., 2013). In contrast, Ancistrura seems to employ acousto-vibratory signals extensively during courtship. Furthermore, sound and vibration seem to be temporally closely coupled and the animals freely switch between unimodal and multimodal signalling (Nataša Stritih-Peljhan, unpublished results).

Many bush cricket neurons seem to respond to both sound and vibration (e.g. Ebendt et al., 1994; Kalmring et al., 1978; Sickmann, 1997). However, most previously described multimodal cells are LF neurons that also respond to vibration; none respond sensitively to frequencies >10 kHz as well as to vibration. DN9 described here is sensitive to both vibration and a broad spectrum of sound frequencies; therefore, it is the first truly multimodal bush cricket neuron in the literature. Consequently, it is uniquely suitable for integrating the newly described multimodal courtship signals in Ancistrura. Future experiments with cell killing in freely behaving animals could further elucidate its relevance for intraspecific signalling.
Summary

*Ancistrura nigrovittata* is a duetting phaneropterine bush cricket species. Males sing with a fixed temporal and spectral pattern, the characteristics of which are important for recognition; females reply to the song with a low latency click (~30 ms). Auditory neurons in the brain have longer spike latencies. Neurons descending from the ear to the wings, gated by a pattern recognition network in the brain, could trigger the female reply directly. Therefore, I investigated the population of descending neurons (DN) in the prothorax, the first level of central auditory processing.

I characterised multiple morphological and physiologically distinct neuron subtypes, some of which have not been previously reported. Lateral DN have weak or no auditory response and are mostly vibratory. These constitute the majority of previous reports in ensiferans. A local descending neuron is structurally similar to other DN but is intraganglionic. It responds to a broad range of frequencies and temporal patterns and could function as a reference neuron. Bushy DN are tuned to frequencies around 20 kHz (peak frequency of the calling song). This group is composed of diverse neurons with only subtle anatomical differences. Another DN is both morphologically and in its sensory responses a mixture of lateral and bushy DN: sensitive to both vibration and sound. It could play a role in near field multimodal courtship behaviour.

Using data from behavioural observations conducted by a collaborator, I describe the nearfield communication of *A. nigrovittata*. It consists of a complex combination of vibration and sound with a consistent use of simultaneous acoustic and vibratory signals when the animals are within close proximity of each other. One DN I describe in the current study could underlie this multimodal behaviour.

Overall, I characterise the last unexplored major group in the local auditory processing network of a species that has been intensively studied. There is a significant stream of descending data with no known function, as well as integration of multiple modalities already on a local level. These findings highlight our limited knowledge about insect sensory processing and the importance of pre-brain areas in distributed nervous systems.
References


Lewis, D.B., 1974. The physiology of the tettigoniid ear: II. The response characteristics of the ear to differential inputs: lesion and blocking experiments. J. Exp. Biol. 60, 839–851. https://doi.org/10.1242/jeb.60.3.839


The Cricket as a Model Organism. Springer Verlag, Tokyo, Japan, pp. 155–167. https://doi.org/10.1007/978-4-431-56478-2_11


Watson, A.H.D., Hardt, M., 1996. Distribution of synapses on two local auditory interneurones, ON1 and ON2, in the prothoracic ganglion of the cricket: relationships with GABA-


## Appendix A: Chemicals, solutions & equipment

### Equipment used in acousto-vibratory recordings by Nataša Stritih Peljhan

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### Chemicals

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Agar LV Hardener VH1 | Agar Scientific | Stansted, UK | - 
Agar LV Hardener VH2 | Agar Scientific | Stansted, UK | - 
Agar LV Accelerator | Agar Scientific | Stansted, UK | - 
Streptavidin Cy3 conjugate | Jackson ImmunoResearch | West Grove/PA, USA | 90637 
 | Molecular Probes | Eugene/OR, USA | 0200J

**Solution/mixes**

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<td>Modified Fielden saline (for 1 l)</td>
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<td>4% paraformaldehyde (for 100 ml)</td>
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<td>PBST</td>
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<td>5% blocking buffer</td>
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<td>DABCO solution (for 100 ml)</td>
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<td>Embedding medium (sagittal)</td>
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### Equipment & software

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Appendix B: Calibration of vibration stimuli

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Acknowledgements

I am truly grateful on a daily basis for all the lovely people in my life, but this work has only been possible thanks to the invaluable and unwavering support and guidance of Andreas Stumpner in all aspects of life in the last four years. You have been the best possible supervisor I could have imagined and so much more; I understand why a supervisor is called Doktorvater thanks to you. I do not know how I could thank you enough or repay my debt of gratitude for all your help and advice and tolerating my all-too-often mental breakdowns and last-minute cramming (including this thesis). I am so thankful to have made the decision to squeeze in a lab rotation with you before leaving for Madagascar back at a time when I did not even want to go into neuroscience.

Elif and Hazar have been my absolute helpers and companions and carried me through the worst of times in these years. You have been my lifeline through some of the truly worst phases of my life, thank you both so much for your friendship and your constant presence.

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